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Original Articles

CLOCK/BMAL1 is Involved in Lipid Metabolism via Transactivation of the Peroxisome Proliferator-activated Receptor (PPAR) Response Element

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Lipid absorption and metabolism are regulated by feeding and by the circadian system. It has been suggested that the expression of enzymes involved in lipid metabolism is directly controlled by the clock system. This study was designed to examine whether or not the CLOCK/BMAL1 heterodimer has transcriptional activity for genes via the peroxisome proliferator-activated receptor response element (PPRE). Male mice 8-12 weeks old were maintained under a 12:12 hour light-dark cycle for at least two weeks before the day of the experiment. The mRNA profiles of BMAL1 and of the PPAR target genes acyl-CoA oxidase (AOX), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and cellular retinol binding protein II (CRBPII) were measured in intestine. The direct effects of CLOCK/BMAL1 on the promoter activities of those three enzymes were assessed *in vitro* by luciferase assay. The expression of PPAR target genes changed in a cyclical manner that followed expression of BMAL1. The promoter activities of the three enzymes were increased by CLOCK/BMAL1 expression. After deletion of the PPRE from the CRBPII construct, CLOCK/BMAL1 did not affect transactivation. CLOCK/BMAL1 transactivates PPAR target genes via the PPRE.*
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Key words: CLOCK, BMAL1, PPARα, acyl-CoA oxidase, HMG-CoA synthase, Cellular retinol binding protein II (CRBP II)

Introduction

The peroxisome proliferator-activated receptor (PPAR) is a member of the nuclear receptor superfamily (1). Three types of PPARs have been described in rodents, humans and amphibians: PPARα, PPARβ/PPARδ and PPARγ. We have shown that these subtypes are involved in lipid homeostasis in the vascular system (2–4). PPARα is expressed most abundantly in the liver where oxidation occurs in the mitochondria and peroxisomes (5). PPARβ/δ, which is expressed ubiquitously (6), activates genes of fatty acid oxidation (7) and is a target for the treatment of metabolic syndrome (8), cardiomyopathy (9), cholesterol metabolism (10), colon carcinogenesis (11), and obesity (12). PPARγ is a transcription factor expressed selectively in adipose tissue (13) and seems to be associated with adipocyte differentiation. Activation of PPARγ has been reported to result in lipid filling of macrophages by induction of CD36 (14), a class B scavenger receptor.

PPARα interacts not only with the nuclear hormone receptor superfamily but also with basic helix-loop-helix (bHLH)-PAS circadian transcription factors (15). CLOCK and BMAL1 are circadian genes encoding bHLH-PAS transcription factor (16). It has been demonstrated that

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the CLOCK/BMAL1 heterodimer plays an important role in lipid homeostasis by transactivating PPARα in mice and that the circadian expression of PPARα is directly regulated by CLOCK protein (17).

It is believed that the CLOCK/BMAL1 heterodimer drives transcription of *mPer* and *mCry* by binding to consensus E-box elements (18). On the other hand, Oishi *et al.* showed that the CLOCK/BMAL1 heterodimer transactivates the PPARα gene via an E-box-rich region located in the second intron (17).

Recently, McNamara *et al.* reported that RXRα can interact with CLOCK (9). RXRα has been shown to bind to PPARs, resulting in the formation of a heterodimer, which binds to the peroxisome proliferator response element (PPRE), followed by the activation of targets such as acyl-CoA oxidase (AOX), cellular retinol binding protein II (CRBPII), and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase (19, 20).

Therefore, by using the promoters of those three enzymes as reporters, we determined whether or not CLOCK/BMAL1 regulates PPRE-controlled target genes via transcriptional activity.

Materials and Methods

Mice

Male mice 8–12 weeks old were maintained under a 12:12 h light-dark cycle (lights on at 0:00 and lights off at 12:00) for at least two weeks before the day of the experiment. Dissected tissues were quickly frozen and stored at – 80°C.

Reverse transcription polymerase chain reaction (RT-PCR)

We performed real time RT-PCR (ABI PRISM 7700 and SYBR Green Reagents; Applied Biosystems, USA) to examine expression levels of the mRNAs for AOX, CRBPII, HMG-CoA synthase, and BMAL1. Total RNA, isolated from mouse small intestine with a commercial kit (Isogen, Nippon Gene, Japan), was employed as a template for DNA synthesis using oligo(dT) primer and a DNA cycle kit (GeneAmp RNA PCR Kit; Perkin Elmer, USA) according to the manufacturer's instructions. The RT reaction was performed at 42°C for 15 min to maximize cDNA synthesis and was terminated by heating at 95°C for 5 min. The resulting cDNA was used as the template for PCR. Oligonucleotide primers for RT-PCR of acyl-CoA oxidase, CRBPII, HMG-CoA synthase, and BMAL1 were designed to amplify partial cDNA sequences. The primers used for acyl-CoA oxidase were 5'-ATATTTACGTCACGTTTACCCCGG-3' for the forward primer and 5'-GGCAGGTCATTCAAGTACGACAC-3' for the reverse primer. The primers used for CRBPII were 5'-GTCCATATCAAACCAGAGGCCA-3' for the forward primer and 5'-TCTAGGGCCTTCATGTAGCCTT-3' for the reverse primer. The primers used for HMG-CoA synthase were 5'-AGGACATCAACTCCCTGTGCCT-3' for the forward primer and 5'-TGGAACAGTTCCATGAGCACTG-3' for the reverse primer. The primers used for BMAL1 were 5'-GGTTCTTTTATCACGCTACG-3' for the forward primer and 5'-GAGGCGTACTTGTGATGTTC-3' for the reverse primer. The PCR conditions were as follows: 10 min at 95°C, then 45 cycles of 15 s at 95°C, 60 s at 60°C and 1 min at 72°C.

Subcloning of open reading frame cDNAs encoding human CLOCK and BMAL1

We generated human ORF cDNAs for CLOCK and BMAL1 by the method reported previously (14). For both CLOCK and BMAL1, the fragment was subcloned into the *Sal* I/*Not* I site of pcDNA3 (Invitrogen Corporation, Carlsbad, CA, USA), and the vectors were named pcDNA-CLOCK and pcDNA-BMAL1, respectively. The sequences of the constructs described above were verified by automated sequencing.

Reporter assay for activation by CLOCK/BMAL1

Transient transfections for the reporter assay were performed with Tfx™-50 reagent (Promega) according to the manufacturer's recommended protocol. Human kidney 293T cells (293T cells) and CV-1 monkey kidney cells (CV-1 cells) were used because of their high transfection efficiency. First, we measured transcriptional activation of PPRE-containing genes by CLOCK/BMAL1. We isolated the promoters of AOX, CRBPII, and HMG-CoA synthase, which were ligated into the *Kpn* I/*Nco* I sites of the pGL3-Basic vector (Promega) upstream of the luciferase gene and termed pAOX-Luc, pCRBPII-Luc and pHMG-Luc, respectively. pAOX-Luc, pCRBPII-Luc, pHMG-Luc and pRL-TK, in the presence or absence of pcDNA-CLOCK and pcDNA-BMAL1, were co-transfected into cells, which were grown in 24-well plates. The total amount of DNA transfected (0.6 μg) was normalized with a carrier DNA (pcDNA3). Finally, luciferase activity for pAOX-Luc, pCRBPII-Luc and pHMG-Luc was normalized to *Renilla* luciferase activity. Both activities were measured according to the manufacturer's instructions (Promega).

Deletions in the CRBPII-Luc plasmid

The promoter of CRBPII contains four repeat sequences (5'AG(G/T)TCA-3')(5'-gtgtcccactctgctgtcacAGGTCAcAGGTCAcAGGTCAcAGTTCAtttacctgtctctgtc-3', from – 670 to + 63) (19). We generated the CRBPII-Luc plasmid (from – 670 to + 63) without this four-repeat sequence, naming it del-0-pCRBPII-Luc (5'-ctttacctgtctctgtc-3', from – 599 to + 63).

DNA sequencing

Direct sequencing of PCR products was performed with

an automated sequencer (ABI PRISM 310 Genetic Analyzer; Perkin Elmer, Forster City, CA, USA). All DNA sequences were confirmed by reading both DNA strands.

Statistical analysis

Experiments were performed in triplicate, and four independent experiments were performed. Parametric data are expressed as means ± SD. Differences between groups were evaluated with Scheffe's F test.

Results

To examine the expression profile of mRNA for AOX, HMG-CoA synthase, CRBPII and BMAL1 in mouse intestine, we performed real time RT-PCR, and attempted to demonstrate a daily rhythm in the expression of these mRNAs. The levels of the mRNAs were indeed found to change in a cyclical manner (Fig. 1A–D). The peak of BMAL1 was earlier than that of AOX, HMG-CoA synthase or CRBPII. Among the three lipid metabolic enzymes, mRNA for CRBPII was expressed most intensely.

As the CLOCK/BMAL1 heterodimer modulates transcriptional activity, we investigated the effect of CLOCK/BMAL1 on the promoter activities of these three genes. Using PCR, we constructed the promoters of AOX (Fig. 2A), HMG-CoA synthase (Fig. 2B), and CRBPII (Fig. 2C), and ligated them into the *Kpn* I/*Nco* I sites of the pGL3-Basic vector (Promega) upstream of the luciferase gene, as described in Materials and Methods. Interestingly, the transcriptional activity of the promoters for all three enzymes was increased by addition of CLOCK/BMAL1 genes (Fig. 3A–C). Next, we investigated the effect of CLOCK/BMAL1 on the CRBPII promoter genes without four repeat sequences (5'AG(G/T)TCA-3') corresponding to PPRE, which is necessary for transactivation of the PPAR target gene. Transcriptional activity of CRBPII by CLOCK/BMAL1 was abolished after deletion of these four repeat sequences (Fig. 4).

Fig. 1. Temporal variation of mRNA levels for A: AOX, B: HMG-CoA synthase, C: CRBPII and D: BMAL1 in mouse intestine. The levels of mRNAs changed in a cyclical manner. Experiments were performed in triplicate, and four independent experiments were conducted. All data are means ± SD. *$p < 0.05$ vs Zeitgeber Time (ZT) 0.

Discussion

We demonstrated a circadian rhythm in the expression of AOX, HMG-CoA synthase and CRBPII mRNAs in mouse intestine (Fig. 1). These three enzymes are targets of PPARα and participate in lipid homeostasis (19, 20). Little is known about regulators of the circadian rhythm of lipid metabolism in mouse intestine. The ex-

Fig. 2. Construction of the promoters of AOX, HMG-CoA synthase and CRBPII by using PCR. These promoters were ligated into the *Kpn* I/*Nco* I sites of the pGL3-Basic vector (Promega) upstream of the luciferase gene. The sequences of the AOX promoter region (A), the HMG-CoA synthase promoter regions (B) and the CRBPII promoter region (C) are shown. Consensus sequences are underlined.

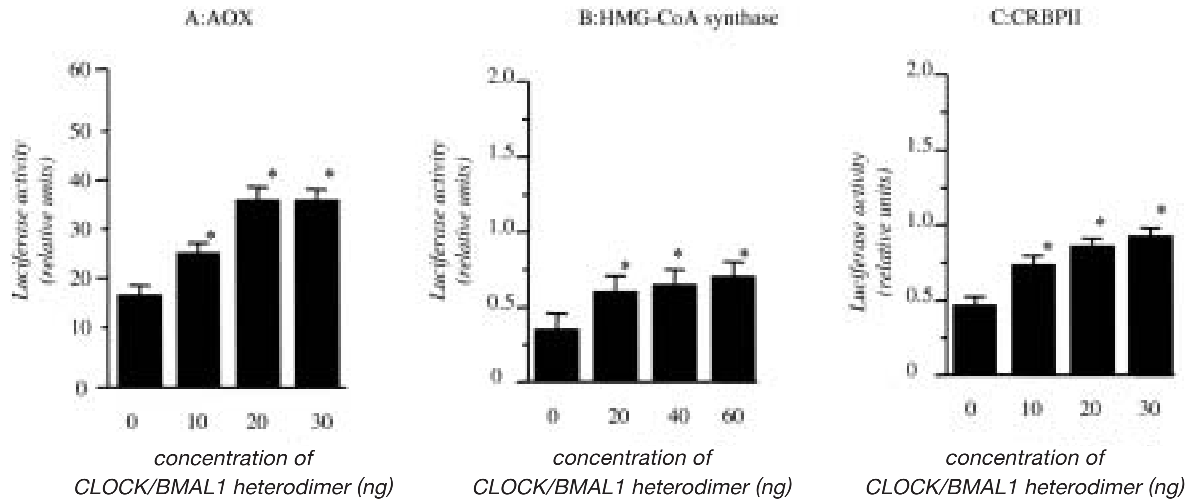

Fig. 3. Effect of CLOCK/BMAL1 on transcriptional modulation of the AOX promoter (in human kidney 293T), the HMG-CoA synthase promoter (in CV-1 monkey kidney cells) and the CRBPII promoter (in human kidney 293T). A: Transcriptional modulation of AOX by CLOCK/BMAL1, B: Transcriptional modulation of HMG-CoA synthase by CLOCK/BMAL1, C: Transcriptional modulation of CRBPII by CLOCK/BMAL1. Luciferase activity for pAOX-Luc, pHMG-Luc and CRBPII-Luc was normalized to luciferase activity for pRL-TK. Experiments were performed in triplicate, and four independent experiments were conducted. All data are means ± SD. $*p < 0.05$ vs cells not treated with the CLOCK/BMAL1 gene.

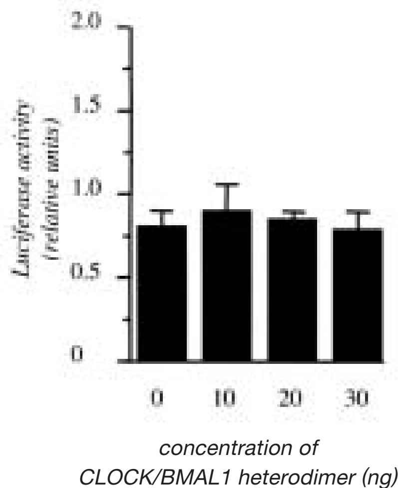

Fig. 4. Effect of CLOCK/BMAL1 on transcriptional modulation of the CRBPII promoter after deletion of the peroxisome proliferator response element (PPRE) in human kidney 293T cells. Luciferase activity for pCRBPII-Luc (deletion) was normalized to luciferase activity for pRL-TK. Experiments were performed in triplicate, and four independent experiments were conducted. All data are means ± SD.

pression of PPARα mRNA in the liver has a robust circadian rhythm that peaks at 8:00 in rats (21) and mice (22). In addition, Fu *et al.* have reported that PPARα has a circadian expression in mouse intestine similar to that in the liver (23). Taken together with these previous studies, our results suggest that there may be a relationship between the daily rhythm of the PPARα gene and of PPARα target genes such as AOX, HMG-CoA synthase and CRBPII.

Next, we investigated regulators of the circadian rhythm of these genes. Clock genes, which themselves have circadian rhythms, regulate several genes as transcription factors. We previously reported a robust circadian rhythm of rat BMAL1 expression at the SCN, the site of the circadian clock, with the highest level in the subjective night (24). Interestingly, our study indicates a circadian rhythm of BMAL1 mRNA expression in mouse intestine, with its peak preceding that of the mRNA for AOX, HMG-CoA synthase or CRBPII (Fig. 1).

It is believed that BMAL1 binds DNA and modulates transcription after dimerization with CLOCK (18). We determined by use of the luciferase assay whether or not CLOCK/BMAL1 affects transactivation of the promoters of AOX, HMG-CoA synthase and CRBPII. As shown in Fig. 3, CLOCK/BMAL1 dose-dependently transactivates the promoters of AOX, HMG-CoA synthase and CRBPII. These results suggest that CLOCK/BMAL1 is able to regulate via transactivation PPARα target genes involved in lipid metabolism.

The promoter of PPARα target genes such as AOX, HMG-CoA synthase and CRBPII contains a PPRE (5'AG(G/T)TCA-3') (Fig. 2). Transcriptional activation of CRBPII by CLOCK/BMAL1 (Fig. 3C) was abolished after deletion of the PPRE (5'AG(G/T)TCA-3') (Fig. 4), indicat-

ing that CLOCK/BMAL1 transactivates the genes of those three enzymes via the PPRE. Recently, Oishi *et al.* reported that the CLOCK/BMAL1 heterodimer transactivates the PPARα gene via an E-box-rich region that is located in the second intron of PPARα (17). CLOCK/BMAL1 may control splicing of PPARα via the E-box-rich region, and in turn PPARα might regulate PPARα target genes such as AOX, HMG-CoA synthase and CRBPII via the PPRE. The present study clearly demonstrates that the clock genes CLOCK/BMAL1 are directly associated with the circadian expression of these PPRE-controlled target genes. We have proposed that PPARα regulates lipid metabolism and oxidative stress in the vascular wall (2–4, 25, 26). It is well established that the intestine is a model for the microvascular system (27). Thus, CLOCK/BMAL1-regulated circadian transactivation of the PPARα gene might play a key role in circadian changes in the physiological responses to PPARα in the microvasculature. Our findings with CLOCK/BMAL1 may provide new insights into circadian changes involving lipid metabolism. Experiments with clock gene knockout or transgenic mice will be needed to establish the details of the relationship between clock genes and lipid metabolism.

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