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Reciprocal Regulation of Brain and Muscle Arnt-Like Protein 1 and Peroxisome Proliferator-Activated Receptor α Defines a Novel Positive Feedback Loop in the Rodent Liver Circadian Clock

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Recent evidence has emerged that peroxisome proliferator-activated receptor α (PPARα), which is largely involved in lipid metabolism, can play an important role in connecting circadian biology and metabolism. In the present study, we investigated the mechanisms by which PPARα influences the pacemakers acting in the central clock located in the suprachiasmatic nucleus and in the peripheral oscillator of the liver. We demonstrate that PPARα plays a specific role in the peripheral circadian control because it is required to maintain the circadian rhythm of the master clock gene brain and muscle Arnt-like protein 1 (*bmal1*) *in vivo*. This regulation occurs via a direct binding of PPARα on a potential PPARα response element located in the *bmal1* promoter. Reversely, BMAL1 is an upstream regulator of *PPARα* gene expression. We further demonstrate that fenofibrate induces circadian rhythm of clock gene expression in cell culture and up-regulates hepatic *bmal1 in vivo*. Together, these results provide evidence for an additional regulatory feedback loop involving BMAL1 and PPARα in peripheral clocks. (*Molecular Endocrinology* 20: 1715–1727, 2006)

CIRCADIAN RHYTHMS ENABLE numerous organisms to adapt to daily environmental changes such as light, temperature, and social communication and serve to synchronize multiple molecular, biochemical, physiological, and behavioral processes. Circadian rhythms persist with an approximate 24-h periodicity even in temporally isolated subjects, indicating the presence of an autonomous time-keeping system called circadian clock. In mammals, circadian rhythms are generated by the main pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus (1). To ensure that internal time coincides with environmental time, the clock must be adjusted, a process known as entrainment. In mammals, light received by the eyes synchronizes the oscillator through the retino-hypothalamic tract and hence synchronizes the behavior of the organism with the daily 24-h light-dark (LD) cycle (for review, see Refs. 2–6).

In addition to the SCN, other peripheral tissues such as liver, heart, kidney (7, 8), as well as isolated cells (9) express clock genes giving rise to circadian rhythms with a different phase from that observed in the SCN. Interestingly, these peripheral clocks can be resetted by alternative routes independently of the SCN, for example by forced change of feeding time (10, 11). Several lines of evidence suggest that the peripheral circadian clocks are not SCN independent but require inputs from the SCN to drive the rhythmicity and ensure an ordered response of the organism to environmental changes (12, 13). Thus, the SCN is believed to coordinate rhythms in the brain and body via a combination of neural and humoral diffusible and synaptic signals (7, 14, 15).

Genetic analyses have identified master clock genes such as *clock*, *bmal1*, *period (per1, 2)*, and *cryptochrome* genes (*cry1, 2*), as well as the orphan nuclear receptor genes, *rev-erbα* and *rorα* (16, 17). Other transcription factors functioning in the circadian regulation of gene expression, including DBP-related factors

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Abbreviations: BMAL1, Brain and muscle Arnt-like protein 1; ChIP, chromatin immunoprecipitation; CT, circadian time; DD, constant darkness; LD, light-dark; 12L:12D, 12-h light, 12-h dark; PPARα, peroxisome proliferator-activated receptor α; PPRE, peroxisome proliferator response element; PSG, penicillin/streptomycin/glutamine; SCN, suprachiasmatic nucleus; WT, wild type; ZT, Zeitgeber time.

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(18), Rev-erbβ, RORβ and -γ (19) have also been identified. The clock mechanism mainly involves an integrated network of interacting self-sustained transcriptional-translational feedback loops, composed of positive and negative regulators, which drive their own rhythmic expression and the one of clock-controlled genes to perform a fine tuning of circadian gene expression (20).

Recent reports have highlighted the interplay between circadian oscillators, metabolism, and physiology. Whereas genes involved in the glucose and lipid metabolism are known to exhibit circadian variations (21–23), molecular studies have revealed a critical role for *bmal1* and *clock* genes in regulating glucose homeostasis (24) and lipid metabolism (25–28). Moreover, the cross talk between RORα and Rev-erbα was shown to be physiologically important for the control of cholesterol and triglyceride metabolism (29–31). In turn, the peripheral clocks can be coordinately regulated by multiple circulating factors, which are affected by the metabolic status of the organism. Indeed, glucose, one of the major food metabolites that exhibits a plasma diurnal rhythm, is a direct resetting signal in cultured cells by down-regulating *per1* and *per2* RNA levels (32). The levels of glucose-regulated hormones such as insulin or glucagon immediately up-regulate *per1* and *per2* expression (13). In addition, other studies have revealed an important role of glucocorticoids and retinoids in the resetting of peripheral clocks (33, 34). Although the elucidation of the mechanisms that govern the connection between metabolism and circadian clock has just begun, it appears that several members of the nuclear receptor family are involved in this pathway.

Evidence has emerged that peroxisome proliferator-activated receptor α (PPARα), a member of the nuclear receptor superfamily that regulates the expression of numerous genes involved in lipid metabolism and energy homeostasis, can play a role in the normal circadian regulation. First, *PPARα* has been identified as a circadian clock-controlled gene with a diurnal rhythm at the mRNA and protein levels in rats and mice in many peripheral organs such as liver, heart, kidney, and to a lesser extent in the SCN, where the central pacemaker is located (7, 35, 36). This circadian expression of *PPARα* may be in part controlled by hormonal factors, because insulin and glucocorticoids regulate its mRNA expression (35, 37–39). A recent study has also shown that the circadian expression of *PPARα* mRNA is regulated by the peripheral oscillators in a CLOCK-dependent manner (27). Second, because daily variations in lipogenic and cholesterogenic gene expression are attenuated or abolished in mice in which the *PPARα* gene has been disrupted, PPARα may be an important mediator for the circadian regulation of lipid metabolism (40, 41). It is now believed that PPARα has a wider general role in transducing hormone messages involved in dietary status (42). These observations thus suggest that PPARα may be required in the control of circadian food-dependent fluctuations in gene expression. Third, PPARα is connected to the regulation of other nuclear hormone receptors such as Rev-erbα, because fenofibrate, a PPARα agonist, induces human and rat *rev-erbα* expression in liver through the direct binding of PPARα on an atypical DR2 element located in the *rev-erbα* promoter (30, 43). It was recently shown that CLOCK plays an important role in lipid homeostasis by regulating the circadian transactivation of potential PPARα response element (PPRE)-controlled target genes (26) and of *PPARα* gene itself *via* an E-box rich region *in vivo* and *in vitro* (27). Fourth, the partner of PPARα, RXRα, interacts with CLOCK protein in a ligand-dependent manner and inhibits CLOCK/brain and muscle Arnt-like protein 1 (BMAL1)-dependent activation via an E-box element (34). Other results have also suggested that *PPARα* deficiency disturbs the normal circadian regulation of certain SREBP-sensitive genes in the liver (40, 44).

Despite much evidence supporting a role of PPARα in metabolic control and energy homeostasis (45) and the accumulation of data connecting metabolism and circadian biology (3, 46, 47), little is known concerning the influence of PPARα on the circadian clock. In the present study, we investigated the mechanisms by which PPARα can influence the pacemakers acting in the SCN and in the liver. We report that *PPARα*-deficient (*PPARα*$^{-/-}$) mice present similar locomotor activity with wild-type (WT) mice without any molecular alteration of clock gene expression in the SCN. Interestingly, we show for the first time that PPARα is a direct regulator of *bmal1* expression in liver via its direct binding on a PPRE located on the *bmal1* promoter. This regulation is required to maintain the normal circadian oscillation of *bmal1 in vivo*. Stressing the importance of the regulatory pathway that exists between PPARα and the peripheral clock genes, we show that fenofibrate up-regulates *bmal1* gene expression in murine liver and induces circadian rhythm of clock gene expression in cell culture in a PPARα-dependent manner. Reversely, we also observe that BMAL1 is an upstream regulator of the *PPARα* gene expression. Taken together, our data implicate PPARα in a new regulatory loop that controls peripheral circadian clocks.

RESULTS

PPARα$^{-/-}$ Mice Display Normal Circadian Locomotor Activity and Clock Gene Expression in the SCN

To evaluate the influence of PPARα on the function of the central circadian oscillator, behavioral analysis of the circadian rhythm was carried out using *PPARα*$^{-/-}$ and WT littermate mice. Animals were first synchronized for 2 wk to a 12-h light, 12-h dark cycle (12L:12D). Under these light conditions, WT and *PPARα*$^{-/-}$ mice entrained normally and consolidated their loco-

motor activity to the dark period of the LD cycle (Fig. 1A). No difference in the total amount of daily activity was observed between WT and *PPARα*$^{-/-}$ mice (Fig. 1B). When placed in constant darkness, the *PPARα*-deficient mice do not display an arrhythmic behavior with endogenous period similar between both genotypes (24.11 ± 0.11 and 24.12 ± 0.08 h, respectively, in *PPARα*-deficient and WT mice).

To determine whether the inactivation of *PPARα* can alter the master oscillator in the SCN at the molecular level, we further compared the circadian expression profiles of clock genes in the SCN of WT and PPARα mutant mice (Fig. 1C). There is no significant difference in the amplitude and in the phase of the circadian expression of the tested clock genes (*bmal1*, *per2*, *per3*, *cry2*, and *rev-erbα*). This clearly suggests that

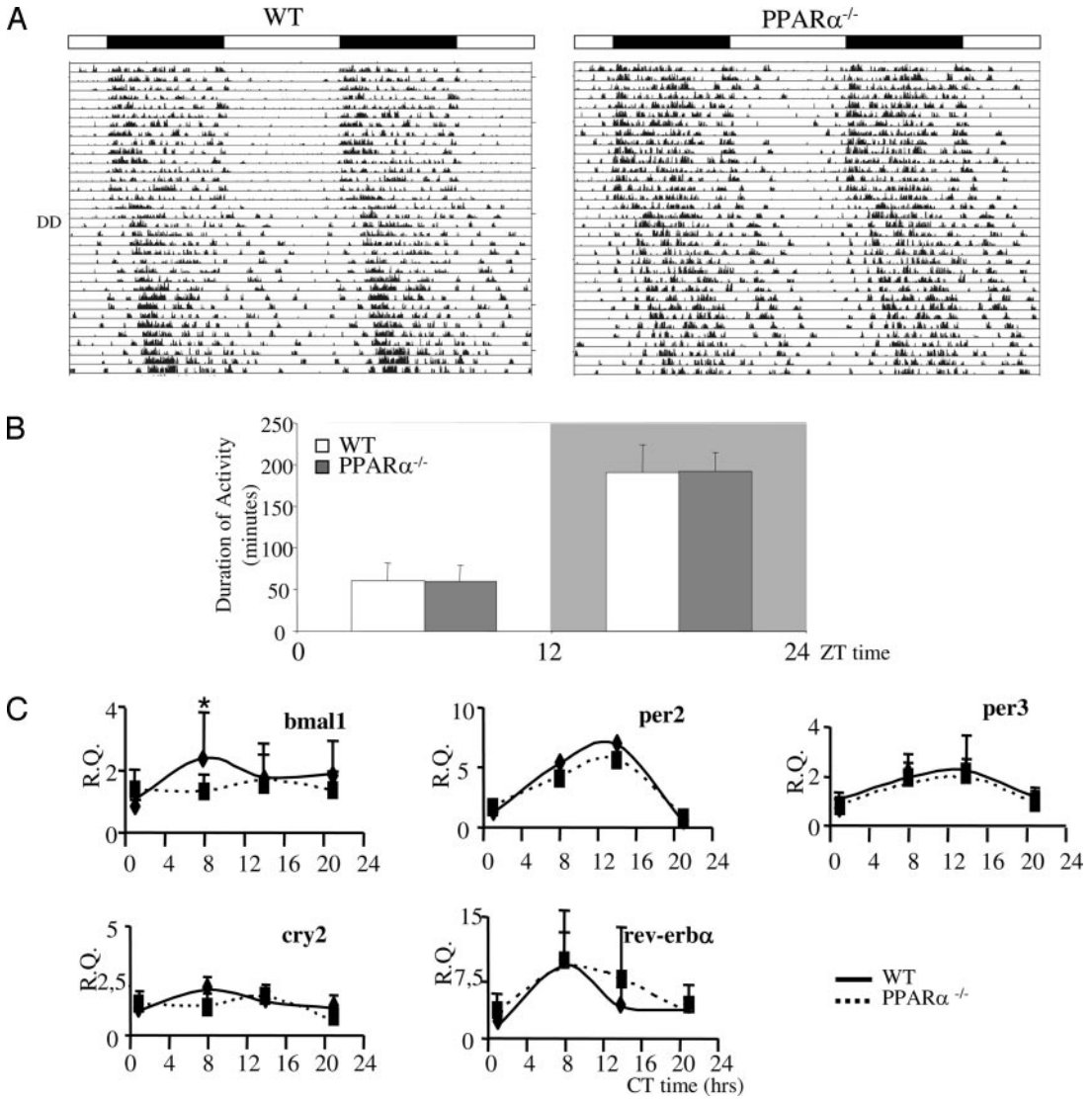

Fig. 1. Entrainment and Free Running Locomotor Activity of WT and *PPARα*$^{-/-}$ Mice

A, Representative actograms of locomotor activity for WT and *PPARα*$^{-/-}$ mice under 12L:12D cycle and DD conditions. After 2 wk of entrainment under a 12L:12D cycle, mice were placed in DD. *Horizontal bar* at the top of each actogram depicts the lighting conditions of LD cycles. Time spans in darkness are marked by *gray shadowing*. B, Twenty-four-hour profiles of spontaneous locomotor activity of WT (□) and *PPARα*-deficient mice (▨). The distribution of activity was determined every 12 h during LD cycle. *Shadowed areas* indicate the dark period. Results are expressed as the means ± SEM of values from eight animals per group. C, Circadian expression of *bmal1*, *per2*, *per3*, *cry2*, and *rev-erbα* mRNAs in SCN of WT (♦) and *PPARα*$^{-/-}$ mice (■). Real-time PCR was used to determine transcript levels at four circadian times (CT1, CT8, CT13, CT21). Transcript levels are displayed as relative quantity (RQ) after normalization to the noncyclic 36B4 expression levels in the same sample. Results are expressed as the means ± SEM of values of two independent experiments, each realized with three animals for both genotypes at each time point. Statistically significant differences between WT and PPARα-deficient mice are indicated by an *asterisk* ($P < 0.05$).

there is no major molecular effect of the *PPARα* deletion at the central clock level and that the expression of *PPARα* in the SCN is not essential for the basal maintenance of the central circadian timing system.

PPARα Is Required to Maintain the Amplitude of the Circadian Expression of *bmal1* in the Murine Liver

To evaluate the role of PPARα on the circadian system of the liver (a peripheral clock), where it is mainly expressed, we analyzed the circadian expression of several clock genes in liver isolated from WT and *PPARα* mutant mice. As already reported, PPARα expression effectively follows a circadian rhythm in peripheral tissues such as liver, kidney, and muscle (data not shown). Figure 2 shows that all the clock genes tested (*bmal1*, *per1*, *per2*, *per3*, *cry2*, and *rev-erbα*) are expressed in a circadian manner with no modification in the phase of their rhythm between both genotypes. By contrast, the amplitudes of *bmal1* and *per3* expression are drastically affected in *PPARα*-deficient mice by comparison with the WT, with a significant decrease at circadian time 1 (CT1) and CT21 for *bmal1* (where CT0 is subjective day beginning at 0700 h, and CT12 is subjective night beginning at 1900 h) and an increase at CT8 for *per3*. These data suggest that PPARα does not influence the phase synchronization properties of the liver clock but affects the amplitude of two major clock genes *bmal1* and *per3*.

Food-Induced Phase Resetting Entrains Circadian PPARα Expression and Is Globally Maintained in Liver of PPARα$^{-/-}$ Mice

Although the day-night cycle is the most obvious time cue, animals can also respond to other synchronizing signals, and feeding time appears to be a potent temporal cue, or Zeitgeber, for the liver clock (10, 11). According to PPARα role in the hepatic lipid metabolism during starvation, we hypothesized its potential role in food phase resetting of the liver clock. To examine whether the daily feeding time can affect the phase of the *PPARα* circadian expression in the liver, WT mice were fed for 2 wk exclusively during the day or during the night. As expected, mice fed exclusively during the day displayed an inversed phase in circadian hepatic expression of *bmal1*, *per1*, *per3*, and *rev-erbα* gene by comparison to mice fed only during the night. Similarly, feeding during the day entirely inversed the phase of liver *PPARα* expression (Fig. 3A), a result that is in agreement with the demonstration that *PPARα* is a clock-controlled gene in the liver (7, 36). Control mice fed only during the night displayed a similar phase of hepatic clock gene expression and *PPARα* than mice fed *ad libitum*.

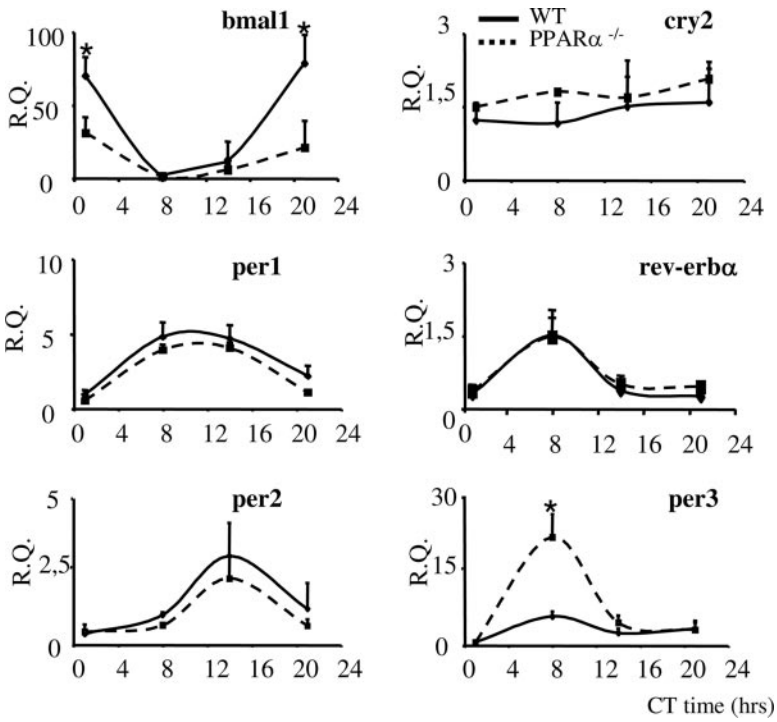

Fig. 2. Circadian Expression of *bmal1*, *per1*, *per2*, *per3*, *cry2*, and *rev-erbα* mRNAs in Liver of WT (♦) and *PPARα*$^{-/-}$ Mice (■) using Real-Time PCR

Transcript level values are expressed as relative quantity (RQ) after normalization to the corresponding noncyclic 36B4 expression levels. Results are shown as the mean ± SEM of values of two independent experiments, each with three animals for both genotypes at each time point. There were significant variations between the two genotypes at CT1 and CT21 for *bmal1* and CT8 for *per3* as indicated by an *asterisk* ($P < 0.01$).

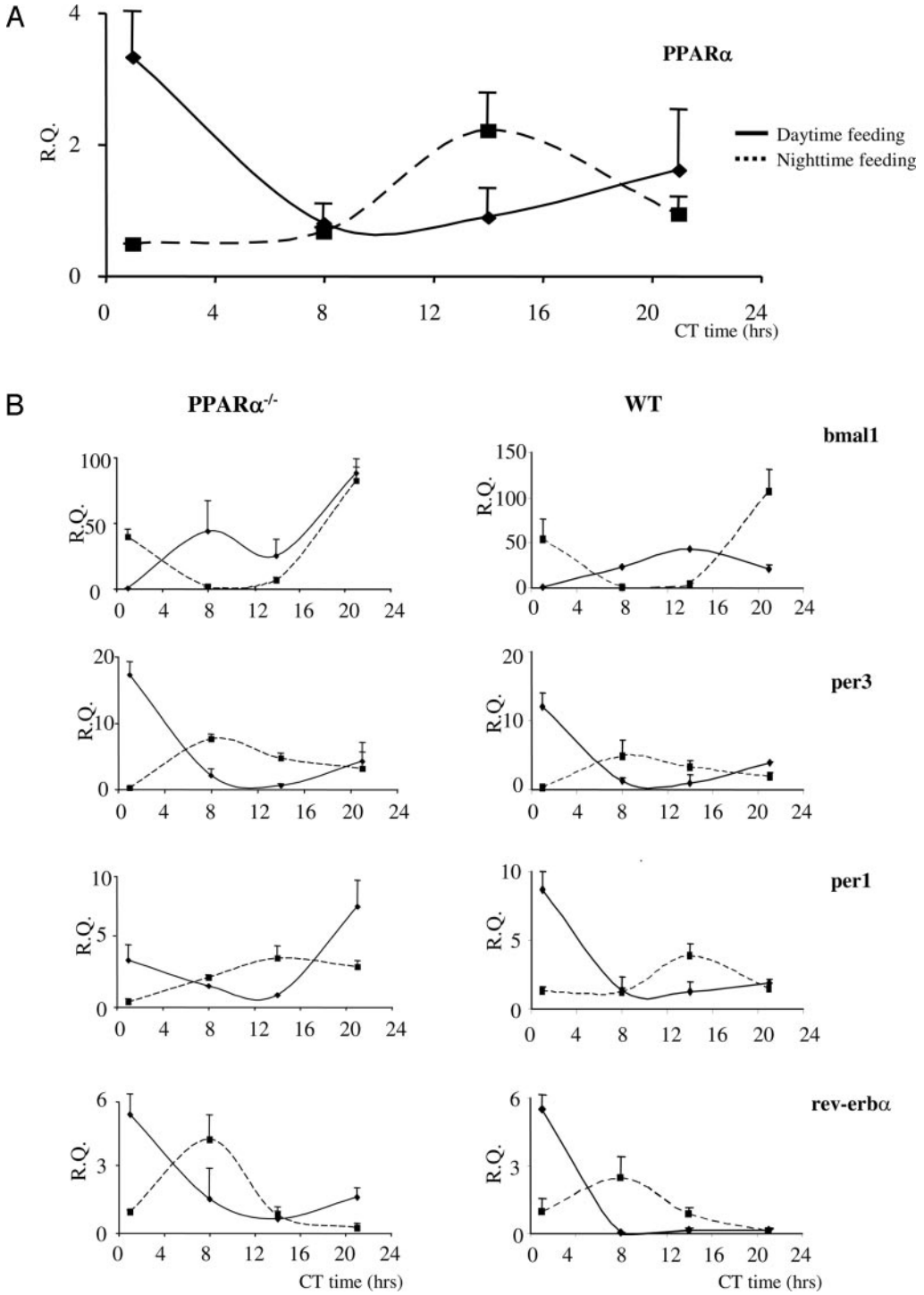

Fig. 3. Circadian Gene Expression in Liver after Restricted Feeding in WT and *PPARα*$^{-/-}$ Mice

A, *PPARα* expression in the liver of food-entrained WT mice (daytime (-♦-) or nighttime feeding (■). B, Circadian accumulation of *bmal1*, *per1*, *per3*, and *rev-erbα* mRNAs in liver of WT and *PPARα*$^{-/-}$ mice as a function of daytime (♦) or nighttime feeding (■). Transcript levels are displayed as relative quantity (RQ) after normalization to the corresponding noncyclic 36B4 expression levels. The presented values are expressed as means ± SEM of duplicates of the same reaction for six different mice per genotype.

Next, we studied whether feeding time can also reset the phase of *bmal1*, *per1*, *per3*, and *rev-erbα* expression in the absence of PPARα (Fig. 3B). Except the *bmal1* expression, which was refractory to resetting, *per1*, *per3*, and *rev-erbα* genes showed an inversed rhythm of their expression in the liver after daytime-restricted feeding compared with nighttime feeding in the *PPARα* knockout context. The present

results show that feeding time can reset the expression of *per1*, *per3*, and *rev-erbα* in the liver of *PPARα*$^{-/-}$ mice and suggest that *bmal1* expression might be controlled by PPARα.

Fenofibrate Induces Expression of Clock Genes in Rat-1 Fibroblasts and Up-Regulates *bmal1* Gene Expression in Liver

Because PPARα presents a circadian expression in liver, we first studied whether a shock with a serum-rich medium is able to induce an oscillation of *PPARα* in the well-established *in vitro* model Rat-1 fibroblasts (9, 13, 48–50). As shown in Fig. 4A, *PPARα* gene expression is induced by a serum shock in Rat-1 fibroblasts with a maximal level reached 12 h after the beginning of the treatment in comparison with the control. The induced-oscillating expressions of *rev-erbα* and *cry1* after serum shock were in accordance with previously reported data (9, 51). In addition, *rev-erbα* expression is delayed (peak at 16 h after the serum shock treatment) compared with *PPARα* expression, which suggests that in Rat-1 fibroblasts *PPARα* induces *rev-erbα* expression. Similarly to these observations, it seems likely that serum largely participates in the synchronization of the circadian oscillation of *PPARα* mRNA in fibroblast cultures.

Fibrates are well-known activators of *PPARα* expression and are classical drugs used in the treatment of dyslipidemias. Using the same *in vitro* model, we then evaluated whether fenofibrate can induce expression of *PPARα* gene. The addition of fenofibrate into the serum-free medium triggered a rhythmic expression of *PPARα* peaking 12–14 h as observed after a serum treatment (Fig. 4B). Fenofibrate induced a *PPARα* expression 6-fold higher than serum (Fig. 4, A and B).

Fenofibrate can also induce a rhythmic expression of clock genes. The temporal induction by fenofibrate of *cry1, rev-erbα*, and *bmal1* expression into the culture medium was almost similar to that observed after a serum-shock: *cry1* mRNA level peaked at Zeitgeber time 8 (ZT8) to ZT10 (where ZT0 is time when the light switched on at 0700 h, and ZT12 is time when the light switched off at 1900 h), *rev-erbα* at ZT16–ZT20, and *bmal1* at ZT4 (Fig. 4C). Thus, fenofibrate can act as a Zeitgeber in cell culture and trigger a rhythm of clock gene expression.

To determine whether fenofibrate can also reset the liver clock *in vivo*, the response of *bmal1* and *rev-erbα* genes to fenofibrate treatment was compared in liver of WT and *PPARα*$^{-/-}$ mice (Fig. 4D). In accordance with our previous results, fenofibrate was also able to markedly induce *bmal1* mRNA levels in control mice but not in *PPARα*-deficient mice, suggesting that *bmal1* induction by fenofibrate is effectively mediated by PPARα. Similarly, as it was previously reported in rat liver and human hepatocytes (30, 43), administration of fenofibrate in WT mice significantly increased the *rev-erbα* mRNA levels. As expected, *rev-erbα* expression in *PPARα* knockout mice was not induced by fenofibrate, confirming that murine *rev-erbα* induction by fenofibrate is mediated by PPARα. Taken together, these data strongly suggest that fenofibrate can alter the endogenous rhythm of the liver peripheral clock and *in vivo.*

PPARα Is a Direct Regulator of *bmal1* Expression

To specify the regulation of *bmal1* gene expression by PPARα suggested by our analysis of *bmal1* expression in the *PPARα*$^{-/-}$ mice, we analyzed the interaction of PPARα with the *bmal1* promoter. First, we performed a bioinformatic research of potential PPARα binding sites (PPREs) on the *bmal1* promoter region using the Nubiscan software. Two major PPREs were predicted at the positions −1519 (+, TGGACATGGGTCA) and −4943 (−, AGGGCTGAGGACA), the start site corresponding to the one identified in mouse testis (52). To evaluate whether PPARα binds to the *bmal1* gene promoter *in vivo*, the occupancy of the potential PPRE binding sites by PPARα was analyzed using chromatin immunoprecipitation (ChIP) assays performed on hepatocyte DNA using an anti-PPARα antibody (Fig. 5A). As previously shown *in vitro*, the DNA encompassing the *rev-erbα* Rev-DR2 site (position −45) was precipitated *in vivo* by the anti-PPARα antibody after fibrate treatment (Fig. 5A, *lower panel*, lanes 7 and 9), in accordance with a PPARα-Rev-erbα cross talk through competition for binding to the same Rev-DR2 site (43). Moreover, an amplification product was observed when the same DNA samples were PCR-amplified using primers covering the PPRE located at the position −1519 in the *bmal1* promoter (Fig. 5A, *upper panel*, lanes 7 and 9). No amplification product was obtained using primers flanking the site at the position −4943 (*middle panel*). These data further demonstrate that in mice *in vivo*, PPARα directly binds to the PPRE site located at the position −1519 of the *bmal1* promoter in peripheral oscillators and therefore that *bmal1* is a direct PPARα target gene.

PPARα mRNA Expression Is Severely Down-Regulated in the Liver of *bmal1*$^{-/-}$ Mice

To test the hypothesis that the regulation of circadian *PPARα* expression involves *bmal1* in the liver, we analyzed the daily accumulation of *PPARα* mRNA in the liver of *bmal1*$^{-/-}$ mice by quantitative PCR. *PPARα* mRNA expression is rhythmic in *bmal1*$^{-/-}$ mice with a maximum around ZT12 as observed in WT mice, whereas the amplitude of the peak of expression is significantly dampened (Fig. 5B). This result suggests that BMAL1 is an upstream regulator of *PPARα* gene and is consistent with the recent observation that *PPARα* expression is also CLOCK dependent (27).

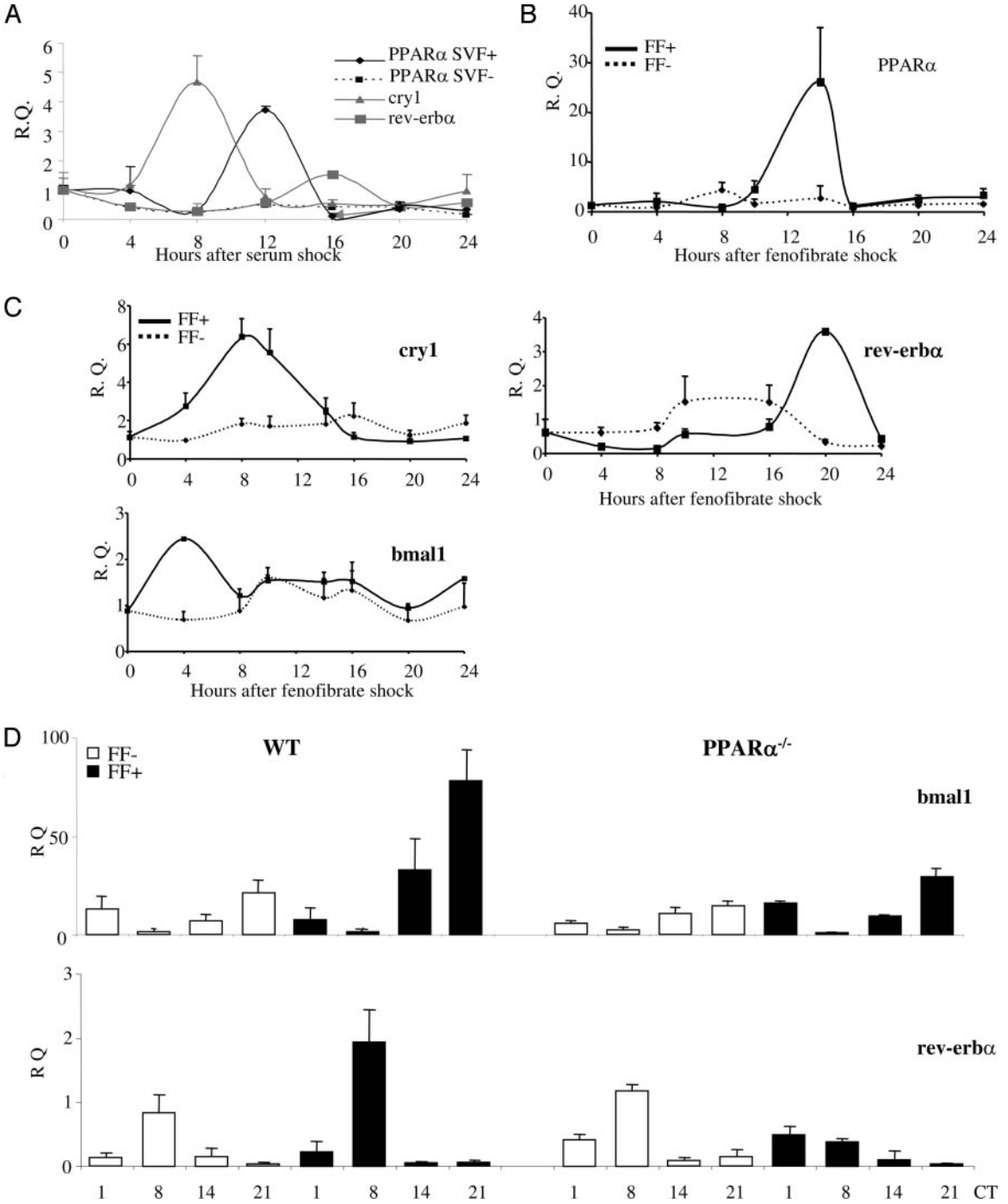

Fig. 4. Effect of Serum Shock or Fenofibrate on Clock Gene Expression

A, Accumulation of *PPARα* (♦), *cry1* (▲), and *rev-erbα* (■) mRNAs in Rat-1 fibroblasts shocked with 50% of horse serum. *PPARα* accumulation in absence of horse serum (■) is reported as a control of noninduction. The presented values are means ± SEM of duplicates of the same reaction for three different experimental points. B, Accumulation of *PPARα* mRNAs in Rat-1 fibroblasts shocked with 50 μM fenofibrate (■) or with vehicle (♦) as controls. The presented values are means ± SEM of duplicates of the same reaction for three independent experimental points. C, Accumulation of *bma1l*, *cry1*, and *rev-erbα* mRNAs in Rat-1 fibroblasts shocked with fenofibrate (■) or with vehicle (♦) as controls. The presented values are means ± SEM of duplicates of three independent experimental points. D, Circadian accumulation of *bmal1* and *rev-erbα* mRNAs in liver of WT and *PPARa*$^{-/-}$ mice treated (■) or not (□) with fenofibrate. In this experiment, mice were treated for 2 wk with fenofibric acid (vehicle DMSO) mixed in the drinking water at the final concentration of 7 mM. Control animals were treated with the vehicle in the drinking water. Real-time PCR was used to determine transcript levels. Transcript level values are displayed as relative quantity (RQ) after normalization to the noncyclic 36B4 expression levels in the same sample. Results are expressed as the means ± SEM of values from eight animals for both genotypes at each time point.

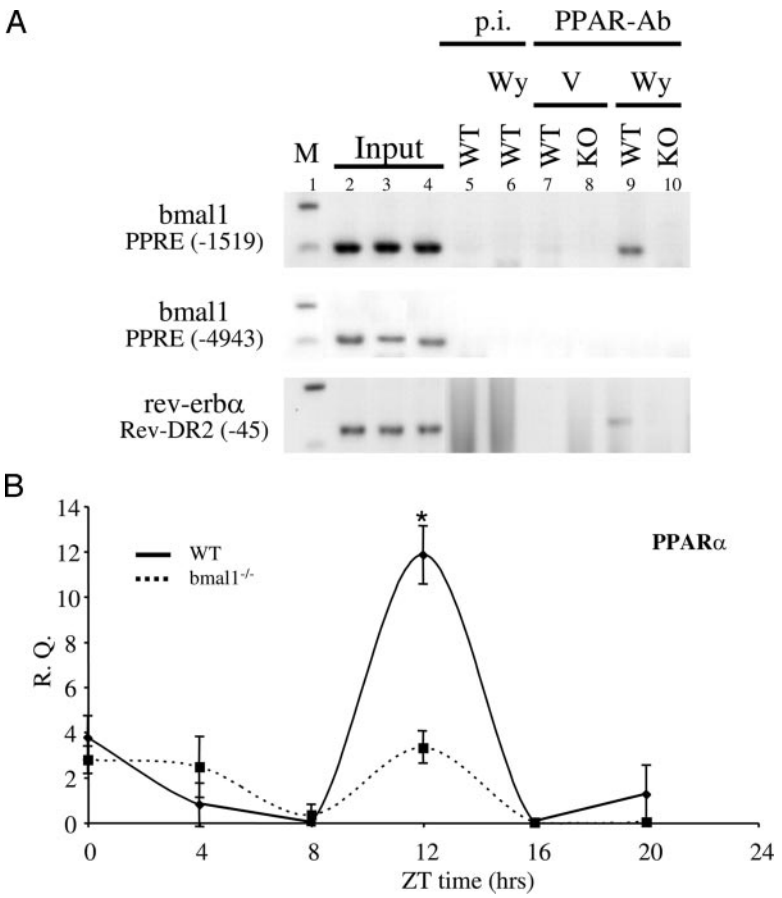

Fig. 5. Reciprocal Regulation of BMAL1 and PPARα

A, ChIP of the potential *bmal1* and *rev-erbα* PPRE elements with PPARα antibodies. WT and *PPARα*$^{-/-}$ mice (n = 3 for both genotypes) were fed for 5 d with either Wy14,643 or vehicle (V). ChIP of liver extracts was performed with a PPARα antibody (PPAR-Ab) and analyzed by PCR for enrichment of the PPRE element of the *bmal1* promoter (*top panels*) and of the *rev-erbα* promoter Rev -DR2 (*bottom panel*). p.i., Preimmune serum; Input, nonprecipitated genomic DNA. B, Daily expression of *PPARα* mRNAs in WT (♦) and *bmal1* mutant mice (■) using real-time PCR. Transcript levels were normalized against the noncyclic 36B4 transcript level in the same sample. Results are expressed as the means ± SEM of values from three to five animals at each time point. Statistically significant differences between WT and deficient mice are indicated by an *asterisk* ($P < 0.0001$).

DISCUSSION

A New Regulatory Feedback Loop Involved BMAL1 and PPARα in Peripheral Clocks

Molecular dissection of the mechanisms by which the clock oscillating system is controlled remains one of the most important challenges to assess the importance of the circadian regulation in diverse physiological and metabolic processes in mammals. In the present study, we have integrated the *PPARα* gene and its protein into a new positive-regulatory feedback loop in the liver. A model summarizing our main results is shown in Fig. 6. We show that *PPARα* plays an important role in the endogenous rhythmic property of peripheral clocks *in vivo*, whereas in the central clock *PPARα* deficiency does not alter the circadian expression of clock genes. These clock gene expressions are only affected in the liver of *PPARα*-deficient mice. First, *PPARα* is not essential to drive the central circadian system because *PPARα*$^{-/-}$ mice entrained normally without arrhythmic behavior in constant darkness and displayed no alteration in the amplitude and the phase of circadian expression of the clock genes (*bmal1*, *per2*, *per3*, *cry2*, and *rev-erbα*) compared with WT mice. The lack of PPARα-dependent clock regulation *in vivo* in the SCN can be due either to the absence of a circadian PPARα function in the central clock or to the presence of another isotype of PPAR (β, γ) or other nuclear receptors that are able to bind to the PPRE site and to exert a compensatory effect. Second, the expression of *bmal1*, an essential gene of the molecular oscillator, is drastically reduced in the liver of *PPARα*-deficient mice. This regulation of *bmal1* transcription by PPARα is likely through a direct binding of PPARα to the PPRE element located at the position −1519 in the *bmal1* promoter. In addition to the established transactivation of the circadian promoter *rev-erbα* by PPARα (43), our data largely support the view that PPARα also associates with the

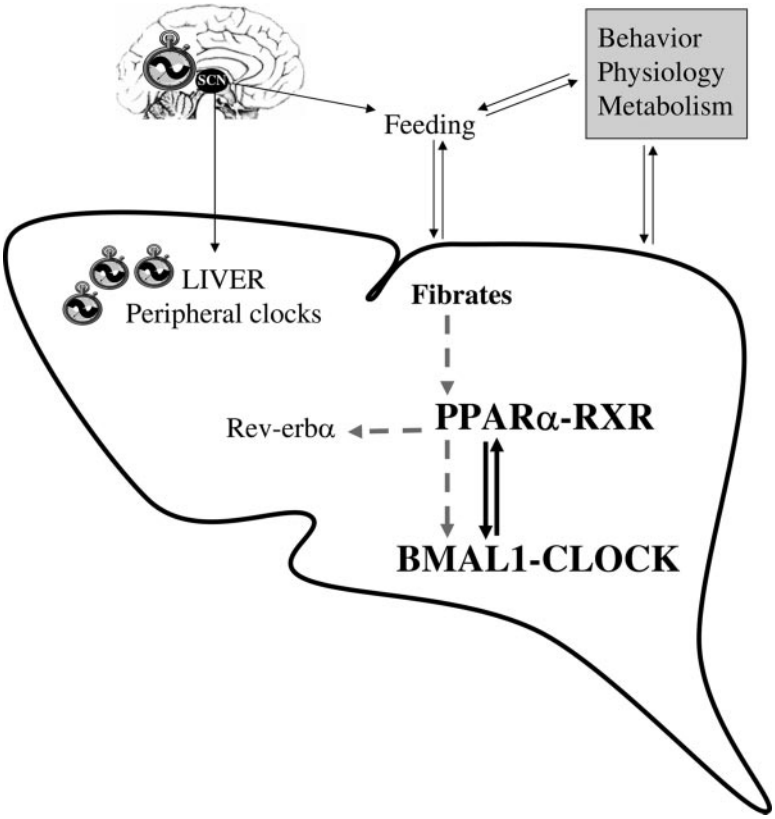

Fig. 6. Model of Cross Talk between PPARα and Circadian Pathways Depicting the Control of Circadian Regulation by PPARα in Peripheral Clocks

In mammals, circadian rhythms are generated by the main pacemaker located in the SCN of the hypothalamus, which synchronizes the peripheral oscillators and ensure an ordered response of the organism in terms of physiology, metabolism, and behavior to environmental changes. These peripheral clocks can be resetted by alternative routes such as feeding time. Herein, we show that PPARα is entirely resetted by feeding time. We propose that *bmal1* transcription is directly positively regulated by PPARα and that BMAL1 imposes circadian regulation on *PPARα* transcription (*bold arrows*). The amplified accumulation of PPARα under fibrate treatment leads in turn to a higher level of expression of *bmal1* gene (*dashed arrows*).

circadian *bmal1* promoter *in vivo*. Interestingly, *PPARα* expression is strongly decreased in the liver of *bmal1*-deficient mice compared with WT mice. Because no significant change in hepatic CLOCK level is found in *bmal1*-deficient mice compared with WT mice (53), this suggests that BMAL1 itself is in return involved in the circadian transactivation of *PPARα* gene at the level of peripheral oscillators in mice. Other observations have also revealed that CLOCK is involved in the circadian transactivation of *PPARα* (27) and interacts with its partner RXR (34). We propose that *bmal1* is directly and positively regulated by PPARα and that BMAL1 imposes in return a circadian regulation on *PPARα* transcription.

PPARα Expression Is Resetted by Feeding in Peripheral Clocks

Liver is known to be the organ reacting most rapidly to the temporal feeding regimen because it plays a dominant role in the metabolism and processing of food components, such as proteins, lipids, and carbohydrates. It was clearly established that the circadian gene expression in peripheral hepatic cells is intimately connected to feeding (10, 11) and that PPARα is involved in food processing and energy homeostasis (45). A connection between circadian gene regulation, metabolism and energy homeostasis was also established. For example, glucose metabolism (*e.g.* 6-phosphofructokinase-2, aldolase, and glucose phosphate isomerase) is under the control of the circadian time-keeping system (23, 24). Rhythmic expression of numerous enzymes and transcription factors involved in protein and amino acid metabolism (*e.g.* serine dehydratase, DBP, and 3-hydroxy-3-methylglutaryl coenzyme A reductase) (7, 54), in fat metabolism (*e.g.* cholesterol 7α-hydroxylase, PPARα, HMG-CoA lyase and reductase) (7, 35, 55), or in detoxification process [*e.g.* steroid 15α-hydroxylase (Cyp2a4) and coumarin 7-hydroxylase (Cyp2a5), Cyp2e1, Cyp17, and glutathione-*S*-transferase θ2] (23, 56) was also observed in liver. Herein, we show that the circadian expression of *PPARα* is resetted by feeding time and that, in the absence of PPARα, clock

gene expression in liver is entrained by reversed feeding time, suggesting that PPARα does not play a major role in food resetting. We underline that, after an extended duration of daytime feeding, the food-imposed reversed phase of circadian gene expression in peripheral liver is similar between WT and *PPARα*-deficient mice. This is probably due to PPARα-independent signaling pathway that plays a major role in the phase resetting of circadian gene expression by feeding time.

Fibrates as a Resetting Signal in Cell Cultures and Peripheral Clocks

Previous studies have shown that *PPARα* expression is positively controlled by glucocorticoids and fibrates and negatively by insulin (37–39). Fibrates are also known to up-regulate the expression of *rev-erbα* in the rat liver and in both rat and human primary hepatocyte cultures (30, 43). *In vitro*, *PPARα* mRNA was induced in rat fibroblasts culture after both a serum shock and, more interestingly, a fenofibrate treatment. Moreover, fenofibrate efficiently stimulates the rhythmic expression of several clock genes such as *cry1*, *bmal1*, and *rev-erbα*. This suggests that fenofibrate is able to entrain rhythmic *PPARα* and clock gene expressions in Rat-1 fibroblasts. *In vivo*, *rev-erbα* mRNA level is up-regulated by fenofibrate in the mouse liver of WT animals. Because this up-regulation of *rev-erbα* gene by fibrates is not observed in the *PPARα-deficient* mice, we confirm *in vivo* that the induction of *rev-erbα* gene expression by fibrates is mediated by PPARα at the transcriptional level. Interestingly, the increased accumulation of PPARα after fibrate treatment leads in turn to a higher level of expression of *bmal1* in WT mice. Other convincing evidence that the fibrate effect on the *bmal1* expression is exerted at the transcriptional level via the PPARα protein is that fibrates have no effect on the *bmal1* expression in the *PPARα*$^{-/-}$ mice. Taken together, these results suggest that PPARα could play a role in integrating chemical signals inside the liver.

How Circadian Rhythm Might Influence a Fibrate Therapy?

To date, fibrates are clinically used as hypolipidemic drugs that lower plasma cholesterol and triglycerides. They exert their effect by regulating the expression of several key genes implicated in lipid metabolism via PPARα activation. Interestingly, fibrate therapy represents a cost-effective approach in the clinical management and the prevention of cardiovascular diseases in a growing population suffering from lifestyle-induced metabolic dysfunctions such as obesity, insulin resistance, and diabetes (57). Although important differences in lipid metabolism exist between mice and human, including the function of PPARα (58, 59), our *in vitro* and *in vivo* studies suggest that the regulation by fibrates of the circadian expression of clock genes may influence the success of a treatment because it would suggest a potential induction and subsequently a dysfunction of their expression after fibrate supply in patients. The validity of this hypothesis remains to be demonstrated in humans. Therefore, one additional question to address is how fibrate administration at a selected time of the day can impact the efficacy and the success of the treatment. It will be of interest to decipher the molecular mechanisms involved in the circadian expression of clock genes and PPARα-regulated genes in presence or absence of fibrates to provide new insight in the downstream circadian physiological and cellular processes governed by PPARα itself. Similarly, such understanding should lead to new strategies for pharmacological manipulation of the human clock to improve the treatment of dyslipidaemias.

In summary, our data indicate that PPARα is a specific element of the liver oscillatory clock in mammals and plays an important role in integrating signals into the clock machinery. We clearly demonstrate that, *in vivo*, PPARα is required to maintain normal circadian oscillation of the master clock gene *bmal1* in liver. This regulation occurs via a direct binding of PPARα on a PPRE located in the *bmal1* promoter. In addition, BMAL1 is an upstream regulator of the *PPARα* expression. This finding provides a new regulatory pathway for the circadian system and suggests that some transcription factors may have a specific role in the peripheral clocks. Further studies are now required to determine the exact impact of circadian rhythms on the metabolic processes governed by PPARα in peripheral organs. This can be addressed by investigating the circadian regulation of PPARα target genes and some of the downstream targets in the peripheral clock in WT, *PPARα*$^{-/-}$, and other clock mutant mice. The complete elucidation of the signaling elements involved in the interactions between central and peripheral clocks and the mechanisms that govern the interplay between metabolism and circadian oscillators will also have a major impact on the circadian field in the next future.

MATERIALS AND METHODS

Cell Culture, Serum, and Fibrate Shock

Rat-1 fibroblasts were grown in DMEM supplemented with 5% fetal calf serum and a mixture of penicillin/streptomycin/glutamine (PSG). The serum shock was done as described elsewhere using 50% horse serum (9). For the fibrate shock, the medium was exchanged with DMEM-PSG supplemented with 50 μM fenofibrate (Sigma, St. Louis, MO) after the cells reach confluence (time 0). This medium was replaced with fenofibrate-free DMEM-PSG medium after 2 h. At 0, 4, 8, 12, 16, 20, 24 h after shock, cells were lysed and kept at −70 C until RNA extraction. Whole-cell RNAs were extracted using GenElute Mammalian Total RNA Extraction kit from Sigma.

Animal Experiments

Purebred WT and homozygous *PPARα*$^{-/-}$ mice on an SV129 background were used. All experiments were done with male mice between 6 and 8 wk of age. Animals were kept under a 12L:12D cycle, and food and drinking water were available *ad libitum*, except when indicated otherwise. The experimental protocols of the current research were approved by the rules and regulations of French veterinary services.

Locomotor Activity Recording

Adult male mice (n = 8 for both WT and *PPARα*$^{-/-}$) were exposed to 12L:12D cycle for at least 2 wk. For monitoring locomotor activity, mice were housed individually in cages equipped with infrared motion captors placed over the cages and a computerized data acquisition system (Circadian Activity Monitoring System, Institut National de la Santé et de la Recherche Médicale, France). Activity records were analyzed with the Clocklab software package (Actimetrics, Evanston, IL). For each animal, the total duration of activity was determined every 2 or 12 h during the LD cycle and then averaged for WT and knockout mice. Animals were then allowed to free run in constant darkness (DD) for at least 15 d. The endogenous period in DD was subsequently determined using the Clocklab software.

Circadian Expression of Clock Genes in Wild-Type *vs.* PPARα$^{-/-}$ Mice

Twelve WT and 12 *PPARα*$^{-/-}$ mice were maintained for a period of 2 wk in a 12L:12D cycle and transferred in DD the day before the kill. Livers and SCN were removed at CT1, CT8, CT14, and CT21; stored at −70 C until RNA extraction; and analyzed by quantitative RT-PCR. The experiment was done twice.

Restricted Feeding

Mice (n = 24 both for WT and *PPARα*$^{-/-}$) fed during the day received food when light was on (0700 to 1900 h), whereas mice (n = 24 both for WT and *PPARα*$^{-/-}$) fed during the night received food from 1900 to 0700 h for 2 wk. Water was freely available over the experimental period. As controls, WT and PPARα$^{-/-}$ mice (n = 24 for both) were fed *ad libitum*. Mice were transferred in DD the day before killing. Livers were dissected at indicated circadian times (CT1, CT8, CT14, CT21), stored at −70 C until RNA extraction, and analyzed by quantitative-RT-PCR.

Fenofibrate Response

In this experiment, mice (n = 32 both for WT and *PPARα*$^{-/-}$) were treated for 2 wk with fenofibric acid (vehicle DMSO) mixed in the drinking water at the final concentration of 7 mM. Control animals (n = 32 both for WT and *PPARα*$^{-/-}$) were treated with vehicle in the drinking water. Livers were removed at CT1, CT8, CT14, and CT21, stored at −70 C until RNA extraction, and analyzed by quantitative RT-PCR.

Analysis of *bmal1*$^{-/-}$ Mice

WT and homozygous *bmal1*$^{-/-}$ mice on B6 background (both females and males aged from 8–14 wk provided by C. Bradfield, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI) were kept under 12L:12D cycles and fed *ad libitum*. Livers were removed at indicated Zeitgeber time. Reverse-transcribed total RNAs from three to five animals per time point were analyzed by quantitative RT-PCR as described below.

RNA Extraction and Quantitative RT-PCR

Total RNAs were prepared from cells or from organs according to the manufacturer's instructions (Sigma) and reverse transcribed using random primers and MMLV Reverse Transcriptase (Invitrogen, San Diego, CA). cDNA were then used as template for a quantitative real-time PCR assay using the QuantiTect SYBR Green PCR reagents (Qiagen, Valencia, CA) and the DNA Engine Opticon system (MJ Research, Cambridge, MA). Each couple of oligonucleotides used were designed to hybridize on different exons. The sequences of forward and reverse primers were as follows: bmal1, forward, 5′-CCAAGAAAGTATGGACACAGACAAA-3′; bmal1, reverse, 5′-GCATTCTTGATCCTTCCTTGGT-3′; cry1, forward, 5′-CTGGCGTGGAAGTCATCGT-3′; cry1, reverse, 5′-CTGTCCGCCATTGAGTTCTATG-3′; cry2, forward, 5′-TGTCCCTTCCTGTGTGGAAGA-3′; cry2, reverse, 5′-GCTCCCAGCTTGGCTTGAA-3′; per1, forward, 5′-GGAGACCACTGAGAGCAGCAAG-3′; per1, reverse, 5′-CGCACTCAGGAGGCTGTAGGC-3′; per2, forward, 5′-ATGCTCGCCATCCACAAGA-3′; per2, reverse, 5′-GCGGAATCGAATGGGAGAAT-3′; per3, forward, 5′-GGCGTTCTACGCGCACACTGC-3′; per3, reverse, 5′-CGCTGGTGCACATTCATACTGCG-3′; pparα, forward, 5′-CGCTATGAAGTTCAATGCCTT-3′; pparα, reverse, 5′-TGCAACTTCTCAATGTAGCC-3′; rev-erbα, forward, 5′-CATGGTGCTACTGTGTAAGGTGTGT-3′; rev-erbα, reverse, 5′-CACAGGCGTGCACTCCATAG-3′; 36B4, forward, 5′-ACCTCCTTCTTCCAGGCTTT-3′; and 36B4, reverse, 5′-CCCACCTTGTCTCCAGTCTTT-3′.

The efficiency (>95%) and the specificity of the amplification were controlled by generating standard curves and carrying out melting curves and agarose gels of the amplicons, respectively. The relative levels of each RNA were calculated by 2^{-CT} (CT standing as the cycle number in which SYBR Green fluorescence exceeds a constant threshold value) and normalized to the corresponding noncyclic 36B4 RNA levels. The presented values are means ± SEM of duplicates of the same reaction for at least three different mice or three experimental points.

The significance of differences was assessed by distribution-free two-way ANOVA. Paired Student's tests were used to compare WT and *PPARα*$^{-/-}$ or *bmal1*$^{-/-}$ mice. Results are presented as mean ± SEM. Differences were considered significant when $P < 0.05$.

ChIP Assays

ChIP experiments were performed as already described in Ijpenberg *et al.* (60). Briefly, WT and *PPARα*$^{-/-}$ mice (n = 3) were fed for 5 d with either Wy14,643 (50 mg/kg·d) or vehicle. Immunoprecipitation of liver extracts was done using a PPARα antibody and the immunoprecipitated DNA was PCR amplified using primers flanking either the *rev-erbα* Rev-DR2 (GTGTCACTGGGGC) or potential PPRE (usually AGGTCANAGGTCA) on the *bmal1* promoter predicted using the computer program NUBISCAN available at the following website: www.nubiscan.unibas.ch (61). An equal volume of nonprecipitated genomic DNA (Input) was amplified as positive control. One fifth of PCR products were separated on an ethidium bromide-stained 2% agarose gel.

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