Clock mutation facilitates accumulation of cholesterol in the liver of mice fed a cholesterol and/or cholic acid diet

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Kudo T, Kawashima M, Tamagawa T, Shibata S. Clock mutation facilitates accumulation of cholesterol in the liver of mice fed a cholesterol and/or cholic acid diet. Am J Physiol Endocrinol Metab 294: E120–E130, 2008. First published October 30, 2007; doi:10.1152/ajpendo.00061.2007.—Cholesterol (CH) homeostasis in the liver is regulated by enzymes of CH synthesis such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and catabolic enzymes such as cytochrome P-450, family 7, subfamily A, and polypeptide 1 (CYP7A1). Since a circadian clock controls the gene expression of these enzymes, these genes exhibit circadian rhythm in the liver. In this study, we examined the relationship between a diet containing CH and/or cholic acid (CA) and the circadian regulation of Hmgcr, low-density lipoprotein receptor (Ldlr), and Cyp7a1 gene expression in the mouse liver. A 4-wk CA diet lowered and eventually abolished the circadian expression of these genes. Not only clock genes such as period homolog 2 (Drosophila) (Per2) and brain and muscle arnt-like protein-1 (Bmal1) but also clock-controlled genes such as Hmgcr, Ldlr, and Cyp7a1 showed a reduced and arrhythmic expression pattern in the liver of Clock mutant mice. The reduced gene expression of Cyp7a1 in mice fed a diet containing CA or CH + CA was remarkable in the liver of Clock mutants compared with wild-type mice, and high liver CH accumulation was apparent in Clock mutant mice. In contrast, a CH diet without CA only elevated Cyp7a1 expression in both wild-type and Clock mutant mice. The present findings indicate that normal circadian clock function is important for the regulation of CH homeostasis in the mouse liver, especially in conjunction with a diet containing high CH and CA.

Circadian rhythm is a physiological phenomenon, such as body temperature, food intake, and the sleep-wake cycle, controlled by a circadian clock (15). The molecular mechanism of the circadian oscillator is believed to be operated by a transcriptional-translational feedback loop of so-called clock genes (18). In addition to clock genes (31, 37), clock-controlled genes (29, 31, 36) have been discovered through DNA microarray analysis, and hundreds of genes showing circadian oscillation in their expression pattern have been revealed (31). Of these genes, CH metabolism-related genes such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), a rate-limiting enzyme of cholesterol (CH) synthesis (6), LDL receptor (Ldlr), responsible for CH uptake into the liver (5), and cholesterol-7a-hydroxylase (Cyp7a1), associated with CH degradation, can be found in the mouse liver (3, 31, 36). It has been well established that in rats maintained on a light-dark cycle, bile acid synthesis and CYP7A1 expression exhibit a circadian cycle in which expression is greatest just prior to the lights-off time (9). Throughout the circadian cycle, transcription of the Cyp7a1 gene correlates with hepatic expression of the basic leucine zipper transcription factor Dbp (albumin promoter D-site binding protein) (17). Thus, several papers have noted an important relationship between circadian clock function and CH homeostasis in the liver.

The synthesis and excretion of bile acids comprise the major pathway of CH catabolism in mammals. Bile acid synthesis is tightly regulated via a negative feedback mechanism to ensure that sufficient amounts of CH are catabolized to maintain homeostasis. The accumulation of bile acids causes a decrease in the transcription of Cyp7a1, which encodes the first and rate-limiting enzyme in the major biosynthetic pathway (23), and the loss of bile acids results in enhanced transcription from this gene and a subsequent increase in biosynthetic output. On the other hand, Cyp7a1 gene expression is known to be positively regulated by CH-rich diets (32), resulting in the production of bile acids in response to CH diets. Because a previous paper reported that a 30-day CH-rich diet enhances liver and serum CH (32), we decided to investigate CH, cholic acid (CA), and CH + CA diets for 4 wk in the present experiment to better understand how these diets impact the circadian pattern of Hmgcr, Ldlr, and Cyp7a1 gene expression in the liver of intact mice.

We also examined the gene expression of Liver X receptor-α (Lxra) and sterol regulatory element-binding protein 2 (Srebp2) in the liver of mice fed CH + CA diets. Lxra is activated by oxysterols acting as ligands (34) to mediate Cyp7a1 transactivation by binding to an LXR regulation element in the promoter (13). Srebp2 is known to regulate a large pool of target genes, such as Hmgcr in the liver (1), involved in CH homeostasis. The DNA binding specificity of SREBP is not restricted to the sterol response element. Instead, specificity extends even further to include a short palindromic repeat known as the E-box, which is, interestingly, the main hexameric sequence driving circadian control of clock and clock-controlled gene transcription (14, 22). Because the Srebp2 promoter has an E-box, it is possible that Srebp2 gene expression shows a circadian rhythm (33).

The liver and intestine are essential to normal high-density lipoprotein (HDL) CH production in two ways: the production and the stabilization of apolipoprotein A-I (Apoa1) in HDL particles. Apoa1 and ATP-binding cassette, subfamily A, member 1 (Abca1) are major HDL-modulating genes. To prevent the toxicity associated with CH overload, cells transport excess CH across the plasma membrane in part through the ABCA1 lipid transporter (2). In relation to serum HDL content, we...
examined the expression of Apoal and Abca1 genes in mice fed a CH + CA diet. Since the transcription of Apoal and Abca1 is controlled by nuclear receptors (7, 24), a circadian rhythm may be evident.

Clock mutant mice have been used as circadian-abnormal mice in the fields of reproduction (12, 21) and lipid homeostasis (27, 35). Many studies have demonstrated that Clock mutant mice show a low and arrhythmic pattern of Per1, Per2, and Dbp gene expression in the liver and heart (11, 28). To elucidate whether CH homeostasis is still negatively or positively regulated by a CH + CA diet under Clock mutation, we examined the effects of CH, CA, and CH + CA diets on the circadian pattern of Hmger, Ldlr, and Cyp7a1 gene expression in the liver of Clock mutant mice. In this experiment, we used CH content in the liver and serum as an index of abnormality for CH homeostasis.

There are two working hypotheses for the regulation of Hmger, Ldlr, and Cyp7a1 gene expression in mice fed a CA or CH + CA diet. One hypothesis is that a CH + CA diet reduces Hmger, Ldlr, and Cyp7a1 gene expression by targeting clock gene expression. The other is that a CH + CA diet targets directly the expression of these three genes. To test these hypotheses, we examined liver Per2, brain and muscle arnt-like protein-1 (Bmal1), and Dbp gene expression in mice fed a CH + CA diet.

MATERIALS AND METHODS

General conditions of animals and housing. Clock mutant mice were purchased from The Jackson Laboratory (stock no. 002923; Bar Harbor, ME), and 6- to 8-wk-old male wild-type and Clock mutant mice were backcrossed using a C57-ICR background more than eight times. Mice were maintained on a 12:12-h light-dark cycle (lights on at 8:00 AM, room temperature of 23 ± 1°C). During the light period, light intensity was 100–150 lux at cage level. Mice were fed a normal diet (ND) and allowed water ad libitum.

Diet composition (normal, CA, CH, and CH + CA diets). The nutraceutical composition of a standard diet (ND; Oriental Yeast, Tokyo, Japan) is composed of 6.1% carbohydrate, 23.6% protein, and 5.3% lipid. Control mice were fed an ND, and experimental mice were fed a CA diet (0.25% CA, wt/wt added to the standard diet), CH diet (2% lipid), or CH + CA diet (0.25% CA, wt/wt added to the standard diet) and allowed water ad libitum.

Description of the time course of CH accumulation. To examine the time course of CH accumulation after a CH + CA diet, mice (wild type: n = 16; Clock mutant: n = 17) were placed on a CH + CA diet for 0 (wild type: n = 3; Clock mutant: n = 4), 1 (wild type: n = 4; Clock mutant: n = 4), 2 (wild type: n = 4; Clock mutant: n = 4), and 4 wk (wild type: n = 5; Clock mutant: n = 5). The liver and serum were then obtained to measure CH.

Animal groups and treatment. Wild-type and Clock mutant mice were fed an ND (wild type: n = 30; Clock mutant: n = 30) or a CA diet (wild type: n = 30; Clock mutant: n = 30; CH + CA diet: wild type: n = 30; Clock mutant: n = 30), CH (wild type: n = 30; Clock mutant: n = 30), or CH + CA diet (wild type: n = 30; Clock mutant: n = 30) diet for 4 wk. After 4 wk, the liver and serum from each mouse were collected every 4 h at zeitgeber times (ZT0, 4, 8, 12, 16, and 20 (ZT0 was lights on and ZT12 was lights off).

Statement of animal care regulations. Animal care and experiments were treated in accordance with the Regulations for Animal Experimentation at Waseda University and were approved by the Experimental Animal Welfare Committee at the School of Science and Engineering at Waseda University (permission no. 05G19).

RNA isolation and real-time RT-PCR. Mice were deeply anesthetized with ether, and their livers were rapidly isolated. Total RNA was extracted using ISOGEN Reagent (Nippon Gene, Tokyo, Japan). Fifty nanograms of total RNA was reverse transcribed and amplified using the One-Step SYBR RT-PCR Kit (TaKaRa, Otsu, Japan) in the iCycler (Bio-Rad, Hercules, CA). Specific primer pairs were designed on the basis of the following published data on the Abca1, Apoal, β-actin, Bmal1, Cyp7a1, Dbp, Hmger, Ldlr, Lxrα, Per2, and Srebp2 genes in GenBank: Abca1 (103 bp, GenBank NM_013454, 4,015–4,117); 5′-ATTGCCAGACGGGGCCG-3′ (forward) and 5′-TGGCCAAAGGTGGCACCA-3′ (reverse); Apoal (129 bp, GenBank NM_009692, 16–144); 5′-CTGGCCCGTGT-GCTGTCTCCTC-3′ (forward) and 5′-GCTGTCTTGTACCTGGCACCA-3′ (reverse); β-actin (131 bp, GenBank AK075973, 1,009–1,139); 5′-TGACAGATTGCGAGAAGG-3′ (forward) and 5′-GCTGGAGGGTGACGTAG-3′ (reverse); Bmal1 (71 bp, GenBank AB014494, 2,407–2,477); 5′-CCACCTCGAGCCCAT-TGAATA-3′ (forward) and 5′-GAGCGGTTAGTTGACCUA-3′ (reverse); Cyp7a1 (244 bp, GenBank NM_007824, 746–988); 5′-AGACCGCATAAAGCGCCG-3′ (forward) and 5′-CTTCTTCGCTTGAGCGGCTC-3′ (reverse); Dbp (105 bp, GenBank NM_016974, 984–1,087); 5′-CCGCTGGAGGTGCAAATGACCT-3′ (forward) and 5′-CCTCTGAGAAGCGGTTGT-3′ (reverse); Hmger (201 bp, GenBank NM_008255, 2,303–2,503); 5′-TACAACCGCCAGCGACAA-3′ (forward) and 5′-ACACACCTTCTACTCAACGCAA-3′ (reverse); Ldlr (142 bp, GenBank NM_010700, 2,062–2,203); 5′-AGCATTATTCCAGTGGC-3′ (forward) and 5′-GAGGGGCTGTGTGTCACAT-3′ (reverse); Per2 (142 bp, GenBank AF036893, 5,563–5,704); 5′-TGCTGTCTCACCGCGGTG-3′ (forward) and 5′-ACGGTTGTGTTTGGCGCATG-3′ (reverse); Srebp2 (131 bp, GenBank NM_033218, 23–153); 5′-GGCTGTCCGAGCATGCTGA-3′ (forward) and 5′-ACAAAGTGTGCTTGAACAAATC-3′ (reverse). RT-PCR was executed under the following conditions: cDNA synthesis at 42°C for 15 min followed by 95°C for 2 min, PCR amplification for 40 cycles with denaturation at 95°C for 5 s, and annealing and extension at 60°C for 20 s. The relative light unit of the target gene PCR products was normalized to that of β-actin. A melt curve analysis was then performed.

Assay for liver or serum CH content. An assay for liver CH content was performed on the basis of the Yokode et al. (38) method. After

Fig. 1. Time course for accumulation of cholesterol (CH) in the serum and liver. Six- to 8-wk-old wild-type and Clock mutant mice were fed a CH + cholic acid (CA) diet for 0, 1, 2, or 4 wk. Mouse liver and serum were collected every 4 h at zeitgeber times (ZT0, 4, 8, 12, 16, and 20 (ZT0 was lights on and ZT12 was lights off), and 5′-GGCTGTCCGAGCATGCTGA-3′ (forward) and 5′-ACAAAGTGTGCTTGAACAAATC-3′ (reverse). RT-PCR was executed under the following conditions: cDNA synthesis at 42°C for 15 min followed by 95°C for 2 min, PCR amplification for 40 cycles with denaturation at 95°C for 5 s, and annealing and extension at 60°C for 20 s. The relative light unit of the target gene PCR products was normalized to that of β-actin. A melt curve analysis was then performed.

Assay for liver or serum CH content. An assay for liver CH content was performed on the basis of the Yokode et al. (38) method. After

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mice were anesthetized with ether and killed. 0.2 g of liver tissue was homogenized in a Polytron homogenizer with 4 ml chloroform-methanol (2:1, vol/vol), and then 0.8 ml of 50 mM sodium chloride was added. A sample (50 μl) of the organic phase was mixed with 7.5 mg of Triton X-100. After evaporation of the organic solvents, the lipid in the detergent phase was used to measure total and unesterified CH content with the Cholesterol/Cholesteryl Ester Quantitation Kit (Calbiochem, Darmstadt, Germany). Blood samples (500 – 750 μl) from each mouse were centrifuged, and serum was obtained. A sample (20 μl) of the serum from each mouse was used to obtain total and HDL CH content with the Cholesterol E-test Wako (Wako Pure Chemical Industries, Osaka, Japan) or the HDL Cholesterol E-test Wako (Wako Pure Chemical Industries). Total CH was measured by a CH oxidase-3,5-dimetoxy-N-ethyl-N-(2-hydroxy-3-sulfopropyl)aniline sodium (DAOS) assay. In brief, CH ester was cleaved into CH and fatty acid by CH esterase. CH was oxidized by CH oxidase and produced hydrogen peroxide. Substrates produced blue pigments from hydrogen peroxide and peroxidase. Optical density was measured as total CH concentration. For HDL CH measurements, non-HDL CH was first selectively precipitated out by phosphotungstic acid and magnesium salt, and then HDL CH was measured by the CH oxidase-DAOS assay. Wako kits can measure serum total and HDL CH. Non-HDL CH values were obtained by subtracting HDL CH from total CH. These kits were widely used for the experiments of rodents.

Statistical analysis. Values are expressed as means ± SE. For statistical analysis, Student’s t-test and one- or two-way ANOVA was applied and post hoc analysis conducted in addition to the Fisher protected least significant difference (PLSD) test.

RESULTS

Time course of CH accumulation in the liver and serum. We examined the time course of liver and serum CH content in mice fed a CH + CA diet for 0, 1, 2, and 4 wk. CH content was time-dependently elevated in the liver of both wild-type and Clock mutant mice fed CH + CA; however, liver CH was higher in Clock mutant mice compared with wild-type mice during the 4-wk CH + CA diet period (Student’s t-test, 4 wk: \( P < 0.05 \); Fig. 1A). Although there were no differences in unesterified CH content between genotypes (Fig. 1B), Clock mutant mice showed higher values of esterified CH content at 4 wk compared with wild-type mice (Student’s t-test, 4 wk: \( P < 0.05 \); Fig. 1C). Concerning total CH, HDL CH, and non-HDL CH in the serum, there were no differences between the two genotypes (Fig. 1, D, E, and F).

Effect of a CA diet on liver Hmgcr, Ldlr, Cyp7a1, and Srebp2 gene expression. The expression pattern of liver Hmgcr, Ldlr, and Cyp7a1 genes and the upstream Srebp2 gene were inspected in mice fed an ND or CA diet. Regarding the Hmgcr gene in wild-type mice, ND-fed mice showed a significant rhythmicity (1-way ANOVA, \( F[5,24] = 3.106, \ P < 0.05 \); Fig. 2A).
Fig. 2A), but in CA-fed mice this rhythmicity completely disappeared (1-way ANOVA, \(F[5, 12] = 2.429\), no significance (NS); Fig. 2A). With regard to Hmger gene expression in Clock mutant mice, ND- and CA-fed mice did not have any notable rhythmicity (1-way ANOVA: ND, \(F[5, 24] = 1.023\), NS; CA, \(F[5, 12] = 2.429\), NS; Fig. 2B). Both wild-type and Clock mutant mice fed a CA diet had remarkably decreased mean daily levels of Hmger gene expression (Fisher PLSD test: ND, \(P < 0.01\); CA, \(P < 0.01\); Fig. 2C). Hmger gene expression in Clock mutant mice was significantly less than that in wild-type mice fed an ND (Fisher PLSD test: ND, \(P < 0.05\); Fig. 2C).

Wild-type mice exhibited clear rhythmicity in Ldlr gene expression on an ND (1-way ANOVA: \(F[5, 24] = 7.443\), \(P < 0.01\); Fig. 2D), but this rhythmicity was lost on a CA diet (1-way ANOVA: \(F[5, 12] = 1.765\), NS; Fig. 2D). In Clock mutant mice of both ND- and CA-fed groups, there was no noteworthy rhythmicity of Ldlr gene expression (1-way ANOVA: ND, \(F[5, 24] = 0.446\), NS; CA, \(F[5, 12] = 1.757\); NS; Fig. 2E). CA diet led to a remarkably decreased mean daily level of Ldlr gene expression in wild-type and Clock mutant mice (Fisher PLSD test: wild type, \(P < 0.01\); Clock mutant, \(P < 0.01\); Fig. 2F). Ldlr gene expression in Clock mutant mice was significantly lower than that in wild-type mice on an ND or CA diet (Fisher PLSD test: ND, \(P < 0.01\); CA, \(P < 0.01\); Fig. 2F).

ND-fed wild-type mice displayed significant rhythmicity in Cyp7a1 gene expression (1-way ANOVA: \(F[5, 24] = 0.937\), \(P < 0.05\); Fig. 2G) compared with CA-fed wild types (1-way ANOVA: \(F[5, 12] = 2.370\), NS; Fig. 2G). In Clock mutant mice, both ND- and CA-fed groups did not show any rhythmicity in Cyp7a1 gene expression (1-way ANOVA: ND, \(F[5, 24] = 1.144\), NS; CA, \(F[5, 12] = 1.801\), NS; Fig. 2H). Mean daily levels of Cyp7a1 gene expression were strongly reduced in wild-type and Clock mutant mice on a CA diet (Fisher PLSD test: wild type, \(P < 0.01\); Clock mutant, \(P < 0.01\); Fig. 2I), and Cyp7a1 gene expression in Clock mutant mice was significantly smaller than that in wild-type mice on an ND (Fisher PLSD test: ND, \(P < 0.01\); Fig. 2I).

ND- and CA-fed wild-type mice exhibited a significant rhythmicity in Srebp2 gene expression (1-way ANOVA: ND, \(F[5, 24] = 11.316\), \(P < 0.01\); CA, \(F[5, 12] = 6.682\), \(P < 0.01\); Fig. 2J). In Clock mutant mice, both ND- and CA-fed groups did not show any rhythmicity in Srebp2 gene expression (1-way ANOVA: ND, \(F[5, 24] = 0.467\), NS; CA, \(F[5, 12] = 1.561\); NS; Fig. 2K). Mean daily levels of Srebp2 gene expression were significantly dropped in wild-type and Clock mutant mice on a CA diet (Fisher PLSD test: wild type, \(P < 0.01\); Clock mutant, \(P < 0.01\); Fig. 2L), and Srebp2 gene expression in Clock mutant mice was significantly less than that in wild-type mice on a CA diet (Fisher PLSD test: ND, \(P < 0.01\); Fig. 2L).
wild-type mice on an ND or CA diet (Fisher PLSD test: ND, P < 0.01; CA, P < 0.01; Fig. 2L).

Effect of a CA diet on CH content. Irrespective of food conditions, wild-type and Clock mutant mice did not show any rhythmicity in serum CH (1-way ANOVA: ND wild type, F[5, 12] = 2.771, NS; ND Clock mutant, F[5, 12] = 0.217, NS; CA wild type, F[5, 12] = 1.814, NS; CA Clock mutant, F[5, 12] = 1.974, NS; Fig. 3, A and B). Serum CH concentration did not differ between wild-type and Clock mutant groups fed a CA diet (Fisher PLSD test: wild type, P > 0.05; Clock mutant, P > 0.05; Fig. 3C).

Regardless of the food conditions, serum HDL CH was unaffected by Clock mutation (1-way ANOVA: ND wild type, F[5, 12] = 1.158, NS; ND Clock mutant, F[5, 12] = 1.741, NS; CA wild type, F[5, 12] = 0.679, NS; CA Clock mutant, F[5, 12] = 0.942, NS; Fig. 3, D, E, and F). Furthermore, wild-type and Clock mutant mice did not possess any clear rhythmicity in serum non-HDL CH (1-way ANOVA: ND wild type, F[5, 12] = 2.594, NS; ND Clock mutant, F[5, 12] = 0.382, NS; CA wild type, F[5, 12] = 1.663, NS; CA Clock mutant, F[5, 12] = 2.037, NS; Fig. 3, G and H). Serum non-HDL CH concentration did not show any changes in wild-type or Clock mutant mice fed a CA diet (Fisher PLSD test: wild type, P > 0.05; Clock mutant, P > 0.05; Fig. 3I), but liver CH content was significantly elevated in both groups of mice (Fisher PLSD test: wild type, P < 0.01; Clock mutant, P < 0.01; Fig. 3J). Despite the fact that there were no differences in liver CH between genotypes fed an ND, liver CH in Clock mutant mice was significantly higher than that in wild-type mice on a CA diet (Fisher PLSD test: ND, NS; CA, P < 0.01; Fig. 3J).

Effect of a CH + CA diet on liver Hmgcr, Ldlr, and Cyp7a1 gene expression. To clarify the impact of a CH + CA diet, we examined Hmgcr, Ldlr, and Cyp7a1 gene expression in the liver of mice on a CH + CA diet. With a CH + CA diet, Hmgcr gene expression in wild-type mice was weak but of significant rhythmicity (1-way ANOVA: F[5, 24] = 3.106, P < 0.01; Fig. 4A), and the amplitude was reduced compared with ND-fed mice. When fed the same CH + CA diet, Clock mutant mice did not display rhythmicity (1-way ANOVA: F[5, 24] = 2.283, NS; Fig. 4B). Mean daily levels of Hmgcr gene expression were considerably less in both wild-type and Clock mutant mice on a CH + CA diet (Fisher PLSD test: wild type, P < 0.01; Clock mutant, P < 0.01; Fig. 4C). Hmgcr gene expression in Clock mutant mice was significantly less than that in wild-type mice on an ND (Fisher PLSD test: P < 0.01; Fig. 4C).

Ldlr gene expression in wild-type mice fed a CH + CA diet had no rhythmicity (1-way ANOVA: F[5, 24] = 2.265, NS; Fig. 4D), in contrast to that in Clock mutant mice (1-way ANOVA: F[5, 24] = 3.696, P < 0.05; Fig. 4E). Mean daily level of the Ldlr gene was significantly reduced in wild-type and Clock mutant mice on a CH + CA diet (Fisher PLSD test: wild type, P < 0.01; Clock mutant, P < 0.01; Fig. 4F). Ldlr gene expression in Clock mutant mice was significantly less than that in wild-type mice on an ND or CH + CA diet (Fisher PLSD test: ND, P < 0.01; CH + CA, P < 0.01; Fig. 4F).

Cyp7a1 gene expression in wild-type mice still had significant rhythmicity under CH + CA diet conditions (1-way
ANOVA: \( F[5, 24] = 4.180, P < 0.01 \); Fig. 4G), but expression in *Clock* mutant mice did not show any rhythmicity (1-way ANOVA: \( F[5, 24] = 1.993, \text{NS}; \) Fig. 4H). Mean daily level of *Cyp7a1* gene expression was significantly lower in both wild-type and *Clock* mutant mice under a CH + CA diet (Fisher PLSD test: wild type, \( P < 0.01 \); *Clock* mutant, \( P < 0.01 \); Fig. 4I). *Cyp7a1* gene expression in *Clock* mutant mice was less than that in wild-type mice on ND and CH + CA diets (Fisher PLSD test: ND, \( P < 0.01 \); CH + CA, \( P < 0.05 \); Fig. 4I).

Effect of a CH + CA diet on liver *Lxra*, *Abca1*, and *Apoal* and *Srebp2* gene expression. The expression pattern of liver *Lxra*, *Abca1*, *Apoa1*, and *Srebp2* genes was examined in mice fed an ND or CH + CA diet. There was no rhythmicity to *Lxra* gene expression in wild-type mice (1-way ANOVA: ND, \( F[5, 24] = 0.224, \text{NS}; \) CH + CA, \( F[5, 24] = 1.158, \text{NS}; \) Fig. 5A) or *Clock* mutant mice (1-way ANOVA: ND, \( F[5, 24] = 0.991, \text{NS}; \) CH + CA, \( F[5, 24] = 0.373, \text{NS}; \) Fig. 5B). A CH + CA diet led to a reduced mean daily level of *Lxra* gene expression in *Clock* mutant mice (Fisher PLSD test: \( P < 0.01 \); Fig. 5C).

Regarding the *Abca1* gene, there was no rhythmicity in wild-type mice (1-way ANOVA: ND, \( F[5, 24] = 1.683, \text{NS}; \) CH + CA, \( F[5, 24] = 1.198, \text{NS}; \) Fig. 5D) or *Clock* mutant mice (1-way ANOVA: ND, \( F[5, 24] = 0.413, \text{NS}; \) CH + CA, \( F[5, 24] = 0.561, \text{NS}; \) Fig. 5E). A CH + CA diet led to a strong decrease in the mean daily levels of *Abca1* gene expression in *Clock* mutant mice (Fisher PLSD test: \( P < 0.01 \); Fig. 5F).

As shown in Fig. 5, G–I, the expression pattern of *Apoa1* was similar to that of *Abca1*. There was no significant rhythmicity in wild-type mice (1-way ANOVA: ND, \( F[5, 24] = 0.708, \text{NS}; \) CH + CA, \( F[5, 24] = 0.308, \text{NS}; \) Fig. 5G) or *Clock* mutant mice (1-way ANOVA: ND, \( F[5, 24] = 0.708, \text{NS}; \) CH + CA, \( F[5, 24] = 0.308, \text{NS}; \) Fig. 5H). A CH + CA diet resulted in reduction of mean daily levels of *Apoa1* gene expression in wild-type and *Clock* mutant mice (Fisher PLSD test: wild type, \( P < 0.01 \); *Clock* mutant, \( P < 0.01 \); Fig. 5I).

*Srebp2* gene expression rhythmicity was not significant in wild-type mice (1-way ANOVA: \( F[5, 24] = 0.710, \text{NS}; \) Fig. 5J) or *Clock* mutant mice (1-way ANOVA: \( F[5, 24] = 1.702, \text{NS}; \) Fig. 5K) fed a CH + CA diet. Mean daily level of *Srebp2* gene expression was significantly reduced in both wild-type and *Clock* mutant mice on a CH + CA diet (Fisher PLSD test: wild type, \( P < 0.01 \); *Clock* mutant, \( P < 0.01 \); Fig. 5L). *Srebp2* gene expression in *Clock* mutant mice was significantly lower than that in wild-type mice in both the ND and CH + CA conditions were compared using Student’s *t*-test or 1-way ANOVA. \(*P < 0.05, **P < 0.01 \) (wild type vs. *Clock* mutant). \( 
\text{\##}P < 0.01 \) (ND vs. CH + CA). The ND data are the same as that for *Srebp2* in Fig. 2.

**Fig. 5.** Effect of a CH + CA diet on liver gene expression. The sample for this experiment was exactly the same as for Fig. 4, except that *Lxra* (A–C), ATP-binding cassette, subfamily A, member 1 (*Abca1*; D–F), apolipoprotein A-I (*Apoa1*; G–I), and *Srebp2* (J–L) gene expression was examined in the liver. Values represent means ± SE. Genotypes and food conditions were compared using Student’s *t*-test or 1-way ANOVA. \(*P < 0.05, **P < 0.01 \) (wild type vs. *Clock* mutant). \( 
\text{\##}P < 0.01 \) (ND vs. CH + CA). The ND data are the same as that for *Srebp2* in Fig. 2.
groups (Fisher PLSD test: ND, P < 0.01; CH + CA, P < 0.01; Fig. 5L).

Effect of a CH + CA diet on CH content. Wild-type and Clock mutant mice fed a CH + CA diet did not show any significant rhythmicity in serum CH (1-way ANOVA: wild type, F[5, 12] = 0.878, NS; Clock mutant, F[5, 12] = 1.099, NS; Fig. 6A). Serum CH concentration did not differ between wild-type and Clock mutant groups of mice on a CH + CA diet (Fisher PLSD test: wild type, P > 0.05; Clock mutant, P > 0.01; Fig. 6B).

Wild-type and Clock mutant mice did not display any rhythmicity in serum HDL CH (1-way ANOVA: wild type, F[5, 12] = 1.491, NS; Clock mutant, F[5, 12] = 1.572, NS; Fig. 6C). Serum HDL CH concentration values were almost the same for both wild-type and Clock mutant groups of mice fed a CH + CA diet (Fisher PLSD test: wild type, P > 0.05; Clock mutant, P > 0.05; Fig. 6D).

With the CH + CA diet, wild-type and Clock mutant mice did not display a significant rhythmicity in serum non-HDL CH (1-way ANOVA: wild type, F[5, 12] = 0.956, NS; Clock mutant, F[5, 12] = 0.983, NS; Fig. 6E). Serum non-HDL CH concentration did not show any changes in both wild-type and Clock mutant groups of mice (Fisher PLSD test: wild type, P > 0.05; Clock mutant, P > 0.05; Fig. 6F).

Liver CH content was significantly elevated in both wild-type and Clock mutant mice on a CH + CA diet (Fisher PLSD test: wild type, P < 0.01; Clock mutant, P < 0.01; Fig. 6G), and Clock mutant mice showed significantly higher CH content in the liver compared with wild-type mice (Fisher PLSD test: P < 0.01; Fig. 6G).

Effect of a CH + CA diet on liver clock gene expression. With ND conditions, Per2, Bmal1, and Dbp gene expression in the liver of wild-type mice had a significant rhythmicity (1-way ANOVA: Per2, F[5, 24] = 11.979, P < 0.01; Bmal1, F[5, 24] = 5.947, P < 0.01; Dbp, F[5, 24] = 19.705, P < 0.01; Fig. 7, A–C), as was found to be the case with CH + CA diet conditions (1-way ANOVA: Per2, F[5, 24] = 12.548, P < 0.01; Bmal1, F[5, 24] = 6.385, P < 0.01; Dbp, F[5, 24] = 18.964, P < 0.01; Fig. 7, A–C).

Similar to findings from previous papers (11, 28), Per2, Bmal1, and Dbp gene expression in the liver of Clock mutant mice did not exhibit any circadian rhythmicity (1-way ANOVA: Per2, F[5, 24] = 0.943, NS; Bmal1, F[5, 24] = 1.316, NS; Dbp, F[5, 24] = 1.816, NS; Fig. 7, D–F). Per2 and Bmal1 were slightly decreased in Clock mice fed a CH + CA diet (Fig. 7, D and E), whereas Dbp gene expression in these mice was strongly diminished (Fig. 7F).

Effect of a CH diet on liver gene expression. We examined whether a CH diet caused an opposite effect on Hmgcr, Ldlr, Cyp7a1, and Srebp2 gene expression compared with a CA diet. Hmgcr gene expression in wild-type mice fed a CH diet showed an increase at ZT16 (Fisher PLSD test: ZT4, NS; ZT16,
CH diet and liver gene expression

**Hmgcr**

Clock +/-  

Clock -/

Daily levels

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**Ldlr**

Clock +/-  

Clock -/

Daily levels

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**Cyp7a1**

Clock +/-  

Clock -/

Daily levels

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**Srebp2**

Clock +/-  

Clock -/

Relative mRNA levels

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Fig. 8. Effect of a CH diet on liver gene expression. Six- to 8-wk-old wild-type and Clock mutant mice were fed a CH diet for 4 wk. Mouse liver and serum were collected at zeitgeber time (ZT)4 and ZT16. Hmgcr (A–C), Ldlr (D–F), Cyp7a1 (G–I), and Srebp2 (J–L) gene expression was examined in the liver (A, B, D, E, G, H, J, and K: n = 3–5 for each time point; C, F, I, and L: n = 3–5 or 6–10). Values represent means ± SE. Time and food conditions were compared using 1-way ANOVA. *P < 0.05 (ZT4 vs. ZT16). #P < 0.05. ##P < 0.01 (ND vs. CH). "+/+ vs. −/−.

P < 0.05; Fig. 8A), whereas that in Clock mutant mice did not differ (Fisher PLSD test: ZT4, NS; ZT16, NS; Fig. 8B). Concerning Hmgcr gene expression in daily levels, there were no variances between food conditions (Fisher PLSD test: wild type, NS; Clock mutant, NS; Fig. 8C). Ldlr gene expression in both wild-type mice (Fisher PLSD test: ZT4, NS; ZT16, NS; Fig. 8D) and Clock mutant mice (Fisher PLSD test: ZT4, NS; ZT16, NS; Fig. 8E) did not show day-night differences.

A CH diet increased Cyp7a1 gene expression slightly in wild-type mice (Fisher PLSD test: ZT4, NS; ZT16, NS; Fig. 8G) and strongly in Clock mutant mice at ZT14 (Fisher PLSD test: ZT4, P < 0.01; ZT16, NS; Fig. 8H). The mean daily level of Cyp7a1 gene expression was elevated in both wild-type and Clock mutant mice on a CH diet (Fisher PLSD test: wild type, P < 0.01; Clock mutant, P < 0.01; Fig. 8I). Regarding Srebp2 gene expression in wild-type mice, there were no contrasts between food conditions (Fisher PLSD test: ZT4, NS; ZT16, NS; Fig. 8J), but Clock mutant mice on a CH diet showed significantly lowered Srebp2 expression at ZT16 (Fisher PLSD test: ZT4, NS; ZT16, P < 0.01; Fig. 8K). For Srebp2 gene expression in daily levels, there were no significant differences between food conditions (Fisher PLSD test: wild type, NS; Clock mutant, NS; Fig. 8L).

**Effect of a CH diet on CH content.** Under a CH diet, serum CH was not increased in wild-type mice (Fisher PLSD test: ZT4, P > 0.05; ZT16, P > 0.05; Fig. 9A) or Clock mutant mice (Fisher PLSD test: ZT4, P > 0.05; ZT16, P > 0.05; Fig. 9B) at both ZT4 and ZT16. There were no significant differences in daily levels of serum CH in relation to food conditions in wild-type and Clock mutant mice (Fisher PLSD test: wild type, P > 0.05; Clock mutant, P > 0.5; Fig. 9C). Concerning serum HDL CH, there were no significant differences between food conditions at ZT4 and ZT16 in either wild-type mice (Fisher PLSD test: ZT4, NS; ZT16, NS; Fig. 9D) or Clock mutant mice (Fisher PLSD test: ZT4, NS; ZT16, NS; Fig. 9E). For the daily levels and serum HDL CH, there were no significant differences according to food conditions in wild-type or Clock mutant mice (Fisher PLSD test: wild type, NS; Clock mutant, NS; Fig. 9F). Serum non-HDL CH (Fig. 9G–I) was increased with a CH diet; however, there were no significant differences between ND and CH diet groups. Liver CH content was remarkably elevated in both wild-type and Clock mutant mice on a CH diet (Fisher PLSD test: wild type, P < 0.01; Clock mutant, P < 0.01; Fig. 9J).

**DISCUSSION**

The most important finding in this experiment was the CH + CA diet-related increase of CH content in the liver of Clock mutant mice compared with wild-type mice. In addition, with
a CA diet, CH content in the liver of Clock mutant mice was also significantly higher than that of wild-type mice, although the increase was only about one-third of that seen with the CH/H11001 CA diet. CH accumulation in the liver was augmented by Clock mutation combined with at least a 4-wk CA or CH/H11001 CA diet. Thus, CA in a diet was seen to facilitate CH accumulation in the liver of Clock mutant mice. There were significant differences between the two genotypes in liver-esterified CH, but there were no differences between genotypes in liver-unesterified CH. Mari et al. (20) reported that excessive mitochondrial free CH causes the progression of steatosis in more severe liver disease. In the present experiments, both wild-type and Clock mutant mice accumulated substantial amounts of unesterified CH, suggesting that some degree of liver toxicity cannot be ruled out in either group.

Presently, the reason underlying high CH accumulation in the liver of Clock mutant mice fed a CA or CH + CA diet remains obscure. We found lower Cyp7a1 expression in Clock mutant mice (12% in mice on an ND vs. CA diet, 11% in mice on an ND followed by a CH + CA diet) compared with wild-type mice (19% in mice on an ND followed by a CA diet, 18% in mice on a ND followed by a CH + CA diet). CYP7A1 is known as the key enzyme for CH catabolism in the liver (23), and Cyp7a1 expression in the liver is found to be strongly suppressed by CA application (19). Therefore, the CA in a CA or CH + CA diet may suppress CH catabolism in the liver of Clock mutant mice, resulting in the facilitation of CH accumulation in the liver. Contrary to a CA or CH + CA diet, a CH diet increased Cyp7a1 expression in the liver to a level that was almost identical in Clock mutant and wild-type mice. Such an observed increase in the present experiment supports a previous paper (32) that demonstrated a facilitatory effect of CH on Cyp7a1. Thus, a CH diet may affect CH accumulation and Cyp7a1 regulation equally in both wild-type and Clock mutant mice.

Among Per2, Bmal1, and Dbp genes, only Dbp expression was strongly suppressed in the liver of Clock mutant but not wild-type mice fed a CH + CA diet, although future experimentation is necessary to elucidate why. Because the promoter of the Cyp7a1 gene possesses the Dbp binding motif, the reduction of Dbp expression in CH/H11001 CA-fed Clock mutant mice may further reduce the expression of Cyp7a1 in the liver. Recently, Noshiro et al. (26) used Clock mutant mice to examine multiple mechanisms that regulate circadian expression of the Cyp7a1 gene and found that Dbp, Rev-erbA, and Dec2, which are all dominantly regulated by the CLOCK-BMAL1 heterodimer through the E-box, were abolished. In contrast, Rev-erbA, Lxr, Ppara, and E4bp4 expression remained in this mutant mouse. In their experiment, Dec2 expression was lowered, Lxra expression was enhanced, and Cyp7a1 expression was upregulated. In our experiment, the expression of E4bp4, Ppara (data not shown), and Lxra (Fig. 5) was unaffected by clock mutation, but Dec2 expression was lowered (data not shown) and Cyp7a1 expression reduced.

**Fig. 9.** Effect of a CH diet on CH content. Six- to 8-wk-old wild-type and Clock mutant mice were fed a CH diet for 4 wk. Mouse liver and serum were collected at ZT4 and ZT16. Serum CH (A–C), serum HDL CH (D–F), serum non-HDL CH (G–I), and liver CH (J) (n = 3–5 or 6–10) were measured. Values represent means ± SE. Time and food conditions were compared using 1-way ANOVA. ##P < 0.01 (ND vs. CH).
This discrepancy in the Cyp7a1 expression of Clock mutant mice may be related to the different Lxra expressions (upregulation in Noshiro’s data vs. downregulation in our data). Lxra is activated by oxysterols acting as ligands (34) to mediate Cyp7a1 transactivation, and dietary or endogenous CH is a source of oxysterols. Therefore, background differences in the Clock mutant mice, BALB/c for Noshiro’s data vs. ICR for our data, may have affected the source of oxysterols. Several studies have demonstrated that different background genes of Clock mutant mice affect lipid metabolism (16, 27, 35). Future examination of the effect of a CH and/or CA diet has on clock-controlled gene expression in Clock mutant mice of different background genes would aid in understanding the true effect of clock genes on CH metabolism.

Contrary to what we expected, there was no direct effect of CA or CH + CA diet on the circadian expression of liver Per2 and Bmal1 genes, although there is still the possibility that an indirect mechanism mediates metabolism-related genes. For example, diet components may somehow interfere in the binding of CLOCK-BMAL1 transcription factor to the E-box site in the promoter of clock-controlled genes such as Hmgcr, Ldlr, and Cyp7a1 or upstream regulators such as Srebp1 and Srebp2. Brewer et al. (4) found SREBP-1 to be a transcriptional integrator of circadian and nutritional cues in the liver when comparing the affinity of SREBP-1 for SRE-like and E-box sequences in the lipogenic gene promoters.

Hmger and Ldrl gene expression is known to be suppressed by CH through SRE-1 activation (10); however, in the present experiment, a CA or CH + CA diet similarly suppressed the expression of these genes in the liver of Clock mutant and wild-type mice. Reduced Hmger and Ldrl expression may be caused by Lxra and Srebpl gene products, since Clock mutant mice exhibited a lower level of gene expression than wild-type mice on a CA or CH + CA diet. Because HMGCR is a rate-limiting enzyme for CH synthesis in the liver, we could not exclude the possibility that CH synthesis through HMGCR liver enzyme function was less impaired and/or upregulated in Clock mutant mice, thereby leading to high CH accumulation in mice fed a CA or CH + CA diet. We believe CH efflux may have been reduced, and this reduced efflux contributed to hepatic CH accumulation.

When Abca1 and Apoa1 gene expression levels were examined, corresponding with the low level of Cyp7a1 gene expression, the gene expression level of Abca1 and Apoa1 was significantly lower in Clock mutant mice than in wild-type mice on a CH + CA diet. Since Abca1 and Apoa1 are known to modulate HDL CH in the serum, we observed serum CH, serum HDL CH, and serum non-HDL CH in wild-type and Clock mutant mice under various food conditions. Abca1 and Apoa1 expressions in Clock mutant mice were significantly lower than in wild-type mice on a CH + CA diet (Fig. 5, F and I), whereas serum HDL CH content in Clock mutant mice was not significantly different than in wild-type mice (Fig. 6D). Thus, the lower levels of Abca1 and Apoa1 expression were not related to serum HDL CH content. LXR activates the transcription of many known ABC CH transporters, including Abca1 (2). In the present experiment, Lxra gene expression was strongly reduced in the liver of Clock mutant mice fed a CH + CA diet, a result that may support the low level of Abca1 in Clock mutant mice. Because HDL particles can circulate and “pick up” additional lipids from extrahepatic tissues through both ABCA1-dependent and ABCA1-independent mechanisms, the ABCA1-independent mechanism may compensate for the reduction of serum HDL CH content in Clock mutant mice. Hepatocyte nuclear factor 4 (Hnf4a) is known to activate Apoa1 gene expression (24) as well as Cyp7a1 (26). In future experimentation, we hope to examine the gene expression of Hnf4a in Clock mutant mice fed CA and/or CH diets.

In the present experiment, Hmger, Ldlr, and Cyp7a1 gene expression was strongly suppressed by Clock mutation, since these genes are clock-controlled genes (3, 31, 36). Surprisingly, similar to the Clock mutation findings, a CA or CH + CA diet strongly suppressed the expression of Hmger, Ldlr, and Cyp7a1 in the liver of wild-type mice, and eventually the daily rhythm of these genes became weak. A so-called masking effect may have played a role in Hmger, Ldlr, and Cyp7a1 gene expression, since a CH + CA diet had no effect on clock genes such as Per2 and Bmal1. This study provides evidence that a CH + CA diet not only reduced the expression level of clock-controlled genes but also hindered the daily rhythm through a potential masking effect.

Clock mutant mice may function as a good animal model for circadian disorders related to reproduction (12, 21), sleep-wakefulness (25), and lipid metabolism (35). They can also model obesity and diabetes onset when placed on a high-fat diet (35) or obesity with an ob gene mutation vs. an intact ob gene (30). On the basis of our current findings, we believe Clock mutant mice may also act as a good animal model for circadian disorders related to CH metabolism.

Debruyne et al. (8) recently reported that Clock gene knockout mice had nearly normal daily cycles of activity, even in total darkness, in contrast to Clock mutant mice. But in Clock knockout mice, it is possible that when CLOCK is absent, neuronal PAS domain protein 2 (NPAS2) can take its place. Thus, further study is needed to elucidate the relationship between the Clock gene and CH metabolism.

In conclusion, our data show decreased Cyp7a1 gene expression in the liver of Clock mutant mice fed a CH + CA diet compared with wild-type mice fed the same diet. In Clock mutant mice, liver CH accumulation, especially esterified CH, was higher than that of wild-type mice. Consequently, the present findings indicate that normal circadian clock function was important for the regulation of CH homeostasis in the mouse liver, especially in conjunction with a diet containing high CH + CA.

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