Ketogenic Diet Disrupts the Circadian Clock and Increases Hypofibrinolytic Risk by Inducing Expression of Plasminogen Activator Inhibitor-1

Katsutaka Oishi, Daisuke Uchida, Naoki Ohkura, Ryosuke Doi, Norio Ishida, Koji Kadota, Shuichi Horie

Objectives—Metabolic disorders such as diabetes and obesity are considered risk factors for cardiovascular diseases by increasing levels of blood plasminogen activator inhibitor-1 (PAI-1). Ketogenic diets (KDs) have been used as an approach to weight loss in both obese and nonobese individuals. We examined circadian changes in plasma PAI-1 and its mRNA expression levels in tissues from mice fed with a KD (KD mice), to evaluate its effects on fibrinolytic functions.

Methods and Results—Two weeks on the kDa increased plasma levels of free fatty acids and ketones accompanied by hypoglycemia in mice. Plasma PAI-1 concentrations were extremely elevated in accordance with mRNA expression levels in the heart and liver, but not in the kidneys of KD mice. Circadian expression of PAI-1 mRNA was phase-advanced for 4.7, 7.9, and 7.8 hours in the heart, kidney, and adipose tissues, respectively, as well as that of circadian genes mPer2 and DBP in KD mice, suggesting that peripheral clocks were phase-advanced by ketosis despite feeding ad libitum under a periodic light-dark cycle. The circadian clock that regulates behavioral activity rhythms was also phase-advanced, and its free-running period was significantly shortened in KD mice.

Conclusions—Our findings suggest that ketogenic status increases hypofibrinolytic risk by inducing abnormal circadian expression of PAI-1. (Arterioscler Thromb Vasc Biol. 2009;29:1571-1577.)

Key Words: clock gene ■ ketosis ■ metabolic disorders ■ weight loss ■ obesity ■ hypofibrinolysis

Plasminogen activator inhibitor-1 (PAI-1), the primary physiological inhibitor of plasminogen activators (PAs), is an important contributor to hypofibrinolysis in the presence of metabolic disorders such as diabetes and obesity. Because the half-life of PAI-1 circulation in the bloodstream is of metabolic disorders such as diabetes and obesity. Because the half-life of PAI-1 circulation in the bloodstream is relatively short (approximately 6 minutes), plasma PAI-1 concentrations are regulated at the level of gene expression. The PAI-1 gene is expressed in several tissues including vessel walls (endothelial and smooth muscle cells), heart, liver, kidney, adipose tissue, and in macrophages. Its expression is regulated by several cytokines, hormones, and metabolic factors such as tumor necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1), insulin, glucocorticoids, angiotensin II, fatty acids, and glucose.

Serious adverse cardiovascular events including myocardial infarction, sudden cardiac death, and stroke have pronounced circadian rhythmicity that peaks during the morning. The circadian oscillation of PAI-1 activity is obvious as it peaks in the early morning, and this might account for the morning onset of myocardial infarctions. Maemura et al described the circadian expression of PAI-1 mRNA in the heart and kidneys of mice, and suggested that the circadian oscillation of PAI-1 gene expression plays an important role in the circadian fluctuation of blood fibrinolytic activity. Basic helix-loop-helix (bHLH)-PAS transcription factors such as CLOCK and BMAL1 are positive regulators of an autoregulatory transcription-translation feedback loop of the molecular circadian clock. Both CLOCK and BMAL1 transactivate other clock genes such as period1 (Per1), Per2, cryptochrome1 (Cry1), and Cry2 and clock-controlled genes such as albumin D-site binding protein (DBP) via E-box (CACGTG) elements in their promoters. Assays in vitro have shown that CLOCK:BMAL2 (CLIF) and CLOCK: BMAL1 heterodimers upregulate PAI-1 gene expression via E-box elements both in humans and in mice.

Ketogenic diets (KDs) comprise high-fat with low-carbohydrate and -protein contents, and they have been used as an approach to weight loss for both obese and nonobese
individuals. Such diets mimic the metabolic conditions of fasting or caloric restriction (CR) and are based on theoretical concepts of the effects of dietary component ratios on energy expenditure.\textsuperscript{14} Although KDs might provide short-term solutions to obesity and type 2 diabetes, the long-term benefits of KDs are questionable.\textsuperscript{15-17} Both kDa and CR produce changes in energy metabolism that involve a shift to lower circulating levels of glucose, which is the most prevalent sugar in the general circulation, and ketone bodies are generated from fatty acids in the blood, such as an abundance of palmitate. This long-chain fatty acid is catabolized in the mitochondria of hepatocytes by β-oxidation and consequently delivered via the bloodstream to extrahepatic tissues such as muscle and the brain. Both KDs and fasting alter blood concentrations of the pancreatic hormones insulin and glucagon and of glucocorticoids that are secreted by the adrenal cortex. Under kDa conditions, blood levels of ketogenesis-inhibiting insulin are low, whereas those of the ketogenesis-promoting glucagon and glucocorticoids are high. Few studies have documented the effect of kDa on fibrinolytic functions, although KDs affect various cardiovascular risk factors such as body weight (BW), fat mass, blood lipids, and hormones both in humans\textsuperscript{14,16-21} and in animal models.\textsuperscript{22-24} To evaluate the effect of kDa on fibrinolytic functions, we examined the temporal expression profile of PAI-1 in tissues from mice fed with a kDa (kDa mice). We discovered that kDa increases hypofibrinolytic risk by inducing abnormal circadian expression of the PAI-1 gene.

### Methods

#### Animals and Diet

Male Jcl:ICR mice (Clea Japan Inc, Tokyo, Japan) at 6 to 7 weeks of age housed under a 12-hour light–12-hour dark cycle (LD 12:12; lights on at 0:00 and lights off at 12:00) were fed with a normal diet (ND; CE-2; Clea Japan Inc) or with a ketogenic diet (kDa; 73.9% fat, 8.3% protein and 0.73% carbohydrates, w/w; modified AIN-93G; Oriental Yeast Co Ltd) ad libitum for 2 weeks. The proportions of calories derived from fat, carbohydrate, and protein were CE-2: 52.9%, 21.3%, and 25.8%; kDa: 91.4%, 4.3%, and 4.3%, respectively. Weekly water consumption was monitored at 5-minute intervals and the BW of kDa mice was significantly decreased from 37.8 ± 1.2 g (F = 1.68, P = 0.001), whereas that of control mice was slightly but not statistically significantly increased from 36.4 ± 1.2 g to 37.2 ± 1.0 g (F = 1.44, P = 0.08). On day 14 of the feeding study, control and kDa mice consumed 502.7 ± 14.7 and 419.0 ± 55.8 calories/kg BW, respectively (F = 17.29, P = 0.001). Plasma levels of total ketones were significantly lower in kDa than in control mice (Table 1). Figure 1 shows that plasma FFA levels peaked at the day to night transition in kDa mice, although these levels increased during the inactive phase (daytime) in control mice. Figure 2 shows the temporal mRNA expression profiles of PAI-1, mPer2, and DBP in tissues from control and kDa mice on day 14, and Table 2 shows the results of cosinor analyses using the NLLS Marquardt-Levenberg algorithm and 1-way ANOVA. Mean PAI-1 mRNA levels (MESOR in Table 2) were significantly increased by 3.3- and 6.4-fold in the heart and liver of kDa mice, respectively, despite identical levels in the kidney. Levels of PAI-1 mRNA were only slightly

| Table 1. Plasma Metabolic Parameters in Mice Fed With a Ketogenic Diet |
|-----------------------------|-----------------------------|
| **CTRL**                 | **KD**                    |
| Glucose, mg/dl             | 226.7 ± 5.4                | 69.4 ± 5.2†                  |
| Free fatty acids, mEq/dl   | 0.53 ± 0.03                | 1.05 ± 0.06†                 |
| Triglyceride, mg/dl        | 161.3 ± 8.2                | 135.3 ± 9.4*                 |
| Total cholesterol, mg/dl   | 77.3 ± 3.0                 | 221.7 ± 14.0†                |
| Total ketones, µmol/l      | 87.9 ± 5.7                 | 1492.2 ± 107.8†              |

KD indicates ketogenic diet. Data are shown as means ± SEM throughout day 14 (n = 24). Significant differences compared with CTRL value are indicated as *P < 0.05, †P < 0.01.

#### Measurement of Blood Metabolic Parameters

Mouse blood was immediately mixed with 0.2 volumes of 65 mmol/L sodium citrate (pH 7.2) and separated by centrifugation for 10 minutes at 5800g. Platelet-poor plasma samples were collected and stored at −80°C. Plasma glucose, free fatty acids (FFA), triglyceride (TG), and total cholesterol (T-Chol) levels were measured using kits (Wako Pure Chemical Industries Ltd). Plasma levels of total ketone bodies were measured using an enzymatic assay kit (Kainos Laboratories).

#### Measurement of Plasma PAI-1

Plasma PAI-1 levels were measured using a total murine PAI-1 ELISA (Molecular Innovations Inc) kit as described.\textsuperscript{25}

#### Statistical Analysis

All values are expressed as means ± SEM. The time-dependence of each parameter was statistically evaluated using 1-way ANOVA. Circadian rhythms were statistically analyzed using the modified cosinor method (nonlinear least-squares [NLLS] Marquardt-Levenberg algorithm).\textsuperscript{26} We defined the function as f(x) = A sin([T(x − φ)]/T(2π)), where T is the period, φ is the acrophase, and A is the amplitude. The cosinor method was used to determine the circadian period (T) and acrophase (φ) for each parameter. Significant differences were determined using Welch’s t-test.
acrophase of circadian mPer2 expression for 5.6, 4.3, 7.6, and 5.5 hours in the heart, liver, kidney and adipose tissues, respectively, of mice, despite ad libitum feeding under a periodic light–dark cycle. The acrophase of circadian DBP expression was phase-advanced for 6.0, 3.9, 7.4, and 7.0 hours in the heart, liver, kidney, and adipose tissues, respectively. The phase-advancing effect in the liver was relatively small compared with that in other tissues.

To determine whether the endogenous circadian clock that governs the behavioral activity rhythm was also phase-advanced, we examined the drinking behavior of kDa mice (Figure 4). The ketogenic diet did not affect activity onset under LD. However, the latter half of nocturnal activity was remarkably reduced, which obviously shortened the duration of daytime activity. The activity onset of kDa mice was immediately advanced by several hours when the mice were transferred from LD to constant darkness (DD), suggesting that the kDa phase-advanced the endogenous circadian clock governing rhythmic behavior. It should be noted that the free-running period of kDa mice (23.646±0.051 hour, n=20) was significantly shorter than that of control mice (23.892±0.018 hour, n=12) under DD (F=13.38, P<0.01).

We also examined the feeding behavior of kDa mice (supplemental Figure I, available online at http://atvb.ahajournals.org), because ketosis suppresses appetite. We found that the daily profile of feeding behavior was identical to that of drinking behavior in kDa mice.

**Discussion**

Ketogenic diets have become increasingly popular for weight loss not only among obese, but also normal individuals. However, the effectiveness and safety of KDs for obese and type 2 diabetic patients with cardiovascular risk factors have been questioned. Here, we demonstrated that a kDa increases hypofibrinolytic risk by inducing the abnormal circadian expression of PAI-1.

Levels of PAI-1 mRNA were remarkably increased in the liver (more than 6-fold) compared with those in the heart (3-fold) and epididymal fat (2-fold) of kDa mice. Hepatic metabolism plays an important part in adaptation and metabolic responses to kDa, such as synthesizing glucose and metabolizing fatty acids into ketones. Hepatocyte PAI-1 expression is induced by various hormones, metabolic factors, and cytokines both in vitro and in vivo. Ketogenic diets enhance the liver supply of FFA from adipose tissues, and fatty acid accumulation seems to contribute to kDa-induced PAI-1 expression in the liver, because fatty acids induce PAI-1 gene expression in human hepatoma HepG2 cells. We showed that hepatic PAI-1 mRNA significantly correlates with blood FFA levels in fasting mice. A range of fatty acids and their derivatives regulate target gene expression via the activation of peroxisome proliferator-activated receptor α (PPARα), a nuclear hormone receptor that is both a sensor and an effector for kDa. The functional peroxisome proliferator responsive element (PPRE)-like cis-element resides in the promoter of the mouse PAI-1 gene as it does in the human gene. We recently discovered that hyperlipidemia induced by a high-fat diet significantly increased the amount of PPARα binding to the PAI-1 promoter (unpub-
lished data). Furthermore, the PPARα ligand enhanced CLOCK:BMAL1-dependent transactivation of the PAI-1 gene in vitro (unpublished data). The present study showed that the mRNA expression of typical PPARα target genes pyruvate dehydrogenase kinase 4 (PDK4) and fibroblast growth factor 21 (FGF21) was also induced in kDa mice (supplemental Figure II). Therefore, PPARα might be involved in fatty acid-induced PAI-1 gene expression in kDa mice.

Levels of plasma PAI-1 and its hepatic mRNA were also obviously increased in kDa mice. Levels of plasma and hepatic PAI-1 closely correlate in leptin-deficient (ob/ob) obese mice, although PAI-1 expression in either intraabdominal or subcutaneous adipose tissues is not associated with levels of plasma PAI-1.\textsuperscript{24} We previously demonstrated that a Clock gene mutation normalizes plasma PAI-1 levels in accordance with hepatic PAI-1 expression in ob/ob mice, although adipose PAI-1 expression levels obviously increased in parallel with adipocyte hypertrophy in Clock-mutated ob/ob mice.\textsuperscript{25} Furthermore, hepatic PAI-1 levels significantly correlate with plasma PAI-1 and FFA levels in fasting obese and fasting normal mice, although plasma and adipose PAI-1 levels are not associated.\textsuperscript{29} Thus, the kDa-induced increase in levels of plasma PAI-1 appeared to be closely related to those of hepatic PAI-1 mRNA rather than to those in other tissues, because the mRNA expression levels were most obviously induced in the liver compared with all other tissues examined.

Recent studies have demonstrated feedback regulation of the circadian clock by metabolic disorders.\textsuperscript{35} Here, we demonstrated the phase-advancing effects of a kDa on the biological clock that governs both the rhythmic expression of circadian genes (including PAI-1) in peripheral tissues and the rhythmic behavioral activity of mice fed ad libitum. Feeding with a kDa did not affect on the activity onset under LD, whereas the active phase of behavior was immediately pushed forward for several hours when the mice were transferred from LD to DD, suggesting that the endogenous circadian clock governing rhythmic behavior was phase-advanced by the kDa. The masking effect of light might be responsible for daytime behavioral inhibition in kDa mice. We could not identify the neuronal and molecular mechanisms of these kDa-induced circadian clock regulations. The kDa-induced phase-advance of the behavioral activity was
Table 2. Cosinor Analysis of PAI-1, mPer2, and DBP mRNA Expression and Plasma PAI-1 Levels in Mice Fed With a Ketogenic Diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MESOR</th>
<th>Amplitude</th>
<th>Acrophase (h)</th>
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<td>CTRL</td>
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<td>7.85±1.11*</td>
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</tr>
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<tr>
<td>CTRL</td>
<td>39.86±5.31</td>
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<tr>
<td>CTRL</td>
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<td>4.48±0.83†</td>
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</tr>
<tr>
<td>KD</td>
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<td>CTRL</td>
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<td>CTRL</td>
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<tr>
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<td>1.73±0.39*</td>
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<td>0.058</td>
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</table>

KD indicates ketogenic diet; CTRL, control diet; MESOR, mean statistics of rhythm; Amplitude, one-half the total peak-trough variation; Acrophase, hours as a delay from 0:00 (lights on). Data are shown as means±SEM. Significant differences compared with CTRL value are indicated as *P<0.05, †P<0.01.

Figure 3. Plasma PAI-1 concentrations in mice fed with a ketogenic diet. Mice were fed with a ketogenic (closed circles) or a control (open circles) diet for 14 days under LD 12:12 (lights on at 0 hour), and then plasma total PAI-1 levels were measured. Values are means±SEM (n=4). Horizontal open and solid bars, day and night, respectively. (Significant differences in circadian parameters between groups are shown in Table 2).

Figure 4. Representative double-plotted actogram of drinking behavior in mice fed with a ketogenic diet. Mice were fed with a control (CTRL) or ketogenic (kDa) diet under a 12-hour light–12-hour dark cycle (LD 12:12; lights on at 0 hour) and then transferred to constant darkness (DD). Dark phase duration is shaded in gray. Horizontal open, shaded, and solid bars, day, subjective day, and night, respectively. Dots represent drinking behavior at 5-minute intervals. Red lines indicate the phase of activity onset. The phase of activity onset was advanced by kDa feeding.

obvious in homozygous Clock mutant mice (supplemental Figure III) that are models of delayed sleep phase syndrome (DSPS), suggesting that the core components of the circadian clock are indispensable for the kDa-induced phase shift. One possible mechanism for these phenomena is that kDa-induced CR phase-shifts the circadian clock. Caloric restriction limits the amount of calories consumed by the animals to 60% to 70% of their ad libitum intake. Several studies have indicated that CR or fasting affects the circadian clock, which regulates rodent behavioral rhythmicity. However, most of these studies provided a time cue for the biological clock because calorically restricted animals rapidly consume all of their food. Froy et al demonstrated changes in the circadian amplitude or phase of clock-controlled output systems such as feeding behavior, body temperature, and circadian gene expression.
expression in spontaneously calorie-restricted αMUPA mice. Mendoza et al. demonstrated that CR with ultradian feeding schedules induces phase advances in rat behavioral and physiological circadian rhythms. Prolonged fasting advances the phase of free-running rhythms such as wheel-running and body temperature. Glucose availability reduced by the administration of 2-deoxy-D-glucose (a competitive inhibitor of glucose metabolism), insulin, or fasting attenuates the light-induced phase delays of the circadian clock. These observations resemble the situation of our kDa mice. We demonstrated the notable phase-advancing effects of an ad libitum kDa on the biological clock that governs both the rhythmic expression of circadian genes in peripheral tissues and rhythmic behavioral activity in mice. Thus, the effect of feeding with a kDa on the circadian clock seems quite similar to that of CR, fasting, and hypoglycemia. Cellular energy status such as the ratio of reduced to oxidized nicotinamide adenine dinucleotides [NAD(P)H/NAD(P)+] might be involved in the kDa-induced phase-shift of the circadian clock.

To determine which brain structures are activated by kDa is important. The central nervous system (CNS) is the primary consumer of ketone bodies under hypoglycemic conditions and epilepsy has been treated by KDs since the 1920s, although the antiepileptic mechanisms remain essentially unknown. Several studies have revealed the functional importance of PPARα in the CNS despite low expression levels, indicating that PPARα is involved in the phase-advancing effect of kDa on the central and peripheral circadian clocks. We previously demonstrated that the PPARα ligand, bezafibrate, phase-advances behavioral rhythms for several hours in mice. The latter half of the nocturnal behavior was remarkably reduced under an LD in the kDa mice (Figure 3), which also resembled the effects of bezafibrate. Hepatic PPARα might indirectly regulate the circadian clock by transactivating hormone-like signal molecules such as FGF21. We found the circadian induction of FGF21 expression in the kDa mouse liver (supplemental Figure 2), which also resembles the effects of bezafibrate administration. Although the molecular mechanisms are not yet fully elucidated, FGF21 seems to play important roles in adaptation to fasting, such as adipose lipolysis, hepatic ketogenesis, and torpor. Notably, FGF21 enters the brain through the blood-brain barrier, suggesting a direct action of FGF21 on the CNS. Direct transcriptional regulation of BMAL1 by PPARα activation might also be involved in the ketogenesis-induced circadian clock regulation determined herein. Another candidate that mediates kDa-induced circadian clock regulation is AMP kinase (AMPK). This enzyme maintains energy homeostasis by sensing the AMP/ATP ratio and it is activated by CR, fasting, hypoglycemia, and kDa. The activation of AMPK induces PER2 protein degradation by activating casein kinase 1ε, resulting in a phase-advance of the circadian clock in vitro.

One revelation in the present study was that kDa significantly shortened the free-running period by about 0.2 hour, suggesting that kDa affects the central clock in the SCN. These observations might account for the phase-advancing effect of ad libitum kDa feeding on peripheral clocks in the photo-insensitive tissues described above. As also noted above, few studies have demonstrated the effects of caloric restriction on circadian clock systems without a daily time cue for eating because animals under such restriction typically consume daily food rations within a few hours. However, the present study demonstrated that feeding with a kDa ad libitum mimics the metabolic state of fasting and thus affects circadian clock systems in mice. Kohsaka et al. demonstrated that a high-fat diet lengthened the free-running period of locomotor activity by about 0.2 hour in mice. Circadian clock disruption by metabolic disorders has not been documented in detail, although many studies have demonstrated metabolic regulation by the mammalian circadian clock.

Keogh et al. recently demonstrated that 8 weeks of a very-low-carbohydrate, high-saturated-fat weight-loss diet reduced plasma PAI-1 levels in severely obese individuals with a body mass index of 33.6±4.1. However, the beneficial effect of this diet seems rather to be a result of weight loss, because plasma PAI-1 levels are also reduced by an isocaloric high-carbohydrate, low-saturated-fat diet in the obese subjects. On the other hand, the effects of kDa on blood fibrinolysis have not been examined in nonobese individuals as far as we know. Our present findings suggest that the nature of kDa with respect to the prevention of thrombosis is a double-edged sword. The morning increase in PAI-1 plays an established role in the mechanisms underlying transient morning resistance to thrombolysis using tissue-type PA, as the activity of PAI-1 is 2- to 4-fold higher in the morning than in the evening. Therefore, the kDa-induced circadian augmentation of PAI-1 expression in a phase advanced manner not only contributes to an increased risk of myocardial infarction but also alters the efficiency of thrombolytic therapy when applied in the morning.

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Disclosures
None.

References
Oishi et al. Ketogenic Diet Augments Circadian PAI-1 Expression


Ketogenic Diet Disrupts the Circadian Clock and Increases Hypofibrinolytic Risk by Inducing Expression of Plasminogen Activator Inhibitor-1

Katsutaka Oishi, Daisuke Uchida, Naoki Ohkura, Ryosuke Doi, Norio Ishida, Koji Kadota and Shuichi Horie

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Supplement Material

Supplemental Figure Legends

Supplemental Figure I
Representative double-plotted actogram of feeding behavior in mice fed with a ketogenic diet.
Mice were fed with a ketogenic diet (KD) under a 12 h light-12 h dark cycle (LD 12:12; lights on at 0 h) and then transferred to constant darkness (DD). Dark phase duration is shaded in gray. Horizontal open, shaded, and solid bars, day, subjective day, and night, respectively. Red lines indicate phase of activity onset, which was advanced by KD feeding.

Supplemental Figure II
Circadian augmentation of PDK4 and FGF21 mRNAs expression in liver of mice fed with a ketogenic diet.
Mice were fed with a ketogenic (closed circles) or a control (open circles) diet for 14 days under LD 12:12 (lights on at 0 h). Total RNA was extracted from the livers of sacrificed mice and then mRNA levels were determined by quantitative RT-PCR. Maximal value for control mice is expressed as 100%. Values are means ± SEM (n = 4). Horizontal open and solid bars, day and night, respectively.

Supplemental Figure III
Representative double-plotted actogram of drinking behavior in homozygous Clock mutant mice fed with a ketogenic diet.
Clock mutant mice were fed with a ketogenic diet (KD) under a 12 h light-12 h dark cycle (LD 12:12; lights on at 0 h) and then transferred to constant darkness (DD). Dark phase duration is shaded in gray. Horizontal open, shaded, and solid bars, day, subjective day, and night, respectively. Red lines indicate phase of activity onset, which was advanced by KD feeding. Free-running period under DD was extremely long in Clock mutant mice compared with that in wild-type mice (Fig. 4). However, the phase-advancing effect was identical between genotypes.
Supplemental Figure II

**PDK4**

![Graph showing mRNA level (% vs Time (h)) for PDK4]

**FGF21**

![Graph showing mRNA level (% vs Time (h)) for FGF21]
Supplemental Figure III

The figure shows a time-course study with 48 hours on the x-axis, labeled as "Hours", and days on the y-axis, labeled as "Days". The study compares conditions labeled "KD" and "DD", with a threshold value of 0.00. The background suggests a detailed analysis with data points across the timeline, indicating changes or patterns over the course of the experiment.
Suppl. Fig. 1. Oishi K et al.