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Original Article

Chronopharmacology of dapagliflozin-induced antihyperglycemic effects in C57BL/6J mice

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ABSTRACT

Chronopharmacology is the study of the varying responses of drugs to changes in biological timing and endogenous periodicities. The selective sodium-glucose cotransporter 2 inhibitor, dapagliflozin, is a globally prescribed antihyperglycemic drug. Although dapagliflozin is usually administered once a day, the specific intake time is generally not mentioned. Therefore, this study aimed at investigating the diurnal effects of dapagliflozin on high-fat diet (HFD)-induced obesity in mice. Five-week-old male C57BL/6J mice were fed a normal (control) diet or HFD for 10 weeks. During the last 2 weeks, the mice were administered olive oil/ethanol emulsion or dapagliflozin (1 mg/kg, p.o.) in the light or dark phase. At the end of the experiment, the mice were euthanized after an 18 h fasting period, and plasma and tissue samples (epididymal white adipose tissues, liver, and kidney) were collected. Dapagliflozin administration in the light phase significantly decreased plasma glucose levels, insulin levels, adipose adipokines, and decreased the size of adipocytes, compared with the HFD group. In contrast, these parameters remained unchanged in the mice treated during the dark phase. Our data therefore suggests that dapagliflozin portrays definite chronopharmacology, which may provide valuable information on the importance of drug administration timing for maximal pharmacological effects.

Introduction

Increases in the number and size of adipocytes have been recognized as a cause of obesity. Obesity is one of the most important risk factors for the development of lifestyle diseases, such as type 2 diabetes (T2D) and hypertension [1]. As for T2D therapy, the first line of treatment is to introduce changes in diet and exercise; while drug administration is the second-line treatment. However, most of the patients are proceeding onto drug therapy. Prescribed medicine for T2D patient are classified either as for insulin injection or as oral anti-hyperglycemic medicines. Various classes of oral anti-hyperglycemic drugs exist, most of which act by increasing insulin secretion or improving the insulin sensitivity of target tissues, such as the liver, adipose tissues, and skeletal muscle [2]. In addition, a new approach that acts through the inhibition of renal glucose reabsorption is drawing much attention. These medicines are known as selective sodium-glucose cotransporter 2 (SGLT2) inhibitors. SGLT2 is a sodium-solute cotransport protein located in the kidney's proximal tubule that reabsorbs the majority of the glomerular-filtered glucose [3]. Dapagliflozin (Dap) is the first of this new class of oral SGLT2 inhibitors, designed for treating T2D patient. In addition to reducing the hyperglycemia, SGLT2 inhibitors, including Dap, have the potential to enable body weight loss [4]. Therefore, Dap is a globally prescribed and popular medicine.

Recent manuscripts have demonstrated that obesity causes insulin resistance by promoting low-grade inflammation [5]. White adipose tissue (WAT) serves not only as a fuel source, but also secretes various adipokines. Physiologically normal adipocytes of normal sizes, which are observed in fat pads of lean individuals, secrete adipokines, such as adiponectin, to enhance insulin sensitivity [6], whereas pathologically enlarged adipocytes in obese individuals secrete other adipokines, such as tumor necrosis factor

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(TNF) α and monocyte chemoattractant protein-1 (MCP-1), which worsen insulin sensitivity [5]. A balance in the secretion of these adipokines with reciprocal functions determines the pathophysiological state of inflammation and insulin resistance [7]. Therefore, it is important to decrease the production of pro-inflammatory mediators, such as TNFα and MCP-1, derived from obese adipose tissues to reduce insulin resistance.

Circadian rhythms are endogenous 24 h oscillations in biological and behavioral patterns common to all living organisms. The circadian clock drives oscillations in a diverse set of biological processes, including locomotion, sleep, blood pressure, blood hormone levels, and body temperature [8,9]. These differences directly affect disease frequency. For example, there are higher occurrences of asthma at midnight and myocardial ischemia in the morning [10,11]. Moreover, it is well reported that circadian time-dependent differences change the pharmacokinetics of medications, such as anticancer drugs and antibiotics [12,13]. It is therefore necessary to consider the chronobiology of such medications during treatment.

Continuous glucose monitoring systems are available for diabetic patients to monitor glucose levels in real-time or over a period of time [14]. They can be used to observe the chronopharmacology of antidiabetic drugs, which are of particular interest in insulin-based therapy. Conversely, chronopharmacological reports on oral anti-hyperglycemic drugs (such as metformin) in experimental animals are very limited [15]. SGLT2 inhibitors (anti-hyperglycemic drugs) other than Dap are usually prescribed as a single dose before or after breakfast. In contrast, although Dap is also prescribed as a single dose, a specific intake time is not mentioned. Therefore, we considered the possibility that the time of Dap administration might vary for each patient. Additionally, Dap-induced pharmacological effects could also change based on the administration time. To address this hypothesis, we investigated the circadian variations in Dap-induced pharmacological effects, on T2D mellitus-related parameters in high fat diet (HFD)-induced obesity model mice.

Materials and methods

Animal treatment

Four-week old C57BL/6J mice (males) were purchased from CLEA Japan Inc. (Tokyo, Japan). The mice were maintained ina controlled environment: temperature (24 ± 1 °C), humidity (55 ± 5%), and light cycle (12 h light [08:00-20:00]:12 h [20:00-08:00] dark). Food and water were given *ad libitum*, and the mice were allowed to acclimatize to laboratory conditions for a week. The mice were 5-weeks-old (18–21 g) at the start of experiments. All experimental procedures were approved by the Institutional Animal Care and Experiment Committee of our institution (No. 158).

Experimental protocol

C57BL/6J mice were randomly divided into 6 groups (5–8 mice per group). Two groups were fed with the normal diet (CE-2 [CLEA]), and 4 groups (obesity model) were fed with HFD60 from Oriental Yeast Co., (Tokyo, Japan): (HFD60). All 6 groups were fed for a total of 10 weeks. After 8 weeks, the mice were further divided into the vehicle-treated groups (control and HFD) which received olive oil/ethanol emulsion, and the Dap-treated groups (HFD + Dap), which received 1 mg/kg (0.05 mL/kg) Dap purchased from Carbosynth Limited (United Kingdom). Olive oil/ethanol emulsion or Dap treatments were administered by oral gavage once daily for 2 weeks. Treatments were administered at 2-specific time points (clock time:10:00 or 22:00), described here as 2-different zeitgeber times (ZT): ZT2 (control, HFD, and HFD + Dap) or ZT14 (control, HFD, and HFD + Dap). Body weights and food intake were measured weekly for 10 weeks. After the 2-week treatment, mice from each group were allowed to fast for 18 h. The mice were euthanized using pentobarbital, and bled to obtain plasma samples, which were stored at −80 °C until use. Epididymal WAT, liver, and kidney weights were also determined. Separate WAT samples were immediatelyfrozen in liquid nitrogen and subsequently kept at −80 °C, or fixed in 15% neutral buffered formalin (pH7.4).

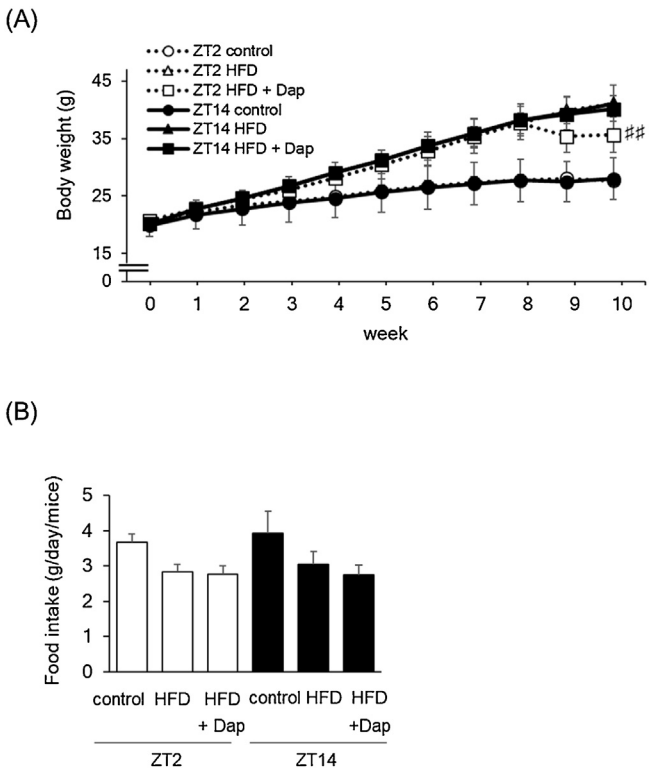

Fig. 1. Diurnal effect of Dap on body weights of HFD mice.
Mice received CE-2 or HFD for 10 weeks. For the last 2 weeks (week 8 to week 10), mice were also administered Dap (1 mg/kg, p.o.) or olive oil/ethanol emulsion at ZT2 or ZT14. (A) The data represent the mean ± SD of body weight results of 5–8 mice per group. "○": control group at ZT2; "●": control group at ZT14; "△": HFD group at ZT2; "▲": HFD group at ZT14; "□": HFD + Dap at ZT2; and "■": HFD + Dap at ZT14. $^{**}p < 0.01$ vs. the control group at ZT2, and $^{\#\#}p < 0.01$ vs. the control group at ZT14. Panel (B) indicate the food intake per day in one mice.

Plasma biochemical analysis

Measuring the plasma glucose and insulin levels were previously described [16].

Histopathological analyses

Epididymal WAT, obtained from each mouse, was formalin-fixed, and embedded in paraffin. Paraffin-embedded liver blocks were cut into 10 μm sections. The sections were stained with hematoxylin and eosin (H&E), as described previously [17].

RNA isolation and quantitative real-time PCR assay

Total RNA was extracted from the epididymal WAT using FastGene RNA Basic kit (Nippon Genetics Co., Ltd., Tokyo, Japan). The procedure for reverse transcription and the PCR conditions were described previously [16]. The amount of each quantified target mRNA was normalized against that of β-Actin. The oligonucleotide sequences of the primers used are:

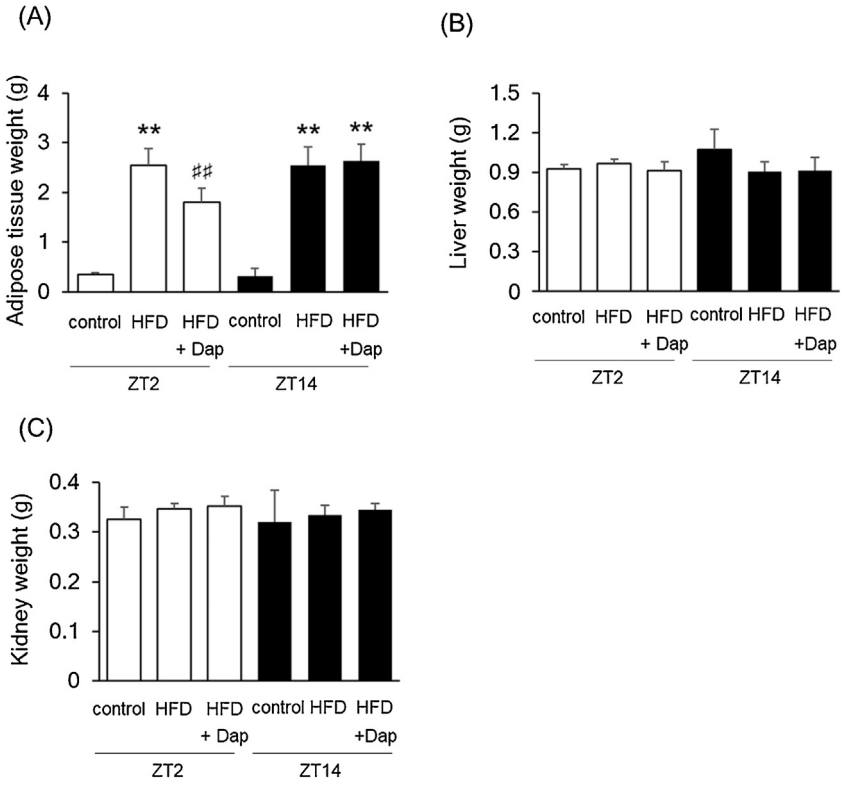

Fig. 2. Dap-induced diurnal effects on organ weights of HFD mice.
Animals were treated as described in Fig. 1. Then, mice in each group were fasted for 18 h. Thereafter, they were euthanized, and their liver, kidney, and epididymal white adipose tissues were isolated and weighed. Panels indicate the weight of (A) epididymal white adipose tissues, (B) liver tissues, and (C) kidney tissues. The data represent the mean ± SD of results of 5–8 mice per group. $^{**}p < 0.01$ vs. the control group at same time. $^{\#\#}p < 0.01$ vs. the HFD group at the same time.

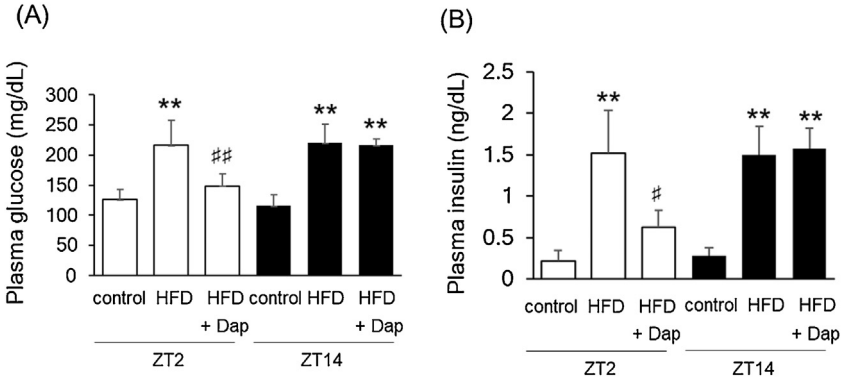

Fig. 3. Diurnal effects of Dap on plasma biochemical parameters.
Animals were treated as described in Fig. 1. Then, mice in each group were fasted for 18 h, euthanized, and bled to obtain plasma. Levels of plasma (A) glucose and (B) insulin are shown. The data represent the mean ± SD of results of 5–8 mice per group. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the control group at same time; and $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ vs. the HFD group at same time.

mouse β-*actin* (NM_007393) sense, 5′-GCAACGAGCGGTTCCG-3′, and antisense, 5′-CCCAAGAAGGAAGGCTGGA-3′; mouse *TNFα* (NM_013693) sense, 5′-ACACTCAGATCATCTTCTCAAAATTCG-3′, and antisense, 5′-GTGTGGGTGAGGAGCACGTAGT-3′; mouse *MCP-1* (NM_011333) 5′- CCCAATGAGTAGGCTGGAGA-3′, and antisense, 5′-TCTGGACCCATTCCTTCTTG-3′; mouse *F4/80* (NM_010130) sense, 5′-CTTTGGCTATGGGCTTCCAGTC-3′, and antisense, 5′-GCAAGGAGGACAGAGTTTATCGTG-3′.

Statistical analyses

Data were presented as the mean ± standard deviation (SD). Statistical significances were determined using one-way analysis of variance (ANOVA) or two-way ANOVA with repeated measures, followed by the Tukey–Kramer's *post hoc* test for multi group comparisons. All statistical analyses were performed using the SPSS software (version 25.0) from IBM Corp. (Armonk, NY, USA).p values <0.05 were considered statistically significant.

Results

Dap administration at ZT2 improves HFD-induced body weight gain

Our initial investigations involved monitoring the effect of Dap (at ZT2 or ZT14) on obesity-related abnormalities. Mice

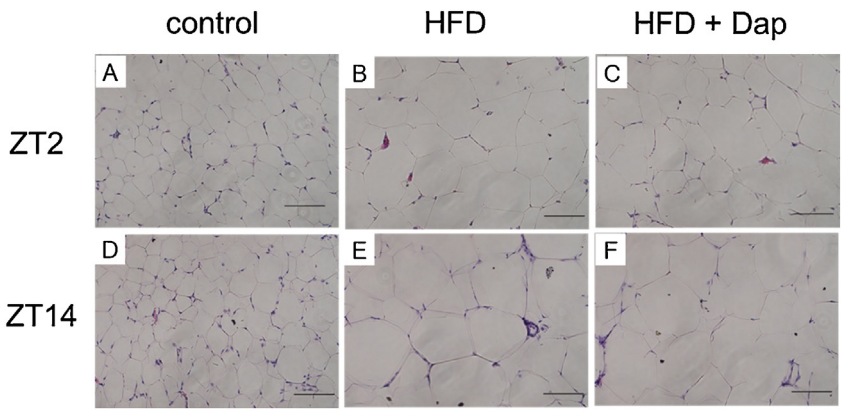

Fig. 4. Diurnal effects of Dap on HFD-induced adipocyte enlargement.
Animals were treated as described in Fig. 1. Then, mice in each group were fasted for 18 h, euthanized, and epididymal white adipose tissues were harvested. They were fixed and processed by standard methods, and the sections were stained with H&E. Panels indicate (A) control group at ZT2, (B) HFD group at ZT2, (C) HFD + Dap group at ZT2, (D) control group at ZT14, (E) HFD group at ZT14, and (F) HFD + Dap group at ZT14. Black arrows indicate small adipocyte compared to HFD; scale bar, 100 μm.

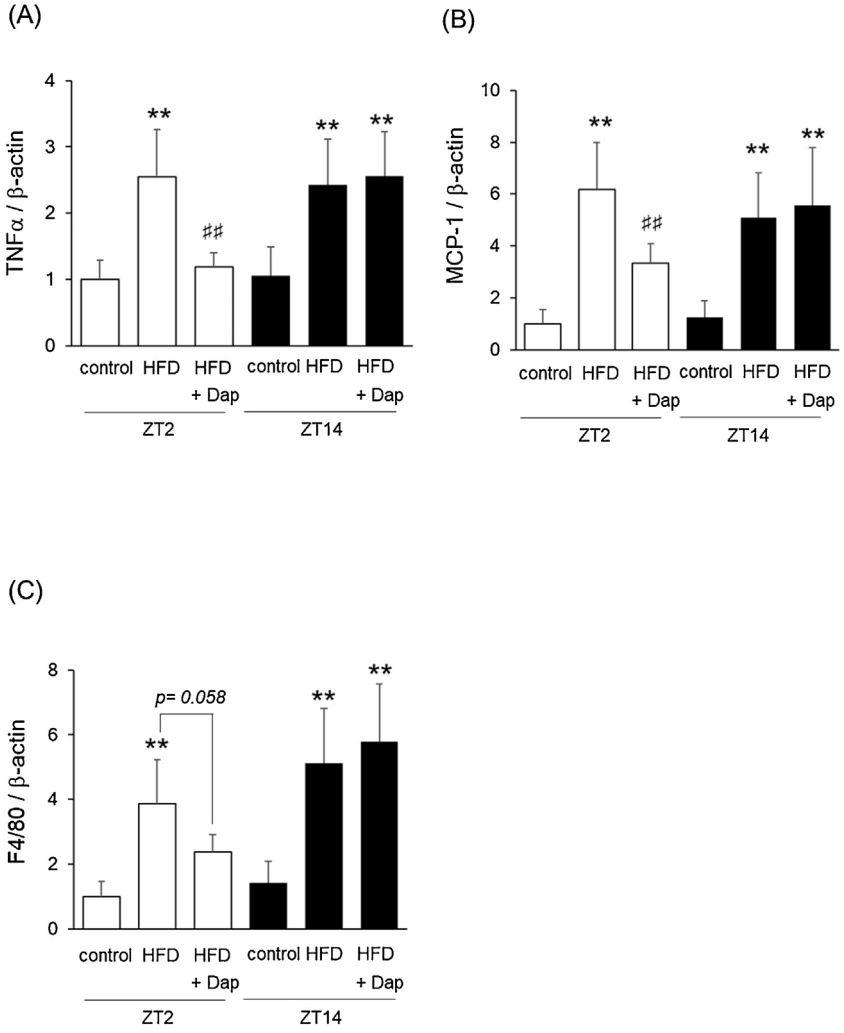

Fig. 5. Diurnal effects of Dap on adipokines.
Animals were treated as described in Fig. 1. Then, mice in each group were fasted for 18 h, euthanized, and epididymal white adipose tissues were isolated. Panels indicate adipocyte *TNF-α* levels at ZT2 (A) and ZT14 (B), *MCP-1* levels at ZT2 (C) and ZT14 (D), and F4/80 levels ZT2 (E) and ZT14 (F). Data are plotted as mean ± SD. $^{*}p < 0.05$ vs. the control group; $^{**}p < 0.01$ vs. the control group; and $^{\#\#}p < 0.01$ vs. the HFD group.

were fed CE-2 (control) or HFD for 10 weeks and administered olive oil/ethanol emulsion or Dap at ZT2 or ZT14 for the last 2 weeks. Changes in body weights are shown in Fig. 1A. The body weight of HFD mice was greater than that of control mice at both times. Dap administration at ZT2 significantly decreased the body weight, while this phenomenon was not observed at ZT14. Food intake was not changed when administrating Dap (Fig. 1B).

To elucidate the cause of body weight alterations, we determined the weights of epididymal WAT, liver, and kidney samples (Fig. 2A–C). At ZT2, the HFD + Dap group had significantly lighter epididymal WAT, compared with the respective tissues in the HFD group. Conversely, this trend was not observed at ZT14 (Fig. 2A). As for the liver and kidney, significant changes were not observed at both times (Fig. 2B and C). These data suggest that the body weight decreases in HFD mice after Dap administration at ZT2 can be attributed to the effects of Dap on the epididymal WAT.

Dap administration at ZT2 decreases the levels of plasma biochemical parameters

To investigate whether Dap reduces obesity-induced hyperglycemia, we determined fasting plasma glucose (Fig. 3A) and insulin levels (Fig. 3B). The HFD groups showed increased plasma glucose levels when compared with the control groupsat ZT2 and ZT14. At ZT2 but not ZT14, the HFD + Dap group had significantly decreased plasma glucose levels compared to those of the HFD group (Fig. 3A). The same trend was observed for plasma insulin levels (Fig. 3B). These results indicate that at ZT2, Dap administration decreased HFD-induced hyperglycemia and hyperinsulinemia, thereby improving the glucose metabolism.

Dap administration at ZT2 decreases adipocyte size

Along with the measurement of plasma biochemical parameters, we conducted histopathological studies on the adipose tissue. Histological analysis of the adipose tissue showed that adipocyte sizes were larger in the HFD group (Fig. 4B and E) than in the controls (Fig. 4A and D) at both times. Adipocyte sizes were smaller in the groups treated with Dap at ZT2 than in the HFD group at ZT2 (Fig. 4C). In contrast, Dap administration at ZT14 did not alter the adipocyte size (Fig. 4F). The data indicate that Dap administration at ZT2 promotes the differentiation of adipocytes.

Dap administration at ZT2 decreases production of adipokines in the adipocyte

Finally, we evaluated whether Dap administration at ZT2 exerts anti-inflammatory effects by regulating the production of obesity-related inflammatory factors, such as *TNFα* (Fig. 5A) and *MCP-1* (Fig. B) because adipokines induce low-grade inflammatory conditions and worsen insulin sensitivity. Dap administration at ZT2 significantly decreased adipose *TNFα* and *MCP-1* mRNA levels in mice, while these recoveries were not confirmed at ZT14. Additionally, we measured *F4/80* as a macrophage marker, which is known to increase obesity [18]. This expression pattern is consistent with previous results (Fig. 5C). These data indicate that Dap administration at ZT2 exerts anti-inflammatory effects in mice by decreasing the production of adipokines in the adipose tissue.

Discussion

In this study, we investigated the circadian variations in Dap-induced pharmacological effects using HFD-induced obesity mice. Daytime Dap administration decreased body weight gain in HFD-fed C57BL/6 mice [4]. Weight loss was observed when Dap was administred in the light phase (ZT2), while this trend was not confirmed in the dark phase (ZT14). The decreased weights of epididymal WAT reflected body weight alterations. These results suggest that Dap exerts its weight loss effect through the suppression of WAT weight.

We observed that HFD-induced plasma glucose and insulin levels, adipocyte enlargement, and adipokines in the adipocytes improved when Dap was administered in the light phase (ZT2), but remained unchanged when it was administered in the dark phase (ZT14). The different Dap-induced glucose metabolism responses observed at different times of the day (morning and evening) suggests that the mechanisms responsible for Dap's pharmacological effects varies according to the time of the day.

The most possible hypothesis for the different pharmacological effect is occurred from hemodynamic action since SGLT2 inhibitors were known to excrete glucose in the urine [3]. Although we measured urine glucose levels when sacrificed, the levels were comparable at either administration time (data not shown). Since we could not calculate total urine volumes and water consumption, we ca not judge our results were correct or not. Further investigations using metabolism cage were necessary to elucidate the association with hemodynamic parameters.

The second hypothesis for the diurnal variation observed in Dap-induced pharmacological effects is it is the effect of circadian rhythm. Clock genes such as brain muscle arnt-like 1 (*Bmal1*), *Clock*, Cryptochrome (*Cry*), and Period (*Per*) play important roles in maintaining the circadian rhythm [19]. Disruption of *Bmal1* and *Clock* is known to cause hyperinsulinemia and diabetes [20]. Moreover, since *Bmal1* knockout mice are reported to have high blood glucose and low insulin levels [21], these clock genes are important for insulin and glucose homeostasis. Pioglitazone is known to improve adipocyte enlargement by activating PPARγ [18]. Additionally, positive correlation with PPARγ and Bmal1/Clock were reported by Nakamura et al [22]. Since *Bmal1/Clock* is expressed at high levels in the morning, PPAR γ-induced adipose differentiation might be strengthened. Not only *Bmal1/Clock*, *Per2* was shown to be inversely correlated with peroxisome proliferator-activated receptor (PPAR) γ [23]. Since *Per2* is expressed at high levels in the night, PPAR γ-induced adipose differentiation might be weakened compared to daytime. Although further investigation is necessary, different expression levels for clock genes may be associated with diurnal variations in Dap administration.

A third hypothesis will be food intake, which is highly interconnected tocircadian clocks [24]. Several medications (such as antibiotics) are known to change their pharmacological effects by changing their absorption efficiency, AUC, and Tmax [13]. Our investigations showed that daily food intake caused no significant change at ZT2 or ZT14 (Fig. 1B). Moreover, Dap-induced AUC was not significantly changed during fasting and after a meal [25]. Since food intake is thought to affect circadian clocks, it can be argued that the food intake conditions were slightly affected. However, this hypothesis may not be very efficient, as this study did not consider the fasting time. Further experiments may be necessary to investigate the effect of diet.

In conclusion, we have demonstrated that mice are sensitive to Dap-induced anti-hyperglycemic effects when administered during the light phase (ZT2). Thus, Dap exhibits definite chronopharmacology. Although we focused on only Dap in the present investigation, it is very important to judge whether the other SGLT2 inhibitors show same tendency or not. We propose that more chronopharmacology studies be carried out on anti-hyperglycemic medications, as they may provide valuable information on the quality of life in the treatment of obesity and other related diseases.

Conflicts of interest

The authors declare no conflicts of interest.

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