Daily Fasting Blood Glucose Rhythm in Male Mice: A Role of the Circadian Clock in the Liver

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Fasting blood glucose (FBG) and hepatic glucose production are regulated according to a circadian rhythm. An early morning increase in FBG levels, which is pronounced among diabetic patients, is known as the dawn phenomenon. Although the intracellular circadian clock generates various molecular rhythms, whether the hepatic clock is involved in FBG rhythm remains unclear. To address this issue, we investigated the effects of phase shift and disruption of the hepatic clock on the FBG rhythm. In both C57BL/6J and diabetic *ob/ob* mice, FBG exhibited significant daily rhythms with a peak at the beginning of the dark phase. Light-phase restricted feeding altered the phase of FBG rhythm mildly in C57BL/6J mice and greatly in *ob/ob* mice, in concert with the phase shifts of mRNA expression rhythms of the clock and glucose production–related genes in the liver. Moreover, the rhythmicity of FBG and *Glut2* expression was not detected in liver-specific *Bmal1*-deficient mice. Furthermore, treatment with octreotide suppressed the plasma growth hormone concentration but did not affect the hepatic mRNA expression of the clock genes or the rise in FBG during the latter half of the resting phase in C57BL/6J mice. These results suggest that the hepatic circadian clock plays a critical role in regulating the daily FBG rhythm, including the dawn phenomenon. **(*Endocrinology* 157: 463–469, 2016)**

An early morning increase in fasting blood glucose (FBG) levels and/or insulin requirements is known as the dawn phenomenon (1). This phenomenon is pronounced among type 1 diabetic patients (1), but the condition occurs also in those with type 2 diabetes (2, 3) and in nondiabetic subjects (4). Clinical studies in the 1980s using pharmacological inhibition suggested that GH, but not cortisol, is responsible for the dawn phenomenon (5–7). Another study revealed that nocturnal surges in GH cause an increased rate of hepatic glucose production during the early morning hours by decreasing hepatic insulin sensitivity (8).

In patients with type 2 diabetes, hepatic glucose production exhibits circadian rhythmicity, which causes daily variation in insulin sensitivity (9). In animals, hepatic gluconeogenesis is increased at the beginning of the active phase, consistent with elevated activity of the rate-controlling enzyme phosphoenolpyruvate carboxykinase (10, 11). In addition, transcripts of numerous components related to glucose metabolism, including glucose 6-phosphatase and glucose transporter type 2, are expressed with a circadian rhythm in the liver (12). Furthermore, a subset of the clock genes that constitute the intracellular circadian pacemaker are reported to mediate gluconeogenesis and glucose export in hepatocytes (13, 14). Collectively, these findings suggest that a hepatic circadian clock mediates the circadian regulation of hepatic glucose production.

Consistent with the rhythm of hepatic glucose production, basal (fasting) blood glucose exhibits an obvious daily rhythm in rats with a peak at the beginning of the active phase (15). The rhythm appears to be regulated by the central clock residing in the hypothalamic suprachiasmatic nucleus because FBG does not exhibit daily vari-

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Abbreviations: BMAL1, brain and muscle Arnt-like protein-1; FBG, fasting blood glucose; ZT, Zeitgeber time.

ation in suprachiasmatic nucleus–lesioned rats (15). However, whether the hepatic circadian clock directly regulates the daily FBG rhythm and the dawn phenomenon remains unclear. Peripheral circadian clocks, including the hepatic clock, are controlled by the central clock, which is reset mainly by light, whereas the hepatic clock rhythm is affected more by feeding (16, 17). Therefore, elucidating the roles of feeding and the hepatic clock in the dawn phenomenon is important and would be clinically useful. In the present study, we found that the daily FBG rhythms in normal and diabetic mice are influenced by feeding time and that the dawn phenomenon is observed in GH-suppressed mice but not in liver-specific circadian clock–disrupted mice.

Materials and Methods

Animals

The protocol for this study was approved by the Institutional Animal Experiment Committee of Jichi Medical University. All animal procedures were performed in accordance with the Institutional Regulation for Animal Experiments and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the MEXT of Japan. All efforts were made to minimize animal suffering.

Six-week-old male C57BL/6J and B6.V-Lep^{ob}/J (*ob*/*ob*) mice were obtained from Charles River Japan. C57BL/6J-background, liver-specific *Bmal1*-deficient (L-*Bmal1*$^{-/-}$) and control (flox/flox) mice (18) homozygous for floxed *Bmal1*, with and without Cre driven by the albumin promoter, respectively, were bred and raised at the Center for Experimental Medicine of Jichi Medical University. All mice were maintained under specific pathogen-free conditions and controlled temperature and humidity with a 12:12-hour light/dark cycle and fed a regular diet (CE-2; CLEA Japan) and water ad libitum.

Experiments

Only male mice were used in this study. Zeitgeber time (ZT) was used to describe the experimental time with ZT 0 defined as lights on and ZT 12 as lights off. To reduce the number of mice, a crossover design was used in experiments 1 and 3 as described below.

Experiment 1

Twelve-week-old C57BL/6J and *ob*/*ob* mice (each $n = 9$) were fasted for 24 hours from ZT 0 or ZT 12 ($n = 4$ and 5, respectively, for each strain). At 15 weeks of age, the mice were fasted again for 24 hours from the other ZT. Glucose concentrations in the blood (taken from the tail) were measured every 6 hours throughout the fasting period using a Glutest Ace R (Sanwa Kagaku Kenkyusyo).

Experiment 2

Twenty-four C57BL/6J mice were divided into 2 groups at 10 weeks of age. Half of the mice were given food only during the light phase (from ZT 0 to ZT 12) and the other half were fed ad libitum. After 14 days, the mice were fasted from ZT 12 and then killed to obtain liver samples at ZT 18, 0, 6, and 12. Other groups of 10-week-old C57BL/6J ($n = 9$) and *ob*/*ob* mice ($n = 5$) were also subjected to the same 2-week time-restricted feeding regimen and then fasted for 24 hours from ZT 12 to investigate daily FBG profiles.

Experiment 3

Twelve- to 14-week-old L-*Bmal1*$^{-/-}$ ($n = 17$) and control (flox/flox) mice ($n = 15$) were fasted from ZT 12 and then killed to obtain liver samples at ZT 18, 0, 6, and 12. To investigate daily FBG rhythms, other groups of L-*Bmal1*$^{-/-}$ ($n = 9$) and control mice ($n = 5$) were fasted for 24 hours twice (from ZT 0 or ZT 12) at an interval of at least 2 weeks from 12 to 15 weeks of age.

Experiment 4

Twelve-week-old C57BL/6J mice ($n = 12$) were divided into 2 groups, injected with 20 mg/kg octreotide (Sandostatin LAR;

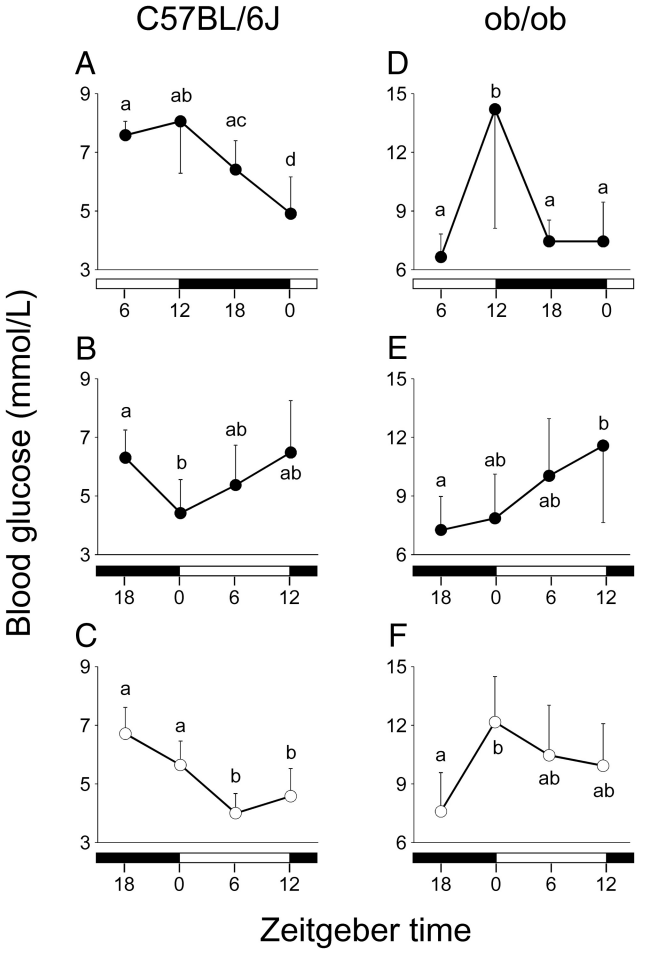

Figure 1. Daily FBG profiles in mice. C57BL/6J (A and B) and *ob*/*ob* (D and E) mice (each $n = 9$) were fasted for 24 hours twice from ZT 0 (A and D) or ZT 12 (B and E) in 3-week intervals. Other groups of C57BL/6J mice (C, $n = 9$) and *ob*/*ob* mice (F, $n = 5$) were subjected to a 2-week light-phase restricted feeding regimen and then fasted for 24 hours from ZT 12. Data represent the means and SD and were analyzed using repeated-measures ANOVA followed by Bonferroni post hoc testing. Different letters indicate significant differences ($P < .05$) between time points. The white and black bars indicate light and dark phases, respectively.

Novartis Pharmaceuticals Japan) or saline intramuscularly at ZT 12 and then fasted for 24 hours. Blood glucose concentrations were measured every 6 hours, and then the mice were killed to obtain blood and liver samples at the end of the fasting period (ZT 12). The blood obtained was mixed immediately with EDTA and aprotinin, and the plasma was stored at −80°C until assayed. Plasma concentrations of GH were determined using an ELISA kit (AKRGH-010; Shibayagi).

Real-time quantitative PCR

RNA extraction and real-time quantitative PCR were performed as described previously (19). Specific sets of primers and TaqMan probes are reported in Supplemental Table 1.

Statistical analyses

Data are presented as means and SD. Differences between 2 datasets were analyzed using the Mann-Whitney *U* test, the Wilcoxon signed rank test, or the Student *t* test. Daily rhythmicity was evaluated with repeated-measures or one-way ANOVA followed by Bonferroni post hoc testing. The calculations were performed using the SPSS software (version 16.0 J for Windows). A *P* value of $<.05$ was considered to indicate statistical significance.

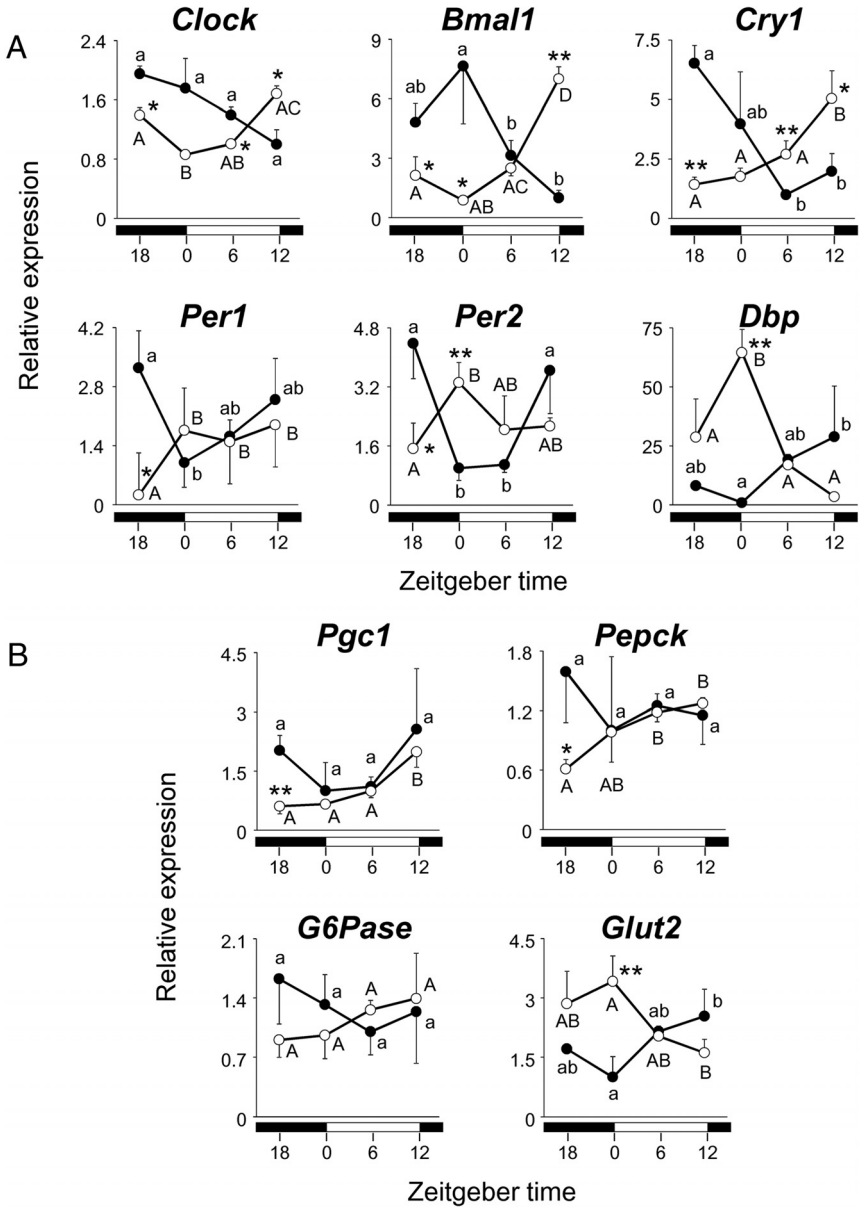

Figure 2. Effects of time-restricted feeding on daily mRNA expression profiles of clock (A) and glucose production–related genes (B) in the liver of C57BL/6J mice. The intervention group (○) was given food only during the light phase for 2 weeks, whereas the control group (●) was fed ad libitum. These mice were fasted from ZT 12 and then killed to obtain liver samples at ZT 18, 0, 6, and 12. The data obtained by real-time PCR were analyzed using the comparative threshold cycle method. Because the *Rplp0* mRNA levels in the mice liver were not rhythmic (19), this gene was used as an endogenous control. Data represent the means and SD of 3 mice per time point and are expressed relative to the lowest mean value in the control group. Different letters indicate significant differences ($P < .05$) between time points in each group (one-way ANOVA followed by Bonferroni post hoc testing). *, $P < .05$; **, $P < .01$ vs the control group at each time point (Student *t* test).

Results

In this study, we defined FBG as a blood glucose level 6 hours or longer after the start of fasting. When fasting was started at the beginning of the light phase, FBG levels increased slightly during the light phase and then decreased significantly during the dark phase in C57BL/6J mice (Figure 1A). In contrast, when fasting was started at the beginning of the dark phase, FBG levels decreased during the dark phase and continued to increase during the following light phase (Figure 1B). In addition, we conducted same experiments using obese diabetic *ob*/*ob* mice, because they have severe insulin resistance with marked hyperinsulinemia and therefore exhibit large fluctuations in blood glucose concentrations (20). As expected, *ob*/*ob* mice showed similar, but more pronounced, daily changes in FBG (Figure 1, D and E), than C57BL/6J mice. In both mouse strains, maximal levels of FBG were observed at the beginning of the dark phase (ie, at "dawn" for the nocturnal animals), regardless of the starting time of fasting.

To examine the involvement of the hepatic clock in the dawn phenomenon, we first investigated

whether time-restricted feeding influences not only the hepatic clock but also the FBG rhythm. As shown in Figure 2A, 2-week restriction of food intake to the light (resting) phase greatly shifted the oscillation phase of hepatic mRNA expression of all of the clock genes examined in C57BL/6J mice under a ZT 12–started fasting condition. In addition, this feeding condition appeared to affect the mRNA expression rhythms of the glucose production–related molecules, although the rhythmicity of the genes other than *Glut2* was not statistically significant probably due to the small number of animals (Figure 2B). FBG dropped to lower levels during the latter half of the light phase in time-restricted–fed mice (Figure 1C), in contrast to the result in control mice (Figure 1B). Moreover, in *ob*/*ob* mice, the acrophase was obviously shifted from the beginning of the dark phase (Figure 1E) to the beginning of the light phase (Figure 1F).

Next, we investigated the daily FBG rhythm in mice with liver-specific deletion of brain and muscle Arnt-like protein-1 (BMAL1), the core and essential component of the intracellular circadian clock (21). As expected, this deletion did not influence the daily feeding rhythm (Supplemental Figure 1) but significantly affected the rhythmic mRNA expression of the clock genes, especially *Bmal1* and *Dbp*, in the liver of mice even under a ZT 12–started fasting condition (Figure 3A). Moreover, consistent with previous results in fed mice (13), daily *Glut2* rhythm disappeared in L-*Bmal1*$^{-/-}$ mice (Figure 3B). Similar to C57BL/6J mice (Figure 1, A and B), control (flox/flox) mice exhibited significant fluctuations in FBG in both ZT 0–started and ZT 12–started fasting trials (Figure 4, A and B). Conversely, L-*Bmal1*$^{-/-}$ mice did not generate any 24-hour rhythmicity in FBG (Figure 4, A and B). These results clearly suggest that the hepatic clock is involved in the daily FBG rhythm.

Because nocturnal secretion of GH is thought to cause the dawn phenomenon in humans (6, 8), we further investigated the effect of octreotide-suppressed GH on elevated FBG levels in C57BL/6J mice. Plasma GH concentrations were suppressed substantially 24 hours after the octreotide administration at ZT 12 (Supplemental Figure 2). Conversely, hepatic mRNA expression of the clock and glucose production-related genes was not affected by this treatment (Supplemental Table 2). Blood glucose levels decreased during the dark phase (ZT 12–ZT 0)

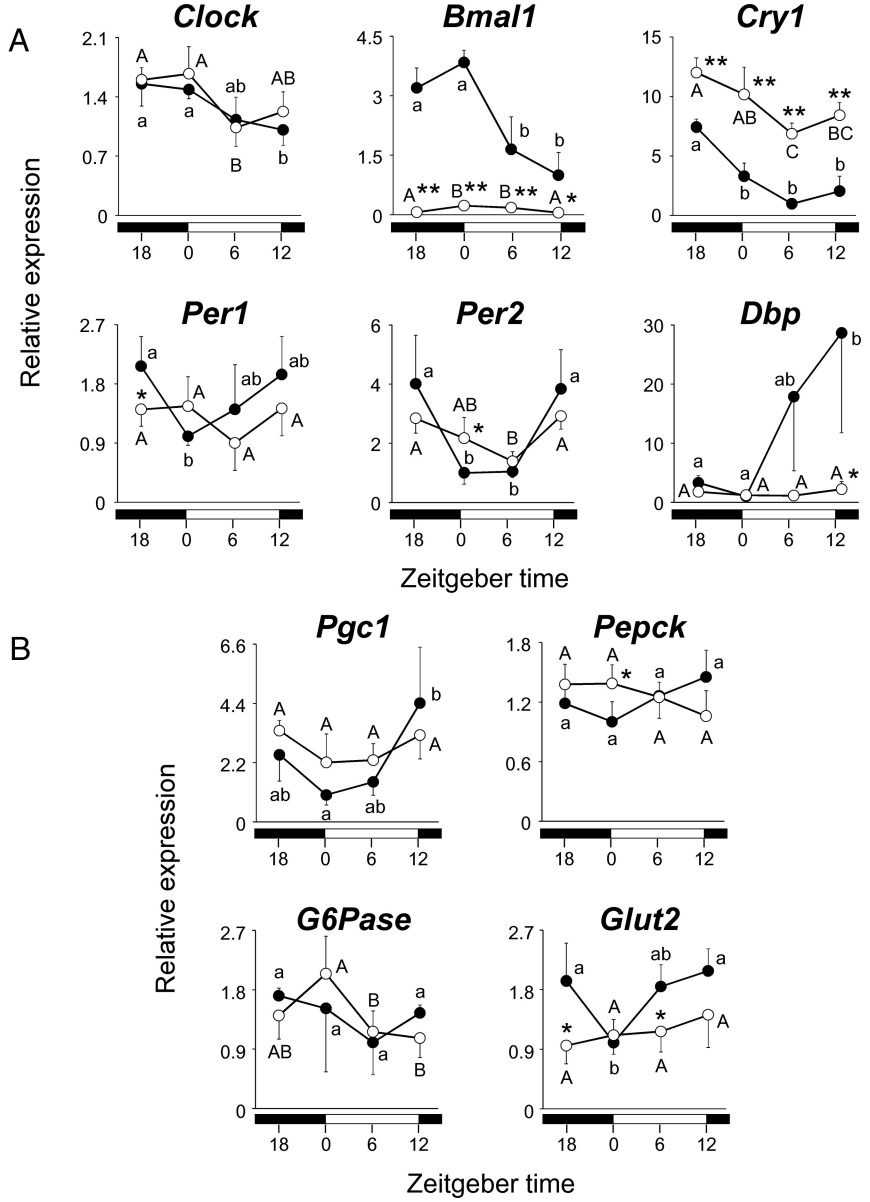

Figure 3. Effects of liver-specific disruption of *Bmal1* on daily mRNA expression profiles of clock (A) and glucose production–related genes (B) in mouse livers. L-*Bmal1*$^{-/-}$ (○) and control (flox/flox) mice (●) were fasted from ZT 12 and then killed to obtain liver samples at ZT 18, 0, 6, and 12. The data obtained by real-time PCR were analyzed using the comparative threshold cycle method with *Rplo0* as an endogenous control. Data represent the means and SD of 3 to 5 mice per time point and are expressed relative to the lowest mean value in the control group. Different letters indicate significant differences ($P < .05$) between time points in each group (one-way ANOVA followed by Bonferroni post hoc testing). *, $P < .05$; **, $P < .01$ vs the control group at each time point (Student *t* test).

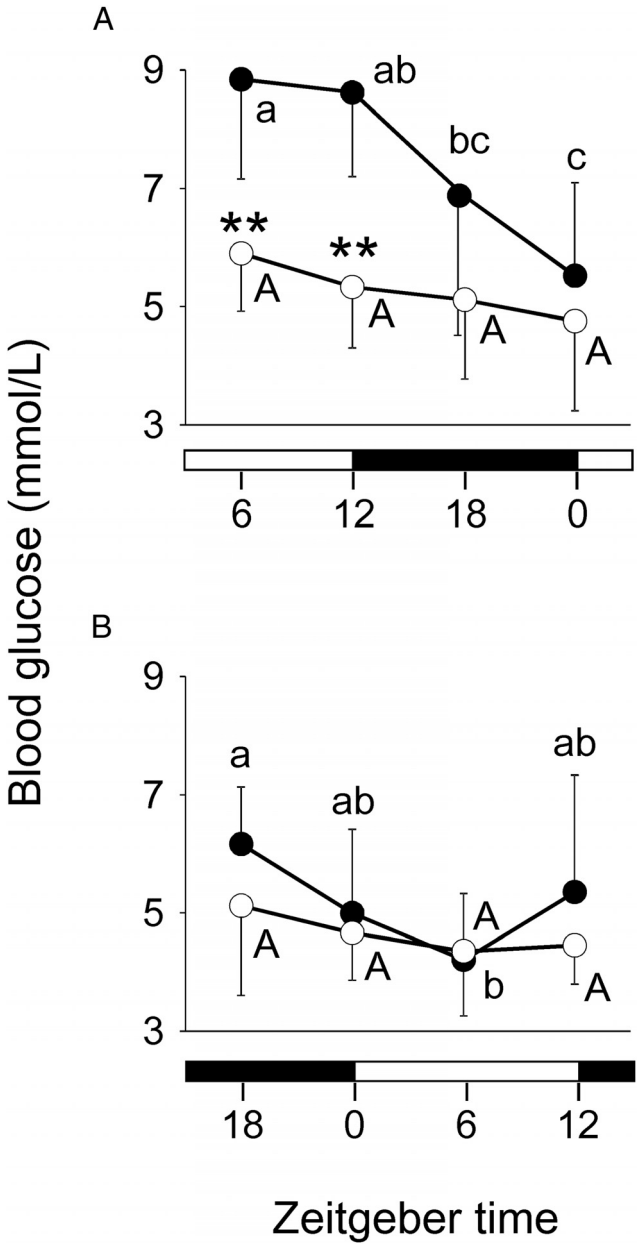

Figure 4. Daily FBG profiles in control and L-*Bmal1*$^{-/-}$ mice. Control (●; $n = 5$) and L-*Bmal1*$^{-/-}$ mice (○; $n = 9$) were fasted for 24 hours twice from ZT 0 (A) or ZT 12 (B) at an interval of at least 2 weeks. Data represent the means and SD and were analyzed using repeated-measures ANOVA followed by Bonferroni post hoc testing. Different letters indicate significant differences ($P < .05$) between time points in each group. **, $P < .01$ vs the control group at each time point.

after the start of fasting and the injection in both octreotide- and vehicle-treated mice (data not shown). In the control group, FBG levels significantly increased from ZT 6 to ZT 12 (Supplemental Figure 3A), similar to those in nontreated mice (Figure 1A). Notably, FBG concentrations were also elevated at the beginning of the active phase in all the mice treated with octreotide (Supplemental Figure 3B). Thus, the dawn phenomenon in the present model was detected even under the GH-suppressed condition.

Discussion

Consistent with previous findings in rats (15), basal blood glucose exhibited daily rhythmicity with a peak at the beginning of the active phase using the simple mouse model used in this study. In this model, mice were fasted for 24 hours, but no mice exhibited severe hypoglycemia (blood glucose level of <3.0 mmol/L) throughout the experiments. Specifically, the FBG trough levels in *ob*/*ob* mice were ~7 mmol/L. Therefore, posthypoglycemic hyperglycemia was probably not involved in increased blood glucose levels in this model. Conversely, the hepatic intracellular clock may play a role in the fluctuation of FBG because L-*Bmal1*$^{-/-}$ mice did not exhibit such rhythmicity. Previous (13, 18) and present studies (Supplemental Figure 1) showed that L-*Bmal1*$^{-/-}$ mice exhibit normal circadian rhythmicity of both locomotor activity and feeding behavior, suggesting that the function of the central clock is intact. Thus, the present study provides further evidence of the crucial role of the hepatic clock in regulating glucose metabolism.

In humans, the dawn phenomenon is caused by increased hepatic glucose production during the early morning hours (8). In this study, elevated FBG levels were observed at the beginning of the active phase in both normal and diabetic mice fed *ad libitum*. In addition, the rising phase of FBG was shifted by the time-restricted feeding procedure, consistent with the alteration of mRNA expression rhythmicity of the glucose production-related molecules. Furthermore, in L-*Bmal1*$^{-/-}$ mice, the temporary increase in FBG levels disappeared, in concert with the disrupted expression rhythmicity of GLUT2, the major glucose transporter of hepatocytes. Based on these results, elevated blood glucose levels observed in this mouse model were considered the dawn phenomenon. Interestingly, the influence of the time-restricted feeding on FBG rhythm was mild in normal mice, compared with that in diabetic mice (Figure 1). This may suggest that some factors such as insulin sensitivity in the muscle and adipose tissues modify the FBG rhythm driven by the hepatic clock.

In patients with type 1 diabetes, suppression of nocturnal spikes of GH secretion by somatostatin results in the disappearance of the dawn phenomenon (6, 8). In contrast, in the present study, treatment with a somatostatin analog did not inhibit the rise of glucose levels in male mice. Reportedly, GH levels are higher during the light phase than during the dark phase in female mice, but similar daily rhythmicity cannot be detected in male mice (22). Collectively, these findings suggest that the dawn phenomenon occurs even in the absence of GH surges during the resting phase, at least in male mice. Nevertheless, because somatostatin suppresses not only GH secretion but

also the secretion of various other hormones, including insulin and glucagon (23), close attention is required to interpret the results of studies using a somatostatin analog. Further studies are needed to clarify the mechanism underlying the discrepancy between the results obtained in human and animal studies.

In the present study, time-restricted feeding affected not only the daily hepatic expression rhythms of the clock genes but also the FBG levels in mice. Consequently, eating habits could possibly affect the dawn phenomenon in humans, especially in diabetic patients. Studies on mice have shown that the hepatic clock phase is affected by both the amount of food and the interval between feeding time points (24). In addition, food composition determines the degree of food entrainment of the hepatic clock (25). Therefore, skipping breakfast, night eating, and/or a severely unbalanced diet might affect the dawn phenomenon in humans. Hopefully, these possibilities will be examined in the near future, because continuous glucose monitoring, which detects the dawn phenomenon even in patients with type 2 diabetes (26), is commonly used.

In conclusion, FBG exhibited an obvious 24-hour rhythm with a peak near the beginning of the active phase in mice. This rhythm, including the temporal rise corresponding to the dawn phenomenon, was altered in concert with the phase shift of the hepatic clock in time-restricted feeding mice and was not detected in liver-specific clock-disrupted mice. Because these results suggest that the hepatic clock plays a critical role in generating rhythmicity of basal blood glucose, further clinical studies are needed to explore the relationship in diabetic patients.

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