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Involvement of adenosine monophosphate–activated protein kinase in the influence of timed high-fat evening diet on the hepatic clock and lipogenic gene expression in mice

Yan Huang[a,b], *Zengyan Zhu*[a,b], *Meilin Xie*[a], *Jie Xue*[a,*]

[a] *Department of Pharmacology, Jiangsu Key Laboratory of Preventive and Translational Medicine for Geriatric Diseases, College of Pharmaceutical Sciences, Soochow University, Suzhou 215123, Jiangsu Province, China*

[b] *Department of Pharmacy, The Affiliated Children's Hospital of Soochow University, Suzhou 215003, Jiangsu Province, China*

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ABSTRACT

A high-fat diet may result in changes in hepatic clock gene expression, but potential mechanisms are not yet elucidated. Adenosine monophosphate–activated protein kinase (AMPK) is a serine/threonine protein kinase that is recognized as a key regulator of energy metabolism and certain clock genes. Therefore, we hypothesized that AMPK may be involved in the alteration of hepatic clock gene expression under a high-fat environment. This study aimed to examine the effects of timed high-fat evening diet on the activity of hepatic AMPK, clock genes, and lipogenic genes. Mice with hyperlipidemic fatty livers were induced by orally administering high-fat milk via gavage every evening (19:00-20:00) for 6 weeks. Results showed that timed high-fat diet in the evening not only decreased the hepatic AMPK protein expression and activity but also disturbed its circadian rhythm. Accordingly, the hepatic clock genes, including *clock*, *brain-muscle-Arnt-like* 1, cryptochrome 2, and period 2, exhibited prominent changes in their expression rhythms and/or amplitudes. The diurnal rhythms of the messenger RNA expression of *peroxisome proliferator–activated receptor α*, *acetyl-CoA carboxylase 1α*, and *carnitine palmitoyltransferase 1* were also disrupted; the amplitude of *peroxisome proliferator–activated receptor γ coactivator 1α* was significantly decreased at 3 time points, and fatty liver was observed. These findings demonstrate that timed high-fat diet at night can change hepatic AMPK protein levels, activity, and circadian rhythm, which may subsequently alter the circadian expression of several hepatic clock genes and finally result in the disorder of hepatic lipogenic gene expression and the formation of fatty liver.

Abbreviations: ACC-1α, acetyl-CoA carboxylase 1α; AMPK, adenosine monophosphate–activated protein kinase; ATP, adenosine triphosphate; Bmal1, brain-muscle-Arnt-like 1; CPT-1, carnitine palmitoyltransferase 1; Cry2, cryptochrome 2; ELISA, enzyme-linked immunosorbent assay; FFA, free fatty acid; p-AMPK, phosphorylated AMPK; PGC-1α, peroxisome proliferator–activated receptor γ coactivator 1α; Per2, period 2; PPARα, peroxisome proliferator–activated receptor α; Real-time, PCR real-time polymerase chain reaction; TG, triglyceride.

* *Corresponding author at:* Department of Pharmacology, College of Pharmaceutical Sciences, Soochow University, 199 Renai Road, Suzhou Industrial Park, Suzhou 215123, Jiangsu Province, China. Tel.: +86 512 6956 6553; fax: +86 512 6588 2089.
E-mail address: xuejie@suda.edu.cn (J. Xue).

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1. Introduction

Mammalian homeostatic systems have adapted to environmental 24-hour day/night cycles via the development of the endogenous circadian clocks, which are located in the suprachiasmatic nucleus of the anterior hypothalamus [1] and some peripheral tissues, such as liver, intestine, and adipose tissue [2-4]. These clock genes mainly consist of positive (clock and brain-muscle-Arnt-like 1 [Bmal1]) and negative components (periods [Pers] and cryptochromes [Crys]). The genes may control some physiologic functions by regulating clock-controlled genes, such as nuclear receptors including peroxisome proliferator–activated receptor α (PPARα) [5]. If the integrity and temporal coordination between these clock genes are disrupted, the risks for developing metabolic disorders are increased [6,7]. Some external stimuli, such as overnutrition, may influence clock function. For example, a long-term high-fat diet across the 24-hour light/dark cycles in mice may lead to alterations in the rhythmic expression of circadian clock genes clock, Bmal1, and Per2 in the liver [8]. Thus, energy metabolism may be closely related to the clockwork system, and further investigation is needed to clarify the pathogenesis of metabolic disorders.

In many countries, usual dietary habits include an evening meal. Currently, few studies have investigated the effects of feeding time on the hepatic circadian clock genes. Our previous studies showed that timed high-fat diet in the evening may result in the changes of hepatic clock gene expression and formation of fatty liver [9]. However, the potential molecular link between energy metabolism and clock genes is not yet elucidated completely. Adenosine monophosphate–activated protein kinase (AMPK) is a serine/threonine protein kinase that is recognized as a key regulator of energy metabolism and is activated by the increment of the AMP/adenosine triphosphate (ATP) ratio [10]. Several works demonstrated that AMPK may be involved in the regulation of some clock genes [11,12]. More recent research data have shown that metformin, a therapeutic drug for diabetes mellitus and an activator of AMPK, may influence the AMPK circadian gene expression in muscle cells [13]. These research findings suggest that the interaction of energy metabolism and clock genes may be mediated by AMPK.

Basing on the aforementioned data, we hypothesized that AMPK may be involved in the alteration of hepatic clock gene expression in mice receiving timed high-fat diet, but findings on this mechanism have not been reported to date. In the present study, we investigated the following in Kunming mice: the effects of a timed high-fat evening diet on the hepatic AMPK and phosphorylated-AMPK (p-AMPK) expression; the relationship between hepatic AMPK and clock genes; and the rhythmic variations of clock-controlled gene PPARα and several lipid metabolism-related genes, including PPAR γ coactivator 1α (PGC-1α), acetyl-CoA carboxylase 1α (ACC-1α), and carnitine palmitoyltransferase 1 (CPT-1).

2. Methods and materials

2.1. Reagents

The assay kit for triglyceride (TG) was obtained from Beijing Beihua Kangtai Clinical Reagent Company (Beijing, China). The assay kit for free fatty acid (FFA) was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The enzyme-linked immunosorbent assay (ELISA) kit for mouse AMPK was the product of Shanghai Xitang Biotechnology Co Ltd (Shanghai, China). Anti-AMPK, anti–p-AMPK, and anti–β-actin antibodies were purchased from Cell Signaling Technology Company (Boston, MA, USA). Anti-Cry2 and anti-Per2 antibodies were purchased from Abcam (Cambridge, United Kingdom) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Trizol was obtained from Invitrogen (Carlsbad, CA, USA). The primers (Table 1) used for amplification by real-time polymerase chain reaction (PCR) were synthesized by Shanghai Sangon Gene Company (Shanghai, China). All other reagents used in this study were of analytic grade.

2.2. Animals and treatments

Male Kunming mice weighing 20 to 22 g were purchased from Shanghai SLAC Laboratory Animal Co Ltd (Shanghai, China) and maintained in regular cages in a room with controlled humidity and temperature with a 12-hour light (8:00-20:00)/12-hour dark (20:00-8:00) cycle. The mice were allowed free access to standard pellet diet (Table 2) and water. They were also allowed to acclimatize to the laboratory environment for 3 days before the study. The animal study was approved by the university ethics committee and conducted according to the regulations for the Use and Care of Experimental Animals at Soochow University.

Seventy-two mice were randomly divided into 2 groups: a high-fat diet group (n = 36) and a control group (n = 36). All mice were allowed free access to standard pellet diet for the entire duration of each 24-hour light/dark cycle during the experiment. Simultaneously, the high-fat diet mice were given high-fat milk at 0.2 mL (containing 1.37 kilojoules from fat and carbohydrate)/10 g body weight per day orally by gavage every evening (19:00-20:00) for 6 weeks. The milk contained 20% lard, 10% cholesterol, 5% saccharose, 0.2% propylthiouracil, 20% propylene glycol, and 20% Tween 80. The control mice were given an equal volume of distilled water in the same manner. The components of fatty acids in the fat milk were 2.5% myristic acid, 26.6% palmitic acid, 10.6% octadecanoic acid, 2.7% palmitoleic acid, 43.7% oleic acid, and 13.9% linoleic acid. After 6 weeks, all of the mice were weighed and euthanized followed by cervical dislocation at a time (6 mice per group) for each of the following time points: 8:00, 12:00, 16:00, 20:00, 24:00, and 4:00. Liver samples were collected for parameter measurements, and partial hepatic tissues were flash-frozen in liquid nitrogen and stored at –80°C for real-time PCR and Western blot analyses.

2.3. Measurement of hepatic TG, FFA, and AMPK levels

Hepatic tissues were obtained and homogenized (10% wt/vol) in cold normal saline. Afterward, tissue homogenates were mixed with a solution of chloroform/methanol (2:1 vol/vol) to a ratio of 1:1 (vol/vol). The prepared samples were then centrifuged at 3000*g* for 10 minutes. The substrata obtained were used to measure TG and FFA contents according to the manufacturer's methods. For the hepatic AMPK measurement, partial hepatic tissues were excised and placed immediately in ice-cold normal saline containing 50 U/mL

Table 1 – Sequences of primers used for the real-time PCR amplification

Gene	Forward 5′-3′	Reverse 5′-3′
Clock	CGCGAGAAAGATGGACAAGTC	CCTGTCGAATCTCACTAGCATCTG
Bmal1	GTCACAGGCAAGTTTTACAGAC	CTGAACCATCGACTTCGTAGC
PPARα	CCCTGTTTGTGGCTGCTATAATTT	GGGAAGAGGAAGGTGTCATCTG
PGC-1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
ACC-1α	TAACAGAATCGACACTGGCTGGCT	ATGCTGTTCCTCAGGCTCACATCT
CPT-1	TTGAATCGGCTCCTAATG	GTCACCTCCAACACATAT
GAPDH	CATCCACTGGTGCTGCCAAGGCTGT	ACAACCTGGTCCTCAGTGTAGCCCA

aprotinin. The tissue homogenates (10% wt/vol) were prepared and then centrifuged at 1200*g* for 10 minutes, wherein the resulting supernatant was used to measure AMPK content by ELISA method according to manufacturer's instructions on a VersaMax plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. Total RNA extraction and messenger RNA expression detection by quantitative real-time PCR

Total RNA was isolated from hepatic tissues, and the concentration and purity of the RNA were determined spectrophotometrically by the ratio of the absorbance at 260 nm to that at 280 nm. For real-time PCR analysis, complementary DNA was synthesized from total RNA by using ReverTra Ace qPCR RT Master Mix (TOYOBO Bio, Osaka, Japan). The complementary DNA product was subjected to PCR analysis with gene-specific primers using SYBR Green (TOYOBO Bio, Osaka, Japan). Quantitative PCR was performed using the ABI-Prism7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers (Table 1) for all genes were tested alongside the normalizing gene GAPDH.

2.5. Western blot analysis for protein expression

An aliquot of appropriate protein from each sample was loaded onto 10% sodium dodecyl sulfonate (SDS)-polyacrylamide gel, subjected to electrophoresis, and then transferred to nitrocellulose membranes. The membrane was blocked with 5% (wt/vol) skimmed milk at room temperature for 2 hours and subsequently incubated with the primary antibodies of AMPK (1:1000), p-AMPK (1:1000), Per2 (1:500), Cry2 (1:500), or β-actin (1:2000) at 4°C overnight.

Table 2 – Proximate analysis and ingredient composition of the standard pellet diet

Component	
Total fat (% kJ)	12.1
Protein (% kJ)	25.6
Carbohydrate (% kJ)	62.3
Energy density (kJ/g)	14.2
Individual components (g/kg)	
Corn starch	260
Soybean powder	247
Flour	340
Fish meal	50
Vegetable oil	23
Alfalfa powder	30
Vitamin and mineral mixture	50

The membrane was then washed and incubated with fluorescent secondary antibodies for 1 hour. The ratio of each protein of interest was subjected to β-actin antibody binding and was densitometrically analyzed by Odyssey infrared imaging system.

2.6. Histologic observation

Mouse liver samples were fixed in 10% formaldehyde solution, embedded in paraffin, sectioned on a microtome for hematoxylin and eosin staining, and then examined under a light microscope. The degree of lipid accumulation was graded by evaluating the proportion of hepatocytes containing fat droplets and expressed as follows: no fat present as "–", less than one-third of the hepatic lobule as "+", one-third to two-thirds as "++", and more than two-thirds as "+++."

2.7. Statistical analyses

Data were expressed as means ± SD. The normally distributed variables were analyzed by 1-way analysis of variance and followed by post hoc least significant difference test. The variables that were not normally distributed and/or that displayed homogeneity of variance were analyzed by 1-way analysis of variance and followed by post hoc Games-Howell test. All statistical analyses were performed using SPSS 20.0, where $P < .05$ was considered statistically significant.

3. Results

3.1. Effects of high-fat milk feeding in the evening on hepatic AMPK and p-AMPK levels

We first initially observed the changes in hepatic AMPK protein levels. Results showed that the hepatic AMPK content exhibited a circadian rhythm; a peak was observed at 12:00 in the control mice, but it was at 20:00 in the high-fat diet mice with an 8-hour delay relative to the matched control mice. In addition, the difference between the amplitudes of the 2 groups at 12:00 and 20:00 was evidently significant (Fig. 1A) ($P < .05$ or $P < .01$). To determine AMPK activity, we observed the changes in p-AMPK protein expression at 12:00 and 20:00. Results showed a decrease in p-AMPK protein expression at 12:00 and an increase at 20:00 for the high-fat diet mice (Fig. 1B) ($P < .05$ or $P < .01$). Notably, the average hepatic AMPK and p-AMPK protein levels were lower in the high-fat diet mice than those in the control mice (Fig. 1C) ($P < .05$). These results indicated that

high-fat diet may not only decrease the hepatic AMPK protein expression and activity but also disturb its circadian rhythm.

3.2. *Effects of high-fat milk feeding in the evening on hepatic clock gene expression*

Compared with the matched control mice, the amplitudes of hepatic Cry2 and Per2 protein expression were decreased in the high-fat diet mice, especially at 12:00, 16:00, 20:00, and 24:00 for Cry2 and 12:00, 16:00, 24:00, and 4:00 for Per2 (Figs. 2A-C) ($P < .05$ or $P < .01$). Meanwhile, the hepatic clock genes, *clock*, and *Bmal1* exhibited circadian oscillation in the control mice (Fig. 2D and E). Administrating a high-fat diet in the evening caused significant enhancement of *clock* and *Bmal1* amplitudes at 8:00 and 12:00 (Fig. 2D and E) ($P < .05$ or $P < .01$), but their circadian rhythms were not affected. These results further verified that the expression rhythms and/or amplitudes of several hepatic clock genes were altered in the high-fat diet mice.

3.3. *Effects of high-fat milk feeding in the evening on lipid metabolism-related gene expression*

Compared with the matched control mice, the amplitudes of the hepatic *PPARα* messenger RNA (mRNA) expression were lower in the high-fat diet mice, especially at 12:00 and 20:00 (Fig. 3A) ($P < .05$ or $P < .01$), causing 4-hour peak value advance (Fig. 3A). The hepatic *PGC-1α* expression in the high-fat diet mice exhibited normal rhythmic variation but with a significant decrease in amplitude at 12:00, 20:00, and 4:00 compared with the matched control mice (Fig. 3B) ($P < .05$ or $P < .01$). The hepatic mRNA expression of *ACC-1α* and *CPT-1* in the high-fat diet mice was significantly altered at 3 time points, namely, at 8:00, 16:00, and 20:00; but these changes between the 2 groups were opposite relative to the matched control mice (Fig. 3C and D) ($P < .05$ or $P < .01$).

3.4. *Effects of high-fat milk feeding in the evening on hepatic lipids and body weight*

Results showed that hepatic steatosis was not observed in the control mice (Fig. 4A), and numerous lipid empty vacuoles in hepatic tissues were observed in the high-fat diet mice (Fig. 4B). This result indicated that hepatic lipids accumulated, and fatty liver developed in mice fed with high-fat diet in the evening. Similarly, the hepatic TG and FFA contents were higher in the high-fat diet mice than in the matched control mice, especially at 8:00, 12:00, 16:00, and 20:00 for TG and at 8:00, 16:00, and 20:00 for FFA (Fig. 5A and B) ($P < .05$ or $P < .01$). However, their circadian rhythms revealed no evident apparent variation. Moreover, the average body weight gain of the high-fat diet mice was higher than that of the control mice (Fig. 5C) ($P < .05$).

4. Discussion

Adenosine monophosphate–activated protein kinase is a suitable candidate for study regarding the link between cellular energy metabolism and clock function [14]. Under normal circumstances, the activation of AMPK is due to increments in the cellular AMP/ATP ratio [10], which may

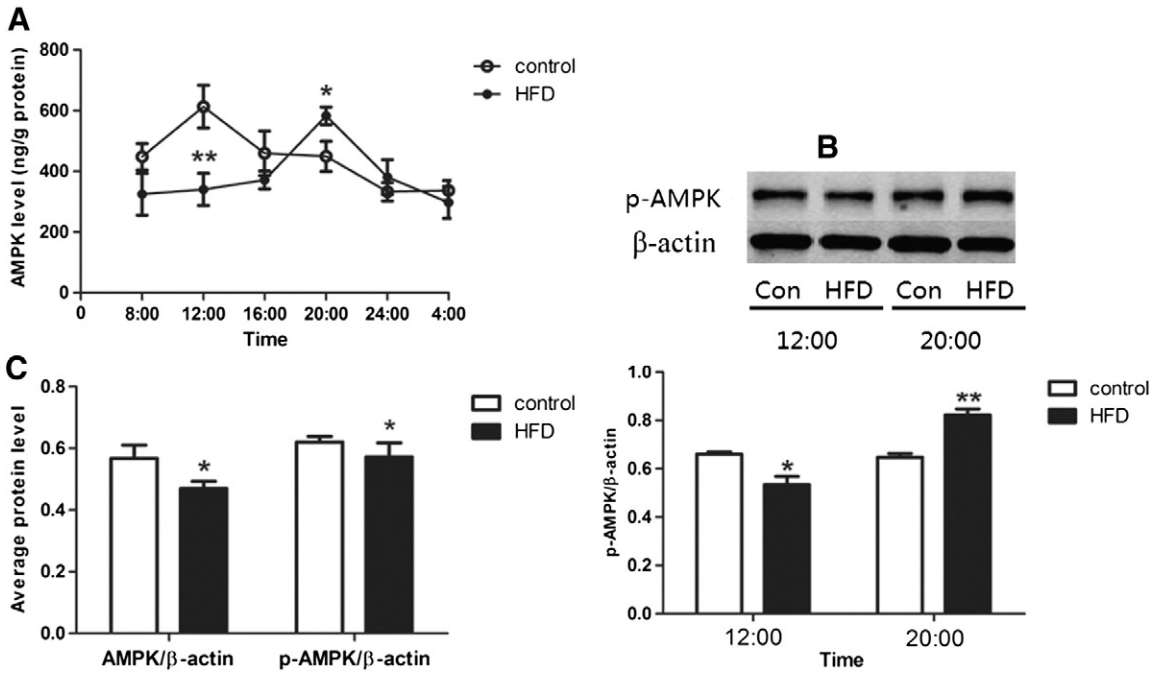

Fig. 1 – Hepatic AMPK and p-AMPK levels in mice fed with high-fat milk every evening for 6 weeks. A, Adenosine monophosphate–activated protein kinase content was detected by ELISA method (n = 6 mice per group). B, Phosphorylated-AMPK expression at 12:00 and 20:00 was determined by Western blot method (n = 3 mice per group). C, Average AMPK and p-AMPK protein expression levels were determined by Western blot method (n = 12 mice per group). Abbreviations: con, control; HFD, high-fat diet. Values are expressed as means ± SD. Protein bands were quantified relative to β-actin. $^{*}P < .05$, $^{**}P < .01$ vs control group at the same time points.

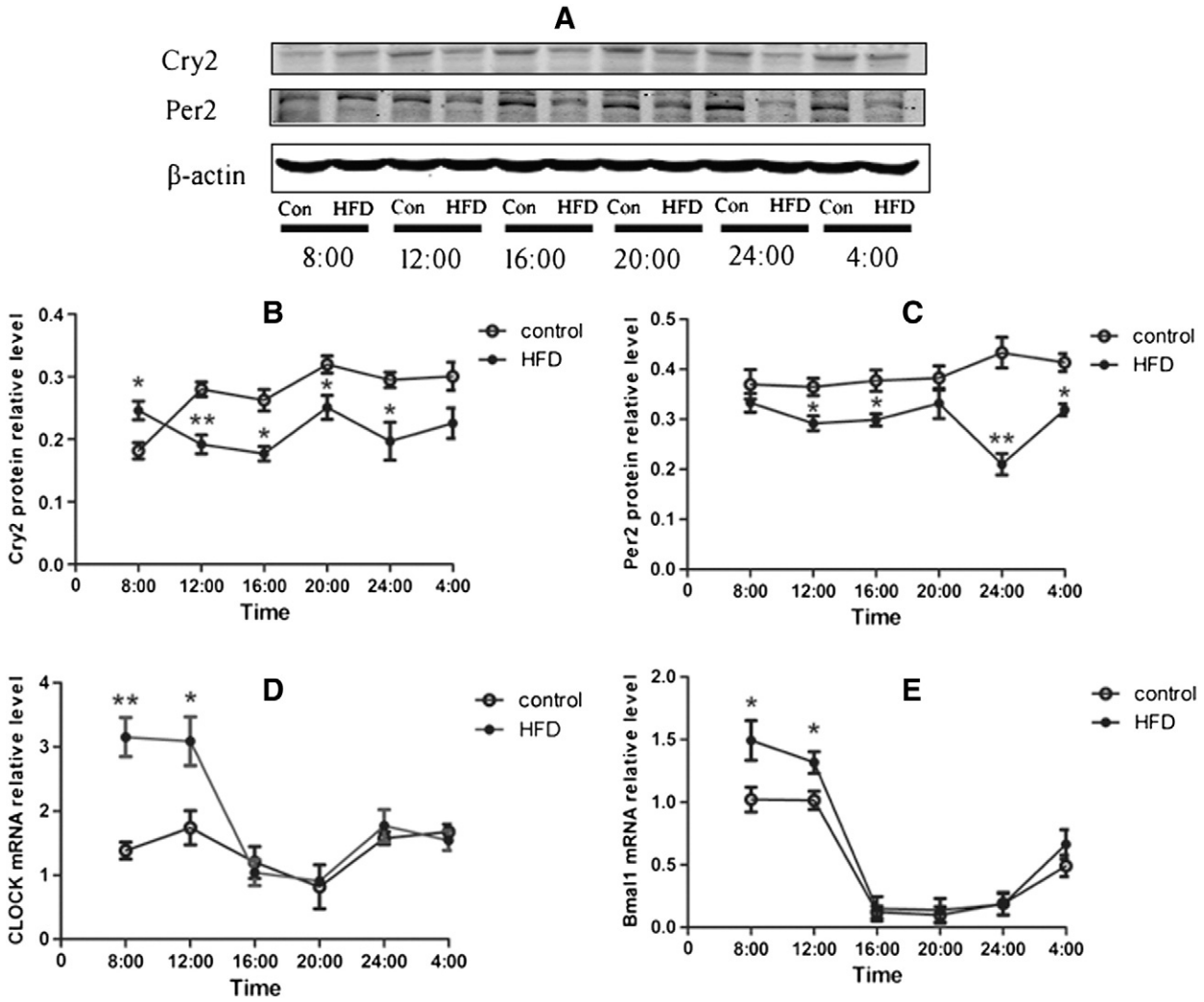

Fig. 2 – Hepatic clock gene mRNA and protein expressions in mice fed with high-fat milk every evening for 6 weeks. A-C, Cryptochrome 2 and Per2 protein expressions were measured by Western blot method (n = 3 mice per group). D and E, *Clock* and *Bmal1* mRNA expressions were measured by real-time PCR method (n = 3 mice per group). Abbreviations: con, control; HFD, high-fat diet. Values are expressed as means ± SD. Protein bands were quantified relative to *ß*-actin, and mRNA expression was normalized to the *GAPDH* level. $^{*}P < .05$, $^{**}P < .01$ vs control group at the same time points.

switch the cellular energy metabolism from ATP consumption to ATP production. Recent studies have demonstrated that the AMPK has normal rhythmic change in the mouse liver and hypothalamus [11,12]. Our present results showed that, in contrast to the control mice, hepatic AMPK expression in the timed high-fat diet mice was lower during the light phase and higher during the dark phase. However, the average hepatic AMPK and p-AMPK levels were all decreased, suggesting that the normal circadian rhythms of hepatic AMPK and p-AMPK are impaired in high-fat diet mice.

Adenosine monophosphate–activated protein kinase modulates the circadian clock genes by modifying the expression of the Crys and casein kinase 1ε [11,15]. The latter may indirectly regulate the clock genes clock and Bmal1 through the degradation of Pers proteins [15]. In the present study, we compared the expression levels of these hepatic clock genes between control and high-fat diet mice. As expected, some hepatic clock genes, including *clock* and *Bmal1*, exhibited prominent changes in amplitude at several time points coinciding with the administration of the high-fat evening diet, but their circadian rhythms were unchanged. Interestingly, the *clock* and *Bmal1* mRNA expression was increased, whereas the Cry2 and Per2 protein expression was decreased during the light phase. As such, we speculated that the reduction in Cry2 and Per2 expression may be attributed to the regulatory attenuation of AMPK signals, which subsequently results in the elevation in *clock* and *Bmal1* expression because of the decrease in the negative feedback loop. However, we also found that the hepatic *clock* and *Bmal1* mRNA expression did not vary significantly with the reduction in Cry2 and Per2 protein expression during the dark phase, and the findings are consistent with our previous results [9]. Hence, how interactions occur among AMPK, clock, Bmal1, Cry2, and Per2 are a valuable issue to investigate. Based on the experimental results, we proposed that Cry2 and Per2 may be partly involved in high-fat diet-induced fatty liver

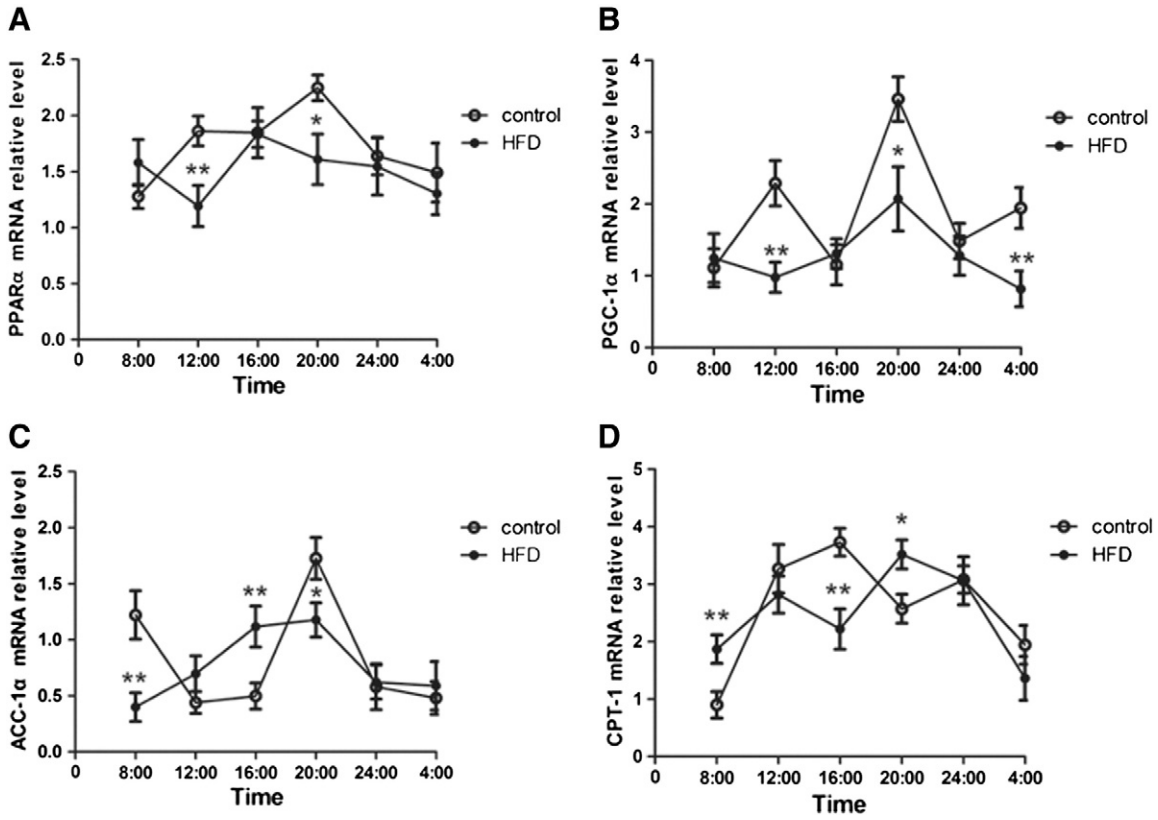

Fig. 3 – Hepatic *PPARα*, *PGC-1α*, *ACC-1α*, and *CPT-1* mRNA expressions in mice fed with high-fat milk every evening for 6 weeks. Each level of gene mRNA expression was measured by real-time PCR method and normalized to the *GAPDH* level. Abbreviation: HFD, high-fat diet. Values are expressed means ± SD of n = 3 mice per group. $^{*}P < .05$, $^{**}P < .01$ vs control group at the same time points.

via modulation of clock and Bmal1 expression and subsequent regulation of clock-controlled and lipid metabolism-related genes.

Previous studies indicated that changes in clock gene expression participate in the development of some metabolic diseases [16-18]. The linking molecules are several nuclear receptors, including PPARα and PGC-1α [19,20]. Peroxisome proliferator–activated receptor α is rhythmically expressed in the liver in a clock/Bmal1-dependent manner [21] and mainly controls lipid metabolism-related genes, such as CPT-1 and ACC-1α [22,23]. Peroxisome proliferator–activated receptor γ coactivator 1α is a nutrient-inducible factor and plays a main role in the maintenance of energy metabolism in numerous tissues via regulation of CPT-1 expression [24]. In this study, amplitudes of *PPARα* and *PGC-1α* expression were decreased in the high-fat diet mice. This decreased expression is consistent with the high expression levels of *clock* and *Bmal1* during the light phase, indicating that the alteration of clock gene expression may result in the reduction in PPARα and PGC-1α expression.

A large body of evidence indicates that FFA is significant to the development of fatty liver and associated lipotoxicity.

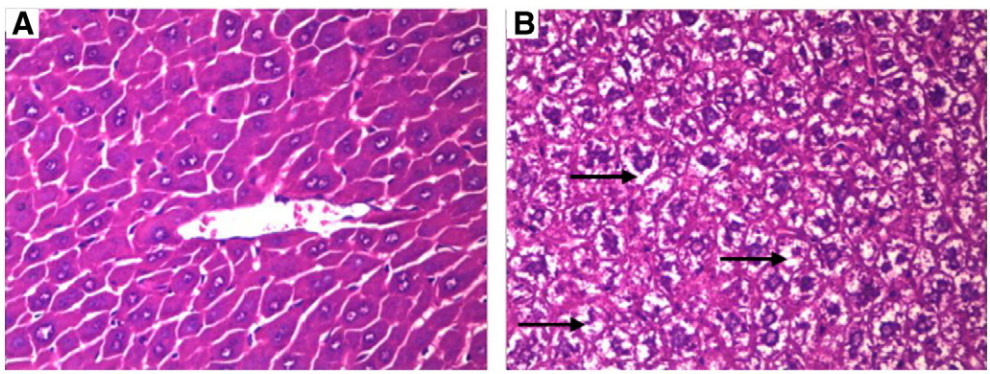

Fig. 4 – Histopathologic changes in mouse hepatic tissues (hematoxylin and eosin staining, ×40). No steatosis was observed in the control group (A), and numerous lipid empty vacuoles were seen and indicated with arrows in the high-fat milk group (B).

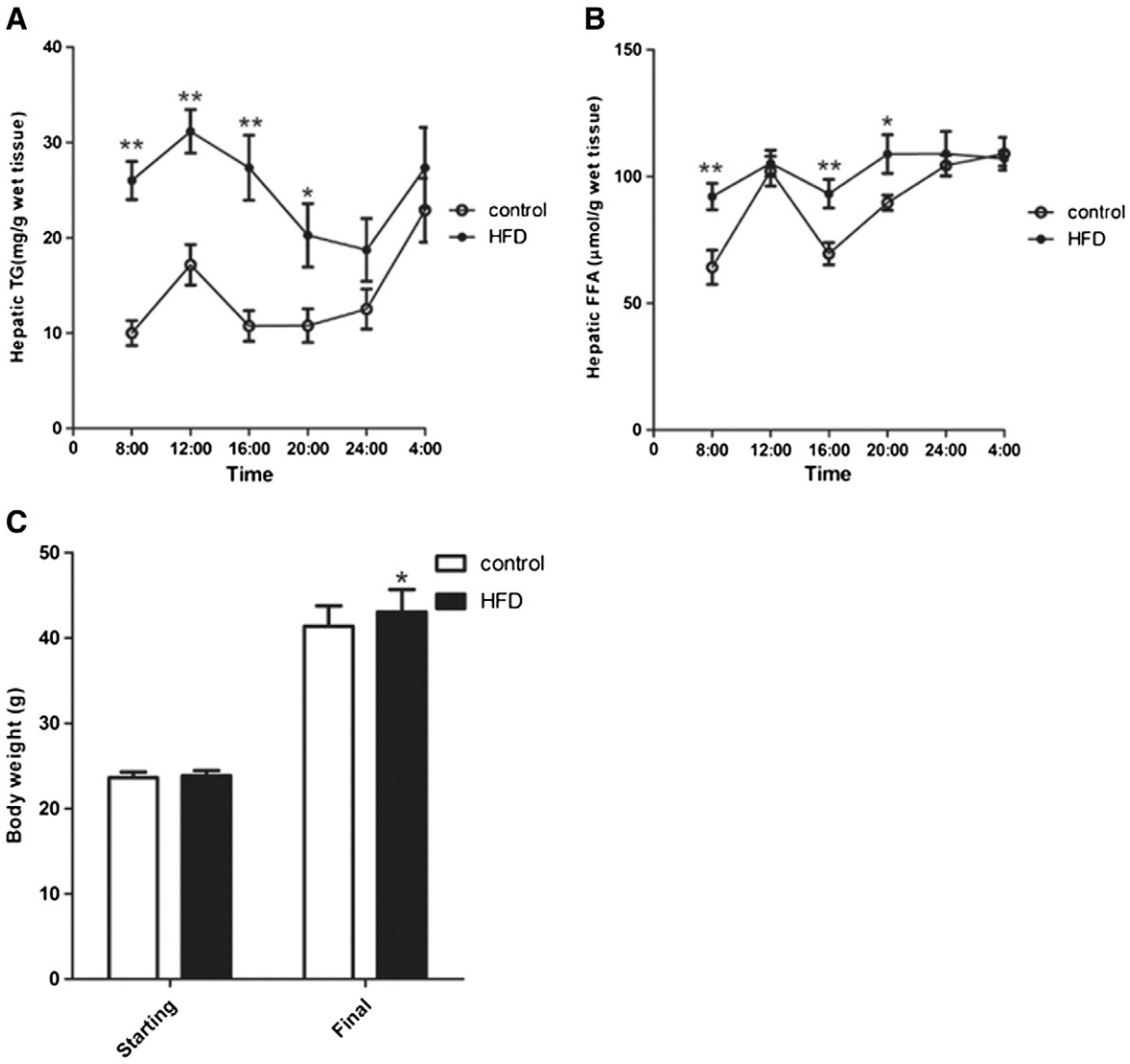

Fig. 5 – Hepatic TG and FFA contents as well as body weight, in mice fed with high-fat milk every evening for 6 weeks. A, Triglyceride content was detected by colorimetric method (n = 6 mice per group). B, Free fatty acid content was detected by colorimetric method (n = 6 mice per group). C, Body weight (n = 36 mice per group). Abbreviation: HFD, high-fat diet. Values are expressed as means ± SD. $^{*}P < .05$, $^{**}P < .01$ vs control group at the same time points.

Acetyl-CoA carboxylase 1α is a prominent regulator of de novo fatty acid synthesis [25], and CPT-1 brings fatty acids to the mitochondria for β-oxidation [26]. In this study, we also found that the changes of *ACC-1α* and *CPT-1* were just opposite over light/dark cycles in the high-fat diet-fed mice, namely, the former increased when the latter decreased, suggesting that the temporal expression between the 2 genes is full coordination in the high-fat diet-fed mice. But total expression levels of *ACC-1α* and *CPT-1* across all 6 time points were not high and low, respectively. This result may be due to organism response to high-fat diet. A previous report revealed that a constant high-fat diet across 24-hour light/dark cycles reduces the expression and activity of ACC-1α [27]. However, hepatic FFA and TG contents were high, and the hepatic lipid empty vacuoles were observed in the high-fat diet mice.

In conclusion, our results demonstrate that timed high-fat diet in the evening can change hepatic AMPK protein levels, activity, and circadian rhythm, which may subsequently alter hepatic clock gene expression and finally result in fatty liver formation. These findings further verify that AMPK may be a potential target for drug action in fatty liver therapy and suggest that avoidance of high-fat diet at night may be favorable for the prevention of fatty liver. However, our research is limited in some aspects. The mechanism by which high-fat diet affects the hepatic AMPK must be investigated further. The exact interactions between AMPK and hepatic clock genes must be also elucidated by possibly using related agonists or gene overexpression methods in cultured hepatocytes in vitro.

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