Impairment of heme biosynthesis induces short circadian period in body temperature rhythms in mice

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Impairment of heme biosynthesis induces short circadian period in body temperature rhythms in mice. Am J Physiol Regul Integr Comp Physiol 303: R8–R18, 2012. First published May 2, 2012; doi:10.1152/ajpregu.00019.2011.—It has been demonstrated that the function of mammalian clock gene transcripts is controlled by the binding of heme in vitro. To examine the effects of heme on biological rhythms in vivo, we measured locomotor activity (LA) and core body temperature (Tb) in a mouse model of porphyria with impaired heme biosynthesis by feeding mice a griseofulvin (GF)-containing diet. Mice fed with a 2.0% GF-containing diet (GF2.0) transiently exhibited phase advance or phase advance-like phenomenon by 1–3 h in terms of the biological rhythms of Tb or LA, respectively (both, P < 0.05) while mice were kept under conditions of constant light/dark (LD) conditions of day and night (17, 37). Circadian rhythms in GF2.0 mice lasted between 1 and 3 wk after the onset of GF ingestion; this finding correlated well with the extent of impairment of heme biosynthesis. When we examined the effects of therapeutic agents for acute porphyria, heme, and hypertonic glucose on the pathological status of GF2.0 mice, it was found that the intraperitoneal administration of heme (10 mg·kg−1·day−1) or glucose (9 g·kg−1·day−1) for 7 days partially reversed (50%) increases in urinary δ-aminolevulinic acids levels associated with acute porphyria. Treatment with heme, but not with glucose, suppressed the phase advance (-like phenomenon) in the diurnal rhythms (P < 0.05) and restored the decrease of heme (P < 0.01) in GF2.0 mice. These results suggest that impairments of heme biosynthesis, in particular a decrease in heme, may affect phase and period of circadian rhythms in animals.

Porphyria; phase advance; core body temperature; resting temperature; δ-aminolevulinic acid

Most organisms, including human beings, have internal biological clocks, i.e., circadian clocks, to adapt to environmental light/dark (LD) conditions of day and night (17, 37). Circadian clocks are composed of several transcription factors as products of what are referred to as clock genes (3, 37). Interactions between these transcription factors form negative feedback loops to produce autonomous circadian oscillations. The clock genes modulate the expression levels of other genes downstream that are involved in biological activities such as cell division, metabolism, and behavioral activities that occur within a circadian period (15, 37).}

Recently, much attention has been paid to heme as a factor that modulates the function of mammalian clock genes. Several studies have demonstrated that heme can directly and indirectly modulate the functions of clock gene products, including positive limbs [circadian locomotor output cycles kaput (CLOCK) and brain and muscle Arnt-kike protein-1 (BMAL1)], which together form the core loop of circadian clocks. For example, CLOCK (26) and its homolog neuronal PAS domain protein-2 (NPAS2) (10) are heme-mediated carbon monoxide (CO)-sensing proteins, and a complex formation of NPAS2/BMAL1, which binds to a specific sequence of DNA, is regulated by CO through the heme-based sensor. Rev-Erbo is another heme-binding clock protein, and inhibition of Bmal1 expression by Rev-Erbo requires the binding of heme (5, 32, 44). Yang et al. (43) reported that the binding of heme to PER2 prevents stable dimer formation of PER2/CRY, resulting in the degradation of PER2. Kaasik and Lee (20) demonstrated interrelationships between heme biosynthesis and biological clocks in vivo based on findings of 1) the repression of per2 expression by heme, 2) decreased Npas2 expression in per2-mutant mice, and 3) decreased gene expression of a rate-limiting enzyme in the heme biosynthetic pathway in Npas2-mutant mice. These lines of evidence clearly indicate the involvement of heme in the regulation of biological clocks in vitro; however, it remains unclear whether heme at physiological levels can regulate circadian clock functions in vivo, since previous reports have used excess amounts of heme to analyze its effects on indices of biological rhythms using live animals. If heme is indeed essential to biological clock function under physiological conditions, it will be necessary to examine how circadian rhythms in live animals are modulated when heme levels are diminished or depleted. Thus we focused on porphyria as a context for examining these effects. Porphyria is a disease in which decreased heme levels are caused by functional disturbances in heme biosynthesis due to either hereditary factors or acquired chemical exposure. Porphyria patients exhibit an accumulation of heme precursors such as ALA and protoporphyrin IX (PPIX) in the liver and in red blood cells,
MATERIALS AND METHODS

Animals and Diets

Male ICR mice (5 wk old) were maintained on a 12 h:12 h LD cycle (ZT: zeitgeber time, ZT12 indicates the time when light is turned off, i.e., 19:00 o’clock in standard method) at ambient temperatures (23 ± 2°C) with food (standard chow pellets, MF; Oriental Yeast, Tokyo, Japan) and water available ad libitum. Mice were acclimated to these conditions for at least 7 days until entrainment to the environmental LD cycle before the initiation of GF ingestion in the LD cycle or the initiation of a change in the environment to conditions of constant darkness (DD). In the experiments under DD conditions, mice were maintained for at least 2 wk before the start of GF ingestion (CT: circadian time, CT12 indicates the daily time of onset of locomotor activity). According to the GF content in the powder chow, the mice were divided into three groups: HC, GF0.5, and GF2.0, the diets of which contained 0%, 0.5%, and 2.0% (wt/wt) GF, respectively. The respective diets were maintained for the entire duration of the experiment. We defined day 1 as the day from the first ZT12 to the next ZT12 after the replacement of the standard chow pellets with the GF-containing powder chow, and day 1 was defined as the day before day 1. All procedures were approved by the Josai International University Animal Care and Use Committee, and all procedures complied with the Guide for the Care and Use of Laboratory Animals.

Experimental Groups

Experimental groups were composed of several combinations of diets and treatments or no treatment. Number of groups in each experiment and number of mice in each group are summarized in Table 1. Data in Table 2, except for rectal temperature, were obtained from mice for other experiments; body weights were monitored in mice for supply of urine and feces samples; liver wet weights were obtained from mice for microsomal preparation (1 wk), mice for rectal temperatures, serum enzymes, and hemoprotein in liver microsomes.

Table 1. Number of groups in each experiment and number of mice in each group in this study

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>HC</th>
<th>GF0.5</th>
<th>GF2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of groups</td>
<td></td>
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</tr>
<tr>
<td>No treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA rhythms under LD cycle</td>
<td>3</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>LA rhythms under DD condition</td>
<td>2</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>Ts rhythms under LD cycle</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Ts rhythms under DD condition</td>
<td>2</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>LA rhythms under LD cycle without any treatment</td>
<td>10</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>LA rhythms under LD cycle with treatment</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Hemoprotein and heme precursors

| Daily change in urinary ALA                      | 2      | 4     |
| Urinary ALA at day 3 or 4                       | 16     | 4     |
| Hemoprotein in liver microsomes                 | 6      | 4     |
| Body weight (1–2 wk)                            | 8      | 4     |
| Rectal temperature (1–2 wk)                     | 8      | 4†    |
| Liver wet weight (1 wk)                         | 6      | 4     |
| Liver wet weight (2 wk)                         | 4      | 4     |
| Liver wet weight (4 wk)                         | 8      | 4     |
| Serum enzymes (4 wk)                            | 4      | 4     |
| Epididymal adipose tissue weight (2 wk)         | 4      | 4     |
| Epididymal adipose tissue weight (4 wk)         | 4      | 4     |

LA, locomotor activity; Ts, core body temperature; LD, light/dark; DD, constant dark; HC, control; GF0.5 and GF2.0, griseofulvin at 0.5% and 2.0%, respectively; Glc, glucose; *n = 1–4 urine samples in each day; †n = 3 at 2 wk; a mouse have never rested around ZT9.

and subsequent excretion of excesses of these precursors in the urine and feces serves as a biochemical marker for the identification of porphyria patients (2, 11). Patients with acute porphyria exhibit psychiatric symptoms such as insomnia, anxiety, depression, and confusion (2, 4, 23, 27), and a markedly low melatonin concentration in the blood of some acute porphyria patients has been reported (6, 31). These findings are closely related to symptoms observed in patients with abnormal circadian rhythms. Therefore, it is likely that porphyria patients may exhibit abnormal circadian rhythms based on functional changes in circadian clocks due to a decrease or lack of heme.

The purposes of this study were to clarify whether it would be possible to observe abnormal circadian rhythms in a mouse model of porphyria and to demonstrate that circadian rhythms can be regulated by heme in intact animals kept under physiological and pathophysiological conditions. In this study, experimental mice were orally administered griseofulvin (GF) to produce a mouse model of GF-induced porphyria (8, 9, 19, 34). Here, we report abnormalities of circadian or diurnal rhythms and physiology in the porphric mice and effects of heme-treatment on their abnormalities.

HEME AND CIRCADIAN RHYTHMS IN MICE

Table 1. Number of groups in each experiment and number of mice in each group in this study

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<tr>
<td>No treatment</td>
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</tr>
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body temperature measurement (2 wk), and mice for activity monitoring with running wheels (4 wk); sera were obtained from mice for activity monitoring with running wheels; epididymal adipose tissues were obtained from mice for rectal body temperature measurement (2 wk) and from mice for activity monitoring with running wheels (4 wk).

Drug Administration

Mice received daily dexamethasone at ZT9 via administration into the abdominal cavity (0, 3, 10, or 30 mg · kg⁻¹ · day⁻¹) or the caudal vein (0 or 10 mg · kg⁻¹ · day⁻¹) for the initial 7 or 8 days starting from the onset of GF ingestion. The first administration of heparin was performed several hours before the mice started ingesting GF, i.e., at ZT9 on day −1. The heme solution was prepared by dissolving hemin (Sigma Chemical, St. Louis, MO) in water according to the method of Freedman et al. (12). In brief, hemin (0, 3, 10, or 30 mg) was dissolved in 200 μl of 1 M KOH and then 1,000 μl of 0.2 M Tris-HCl (pH 8.0) and 2,600 μl of water were added. The pH was adjusted to 7.8 with 1 M HCl. Finally, the solution was diluted to 10 ml with water, and the concentrations of hemin administered to the four groups of mice were 0, 3, 10, or 30 mg · kg⁻¹ · day⁻¹.

Hypertonic glucose (0.5 M) was intraperitoneally administered at 9 g · kg⁻¹ · day⁻¹ into another experimental group of mice for the initial 7 or 8 days starting from the onset of GF ingestion. Because bolus injection was used for the administration of glucose, the total amount of glucose administered was divided into two doses per day, i.e., at ZT3 and ZT9. Thus the first administration of glucose was performed at ZT3 on day −1. The hypertonic sodium chloride solution (NaCl; 0.25 M) exerted the same osmotic pressure as the hypertonic glucose and was used as the control. Both the glucose and the NaCl were dissolved in water to reach the desired concentration.

Locomotor Activity

To assess the daily rhythms of locomotor activity (LA), mice were individually housed in a stainless steel cage (11.5 cm × 11.5 cm × 8 cm) equipped with a running wheel (21 cm id) (Muromachi-Kikai, Tokyo, Japan). The daily profiles of LA were analyzed using the software package CompACT ver. 3 (Muromachi-Kikai), and activity was determined according to the times the mice turned the wheel in 10-min bins. The daily onset of LA was determined as follows, using a modified version of a method discussed in a previous report (7). The onset of activity was defined as the first 10-min bin, in which 10% of the maximal intensity for that cycle was followed by a level of activity that was no less than 10% of the maximal intensity for that cycle in three of the next six bins. The time corresponding to acrophase was determined by fitting the results to the single cosinor model (28). Average acrophase was determined from LA that were averaged in each 10-min per week for each mouse. To assess the changes in LA rhythm without influence of drug administration in the light phase, we introduced a novel parameter TDA20, which was defined as the time to reach 20% of the daily accumulated activity time (activity time, used here, means the 10-min bins in which the intensity was not zero). The daily activity time accumulated each day from ZT12 to ZT28, since LA persisted for several hours, even in the normal mice, after the lights were turned on. The daily circadian period was calculated as the highest χ²-peak as determined by χ²-periodogram (39).

Core Body Temperature

To implant temperature-sensitive radio transmitter probes (Physio-Tel; Data Sciences International, Arden Hills, MN) into the mice, the animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (25 μg/g body wt; Dainippon Pharmaceutical, Osaka, Japan). Each probe was implanted into the abdominal cavity of each mouse, and the muscle wall and the skin of each animal were closed by sutures. Mice were individually housed in exclusive transparent plastic cages (15 cm × 22 cm × 12 cm) with food and water provided ad libitum until recovery from surgery, i.e., for at least 7 days. The core body temperature (Tc) levels of the mice were continuously recorded in 5-min bins using the telemetry system from Data Sciences International (Arden Hills, MN). The mesor (average Tc level) and the time corresponding to acrophase were determined by fitting the results to the single cosinor model (28). Average acrophase was defined as

Table 2. Effects of heme and hypertonic glucose on the development of other physical abnormalities in GF2.0 mice

<table>
<thead>
<tr>
<th>Treat</th>
<th>Diet</th>
<th>Treat</th>
<th>Diet</th>
<th>Diet × Treat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>2.6 ± 0.3</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Heme</td>
<td>2.6 ± 0.3</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.6 ± 0.3</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.6 ± 0.3</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
</tbody>
</table>

Vehicle Heme NaCl Glucose Vehicle Heme NaCl Glucose Diet Treat Diet × Treat

<table>
<thead>
<tr>
<th>Diet</th>
<th>2.6 ± 0.3</th>
<th>2.5 ± 0.1</th>
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</tr>
</thead>
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<tr>
<td>Treat</td>
<td>2.6 ± 0.3</td>
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</table>

Data represent means ± SD. Mice were housed in normal cages and treated with vehicle (iv), heparin (10 mg · kg⁻¹ · day⁻¹), NaCl (ip), or glucose (ip), unless otherwise stated. Statistical differences were analyzed by two-way ANOVA (‡P < 0.05, ¶P < 0.01) with Scheffe’s test (§P < 0.05, ¶¶P < 0.01). Vehicle-treated HC mice vs. others in the same line; §P < 0.01, vehicle-treated GF2.0 mice vs. heme- or glucose-treated GF2.0 mice. *Data from mice that did not receive any treatment (vehicle, heme, NaCl, nor glucose). †Not determined. Wheel-running cages; heme (0 or 10 mg · kg⁻¹ · day⁻¹) ip. Wheel-running cages; heme (0 or 30 mg · kg⁻¹ · day⁻¹) ip.
the average of acrophases from the last 3 days per week for each mouse. The daily circadian period was calculated as the highest x^2-peak as determined by x^2-periodogram (39) from the cosinor fitting curves of Tb under DD conditions. In this case, the Tb values were digitalized to calculate the circadian period: Tb values in excess of the daily mesor were replaced by a value of 1, and other Tb values were represented by a value of 0. To reveal how the circadian period had changed after GF ingestion, the circadian period was calculated by using the Tb data collected for 14 days, with serial sectioning at 7 days, i.e., the circadian period was calculated for three periods; days 1–14, days 8–21, and days 15–28.

To assess decreases in Tb levels as symptomatic of GF-induced porphyria, rectal temperature was measured using a thermistor thermometer (KN-91, Natsume Seisakusyo, Tokyo, Japan) at ZT9 after visual confirmation that the mice had been resting (these results are described in Table 2).

Resting Tb

The LA-independent Tb levels, i.e., “resting Tb,” was determined according to the method of Scheer et al. (36). The resting Tb was defined as the mean Tb determined over 20-min resting epochs, and the resting epochs started in at least six 5-min intervals (30 min) after the initiation of rest (where rest was defined as an LA level of less than 25 infrared movement detected per 5-min interval, as described in a previous report) (35). To calculate resting Tb, LA was monitored continuously by infrared detectors (SUPERMEX, Muromachi-Kikai) located above the individual cages. The locomotion counts were collected in 5-min bins using the software package CompACT AMS ver. 3 (Muromachi-Kikai).

Preparation of Biological Samples

On the indicated day, urine and fecal samples were obtained from four or six mice in each group isolated in metabolic cages during a single DL cycle, and the samples were collected during the final 18 h of examination. Urine samples were purified by centrifugation of 2,000 g at 4°C and were stored at −80°C until use. Fecal samples were stored at −20°C until used for the analysis. Liver tissues and sera were obtained on the indicated days from four mice in each group that had been anesthetized by diethyl ether inhalation. Blood samples were removed from the postcaval vein, and serum separation was carried out by centrifugation to determine enzymatic activity, as described below. Mice were euthanized by exsanguination, and the wet weights of the liver and the bilateral epididymal adipose tissues were measured after their removal.

Urinary δ-Aminolevulinic Acid

δ-Aminolevulinic acid (ALA) levels in the urine were determined by the method of Tomokuni et al. (41). In brief, urine samples (20–100 μl) were diluted to 400 μl with water, and 400 μl of 2 M sodium acetate (pH 4.6) and 80 μl of ethyl 3-oxobutanate were added. After the samples were boiled for 15 min, 1.2 ml of ethyl acetate was added, and 0.8 ml of the organic layer was separated by centrifugation with 2,000 g at room temperature. Samples were reacted with 0.8 ml of modified Ehrlich’s reagent, including 0.5% (wt/vol) N-methylaminobenzaldehyde and 1% (vol/vol) perchloric acid (60%), for 10 min at room temperature, and the absorbance of the ALA reagent was determined by a spectrophotometer at 553 nm. The baseline was determined by the absorbance of each sample in the absence of ethyl 3-oxobutanate. The least detectable dose and the percent coefficient of variation (CV%) in this method were 0.1 mg/l and 4.9%, respectively. The ALA chloride standard was obtained from Wako Pure Chemical (Osaka, Japan).

Urinary concentrations of creatinine were measured by the Jaffé method; a commercially available test kit (Wako Pure Chemical) was used according to the manufacturer’s instructions to normalize the ALA concentration when necessary. The least detectable dose and the CV% in this method were 1.0 mg/dl and 8.3%, respectively.

Fecal Porphyrins

Porphyrin levels in the feces were determined according to the method of Hift et al. (16) with minor modifications (24). In brief, the fecal samples (ca. 50 mg) were homogenized in 3 ml of 5% (wt/vol) ch2so4 in MeOH, and the samples were incubated overnight at ambient temperature (23 ± 2°C) for the methylation of the porphyrins. Diethyl ether was then added to the samples, and the organic layer was dried with Na2so4. After the filtration and evaporation of the organic layer by an N2 flush, the residues were dissolved in MeOH and were separated by HPLC with a 15 cm × 2.0 mm id Gemini-ODS column (GL Sciences, Tokyo, Japan) at 40°C. The eluent was 10% (vol/vol) 1 M ammonium acetate (pH 5.16) in MeOH. The column was equilibrated with the eluent prior to sample injection. The retention time for the dimethylester of PPIX was 13.8 min. The flow rate was 0.2 ml/min. The porphyrin was detected by a fluorescence detector (ex., 400 nm; em., 618 nm). Porphyrin contents were determined as free acids and corrected by feces weight. The least detectable dose and the CV% in this method before the correction by feces weight were 25 ng/ml and 7.6%, respectively. The porphyrin derivative standard was obtained from Wako Pure Chemical.

Hemoproteins in Liver Microsomes

According to the standard method described in the literature (29), liver microsomes were prepared and hemoprotein contents in the microsomes were determined by measuring the spectral difference following CO binding, as the sum of cytochrome P450 and P420 or cytochrome P450 and “P429”, which has maximum absorption at 429 nm. These hemoprotein concentration were calculated by two sets of simultaneous equations; for cytochrome P450 with P420, ∆Absorbance250–490 = 91 (cm-1 mM-1) × [P450] (mM) – 11 (cm-1 mM-1) × [P420] (mM) and ∆Absorbance220–490 = 41 (cm-1 mM-1) × [P450] (mM) + 110 (cm-1 mM-1) × [P420] (mM) (18); for cytochrome P450 with P429, ∆Absorbance250–490 = 91 (cm-1 mM-1) × [P450] (mM) + 24 (cm-1 mM-1) × [P420] (mM) and ∆Absorbance220–490 = 12 (cm-1 mM-1) × [P450] (mM) + 107 (cm-1 mM-1) × [P420] (mM) (29). We chose one of the simultaneous equations by ratio of absorbance height in 420 nm and 429 nm; i.e., if absorbance in 420 nm was higher than that in 429 nm, we chose the former simultaneous equation. Proteins in the microsomes were determined using a BCA assay kit (Sigma-Aldrich, St. Louis, MO) and bovine serum albumin as the standard (the least detectable dose was 5 μg/ml and the CV% was 7.0%).

Enzyme Activity Assay

Serum enzyme activity was evaluated to determine the extent of liver damage. Levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were analyzed according to standard methods described in the literature (21, 22). The least detectable dose and the CV% of ALT in this method were 1 IU/l and 1.0%, respectively, and those of ALP were 1 IU/l and 0.6%, respectively. If limited by range in which obtained the CV% was 2.0 mm id Gemini-ODS column (GL Sciences, Tokyo, Japan) at 40°C. The eluent was 10% (vol/vol) 1 M ammonium acetate (pH 5.16) in MeOH. The column was equilibrated with the eluent prior to sample injection. The retention time for the dimethylester of PPIX was 13.8 min. The flow rate was 0.2 ml/min. The porphyrin was detected by a fluorescence detector (ex., 400 nm; em., 618 nm). Porphyrin contents were determined as free acids and corrected by feces weight. The least detectable dose and the CV% in this method before the correction by feces weight were 25 ng/ml and 7.6%, respectively. The porphyrin derivative standard was obtained from Wako Pure Chemical.

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RESULTS

Circadian Rhythms in Porphyric Mice With Ingestion of Griseofulvin (GF)

Diurnal rhythms phase-advanced under LD conditions. We performed continuous measurement of LA by wheel running and Tb using wireless transceiver system in HC (control diet), GF0.5 (0.5% GF-containing diet), and GF2.0 (2.0% GF-containing diet) mice. The ingestion of GF started at day 1. Top, white and black bars indicate the LD cycle. B: single-plotted actograms and acrophases obtained by wheel-running activity averaged per each week in HC (n = 10) and GF2.0 (n = 8) mice. Shaded area charts, mean LA in HC; closed area charts, mean LA in GF2.0; shaded circles, means ± SD in HC, closed circles, means ± SD in GF2.0 mice. C: rate of activity in the latter half of light phase (ZT6-ZT12) in the day. D: rate of activity in the latter half of dark phase (ZT18-ZT24) in the day. E: weekly changes in daily acrophase of LA rhythms. F: weekly transitions in the averaged activity accumulation curves. Dashed lines indicate the curves in mice before GF ingestion. Arrows indicate shifts in a novel index, TDA20, at each week during a 6-wk period, by comparison to the value before GF ingestion. G: weekly changes in TDA20. Data in C, D, E, and G represent means ± SD (n = 8–13). Open bars or circles, HC; gray bars or triangles, GF0.5; closed bars or squares, GF2.0 mice. Statistical differences were analyzed by Dunnett’s test vs. HC: *P < 0.05, **P < 0.01.

Fig. 1. Circadian rhythms of locomotor activity (LA) in mice kept under 12 h:12 h light/dark (LD) conditions before and during griseofulvin (GF) ingestion. A: double-plotted actograms of LA obtained from a wheel-running trial in HC (control diet), GF0.5 (0.5% GF-containing diet), and GF2.0 (2.0% GF-containing diet) mice. The ingestion of GF started at day 1. Top, white and black bars indicate the LD cycle.

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TDA20 (ΔTDA20) between before and after GF ingestion was used to compare the effects of GF ingestion on the diurnal rhythms of the mice. The ΔTDA20 of GF2.0 mice advanced significantly compared with that of the control mice (Fig. 1G), whereas that of the GF0.5 mice exhibited a slight delay lacking statistical significance. Although diurnal phases of LA in HC and GF2.0 mice cannot be directly compared with each other because of difference of their waveforms, these alternations provided a hypothesis to us that phase of diurnal LA rhythms might be advanced in GF-induced porphyric mice.

Next we examined the diurnal rhythms in Tb. As shown in Fig. 2A, Tb rhythms were maintained with robust amplitude, even when the LA declined in the mice administered the GF diet. However, the Tb of GF2.0 mice increased before the onset of night, whereas the Tb of HC mice concurrently increased with advancing nighttime. Advances in the timing of Tb increases in GF2.0 mice were observed 3 days after GF ingestion; these changes peaked around 3 wk after the onset of GF ingestion, and then normal values were restored by 5 wk. Cosinor analysis of the diurnal Tb rhythms revealed that the acrophases of Tb rhythms in GF2.0 mice advanced by 1.5–3.6 h compared with the corresponding acrophases before GF ingestion; whereas no significant differences were observed in terms of values obtained from GF0.5 and HC mice (Fig. 2B). These results suggest that phase of diurnal Tb rhythms are advanced in GF2.0 mice. The acrophases of resting Tb in GF0.5 mice, i.e., a temperature independent of behavioral activity, significantly advanced, as it did in GF2.0 mice (Fig. 2C). These results also clearly suggest the advancing phase of diurnal Tb rhythms in the porphyric mice.

Period of circadian rhythms shortened under DD conditions. Although we observed an advance-like shift of phase in diurnal LA rhythms by having mice complete a wheel running trial under LD conditions, there remains the possibility that light masking effects on activity may have been involved in producing the obtained results. Therefore, we continuously measured wheel-running activity during a 4- or 5-wk period with the HC (n = 14) and GF2.0 (n = 15) mice kept under DD conditions. As shown in Fig. 3A, the actograms indicated some changes of onset in daily activity rhythms in the GF2.0 mice after the ingestion of GF2.0, whereas the HC mice showed almost constant onset in their activity rhythms. However, there was no statically significant difference in period length by χ²-periodogram between before and after starting of the ingestion of GF2.0 (means ± SD of circadian period in GF2.0 mice; 23.80 ± 0.22, 23.63 ± 0.37, 23.77 ± 0.17, and 23.78 ± 0.20 h at Pre, 1–2, 2–3, and 3–4 wk sections, respectively; an arrhythmic mouse (P > 0.05 in χ²-periodogram) which observed at a 3- to 4-wk section was excluded from the average).

Next we monitored Tb rhythms in HC (n = 8) and GF2.0 (n = 4) mice kept under DD conditions. Cosinor analysis of the Tb rhythms clarified that the GF2.0 mice exhibited circadian periods of shorter length. Acrophases were plotted onto Fig. 3B. According to the results, the shortest period was maintained for 1–2 wk and then the period closed to the original length and maintained stable until the end of the monitoring phase. Analysis of Tb rhythms by χ²-periodogram revealed that the circadian period of GF2.0 mice during the first 2 wk of observation was ~0.3 h shorter than that of HC mice (means ± SD of circadian period; 23.78 ± 0.22 and 23.51 ± 0.05 h in HC and GF2.0 mice, respectively), and this difference was statistically significant (P < 0.01 at 1–2 wk section; Fig. 3C). When resting Tb was used instead of the uncorrected Tb, the circadian period of GF2.0 mice was ~0.4 h shorter than that of HC mice, and this shortening effect was thus more evident than was the case when uncorrected Tb was considered (P < 0.01 at 1–2 wk section; Fig. 3C).

Preventive Treatment in Mice With GF-Induced Porphyria

Treatment with heme or glucose reduced ALA accumulation and decreased hemoprotein levels. In general, medical treatment with heme or hypertonic glucose is utilized for acute attacks in porphyria patients. We analyzed the effects of treatment with heme or glucose for HC (n = 4) and GF2.0 (n = 4–6) mice. Intraperitoneal administration of heme (30 mg·kg⁻¹·day⁻¹) for 8 days completely blocked the increase in urinary ALA in GF2.0 mice (Fig. 4A, n = 4) compared with administration of vehicle (n = 4). The treatment prevented
administration of high-dose heme (30 mg·kg⁻¹·day⁻¹) was effective at inhibiting ALA accumulation, it also caused writhing and, at worst, was lethal to the mice. Therefore, we adopted an approach of intravenous heme delivery (10 mg·kg⁻¹·day⁻¹) for 8 days to elicit therapeutic effects in GF2.0 mice; this mode of administration was not associated with any adverse reactions such as writhing. Moreover, using this approach, complete suppression of urinary ALA to baseline levels was observed on days 4, 8, and 15 after GF2.0 ingestion (means ± SD; 6.77 ± 4.11, 8.72 ± 3.19, 9.97 ± 3.75, and 8.81 ± 1.88 mg/ml on days −1, 4, 8, and 15, respectively; all groups, n = 4).

Intraperitoneal administration of another therapeutic agent, hypertonic glucose (9 g·kg⁻¹·day⁻¹), to GF2.0 mice inhibited increases in urinary ALA on day 4 by half of that of control NaCl-treated GF2.0 mice (Fig. 4B, both groups, n = 4). However, as was the case with the heme treatment, treatment with glucose did not have any effect on the increase in fecal porphyrins in GF2.0 mice (Fig. 4B).

Next we determined hemoprotein contents in liver microsomes at day 7 for index of internal heme level in HC and GF2.0 mice treated with heme (10 mg·kg⁻¹·day⁻¹ iv) or hypertonic glucose (9 g·kg⁻¹·day⁻¹ ip) (n = 4). Hemoprotein contents were significantly decreased in GF2.0 mice. In those mice, administration of heme could completely reverse their heme levels, although hemoprotein contents in glucose-treated GF2.0 mice were exactly the same as nontreated GF2.0 mice (Fig. 4C).

Since treatment with heme or glucose did not exert any influence on urinary ALA or fecal porphyrin levels in control HC mice (data not shown), neither heme nor glucose had any significant effect on heme metabolism in healthy mice maintained under the conditions used here. On the other hand, hemoprotein levels in liver microsomes of HC mice were significantly increased by administration of heme (Fig. 4C).

No heme-induced amelioration of the development of other physical abnormalities in GF2.0 mice. Mice with GF-induced porphyria exhibit a number of symptoms such as liver injury (34), decreases in body weight (34) and T₃ levels (Fig. 2A), and atrophy of adipose tissue (Table 2), in addition to abnormal heme metabolism (34, Fig. 4 in this work). Table 2 summarizes the effects of heme or glucose treatment on these symptoms on this mouse model of porphyria. One-week treatment of GF2.0 mice with glucose tended to ameliorate liver enlargement and decreases in body weight and T₃ levels, whereas heme treatment had no such ameliorative effects. The administration of heme to HC or GF2.0 mice induced enlargement of the liver at 1 wk; however, there was no difference between the groups in terms of liver weight at 4 wk after the beginning of heme administration (0, 3, 10, or 30 mg·kg⁻¹·day⁻¹; data partly shown). In addition, no differences were observed in serum hepatic enzyme (ALT and ALP) activity between vehicle- and heme-treated HC or GF2.0 mice at 4 wk. Atrophy of adipose tissue was clearly observed in GF2.0 mice at 2 and 4 wk after the onset of GF ingestion, regardless of whether the mice had been performing the wheel-running trial. Neither heme nor glucose restored decrease of epididymal adipose tissue weights in GF2.0 mice.

Heme, but not glucose, suppresses abnormal diurnal rhythms in GF2.0 mice. We examined whether preventive treatment with heme or hypertonic glucose would reverse abnormalities in the circadian rhythms of mice with GF-induced porphyria. At first, we

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**Fig. 3. Circadian rhythms of LA and Tₖ in mice kept under constant dark (DD) conditions before and during GF ingestion. A: double-plotted actograms of LA obtained from a wheel-running trial. Left, HC mouse; right, GF2.0 mouse. Black bars at the top of the panels indicate constant darkness. The horizontal length of the black bar is 48 h. B: daily acrophase of uncorrected Tₖ rhythm in HC (left) and GF2.0 (right) mice. The black bars at the top of the upper panels indicate constant darkness. The horizontal length of the black bar is 24 h. The gray-black bars at the top of the lower panels indicate subjective day (circadian time; CT0–12) and subjective night (CT12–24), respectively. The horizontal length of the gray-black bar is the individual circadian period before the GF ingestion. C: weekly changes in circadian periods, calculated from the digitized Tₖ rhythms using a χ²-periodgram. Open circles, HC mice; closed squares, GF2.0 mice: symbols without diagonal, uncorrected Tₖ; symbols with diagonal, resting Tₖ. Data represent means ± SD (HC mice, n = 8; GF2.0 mice, n = 4). The data represent differences in circadian periods from those before GF ingestion in each mouse, and positive values indicate a shortening of this period. Statistical differences were analyzed by Welch’s t-test: **P < 0.01.**
measured wheel-running activity in HC (n = 4) and GF2.0 (n = 4–6) mice that received heme (0, 3, 10 or 30 mg·kg⁻¹·day⁻¹) or hypertonic NaCl or glucose (9 g·kg⁻¹·day⁻¹) via abdominal cavity. Intraperitoneally administered heme (3 or 10 mg·kg⁻¹·day⁻¹) for 7 days prevented a decrease in activity in the latter half of the dark phase, and this effect was dose dependent (Fig. 5A, top). Even in among the control HC mice, the highest dose heme treatment (30 mg·kg⁻¹·day⁻¹) caused a profound decrease in activity in the early half of the dark phase, with a delay in the onset of activity seen by at most 6.1 h after the lights were turned off [means ± SD; −0.4 ± 0.09, −4.7 ± 1.00 (P < 0.01, Dunnnett’s test vs. vehicle-treated HC mice), −0.1 ± 0.26, and −1.9 ± 1.91 h; vehicle-treated HC, heme-treated HC, vehicle-treated GF2.0, and heme-treated GF2.0 mice on day 1, respectively]. On the other hand, intraperitoneal administration of hypertonic glucose (9 g·kg⁻¹·day⁻¹) or its control, hypertonic NaCl, into GF2.0 mice for 7 days was not associated with any amelioration of decreased activity in these mice during the latter half of the dark phase (Fig. 5A, bottom).

Heme treatment administered to GF2.0 mice prevented any advances in Δacrophase and ΔTDA20 until at least 1 wk after GF ingestion, whereas no such effects were seen in HC mice (Fig. 5B). The preventive effects of heme were found to be dose dependent (3, 10 mg·kg⁻¹·day⁻¹). In contrast, the hypertonic glucose treatment exerted no such effects on acrophase and TDA20 in GF2.0 mice (Fig. 5B).

We also examined the effects of heme on diurnal Tb rhythms in HC (n = 2) and GF2.0 mice (n = 3 for vehicle or n = 4 for heme). As a result, the intravenous administration of heme (10 mg·kg⁻¹·day⁻¹) for 7 days restored the advances in the timing of Tb increase in GF2.0 mice, and this effect continued to be observed for at least 2 wk (Fig. 5C). Moreover, heme treatment reversed the advancement of acrophase in diurnal Tb rhythm, even when resting Tb was considered (Fig. 5D). These results clearly demonstrated that treatment with heme, but not glucose, restored to normal the LA- and Tb-diurnal rhythms of these GF-induced porphyric mice.

**DISCUSSION**

Here we demonstrated phase advances in diurnal rhythm and a shortening of the circadian period on Tb rhythms in a GF-induced mouse model of porphyria, and reversal of these abnormalities in diurnal rhythm were seen with heme treatment. Although this is a study only on phenomena, our data clearly provide evidence that heme is a regulator of circadian rhythms in vivo, as had already been demonstrated in vitro.

Previously, Kaasik and Lee (20) reported heme-regulated changes in circadian rhythms in vivo performing wheel-running trials; moreover, they showed that the effects of heme were administration time dependent. However, in the present study, if the same dose (30 mg/kg) of heme was administered to mice at the different time, wheel-running was also suppressed and writhing reaction was induced in some of the mice. Therefore, it is plausible that the suppression of the activity could have been due to the acute toxic effects of high-dose heme treatment.

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GF0.5 mice showed less severe symptoms of porphyria than mice fed higher doses of GF, and the phase advance in resting Tb (although not in the uncorrected Tb) under LD conditions suggests that the LA rhythm, when synchronized to the environmental LD cycle, might be able to mask the phase shift in Tb rhythm, based on changes in intrinsic circadian clocks.

Abnormal circadian or diurnal rhythms in this mouse model of porphyria were primarily observed during 1–3 wk after the onset of GF ingestion, despite the fact that these mice were fed GF during the entire experiment. It was of note that this timing correlates well with the point in time when these mice exhibit abnormalities in heme metabolism (34 and this study). According to our previous reports, the increase in Alas1 mRNA levels in the liver, the decrease in hemoproteins (P450) in liver microsomes, and the increase in fecal porphyrins in porphyric mice are transient and then normalize with time (> 4 wk) after maximum changes are observed at ~1 wk after the onset of GF ingestion. In addition to the above-mentioned abnormalities in circadian rhythm and heme metabolism, mice with GF-induced porphyria exhibit declined body weight, decreased Tb levels, injury and enlargement of the liver, and adipose tissue atrophy. These physical changes were continuously observed during the entire experiment and were distinct from the abnormalities in circadian rhythm and heme metabolism. These findings suggest the possibility that abnormal circadian rhythms in this porphyric mouse might be associated with impaired heme biosynthesis, whereas such abnormalities are unlikely to be related to liver injury or impaired energy metabolism other than abnormal heme metabolism.

To treat episodes of severe abdominal pain in acute porphyria patients, heme (3–4 mg·kg⁻¹·day⁻¹) is generally administered intravenously (2). The infusion of heme replenishes reduced heme levels in porphyria patients, and this replenishment suppresses the accumulation of heme precursors by repressing the mRNA expression of Alas1 (2). Treatment with hypertonic glucose (300–500 g/day) is sometimes utilized to resolve mild attack or to keep symptoms from becoming severe until heme infusion can be implemented (2). Glucose represses Alas1 mRNA induction via
peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) (14), and thus glucose treatment inhibits the accumulation of some heme precursors without replenishment of heme. The results of our study suggest that the administration of heme (10 mg·kg⁻¹·day⁻¹) does not inhibit the accumulation of porphyrins nor does it sufficiently prevent an increase in urinary ALA and decrease in liver microsomal hemoproteins; however, our findings did show a statistically significant amelioration of abnormalities in diurnal rhythm. Although the intraperitoneal administration of glucose (9 g·kg⁻¹·day⁻¹) prevented increases in urinary ALA similar to the suppression by the intraperitoneal administration of heme (10 mg·kg⁻¹·day⁻¹), the administration of glucose failed to ameliorate abnormalities in diurnal rhythm, as determined based on wheel-running activity. Since index calculated from 24-h activity data (e.g., acrophase) could contain artificial factors that were associated with drug administration, we considered to use another index, that is, TDA20, provided without the artificial factors. TDA20, as well as acrophase, was reversed by administration of heme but could not be reversed by administration of glucose. These results strongly indicate the possibility that abnormal circadian or diurnal rhythms in this mouse model of porphyria result from an impairment of heme metabolism, and in particular, from a decrease in heme levels. Furthermore, the administration of heme exerted no ameliorative effects on any of the following: liver injury, enlargement of the liver, increased fecal porphyrins, adipose tissue atrophy, decreased body weight, or decreased Tb levels. These observations also support the notion that such abnormal circadian rhythms result from an impairment of heme biosynthesis but not from an impairment of energy metabolisms other than heme biosynthesis. On the other hand, administration of glucose showed a tendency to reverse liver injury, hypothermia, or body weight loss in the porphyric mice partly with statistical significance. Therapeutic effects of glucose on porphyric symptoms might be due to yet-to-be-defined favorable effectiveness for porphyria other than weak effects on impaired heme metabolism.

The abnormalities in circadian or diurnal rhythm observed in this study were twofold; i.e., a phase advance under LD conditions and a shortening of the circadian period under DD conditions. These phenomena do not contradict one another, when we consider that the biological clock is reset by light to keep time. Thus these phenomena do not contradict one another, when we consider that the biological clock is reset by light to keep time. In this context. However, the present results support the hypothesis that a reduction in heme may elicit an abnormal shortening of the circadian rhythm. These results will be able to help elucidate the site of action of heme involved in the modulating the function of clock genes.

REFERENCES


AUTHOR CONTRIBUTIONS

No conflicts of interest, financial or otherwise, are declared by the author(s).

DISCLOSURES

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Preparation of animals and figures; R.I. and A.M. drafted manuscript; R.I. and Y.W. prepared figures; R.I. and A.M. edited and revised manuscript; R.I., Y.W., H.K., N.K., Y.K., T.M., and A.M. approved final version of manuscript.

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Heme and circadian rhythms in mice


