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# Ketogenic diet and fasting induce the expression of cold-inducible RNA-binding protein with time-dependent hypothermia in the mouse liver☆

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ABSTRACT

**Cold-inducible RNA-binding protein (CIRBP) induced by cold stress modulates the molecular circadian clock *in vitro*. The present study examines the effect of a ketogenic diet (KD) and fasting on *Cirbp* expression in the mouse liver. Chronic KD administration induced time-dependent *Cirbp* expression with hypothermia in mice. The circadian expression of clock genes such as *Bmal1* and *Clock* was phase-advanced and augmented in the liver of mice fed with a KD. Transient food deprivation also induced time-dependent *Cirbp* expression with hypothermia in mice. These findings suggest that hypothermia is involved in the increased expression of *Cirbp* under ketogenic or fasting conditions.**



## 1. Introduction

The central clock that regulates most physiological and behavioral rhythms in mammals is located in the suprachiasmatic nucleus (SCN) of the hypothalamus in the brain [1,2]. The mammalian circadian clock system consists of a master pacemaker in the SCN and peripheral oscillators in most tissues. Many studies at the molecular level have found that circadian oscillators in both the SCN and peripheral tissues are driven by negative feedback loops comprising the periodic expression of clock genes [1,2]. Peripheral oscillators are self-sustained and cell-autonomous because peripheral tissues maintain circadian clock gene expression even *in vitro* [1,2]. However, peripheral clocks are entrained to the central clock in the SCN by systemic time cues such as neural, humoral and other signals including body temperature [1–3].

Cold-inducible RNA-binding protein (CIRBP) has been identified as a protein that is induced by cold stress in cultured cells [4]. Levels of *Cirbp* expression rhythmically fluctuate in the mouse liver independently of the molecular clock, suggesting that changes in body temperature are involved in daily *Cirbp* expression [3,5]. Morf et al. [5] demonstrated direct interaction between CIRBP and transcripts encoding circadian clock proteins *in vitro* and revealed via loss-of-function experiments that CIRBP enhances the amplitude of circadian gene expression.

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 non-obese individuals. Such diets mimic the metabolic conditions of fasting or caloric restriction and are based on theoretical concepts of the effects of dietary component ratios on energy expenditure [6]. We previously demonstrated that circadian clock gene expression is disrupted in peripheral tissues of hypothermic mice fed with a KD (KD mice) [7–9]. We examined the temporal expression profiles of *Cirbp* mRNA in mice under KD feeding and fasting to elucidate the regulation mechanism of CIRBP expression *in vivo*.

## 2. Materials and methods

### 2.1. Animals

Male ICR mice (Japan SLC Inc., Hamamatsu, Japan) aged 7–8 weeks were maintained under a 12:12 h light–dark cycle (lights on at 0:00 and lights off at 12:00). A white fluorescent lamp served as a daytime light source.

In the KD feeding experiment, mice were fed *ad libitum* for two weeks with a normal diet (ND) (CE-2; Clea Japan Inc., Tokyo, Japan) or with a KD (73.9% fat, 8.3% protein and 0.73% carbohydrates, w/w; modified AIN-93G; Oriental Yeast Co. Ltd., Tokyo, Japan). The proportions of calories derived from fat, carbohydrate and protein in ND



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and KD were 12.6%, 58.3% and 29.3%, and 94.8%, 0.1% and 4.8%, respectively. The mice were then sacrificed at 2:00, 6:00, 10:00, 14:00, 18:00, and 22:00, and tissues were dissected, quickly frozen and stored in liquid nitrogen.

Mice were sacrificed under food deprivation at 2:00, 8:00, 14:00, and 20:00 after an overnight fast.

All animal care, handling and experimentation proceeded under the approval of our institutional Animal Care and Use Committee (Permissions #2010–020 and #2012–020).

### 2.2. Monitoring core body temperature

Mice were surgically implanted intra-abdominally with data loggers (TempDisk TD-LAB, Labo Support Co. Ltd., Suita, Osaka, Japan) that were programmed to record $T_b \pm 0.1$ °C every 10 min. The data obtained from each logger were analyzed using RhManager Ver.2.09 (KN Laboratories Inc., Ibaraki, Osaka, Japan). Hourly $T_b$ data were averaged.

### 2.3. Quantitative reverse transcription (RT)-PCR

Total RNA was extracted using RNAiso (Takara Bio Inc., Otsu, Japan). Single-stranded cDNA was synthesized using PrimeScript™ RT reagent kits with gDNA Eraser (Takara Bio Inc., Otsu, Japan). Real-time RT-PCR proceeded using SYBR® Premix Ex Taq™ II (Takara Bio Inc., Otsu, Japan) and a LightCyclerTM (Roche Diagnostics, Mannheim, Germany). The reaction conditions were 95 °C for 10 s followed by 45 cycles of 95 °C for 5 s, 57 °C for 10 s and 72 °C for 10 s. Supplemental Table 1 shows the sequences of the primer pairs. The amount of target mRNA was normalized relative to that of 18S rRNA.

### 2.4. Statistical analysis

All values are expressed as means ± SEM. Data were analyzed using a two-way ANOVA followed by Tukey's multiple comparison test. Circadian rhythms were analyzed using the modified cosinor method (nonlinear least-squares (NLLS) Marquardt–Levenberg algorithm) [10]. We defined the function as $f(x) = M + A\cos(2\pi/T(x - ø))$ and set four variables ($M$, MESOR (mean statistics of rhythm); $A$, amplitude (one-half of the total peak-trough variation); $T$, period; ø, acrophase) as the fit parameters. The circadian period ($T$) was 24 h under LD 12:12. Acrophase is expressed in hours elapsed from 0:00. The significance of the circadian rhythm was tested by the zero-amplitude test; $P < 0.05$ was considered evidence of statistically significant rhythmicity for the given period of the cosine curve approximation. Data from cosinor analyses were compared between groups using Welch's or Student's $t$-test. $P < 0.05$ indicated a statistically significant difference.

## 3. Results

Core $T_b$ fluctuated bimodally with a peak at early night (activity onset) and at the night-to-day transition under ND feeding (Fig. 1). Chronic KD administration significantly decreased $T_b$ particularly at midday and late in the dark period, suggesting the induction of time-of-day-dependent torpor in these mice. Averaged daily $T_b$ declined from 37.3 °C to 36.3 °C during two weeks of KD feeding.

Expression levels of *Cirbp* mRNA fluctuated in a circadian manner and peaked during late nighttime in the liver of ND mice (Fig. 2). The abundance of *Cirbp* mRNA was elevated during late nighttime, which corresponded to daily torpor (Fig. 1). On the other hand, levels of *Cirbp* mRNA expression were continuously elevated in the heart (Supplemental Fig. 1), although the relative abundance of basal *Cirbp* expression was 5.2-fold higher in the liver than in the heart (data not shown).

Expression levels of *Fgf21* (a transcription target of peroxisome proliferator-activated receptor α (PPARα) that plays an important role

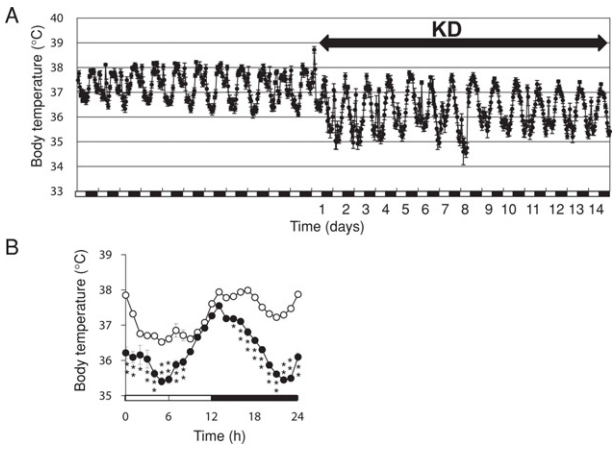


**Fig. 1.** Circadian rhythms of core body temperature ($T_b$) in mice fed with a ketogenic diet (KD). Mice were fed with normal (ND; unfilled circles) or KD (filled circles) diets under 12 h light–12 h dark cycles (LD 12:12; lights on at 0 h). (A) Continuous $T_b$ change during the experiment. (B) Averaged circadian rhythms of $T_b$ under ND and KD feeding. Data are averaged $T_b$ values before (ND; unfilled circles) and after (filled circles) two weeks of KD feeding. Values are shown as means ± SEM ($n = 7$). Significant differences between ND and KD are indicated as $^*P < 0.05$, $^{**}P < 0.01$. Horizontal open and solid bars, day and night, respectively.

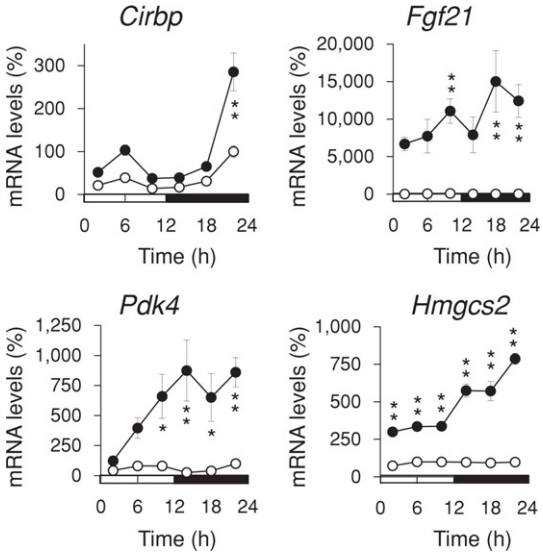


**Fig. 2.** Temporal mRNA expression profiles of *Cirbp* and ketogenesis-related genes in the KD mouse liver. Mice were fed with normal (ND; unfilled circles) or ketogenic (KD filled circles) diets for 14 days under LD 12:12 (lights on at 0 h) and then total RNA was extracted from livers after sacrifice. Messenger RNA levels were measured by quantitative RT-PCR. Maximal value for ND mice is expressed as 100%. Values are shown as means ± SEM ($n = 4$). Significant differences between groups are indicated as $^*P < 0.05$, $^{**}P < 0.01$. Horizontal open and solid bars, day and night, respectively.

in adaptation to fasting and starvation [11]), *Pdk4* (a transcription target of PPARα that suppresses glucose oxidation to increase fatty acid utilization), and *Hmgcs2* (that encodes the rate-limiting enzyme in ketogenesis) were obviously induced in the liver of KD mice (Fig. 2). Notably, the KD-induced expression of these genes and *Cirbp* was considerably enhanced during the nighttime.

The MESOR of *Bmal1*, *Clock*, *Per3*, and *Per2* expression and of the amplitude of *Bmal1* and *Per3* expression were significantly increased in the liver of KD mice (Fig. 3 and Table 1). The KD advanced the acrophase of circadian gene expression by 3.6, 3.8, 3.8, 7.3 and 3.9 h for *Bmal1*, *Clock*, *Per3*, *Per2*, *Dbp*, respectively, in the liver.

The MESOR of circadian mRNA expression in the heart was essentially unaffected by KD except for the *Per2* gene (Supplemental Fig.

**Table 1**
Cosinor analysis of *Bmal1*, *Clock*, *Per3*, *Per2*, and *Dbp* mRNA expression in the liver of mice fed with a ketogenic diet.

| | | MESOR | Amplitude | Acrophase (h) |
|---|---|---|---|---|
| *Bmal1* | ND | 41.8 ± 3.9 | 47.8 ± 5.6 | 22.5 ± 0.44 |
| | KD | 123.4 ± 17.2** | 144.7 ± 24.3** | 18.9 ± 0.64** |
| *Clock* | ND | 62.1 ± 4.9 | 31.2 ± 6.9 | 22.5 ± 0.85 |
| | KD | 141.7 ± 15.5** | 83.9 ± 21.9 | 18.7 ± 1.0* |
| *Per3* | ND | 45.2 ± 7.1 | 42.2 ± 10.0 | 10.6 ± 0.90 |
| | KD | 153.9 ± 12.0** | 143.2 ± 17.0** | 6.8 ± 0.45** |
| *Per2* | ND | 48.8 ± 5.3 | 48.1 ± 7.6 | 16.0 ± 0.60 |
| | KD | 93.8 ± 10.7** | 76.8 ± 15.2 | 8.7 ± 0.75** |
| *Dbp* | ND | 31.1 ± 8.9 | 46.1 ± 12.5 | 9.5 ± 1.0 |
| | KD | 46.1 ± 8.3 | 62.0 ± 11.8 | 5.6 ± 0.73* |

KD, ketogenic diet; ND, normal diet. MESOR, mean statistics of rhythm; amplitude, one-half the total peak-trough variation; acrophase, delay from 0:00 (lights on). Data are shown as means ± SEM ($n = 4$). Significant differences compared with ND value are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$.

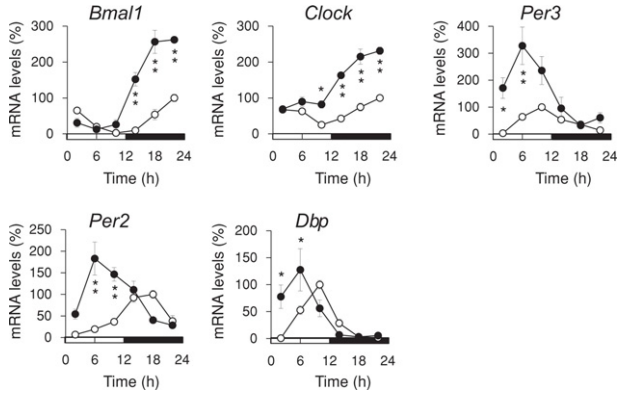


**Fig. 3.** Temporal mRNA expression profiles of circadian clock-related genes in the liver of mice fed with a ketogenic diet (KD). Mice were fed with normal (ND; unfilled circles) or KD (filled circles) diets for 14 days under LD 12:12 (lights on at 0 h) and then total RNA was extracted from livers after sacrifice. Messenger RNA levels were measured by quantitative RT-PCR. Maximal value for ND mice is expressed as 100%. Values are shown as means ± SEM ($n = 4$). Significant differences between groups are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$. Horizontal open and solid bars, day and night, respectively.

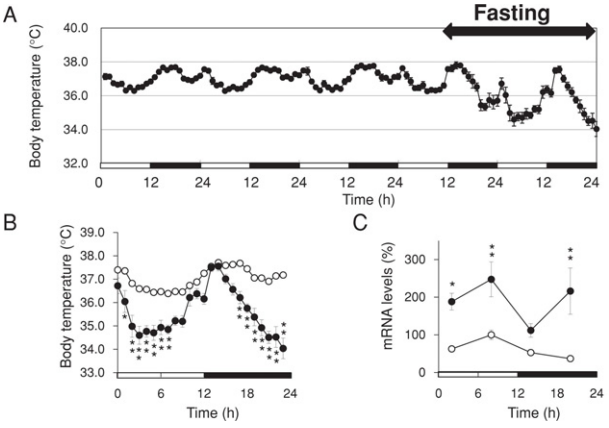


**Fig. 4.** Circadian rhythms of core body temperature ($T_b$) and hepatic *Cirbp* expression of fasted mice. Mice were fed (unfilled circles) or fasted (filled circles) under 12 h light–12 h dark cycles (LD 12:12; lights on at 0 h). (A) Continuous $T_b$ change during the experiment. (B) Averaged circadian rhythms of $T_b$ under fed and fasted conditions. Data are averaged $T_b$ values during fed (unfilled circles) and fasted (filled circles). Values are shown as means ± SEM ($n = 7$). (C) Temporal mRNA expression profiles of *Cirbp* in the liver of fasted mice. Messenger RNA levels were measured by quantitative RT-PCR. Maximal value for fed mice is expressed as 100%. Values are means ± SEM ($n = 4$). Significant differences between fed and fasted groups are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$. Horizontal unfilled and solid bars, day and night, respectively.

1 and Table 2). The amplitude of the circadian mRNA expression of *Bmal1*, *Clock*, *Per3*, and *Per2* was unaffected, whereas that of *Dbp* was significantly decreased in the hearts of KD mice. On the other hand, the phase-advancing effect in the heart was relatively larger (by 6.2, 7.3, 4.8, 5.6, 6.0 h for *Bmal1*, *Clock*, *Per3*, *Per2*, *Dbp*, respectively) than that in the liver.

Food deprivation notably and time-dependently reduced Core $T_b$ (Fig. 4(B)). Fasting decreased $T_b$ particularly at midday and late in the dark period as well as chronic KD administration (Fig. 1). Average daily $T_b$ declined from 37.0 °C to 35.6 °C on the sampling day. Furthermore, hepatic expression levels of *Cirbp* were significantly increased in accordance with the timing of the $T_b$ decrease (Fig. 4(C)).

## 4. Discussion

Mammalian peripheral circadian clocks are self-sustained and cell autonomous, although they are synchronized by the central clock in the SCN. Several circadian genes are expressed in peripheral tissues. Peripheral circadian rhythms are controlled by both local oscillators and systemic cues such as hormones, neural signals, and body temperature [1–3]. Originally identified as a cold stress-induced protein in cultured cells [4], CIRBP was later found to directly interact with circadian RNAs such as *Clock*, *Per3* and *Dbp*, and to maintain robust (high amplitude) circadian gene expression *in vitro* [5].

The present study showed that chronic KD administration and fasting induce time-dependent hepatic *Cirbp* expression with hypothermia in mice. Expression levels of *Cirbp* that were obviously increased late in the dark period corresponded to daily torpor, suggesting that the KD- and fasting-induced mRNA expression of *Cirbp* was a direct effect of hypothermia. To the best our knowledge, this is the first study to demonstrate the induction of *Cirbp* expression *in vivo*.

The magnitude and amplitude of circadian gene expression were obviously increased in the KD mouse liver. Daily body temperature fluctuations are presently considered as possible systemic cues for resetting peripheral clocks [12]. Hypothermia-induced CIRBP expression might be responsible for the KD-induced robustness of peripheral oscillators in the liver, because the depletion of CIRBP by siRNA results in a reduction in the amplitude of circadian gene expression in fibroblasts [5]. The expression of CLOCK is extremely reduced in cells depleted of CIRBP, indicating that CIRBP positively regulates CLOCK [5]. Temperature-dependent peripheral clock regulation might be mediated by CIRBP through the induction of *Clock* gene expression, because CLOCK completely rescues disrupted circadian gene expression in CIRBP-depleted fibroblasts [5].

On the other hand, the magnitude and amplitude of circadian gene expression remained essentially unaffected in the hearts of KD mice, although *Cirbp* expression levels were significantly increased in both tissues. Systemic signals tissue-dependently regulate peripheral oscillators. Non-neuronal signals are sufficient to generate peripheral

rhythms in the liver, whereas neural signals are critical for generating circadian rhythms in the heart [13]. Oscillators in the liver might be more sensitive to changes in $T_b$ than those in the heart.

The phase of circadian gene expression was obviously advanced both in the KD mouse liver and heart as described [8,9]. We demonstrated the phase-advancing effects of a KD on the biological clock that governs rhythmic behavioral activity as well as the rhythmic expression of circadian genes in mouse peripheral tissues [8,9]. Therefore, chronic KD feeding seems to affect the circadian phase of the central nervous systems. Several studies have indicated that caloric restriction or fasting induces a phase-advance and enlarges the amplitude of various circadian rhythms such as locomotor activity, $T_b$ and circadian gene expression [14,15]. Fasting or caloric restriction induces time-dependent hypothermia in mammals [16,17]. Hypothermia-induced CIRBP expression might be involved in the phase-advancing effect of KD or fasting on circadian clocks.

The present study showed that KD and fasting induce time-dependent hepatic *Cirbp* expression accompanied by hypothermia in mice. Further study is required to elucidate direct interactions between hypothermia-induced CIRBP expression and circadian clocks *in vivo*.

## Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2013.03.005.

## References

[1] Reppert S.M., Weaver D.R. (2002) Coordination of circadian timing in mammals. Nature 418, 935–941.
[2] Takahashi J.S., Hong H.K., Ko C.H, McDearmon E.L. (2008) The genetics of mammalian circadian order and disorder: implications for physiology and disease. Nat. Rev. Genet. 9, 764–775.
[3] Kornmann B., Schaad O., Bujard H., Takahashi J.S., Schibler U. (2007) System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. PLoS Biol. 5, e34.
[4] Nishiyama H., Itoh K., Kaneko Y., Kishishita M., Yoshida O., Fujita J. (1997) A glycine-rich RNA-binding protein mediating cold-inducible suppression of mammalian cell growth. J. Cell Biol. 137, 899–908.
[5] Morf J., Rey G., Schneider K., Stratmann M., Fujita J., Naef F. et al. (2012) Cold-inducible RNA-binding protein modulates circadian gene expression posttranscriptionally. Science 338, 379–383.
[6] Astrup A., Meinert Larsen T., Harper A. (2004) Atkins and other low-carbohydrate diets: hoax or an effective tool for weight loss? Lancet 364, 897–899.
[7] Oishi K., Sakamoto K., Konishi M., Murata Y., Itoh N., Sei H. (2010) FGF21 is dispensable for hypothermia induced by fasting in mice. Neuro Endocrinol. Lett. 31, 198–202.
[8] Oishi K., Uchida D., Ohkura N., Doi R., Ishida N., Kadota K. et al. (2009) Ketogenic diet disrupts the circadian clock and increases hypofibrinolytic risk by inducing expression of plasminogen activator inhibitor-1. Arterioscler. Thromb. Vasc. Biol. 29, 1571–1577.
[9] Oishi K., Uchida D., Ohkura N., Horie S. (2010) PPARalpha deficiency augments a ketogenic diet-induced circadian PAI-1 expression possibly through PPARgamma activation in the liver. Biochem. Biophys. Res. Commun. 401, 313–318.
[10] Ohkura N. (2007) Circadian variations in coagulation and fibrinolytic factors among four different strains of mice. Chronobiol. Int. 24, 651–669.
[11] Cuevas-Ramos D., Aguilar-Salinas C.A., Gomez-Perez F.J. (2012) Metabolic actions of fibroblast growth factor 21. Curr. Opin. Pediatr. 24, 523–529.
[12] Saini C., Morf J., Stratmann M., Gos P., Schibler U. (2012) Simulated body temperature rhythms reveal the phase-shifting behavior and plasticity of mammalian circadian oscillators. Genes Dev. 26, 567–580.
[13] Guo H., Brewer J.M., Champhekar A., Harris R.B, Bittman E.L. (2005) Differential control of peripheral circadian rhythms by suprachiasmatic-dependent neural signals. Proc. Natl. Acad. Sci. USA 102, 3111–3116.
[14] Challet E. (2010) Interactions between light, mealtime and calorie restriction to control daily timing in mammals. J. Comp. Physiol. B 180, 631–644.
[15] Froy O., Chapnik N., Miskin R. (2009) Effect of intermittent fasting on circadian rhythms in mice depends on feeding time. Mech. Ageing Dev. 130, 154–160.
[16] Overton J.M., Williams T.D. (2004) Behavioral and physiologic responses to caloric restriction in mice. Physiol. Behav. 81, 749–754.
[17] Tokizawa K., Uchida Y., Nagashima K. (2009) Thermoregulation in the cold changes depending on the time of day and feeding condition: physiological and anatomical analyses of involved circadian mechanisms. Neuroscience 164, 1377–1386.