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Bezafibrate Induces Plasminogen Activator Inhibitor-1 Gene Expression in a CLOCK-Dependent Circadian Manner[S]

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ABSTRACT

A functional interaction between peroxisome proliferator-activated receptor α (PPARα) and components of the circadian clock has been suggested, but whether these transcriptional factors interact to regulate the expression of their target genes remains obscure. Here we used a PPARα ligand, bezafibrate, to search for PPARα-regulated genes that are expressed in a CLOCK-dependent circadian manner. Microarray analyses using hepatic RNA isolated from bezafibrate treated-wild type, *Clock* mutant (*Clk*/*Clk*), and PPARα-null mice revealed that 136 genes are transcriptionally regulated by PPARα in a CLOCK-dependent manner. Among them, we focused on the plasminogen activator inhibitor-1 (*PAI-1*) gene, because its expression typically shows circadian variation, and it has transcriptional response elements for both PPAR and CLOCK. The bezafibrate-induced expression of PAI-1 mRNA was attenuated in *Clk*/*Clk* mice and in PPARα-null mice. The protein levels of PPARα were reduced in *Clk*/*Clk* hepatocytes. However, the overexpression of PPARα could not rescue bezafibrate-induced *PAI-1* expression in *Clk*/*Clk* hepatocytes, suggesting that impaired bezafibrate-induced *PAI-1* expression in *Clk*/*Clk* mice is not due to reduced PPARα expression. Luciferase reporter and chromatin immunoprecipitation analyses using primary hepatocytes demonstrated that DNA binding of both PPARα and CLOCK is essential for bezafibrate-induced *PAI-1* gene expression. Pull-down assays in vitro showed that both PPARα and its heterodimerized partner retinoic acid receptor-α can serve as potential interaction targets of CLOCK. The present findings revealed that molecular interaction between the circadian clock and the lipid metabolism regulator affects the bezafibrate-induced gene expression.

Most living organisms exhibit various biological rhythms with a period length of approximately 24 h. Some of these rhythms are controlled by a self-sustained oscillation mechanism called the circadian clock. The master clock in the suprachiasmatic nuclei of the anterior hypothalamus in mammals is entrained to a 24-h period by a daily light/dark cycle. The master clock, in turn, synchronizes circadian oscillators in peripheral tissues through neural and/or humoral signals (Kalsbeek et al., 1996; Terazono et al., 2003). Synchronized oscillators in the peripheral tissues drive many physiological processes as diverse as energy metabolism, cell division, hormonal secretion, and immune response (Matsuo et al., 2003; Ishida et al., 2005; Shimba et al., 2005; Hashiramoto et al., 2010).

Genetic and molecular approaches have identified a basic mechanism of 24-h rhythms that is governed by interlocked transcription-translation feedback loops: the primary loop is composed of the basic helix-loop-helix transcription factors CLOCK and BMAL1, which drive transcription of the Period (*Per1*, *Per2*) and Cryptochrome (*Cry1*, *Cry2*) genes through an E-box enhancer element (Gekakis et al., 1998; Kume et al., 1999). In turn, the PER

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ABBREVIATIONS: PPARα, peroxisome proliferator-activated receptor α; PAI-1, plasminogen activator inhibitor-1; RXRα, retinoic acid receptor α; RT-PCR, reverse-transcriptase polymerase chain reaction; PCR, polymerase chain reaction; ChIP, chromatin immunoprecipitation; PPRE, peroxisomal proliferator response element; ROR, retinoic acid-related orphan receptor.

and CRY proteins repress CLOCK/BMAL1-mediated gene transactivation, thereby shutting down their own transcription. This allows a new cycle to start. An additional feedback loop that is believed to improve the robustness of that described above involves the nuclear receptors REV-ERBα and retinoic acid-related orphan receptor (ROR)-α. The interlocked loop, consisting of REV-ERBα and ROR, modulate transcriptional activity of the *Bmal1* gene (Preitner et al., 2002). These machineries regulate the 24-h variation in output physiology through the periodic expression of clock-controlled genes.

Besides ROR-α and REV-ERBα, several orphan nuclear receptors might participate in the regulation of circadian physiology by interacting with circadian clock components (McNamara et al., 2001). Peroxisome proliferator-activated receptor α (PPARα) is a member of the nuclear receptor superfamily (Desvergne and Wahli 1999), and its binding to ligands such as fatty acids or fibrate leads to obligate heterodimerization with the 9-cis retinoic acid receptor (RXRα), thereby initiating the transcription of their target genes through the peroxisomal proliferator response element (PPRE). Hepatic PPARα is expressed in a circadian manner at the mRNA and protein levels in the livers of rodents (Lemberger et al., 1996; Patel et al., 2001). The transcription of PPARα is regulated directly by CLOCK protein, and thus circadian PPARα expression is damped in *Clock* mutant (*Clk/Clk*) mice that synthesize a mutant CLOCK protein (CLOCKΔ19) with deficient transcriptional activity (Oishi et al., 2005). The activation of PPARα caused by administering the hypolipidemic PPARα ligand bezafibrate, results in a phase advance of behavioral rhythms in mice (Shirai et al., 2007). Furthermore, a recent in vitro analysis using luciferase reporter constructs has demonstrated that CLOCK/BMAL1 heterodimers modulate PPRE-mediated PPARα transactivation (Nakamura et al., 2008). These facts suggest that PPARα interacts with members of the core circadian clock system. However, whether these transcriptional factors interact to regulate the expression of their target genes remains unknown.

The present study investigates the functional involvement of CLOCK protein in the bezafibrate-induced expression of PPARα-target genes in mice. We initially screened PPARα-regulated genes that are expressed in a CLOCK-dependent manner. DNA microarray analyses using hepatic RNA isolated from bezafibrate-treated wild-type, *Clock* mutant, and PPARα-null mice revealed that 136 genes are transcriptionally regulated by PPARα in a CLOCK-dependent manner. Among them, the *PAI-1* gene has both PPRE and an E-box in its promoter region (Chen et al., 2006; Oishi et al., 2006), and mRNA expression is induced in response to both transient and long-term treatment with bezafibrate. We thus focused on this gene to investigate transcriptional interaction between PPARα and CLOCK.

Materials and Methods

Animals and Manipulation. *Clk/Clk* mutant mice on a Jcl:ICR background, wild-type mice of the same strain, and PPARα-null mice (crossed 129S4SvJae-PPARα^{tm1Gonz}/J with Jcl:ICR) (Oishi et al., 2008) aged 6 to 12 weeks were housed under a 12-h light/dark cycle (lights on at Zeitgeber time 0). Mice were fed with a normal diet (AIN-93M; Oriental Yeast, Tokyo, Japan) with or without 0.5% w/w bezafibrate (Sigma-Aldrich, St. Louis, MO) for 5 days. A white fluorescent lamp provided light (300–500 lux at cage level) during the day. To examine the transient effect of bezafibrate injection on hepatic gene expression, bezafibrate was dissolved in warm (~40°C) sterile corn oil (Sigma-Aldrich) at a concentration of 10 mg/ml and administered intraperitoneally in a single dose of 100 mg/kg body weight at Zeitgeber time 2. Sterile corn oil served as the control injection. All animal experiments and handling proceeded under the permission of the Animal Care and Use Committees of Advanced Industrial Science and Technology (permission number 2009-020) and Kyushu University (Fukuoka, Japan).

Microarray Analysis. To examine which PPARα-regulated genes in mice are expressed in a CLOCK-dependent manner, we performed oligonucleotide microarray analyses at Zeitgeber time 14 when CLOCK/BMAL1 transcriptional activity is maximal (Oishi et al., 2003) using RNA isolated from wild-type ($n = 3$), *Clock* mutant ($n = 3$), and PPARα-null bezafibrate-treated ($n = 3$) and control wild-type ($n = 3$) mice. Total RNA (250 ng) was extracted from livers that were frozen in liquid nitrogen using RNAiso (Takara Bio Inc., Otsu, Japan). The quality was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Hybridizations to GeneChip (Mouse Genome 430 2.0 array; Affymetrix, Santa Clara, CA) arrays were performed by following the GeneChip 3′IVT Express Kit User Manual. Data at the probe level (CEL files) were deposited to the obtained using Affymetrix GCOS software. All microarray data were submitted to the Gene Expression Omnibus (Barrett et al., 2009) at the National Center for Biotechnology Information (accession number GSE20513).

Probe level data were processed using the robust multiarray analysis algorithm (Irizarry et al., 2003) to obtain data at the expression level. All statistical analysis was performed using R (version 2.9.2) and Bioconductor (Gentleman et al., 2004). This produced a gene expression matrix consisting of 45,101 probe sets and 12 samples (4 groups with 3 replicates). We applied three criteria to the selection of putative PPARα-regulated genes that are expressed in a CLOCK-dependent manner in the liver: 1) wild-type (control) < wild-type (bezafibrate); 2) PPARα-null (bezafibrate) < wild-type (bezafibrate); and 3) *Clock* mutant (bezafibrate) < wild-type (bezafibrate). We used the rank products method to detect genes exhibiting each of the three conditions (Breitling et al., 2004). This follows a recent guideline (rank products can produce the best gene ranking for robust multiarray analysis-preprocessed data) for obtaining differentially expressed genes with high sensitivity and specificity (Kadota et al., 2008, 2009).

RT-PCR Analysis. Total RNA was extracted using guanidinium thiocyanate followed by RNAiso (Takara Bio Inc., Otsu, Japan) and then digested with DNase I (Applied Biosystems, Foster City, CA). Single-stranded cDNA was synthesized using the PrimeScript RT reagent kit (Takara Bio Inc.). Real-time RT-PCR proceeded using the SYBR Premix Ex Taq II (Takara Bio Inc.) or THUNDERBIRD (Toyobo Co. Ltd., Osaka, Japan) using a LightCycler (Roche Diagnostics, Mannheim, Germany). The reaction conditions were 95°C for 10 s, followed by 45 cycles at 95°C for 5 s at 57°C for 10 s and 72°C for 10 s. The sequence primer pairs were as follows: mouse *PAI-1*, 5′-GGACACCCTCAGCATGTTCA-3′ and 5′-TCTGATGAGTTCAGCATCCAAGA-3′; mouse *β-actin*, 5′-CACACCTTCTACAATGAGCTGC-3′ and 5′-CATGATCTGGGTCATCTTTTCA-3′. The amount of PAI-1mRNA was corrected relative to that of *β-actin*.

Preparation of Primary Hepatocyte Cultures. Wild-type and *Clk/Clk* mice were anesthetized with urethane, and the liver cells isolated by sequential perfusion with collagenase were purified by density gradient separation. Cells were resuspended in hepatocyte maintenance medium (Lonza Walkersville Inc., Walkersville, MD) and seeded at a density of 2×10^5/well in 24-well tissue culture plates (Nalge Nunc International KK, Tokyo, Japan). After an attachment period of 4 h, the media were changed to Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with insulin

and dexamethasone using an HMM SingleQuots kit (Lonza Walkersville Inc.). Primary hepatocyte cultures were incubated in the presence or absence of bezafibrate for 4 h. Cells were also transfected with PPARα expression constructs. Cells overexpressing PPARα were incubated with bezafibrate for 4 h.

Immunoblotting. Nuclear fractions were prepared from primary cultures of both wild-type and *Clk/Clk* mutant hepatocytes. Fractions containing 20 μg of total protein were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane that was reacted with antibodies against PPARα, RXRα, or ACTIN (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA). Specific antigen-antibody complexes were visualized using horseradish peroxidase-conjugated secondary antibodies and Chemi-Lumi One (Nacalai Tesque Inc., Kyoto, Japan).

Construction of Reporter Plasmids and Expression Plasmids. A 1.0-kilobase pair fragment (-996 to $+43$; $+1$ indicates the putative transcription start site) derived from the 5′-flanking region of the mouse *PAI-1* gene was amplified by PCR from mouse genomic DNA for transcription assays. The PCR products were purified and ligated into the pGL3-Basic luciferase vector (Promega, Madison, WI). The E-box mutant construct of *PAI-1* luciferase reporter vector (*PAI-1*-Luc) was prepared by changing the sequence from CACGTG to GAGCTC (bases -179 to -174). The mutated construct of the PPRE in *PAI-1*-Luc was prepared by changing the sequence from TCCCCCATGCCCT to TCTCGGGTACCAT (bases -206 to -194). We prepared expression constructs by determining and confirming the sequences of the mouse CLOCK, CLOCKΔ19, and BMAL1 coding regions by RT-PCR and ligating them into the pcDNA 3.1 vector. Expression constructs of PPARα and RXRα were a gift from Dr. S. Shimba (Nihon University, Chiba, Japan).

Transcription Assays. Primary cultures of hepatocytes prepared from wild-type mice were transfected with 10 ng of reporter constructs and 2.0 μg (total) of expression vectors using Lipofectamine LTX (Invitrogen, Carlsbad, CA). To correct for variations in transfection efficiency, 0.1 ng of pRL-TK vector (Promega) was cotransfected in all experiments. The total amount of DNA per well was adjusted by adding the pcDNA 3.1 vector (Invitrogen). Cell extracts were prepared 48 h after transfection using 200 μl of passive lysis buffer (Promega), and firefly luciferase and *Renilla reniformis* luciferase were assayed by luminometry in 20-μl portions of the extracts. The ratio of firefly (expressed from reporter construct) to *R. reniformis* (expressed from pRL-TK) luciferase activities in each sample served as a measure of normalized luciferase activity. In addition, 48 h after transfection, cells were also treated with 10 μM bezafibrate for 4 h. Firefly luciferase and *R. reniformis* luciferase were assayed as described above.

Chromatin Immunoprecipitation Assays. Hepatocytes incubated with bezafibrate or vehicle were cross-linked with 4% formaldehyde in phosphate-buffered saline for 20 min, sonicated on ice, and then incubated with antibodies against PPARα (Santa Cruz Biotechnology), CLOCK (Alpha Diagnostic International, San Antonio, TX), and acetyl histone H3 (Upstate, Billerica, MA). We amplified DNA isolated from the immunoprecipitates by PCR using the primer pair for the PAI-1 promoter (from base pairs -252 to -32): 5′-ACCGACCAGCCAAAG-3′ and 5′-AGATGTGAGCCGGAAA-3′. The quantitative reliability of PCR was evaluated as described above. Chromatin immunoprecipitation proceeded in the absence of antibody or in the presence of rabbit IgG as negative controls. Ethidium bromide staining did not detect PCR products from these samples.

Protein-Protein Interaction (FLAG Pull-Down) Assays in Vitro. Cells were transfected with PPARα, RXRα, FLAG-tagged CLOCK, FLAG-tagged CLOCKΔ19, or FLAG-CMV empty vector. Cells were harvested at 48 h after transfection, and FLAG-CLOCK was immunoprecipitated using an anti-FLAG antibody (Sigma-Aldrich) on protein G-agarose beads. Specific bound proteins were released by resuspending the beads in 20 μ1 of SDS loading buffer and then separating the suspension into equal amounts for resolution by SDS-polyacrylamide gel electrophoresis. One gel was immunoblotted using anti-PPARα or anti-RXRα antibodies.

Statistical Analysis. The significance of the 24-h variation in each parameter was tested by analysis of variance. The statistical significance of differences among groups was analyzed by analysis of variance and Tukey's multiple comparison tests. A 5% level of probability was considered significant.

Results

Microarray Analysis to Search for PPARα-Regulated Genes That Are Expressed in a CLOCK-Dependent Manner in Mouse Liver. We applied three criteria to the selection of CLOCK-dependent PPARα-regulated genes (see *Materials and Methods*). We identified 1080, 847, and 774 probe sets that were up-regulated in the "wild-type (bezafibrate)" samples compared with "wild-type (control)," "PPARα-null (bezafibrate)," and "*Clock* mutant (bezafibrate)" samples, respectively (false discovery rate, $<$ 0.05). Among these probe sets, 136 were screened as candidate CLOCK-dependent PPARα-regulated genes. They included 21 and 11 genes that encode cell cycle-related and lipid metabolism-related proteins, respectively. Of these, known target genes of PPARα such as cyclin D1, phospholipid transfer protein, and stearoyl-coenzyme A desaturase 2 were identified. The CLOCK-regulated circadian gene *PAI-1*, the primary physiological inhibitor of plasminogen activators, was included in the PPARα-dependent bezafibrate-induced gene (Fig. 1A). We further examined the molecular mechanisms through which both PPARα and the circadian clock component CLOCK transcriptionally regulate *PAI-1*.

***Clock* Gene Mutation Attenuates Bezafibrate-Induced Expression of the *PAI-1* Gene in Mouse Liver.** Levels of PAI-1 gene mRNA in the livers of wild-mice fed with a normal diet exhibited significant daily rhythms and were higher between the late-light to the early dark phase ($P <$ 0.05, Fig. 1B). Although mRNA levels of the *PAI-1* gene in the livers of bezafibrate-treated mice also oscillated with a significant daily rhythm ($P < 0.05$), they also significantly increased at all investigated time points. On the other hand, the bezafibrate-induced elevation of PAI-1 mRNA levels was quite attenuated in *Clk/Clk* mice. The mRNA levels of PAI-1 in bezafibrate-treated *Clk/Clk* mice were on average 62% lower than those in wild-type mice.

After a single intraperitoneal injection of 100 mg/kg bezafibrate into wild-type mice fed with normal diet, the mRNA levels of PAI-1 in the liver were also transiently increased and reached the maximum level 4 h later (Fig. 1C). By contrast, the transient induction of PAI-1 mRNA by bezafibrate was considerably attenuated in the *Clk/Clk* mouse liver. Peak levels of PAI-1 mRNA in *Clk/Clk* mice were approximately 68% lower than those in wild-type mice.

The induction of mRNA for the other prototypical PPARα target genes *pyruvate dehydrogenase kinase 4* (Oishi et al., 2008) and *fibroblast growth factor 21* (Lundåsen et al., 2007) was also elevated by bezafibrate (Supplemental Data 1), but this induction was not attenuated in *Clk/Clk* mice. These data suggest that the *Clock* mutation does not always attenuate the bezafibrate-induced expression of PPARα target genes.

***Clock* Gene Mutation Attenuates Bezafibrate-Induced Expression of the *PAI-1* Gene in Primary Cultures of Hepatocytes.** Consistent with the observations in vivo, a 4-h incubation with >10 μM bezafibrate caused a significant and dose-dependent induction of PAI-1 mRNA expression in primary cultures of wild-type hepatocytes ($P < 0.05$; Fig. 2A) but not in those of *Clk*/*Clk* hepatocytes in which PAI-1 mRNA expression was significantly lower ($P < 0.05$).

Because CLOCK is a positive regulator of the PPARα gene, *Clk*/*Clk* mice express low levels of PPARα mRNA (Oishi et al., 2005). When *Clk*/*Clk* hepatocytes were transfected with a PPARα expression vector (Fig. 2B, left), PPARα overexpression could not rescue bezafibrate-induced PAI-1 expression in these cells (Fig. 2B, right). These data indicate that attenuation of the bezafibrate-induced expression of PAI-1 mRNA in *Clk*/*Clk* hepatocytes is not attributable to a low level of PPARα expression.

CLOCK Is Involved in PPARα-Mediated Transactivation of the *PAI-1* Gene. To investigate the involvement of PPARα and CLOCK in transcriptional regulation of the *PAI-1* gene, we performed transient transcriptional assays in vitro using mouse *PAI-1* gene luciferase reporter constructs (*PAI-1*-Luc) that contain both PPRE and an E-box (Fig. 3A). Incubating wild-type *PAI-1*-Luc-transfected cells with 10 μM bezafibrate caused a 5.6-fold increase in promoter activity (Fig. 3B), but this was attenuated by a mutation of either PPRE or the E-box, suggesting that both elements are essential for the bezafibrate-induced PAI-1 expression.

Cotransfection of wild-type *PAI-1*-Luc with PPARα/RXRα and CLOCK/BMAL1 resulted in a 6- and 5.5-fold increase in promoter activity, respectively (Fig. 3C). Transcriptional activity was synergistically enhanced by cotransfection with PPARα/RXRα and CLOCK/BMAL1 expression constructs. CLOCK/BMAL1 caused a 6.8-fold increase in PPARα/RXRα-mediated transactivation of the PAI-1 promoter. However, PPARα/RXRα-mediated transactivation was not enhanced in cells cotransfected with CLOCKΔ19 and BMAL1 (Fig. 3C). Bezafibrate-induced transactivation (Fig. 3B) was obviously enhanced in cells transfected with CLOCK/BMAL1 and PPARα/RXRα (Fig. 3D). A similar synergistic effect of CLOCK/BMAL on the PPARα/RXRα-mediated transactivation of the PAI-1 promoter was also found under the presence of bezafibrate (Fig. 3D).

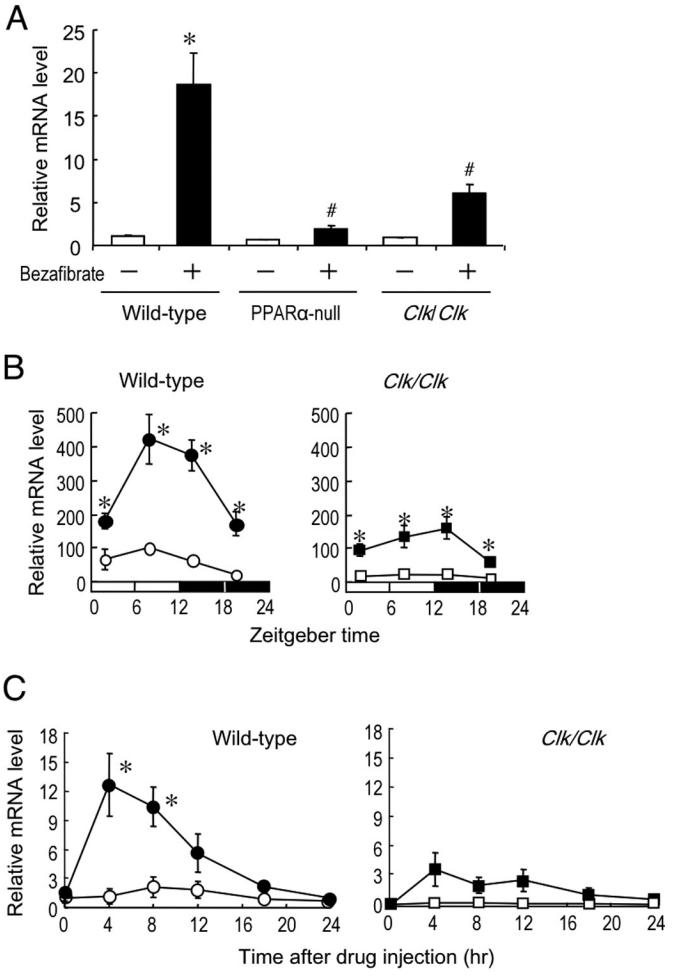

Fig. 1. Influence of *Clock* gene mutation on the bezafibrate-induced expression of mouse PAI-1 mRNA in mouse liver. A, levels of PAI-1 mRNA in livers of wild-type and *Clock*/*Clock* (*Clk*/*Clk*) mice administered orally with bezafibrate for 5 days. B, temporal expression profiles of PAI-1 mRNA in livers of wild-type and *Clk*/*Clk* mice administered orally with bezafibrate. Mice were fed with normal diets without (open symbols) or with 0.5% w/w bezafibrate (closed symbols) for 5 days. C, time course of mRNA levels of PAI-1 in the liver of wild-type (open symbols) and *Clk*/*Clk* (closed symbols) after a single injection of 100 mg/kg i.p. bezafibrate. Both genotypes of mice were fed with a normal diet without bezafibrate. All values are shown as means ± S.E.M. of three to six mice. *, $P < 0.05$ compared with vehicle-treated wild-type group. #, $P < 0.05$ compared with bezafibrate-treated wild-type mice.

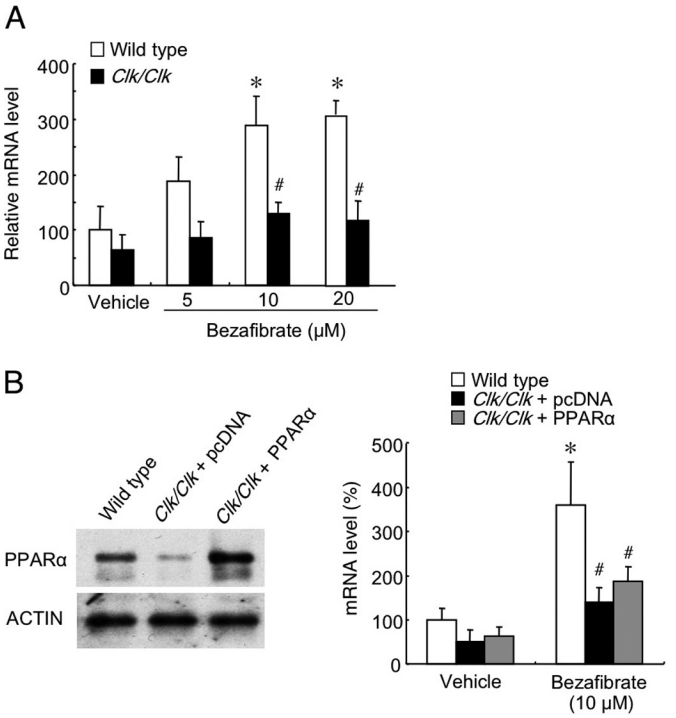

Fig. 2. Influence of *Clock* gene mutation on the bezafibrate-induced expression of mouse PAI-1 mRNA in primary culture of hepatocytes. A, effect of bezafibrate on levels PAI-1 mRNA gene in primary cultures of hepatocytes prepared from wild-type and *Clk*/*Clk* mice (□ and ■, respectively). B, left, protein levels of PPARα in wild-type and *Clk*/*Clk* hepatocytes transfected with PPARα expression or empty (pcDNA) constructs. Right, PPARα overexpression does not enhance bezafibrate-induced expression of PAI-1 mRNA in *Clk*/*Clk* hepatocytes. All values are shown as means ± S.E.M. of four independent experiments. *, $P < 0.05$ compared with vehicle-treated group. #, $P < 0.05$ compared with bezafibrate-treated group at corresponding dosage.

***Clock* Gene Mutation Attenuates Bezafibrate-Induced Acetylation of Histone H3 in the *PAI-1* Gene Promoter of Primary Cultured Hepatocytes.** The results of the chromatin immunoprecipitation (ChIP) assays showed that PPARα binding to the *PAI-1* gene promoter in wild-type hepatocytes was significantly increased by a 4-h incubation with 10 μM bezafibrate ($P < 0.05$; Fig. 4). Bezafibrate also enhanced histone H3 acetylation but had little effect on CLOCK binding to the *PAI-1* gene promoter. On the other hand, the amount of PPARα binding to the *PAI-1* gene promoter in bezafibrate-treated hepatocytes was significantly lower than that in wild-type hepatocytes ($P < 0.05$; Fig. 4). The acetylation of histone H3 in the bezafibrate-treated *Clk/Clk* hepatocytes was similarly attenuated.

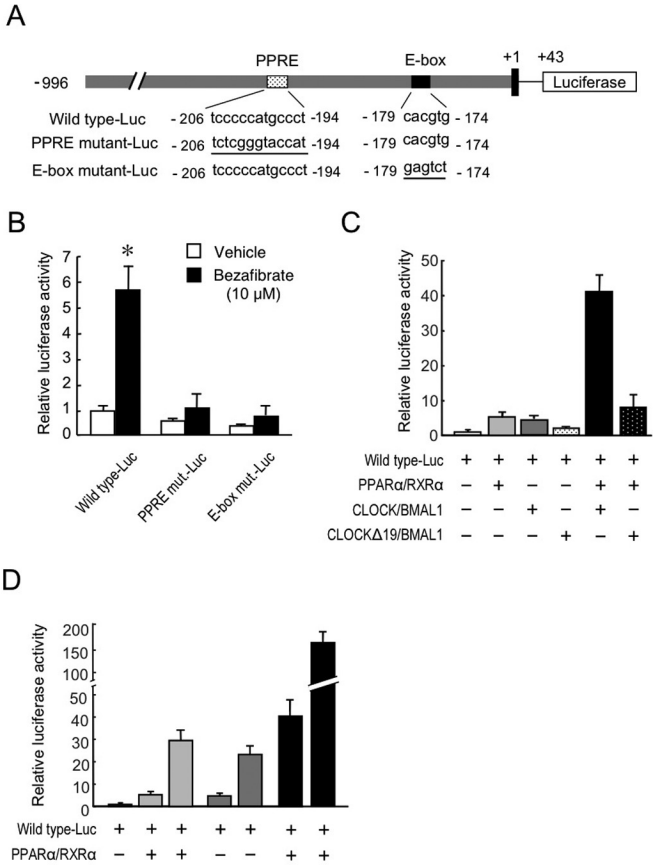

Fig. 3. Influence of *Clock* gene mutation on the PPARα-mediated transactivation of mouse PAI-1 promoter. A, schematic representation of mouse PAI-1 promoter. Numbers below boxes are nucleotide residues in which PPRE and E-box are positioned relative to transcription start site (+1). Underlined nucleotide residues indicate mutated sequence of PPRE and E-box. B, mutation of PPRE and E-box abrogates bezafibrate-induced PAI-1 promoter activity. Primary cultures of hepatocytes were transfected with 0.1 μg each of wild-type-Luc, PPRE-mut-Luc, and E-box-mut-Luc. Cells transfected with reporter constructs were incubated with or without 10 μM bezafibrate for 4 h. C, enhancement of PPARα/RXRα-mediated transcription of mouse *PAI-1* gene by CLOCK/BMAL1. Presence (+) or absence (−) of plasmids (0.1 μg of wild-type-Luc; 0.5 μg for each of CLOCK, CLOCKΔ19, BMAL1, PPARα, and RXRα) is denoted. D, influence of CLOCK/BMAL1 on the PPARα/RXRα-mediated transcription of mouse PAI-1 gene under the presence of bezafibrate. At 48 h after transfection, cells were treated with 10 μM bezafibrate for 4h. Presence (+) or absence (−) of plasmids (0.1 μg of wild-type-Luc; 0.5 μg for each of CLOCK, BMAL1, PPARα, and RXRα) is denoted. Treatment of cells with 10 μM bezafibrate (+) or vehicle (−) was also denoted. Values in B, C, and D represent means ± S.E.M. of four independent experiments.

Interaction of CLOCK with PPARα. To obtain further insight into the mechanism of the synergistic effect of CLOCK on PPARα-induced PAI-1 promoter activity, we explored the notion that CLOCK interacts with PPARα. We confirmed protein-protein interaction between CLOCK and RXRα as described by McNamara et al. (2001) (Fig. 5, left), and immunoprecipitation experiments also showed that CLOCK protein precipitated with PPARα (Fig. 5, right). Although CLOCKΔ19 protein could not enhance PPARα/RXRα-mediated transactivation of the *PAI-1* gene (Fig. 3C), the mutated protein precipitated together with both RXRα and PPARα. These interactions between the lipid metabolism regulators and circadian clock components might enhance *PAI-1* gene transcriptional activity.

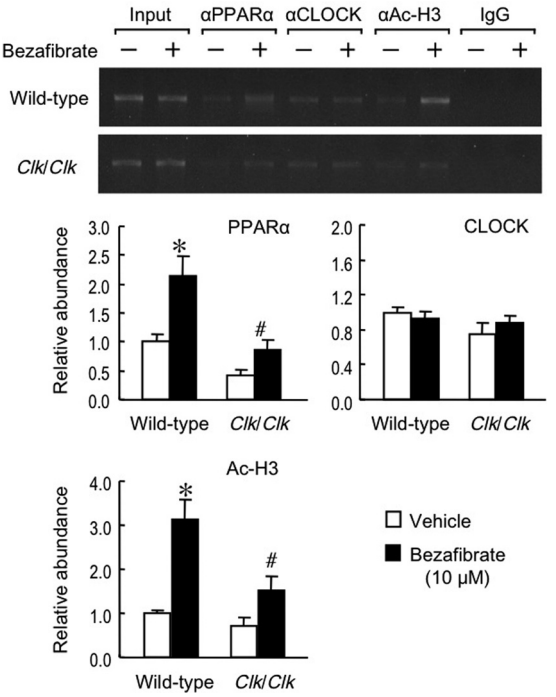

Fig. 4. *Clock* gene mutation attenuates bezafibrate-induced of mouse PAI-1 mRNA expression. Primary cultures of hepatocytes from wild-type or *Clk/Clk* mice were incubated with 10 μM bezafibrate for 4 h. Amounts of PPARα and CLOCK bound to *PAI-1* gene promoter and of histone H3 (Ac-H3) acetylation were assessed using ChIP assays. Values represent means ± S.E.M. of four independent experiments. *, $P < 0.05$ compared with vehicle-treated group. #, $P < 0.05$ compared with bezafibrate-treated wild-type hepatocytes.

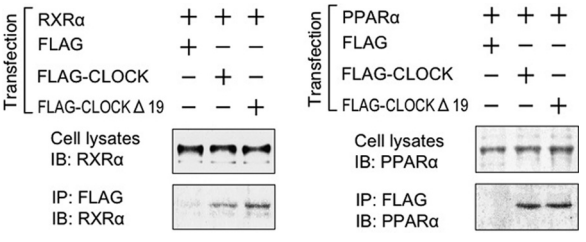

Fig. 5. Interaction of PPARα with CLOCK. Primary cultures of hepatocytes were transfected with indicated expression vectors. At 48 h after transfection, cell lysates were immunoprecipitated (IP) with anti-FLAG antibody, and immunoblotted (IB) with antibodies against PPARα (left) or RXRα (right). Portions of total cell lysates were also immunoblotted with each antibody to confirm expression of CLOCK, PPARα, or RXRα proteins.

Discussion

The present findings demonstrate that PPARα and CLOCK interact to regulate their common target gene expression. The results of microarray analyses using hepatic RNA isolated from bezafibrate-treated wild-type *Clock* mutant and PPARα-null mice revealed that 136 genes were transcriptionally regulated by PPARα in a CLOCK-dependent manner in the mouse liver. Among them, *PAI-1* was typical of genes with expression that exhibits circadian variation and has both PPRE and an E-box element in the promoter region (Chen et al., 2006; Oishi et al., 2006). We thus further focused on this gene to investigate the transcriptional interaction between PPARα and CLOCK.

Bezafibrate induced a significant elevation of PAI-1 mRNA levels in the liver of wild-type mice but had little effect on PAI-1 expression in PPARα-null mice. Although these data showed that bezafibrate could induce the hepatic expression of PAI-1 mRNA by mediating PPARα, others have found that bezafibrate acts as a negative regulator of PAI-1 expression in cultured primate hepatocytes (Arts et al., 1997; Suzuki et al., 2001). The discrepancy between the present and previous findings might be due to a difference in experimental procedures. The action of bezafibrate on *PAI-1* gene expression is also changed by its dosage and treatment period. In brief, exposing hepatoma or endothelial cells to bezafibrate induces PAI-1 mRNA expression (Mussoni et al., 1996; Nilsson et al., 1999). The present findings indicate that bezafibrate acts as a positive regulator of PAI-1 mRNA expression, at least in the livers of lean mice. PPARα seems to mediate bezafibrate-induced expression of the *PAI-1* gene.

We also showed here that bezafibrate-induced *PAI-1* mRNA expression was attenuated by a *Clock* mutation both in vivo and in vitro. Indeed, CLOCK protein is a positive regulator of PAI-1 mRNA expression (Oishi et al., 2006, 2007). That is, CLOCK/BMAL heterodimers transactivate the *PAI-1* gene via the E-box element(s), and this activation is periodically suppressed by PER and CRY proteins. Retinoic orphan receptor-α and REV-ERBα, which are responsible for lipid metabolism, have also been suggested to participate in the circadian control of *PAI-1* gene expression (Wang et al., 2006). Consequently, *PAI-1* is expressed in a circadian manner not only in vascular endothelial cells but also in the liver, heart, kidney, and adipose tissues (Maemura et al., 2000; Kudo et al., 2004; Oishi et al., 2006). Because it did not induce the mRNA expression of *Period1*, a typical E-box-dependent CLOCK-regulated gene, in primary cultures of both wild-type and PPARα-null hepatocytes (Supplemental Data 2), bezafibrate seems not to up-regulate the expression of PAI-1 mRNA via a direct action on CLOCK or BMAL1. Protein levels of PPARα were lower in *Clk*/*Clk* hepatocytes than in wild-type hepatocytes as described previously (Oishi et al., 2005). However, bezafibrate could not significantly induce PAI-1 mRNA in *Clk*/*Clk* hepatocytes even when the cells overexpressed PPARα. Thus, the attenuation of bezafibrate-induced expression of PAI-1 mRNA in *Clk*/*Clk* hepatocytes is probably not associated with low expression levels of PPARα.

The results of the analysis using a mutated PAI-1 luciferase reporter construct demonstrated that bezafibrate-induced transactivation of the *PAI-1* gene was dependent on both PPRE and the E-box. Accordingly, transcriptional factors that regulate PAI-1 promoter activity by binding to these elements are necessary for bezafibrate-induced expression of the mRNA. A recent study suggests a molecular link between PPARα/RXRα- and CLOCK/BMAL1-dependent transcription (Nakamura et al., 2008). The transcriptional activity of PPARα/RXRα-controlled genes is enhanced by cotransfection with low levels of CLOCK and BMAL1 expression constructs. In this study, CLOCK/BMAL1 synergistically affected PPARα/RXRα-induced PAI-1 promoter activity when cells were cotransfected with the same amount of each expression construct. Therefore, an optimal ratio of CLOCK/BMAL1 to PPARα/RXRα might be required to enhance their transcriptional activity. The E-box element of the mouse *PAI-1* gene is located between nucleotides 179 and 174 upstream of the transcription start site (Oishi et al., 2006), and it is indispensable for the rhythmic expression of PAI-1 mRNA. A putative PPRE of the mouse *PAI-1* gene is located 15 base pairs upstream from the E-box (Chen et al., 2006). The surrounding sequence of the E-box and its location has an obvious influence on the transcriptional activity of CLOCK/BMAL heterodimers. In fact, a CT-rich *cis*-acting element of the mouse vasopressin gene confers robust CLOCK/BMAL responsiveness on an adjacent E-box (Muñoz et al., 2006). Therefore, the putative PPRE (TCCCCCATGCCCT) located near the E-box and upstream in the mouse *PAI-1* gene might act as a permissive site in response to CLOCK/BMAL1 heterodimers. This hypothesis was also supported by the finding that PPARα/RXRα could not enhance the CLOCK/BMAL1-mediated transactivation of mouse *Period1* luciferase reporter constructs containing three E-boxes but no PPRE (Supplemental Data 3). Therefore, PPRE-mediated PPARα/RXRα transactivation is associated with E-box-mediated CLOCK/BMAL regulation.

The results of the ChIP assays revealed that incubating wild-type hepatocytes with bezafibrate resulted not only in enhanced PPARα binding to the *PAI-1* gene promoter but also in histone H3 acetylation. However, the bezafibrate-induced acetylation of histone H3 was significantly attenuated in *Clk*/*Clk* hepatocytes. CLOCK protein contains acetyl-CoA binding motifs within the carboxyl-terminal glutamine-rich region and thus has histone acetyltransferase activity (Doi et al., 2006). By forming a heterodimer, BMAL1 enhances the histone acetyltransferase function to activate the transcription of target genes. The coding region of exon 19 of CLOCK protein contains acetyl-CoA binding motifs (King et al., 1997; Doi et al., 2006). Consequently, CLOCKΔ19 protein is deficient in transcriptional activity, probably resulting from the absence of the histone acetyltransferase function. Taken together, data from the ChIP analysis suggest that the histone acetyltransferase activity of CLOCK protein is required for its synergistic effect on bezafibrate-induced *PAI-1* gene expression. The low level of histone H3 acetylation in *Clk*/*Clk* hepatocytes probably underlies the attenuation of bezafibrate-induced PAI-1 mRNA expression.

Although CLOCK interacted not only with RXRα but also with PPARα, we could not distinguish whether these interactions were caused by direct or indirect binding of CLOCK to PPARα. Several nuclear receptors, including RARα and RXRα, modulate the transcriptional activity of CLOCK

through direct protein-protein interaction (McNamara et al., 2001). Because RXRα is endogenously expressed in primary hepatocyte cultures, CLOCK and PPARα protein-protein interaction might be caused via endogenous RXRα. However, a *Clock* mutation did not attenuate the bezafibrate-induced expression of the PPARα target genes *pyruvate dehydrogenase kinase 4* and *fibroblast growth factor 21* (Supplemental data 1). Therefore, in addition to direct interaction, the distance between PPRE and the E-box might also be important for the synergistic effect of CLOCK on bezafibrate-induced expression of PPARα target genes. Further studies are required to clarify how these transcriptional factors interact to regulate bezafibrate-induced expression of the mouse *PAI-1* gene.

The present findings in this animal model suggest that *PAI-1* is a PPARα-targeted gene that is expressed in a CLOCK-dependent circadian manner. Transcriptional interaction between PPARα and CLOCK seemed to enhance the bezafibrate-induced expression of PAI-1 mRNA in the livers of lean mice. Our present findings reveal a link between the circadian clock and the lipid metabolism regulator and provide a new mechanistic basis for the bezafibrate-induced gene expression.

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