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Chronic Treatment with Prednisolone Represses the Circadian Oscillation of Clock Gene Expression in Mouse Peripheral Tissues

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Although altered homeostatic regulation, including disturbance of 24-h rhythms, is often observed in the patients undergoing glucocorticoid therapy, the mechanisms underlying the disturbance remains poorly understood. We report here that chronic treatment with a synthetic glucocorticoid, prednisolone (PSL), can cause alteration of circadian clock function at molecular level. Treatment of cultured hepatic cells (HepG2) with PSL induced expression of *Period1* (*Per1*), and the PSL treatment also attenuated the serum-induced oscillations in the expression of *Period2* (*Per2*), *Rev-erbα,* and *Bmal1* mRNA in HepG2 cells. Because the attenuation of clock gene oscillations was blocked by pretreating the cells with a *Per1* antisense phosphothioate oligodeoxynucleotide, the extensive expression of *Per1* induced by PSL may have resulted in the reduced amplitude of other clock gene oscillations. Continuous administration of PSL into mice constitutively increased the *Per1* mRNA levels in liver and skeletal muscle, which seems to attenuate the oscillation in the expressions of *Per2*, *Rev-erbα,* and *Bmal1*. However, a single daily administration of PSL at the time of day corresponding to acrophase of endogenous glucocorticoid levels had little effect on the rhythmic expression of clock genes. These results suggest a possible pharmacological action by PSL on the core circadian oscillation mechanism and indicate the possibility that the alteration of clock function induced by PSL can be avoided by optimizing the dosing schedule. (*Molecular Endocrinology* 20: 573–583, 2006)

MANY BIOLOGICAL PROCESSES in mammals are subject to daily oscillations, and some of these are controlled by self-sustained oscillation mechanism called circadian clock. This machinery operates at cellular levels and coordinates a wide variety of physiological processes and behavioral activities (1–3). Recent molecular dissection of the circadian biological clock system has revealed that oscillation in the transcription of specific clock genes plays a central role in the generation of circadian rhythms. Gene products of *Clock* and *Bmal1* form a heterodimer that activates the transcription of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes. Once the PER and CRY proteins have reached a critical concentration, they attenuate CLOCK/BMAL1 transactivation, thereby generating a circadian oscillation in their own transcription (4, 5). The clock genes, consisting of the core oscillation loop, control downstream events by regulating the expression of clock-controlled output genes. Namely, CLOCK:BMAL1 heterodimers act through an E box element of the output genes to activate the transcription, and the activation is inhibited by PER and CRY proteins (6, 7). The expression of the clock genes is also modulated by a second oscillation loop composed of the two orphan nuclear receptors, REV-ERBα and retinoid-related orphan receptor-α, which drive a circadian oscillation in *Bmal1* transcription (8, 9).

In mammals, the master clock in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus is entrained to a 24-h period by daily light-dark cycle. The master clock, in turn, synchronizes circadian oscillators in peripheral tissues through neural and/or humoral signals (10). Recently, it has been reported that retinoic acids, epidermal growth factor receptor, and glucocorticoid hormones function as humoral factors for resetting the peripheral oscillators (11–13). Among these substances, glucocorticoids have been considered to be particularly well suited for *Zeitgeber*

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Abbreviations: DMSO, Dimethylsulfoxide; GRE, glucocorticoid response element; ODN, oligodeoxynucleotide; PSL, prednisolone; SCN, suprachiasmatic nuclei; ZT, *Zeitgeber* time.

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for peripheral clocks. In fact, daily rhythm of glucocorticoid secretion is controlled by the hypothalamus-pituitary-adrenal axis, which in turn is regulated by the SCN (14, 15). Furthermore, a single administration of dexamethasone, a glucocorticoid receptor agonist, strongly induces phase shift of clock gene expression in peripheral tissues (13). These facts suggest that circadian secretion of glucocorticoid hormone acts to synchronize peripheral clocks with the central SCN pacemaker.

Circadian clock system is necessary to adapt endogenous physiological functions to daily variations in environmental conditions. Abnormality in circadian rhythms, such as the sleep-wake cycle and the timing of hormonal secretions, is implicated in various physiological and psychiatric disorders (16, 17). Although a large number of synthetic glucocorticoids are frequently used clinically as immunosuppressive or antiallergic agents, it remains poorly understood how the chronic treatment with synthetic glucocorticoid influences the circadian organization of mammalian clockwork.

In this study, we show that chronic treatment with synthetic glucocorticoid can alter the circadian clock function at molecular levels. Continuous administration of prednisolone (PSL), one of the most frequently used synthetic glucocorticoids for prolonged therapy, repressed the oscillation in the expression of clock genes in peripheral tissues of mice without changing the central SCN clockwork. The action of PSL on *Per1* expression appears to have altered directly the molecular clock function in the peripheral cells. Because effectiveness and/or toxicity of many drugs vary according to their administration time, we explored the possibility that the alteration of clock function induced by glucocorticoid therapy could be overcome by optimizing the dosing schedule.

RESULTS

The Effect of PSL on the Expression of Clock Genes in Cultured Hepatic Cells

We first explored how chronic treatment with glucocorticoids affects the expression of circadian clock genes in cultured hepatic cells (HepG2). Because a brief exposure of cultured fibroblasts to dexamethasone causes transient *Period1* (*Per1*) expression (13), we examined the influence of PSL on *Per1* mRNA levels. As shown in Fig. 1A, treatment of HepG2 cells with PSL for 72 h resulted in an accumulation of *Per1* mRNA in a concentration-dependent manner; the most significant accumulation was seen at a concentration of 0.5 μM. Dexamethasone also produced a similar concentration-dependent accumulation of *Per1* mRNA (data not shown).

Glucocorticoids exert their action on gene expression through activation of cytoplasmic glucocorticoid receptors that bind to glucocorticoid response elements (GREs). A consensus sequence of GRE is located at −2093 to −2079 (AGAACATGATGTTCC) in the 5′-flanking region of human *Per1* gene (18). The GER sequence was also found at a similar location in mouse *Per1* gene (AGAACACGATGTTCC). We therefore investigated whether PSL can directly activate the transcription of *Per1* gene. To this end, we performed *in vitro* transient transcription assays using *Per1*-luciferase reporter constructs driven by the 2207-bp mouse *Per1* promoter (Fig. 1B, *upper panel*). Treatment of the *Per1* luciferase reporter vector-transfected HepG2 cells with 0.5 μM PSL for 48 h resulted in a 7.8-fold increase in the transcriptional activity (Fig. 1B, *lower panel*). This PSL-induced transactivation was thought to be dependent on the GRE, because a mutation of the AGAACACGATGTTCC sequence to AGAACACGATGGCTC decreased the transcriptional activity from 7.8-fold to 2.1-fold.

We also explored whether the PSL-induced accumulation of *Per1* mRNA was caused by changing the stability of *Per1* mRNA transcripts. HepG2 cells were incubated for 48 h in the presence or absence of 0.5 μM PSL. Then 10 μg/ml of actinomycin D was added to the media, and total RNA was extracted at selected time intervals. In vehicle-treated cells, the half-life of *Per1* mRNA was approximately 2.98 h (Fig. 1C). However, the half-life was not obviously prolonged during exposure of the cells to PSL (3.07 h). Taken together, these results suggest that the PSL-induced accumulation of *Per1* mRNA occurs at the transcriptional level rather than as a result of altered mRNA stability.

The chronic treatment with PSL also increased PER1 protein abundance in cultured hepatic cells (Fig. 2A, *left panel*). Because PER1 protein plays a critical role in mammalian circadian clock system through interaction with other clock gene products, we investigated how the PSL treatment influences the expression of other clock genes. Treatment of HepG2 cells with 0.5 μM PSL for 72 h substantially decreased the mRNA levels of *Period2* (*Per2*), *Cryptochrome1* (*Cry1*), *D-site binding protein* (*Dbp*), and *Rev-erb*α, whereas it increased the levels of *Bmal1* mRNA (Fig. 2A, *left panel*). Because mRNA levels of these clock genes were also modulated by transfecting the cells with PER1 expression constructs (Fig. 2A, *right panel*), we investigated whether the PSL-induced accumulation of PER1 resulted in alteration of mRNA levels of *Per2*, *Cry1*, *Dbp*, *Rev-erb*α, and *Bmal1* genes. As shown in Fig. 2B, the inductive effect of PSL on *Per1* expression was blocked by pretreating the cells with the *Per1* antisense phosphothioate oligodeoxynucleotide (ODN). The pretreatment with *Per1* antisense ODNs also blocked the modulatory effect of PSL on mRNA expressions of *Per2*, *Cry1*, *Dbp*, *Rev-erb*α, and *Bmal1*. These data suggest that PSL-induced accumulation of PER1 alters the mRNA levels of other clock gene.

To obtain further insight into the mechanism of PSL-induced modulation of clock gene expression, we investigated how overexpression of PER1 proteins affected the transcriptional activity of *Per2*, *Rev-erb*α, and *Bmal1*. To this end, luciferase reporter plasmids

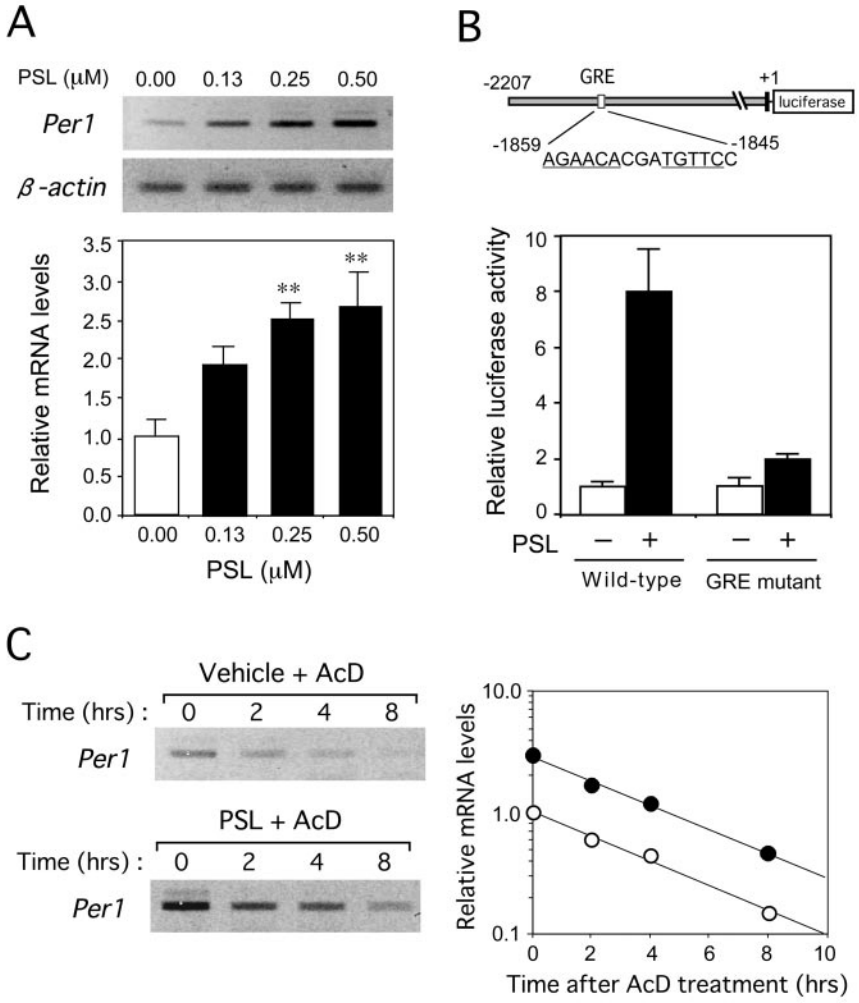

Fig. 1. Influence of PSL on the mRNA Levels of *Per1* in Cultured Hepatic Cells

A, Dose-dependent accumulation of Per1 mRNA by PSL. Cultured HepG2 cells were treated for 72 h with PSL at the indicated concentrations. Total RNA was extracted, and *Per1* mRNA was analyzed by RT-PCR. For plots of intensity, the mean peak value of the untreated group is set at 1.0. Each value represents the mean ± SEM (n = 3). **, $P < 0.01$ compared with the untreated group. B, Transcriptional activation of *Per1* gene by PSL. *Upper panel* shows location of GRE within the 5′-region of the mouse *Per1* gene. The numbers represent distance (bp) from the putative transcriptional start site, marked as +1. *Lower panel* shows the influence of PSL on the transcriptional activity of *Per1* gene. Each value represents the mean ± SEM of three replicates for a single assay. The results shown are representative of three independent experiments. C, Effect of PSL on *Per1* mRNA half-life in HepG2 cells. The cells were incubated for 48 h in the presence or absence of 0.5 μM PSL. Then 10 μg/ml of actinomycin D was added to the media, and total RNA was extracted at selected intervals. For plots of intensity, the value of the vehicle-treated group at 0 h is set at 1.0. AcD, Actinomycin D.

driven by promoters/enhancers of *Per2*, *Rev-erbα*, or *Bmal1* genes were constructed and transfected into HepG2 cells with PER1 expression vectors. As shown in Fig. 2C, the transcriptional activity of *Per2* and *Rev-erbα* was suppressed by cotransfection with PER1, whereas cotransfection of *Bmal1* luciferase reporter with PER1 caused a 2.1-fold increase in the promoter activity. Because overexpression of PER1 had no significant effect on the half-lives of *Per2*, *Rev-erbα,* and *Bmal1* mRNAs (Fig. 2D), the PSL-induced accumulation of PER1 seems to modulate the expression of other clock genes at transcriptional level rather than as a result of their altered mRNA stabilities.

Influence of Chronic Treatment with PSL on the Rhythmic Expression of Clock Genes

We next examined how chronic treatment with PSL affects the rhythmicity of clock gene expression. The oscillation in the expression of clock genes in cultured hepatic cells was triggered by a 2-h treatment with 50% horse serum. Thereafter the cells were incubated in the presence or absence of 0.5 μM PSL. In control cells, which were incubated in the absence of PSL after serum treatment, mRNA levels of *Per1, Per2*, *Rev-erbα,* and *Bmal1* showed obvious circadian oscillations (Fig. 3, A and C). The expression patterns of

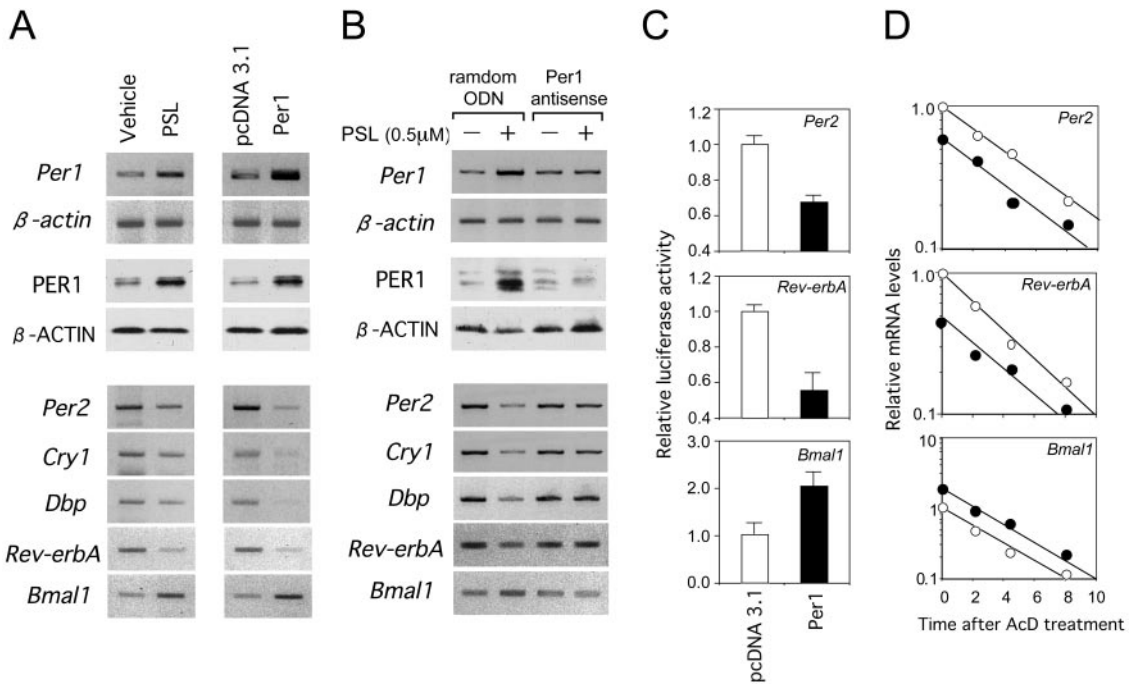

Fig. 2. Influence of PSL on the Expression of Clock Gene in Cultured HepG2 Cells

A, The cells were treated with 0.5 μM PSL (*left panel*) or transfected with 1.0 μg of PER1 expression constructs (*right panel*). Total RNA was extracted from the cells at 72 h after each treatment and subjected to RT-PCR of *Per1, Per2, Cry1*, *Dbp, Rev-erbα*, and *Bmal1* mRNA. Nuclear fractions from HepG2 cells were also prepared at 72 h after each treatment and subjected to Western blotting of PER1. B, Cells were treated in the presence (+) or absence (−) of 0.5 μM PSL after transfection with random ODN or *Per1* antisense. The mRNA levels of clock gene and PER1 protein abundance were analyzed as described in panel A. C, Influence of PER1 on the transcriptional activity of *Per2, Rev-erbα*, and *Bmal1* gene. HepG2 cells were transfected with each reporter gene construct and PER1 expression plasmid. Each luciferase activity was measured. 48 h after transfection. Each value represents the mean ± SEM of three replicates for a single assay. The results shown are representative of three independent experiments. D, Effect of overexpression of PER1 on half-lives of *Per2, Rev-erbα*, and *Bmal1* mRNA in HepG2 cells. The cells were incubated for 48 h in the presence or absence of 0.5 μM PSL. Then 10 μg/ml of actinomycin D was added to the media, and total RNA was extracted at selected intervals. For plots of intensity, the value of the vehicle-treated group at 0 h is set at 1.0. AcD, Actinomycin D.

clock genes were similar to those reported previously in rat-1 fibroblasts and vascular smooth muscle cells (11, 19). By contrast, chronic treatment of the serum-treated cells with 0.5 μM PSL decreased the amplitude of oscillations in clock gene expression. The levels of *Per1* and *Bmal1* mRNA were gradually increased during this treatment period, whereas the oscillations in the levels of *Per2* and *Rev-erbα* mRNAs were severely damped (Fig. 3, B and C). Similar decrease in the amplitude of clock gene oscillations was also observed in liver and skeletal muscle of mice infused continuously with PSL (10 μg/h, sc) using osmotic minipumps (Fig. 4). These results suggest that chronic treatment with PSL can affect the rhythmic expression of clock genes in peripheral tissues. Because the continuous administration of PSL into mice had little effect on the mRNA rhythms of *Per1* and *Per2* in the SCN (supplemental Fig. S1, published as supplemental data on The Endocrine Society's Journals Online web sit at http://mend.endojournals.org), the effects of PSL on the clock genes' expression in peripheral tissues are unlikely to be mediated via master clock function.

To identify the role of PER1 in PSL-induced alteration of the rhythmicity in clock gene expression, we examined the influence of PSL on the rhythmic expression of *Per1, Per2*, *Rev-erbα,* and *Bmal1* mRNAs in PER1 down-regulated cells. The expression of endogenous PER1 proteins was down-regulated by transfecting cells with *Per1* antisense ODNs. The transfected cells were treated with 50% horse serum for 2 h and were subsequently incubated in the presence or absence of 0.5 μM PSL. In the absence of PSL, treatment of random ODN-transfected (control) cells with 50% horse serum resulted in significant time-dependent variations in the mRNA levels of *Per1, Per2*, *Rev-erbα,* and *Bmal1* (Fig, 3D). Although the time-dependent variations in clock gene expression were eliminated when the random ODN-transfected cells were incubated in the presence of PSL after the serum treatment, the inhibitory effects of PSL on the oscillations in the expression of clock genes were not observed in PER1-down-regulated cells (Fig. 3D). The clock gene expression patterns in PSL-treated PER1-down-regulated cells were similar to those observed in control cells. These results suggest that PSL-induced accumulation of PER1 proteins influences the rhythmic expression of other clock genes.

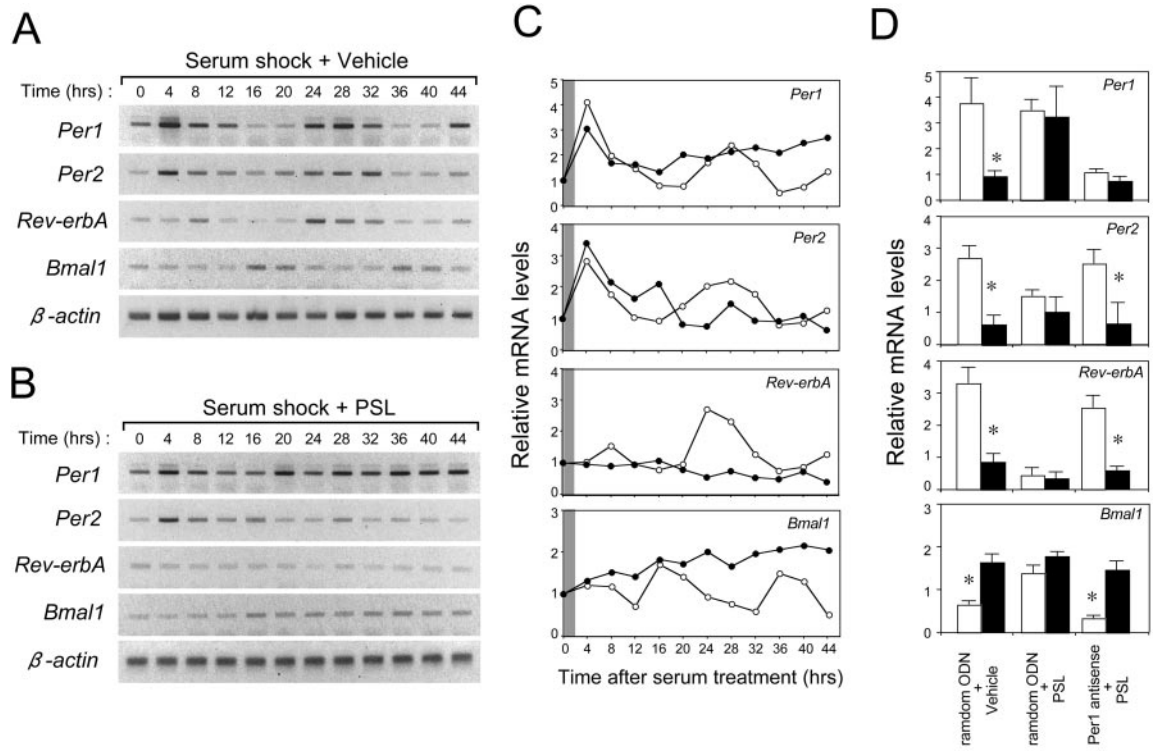

Fig. 3. Influence of Chronic Treatment with PSL on Circadian Gene Expression in PER1-Down-Regulated Cells

A and B, Temporal mRNA expression patterns for *Per1, Per2, Rev-erbα*, and *Bmal1* in vehicle (A)- or PSL (B)-treated cells after serum treatment. C, Quantification of temporal changes in *Per1, Per2, Rev-erbα*, and *Bmal1* mRNA in vehicle (○)- or PSL (●)-treated cells. Data shown were confirmed in two independent vehicle-treated cells and three PSL-treated cells, respectively. Basal levels of each mRNA (at time point 0) were set at 1.0. The *gray bars* indicate the duration of serum treatment. D, Effect of down-regulation of PER1 protein on the PSL-induced modulation of circadian gene expression. *Per1* antisense or random ODN-transfected cells were treated with 50% horse serum for 2 h and subsequently incubated in the presence or absence of 0.5 μM PSL. The mRNA levels of clock genes were determined at 28 h (□) and 40 h (■) after serum treatment. The levels of each mRNA were expressed as relative ratio to time point 0 (set at 1.0). Each value represents the mean ± SEM of three independent experiments. *, $P < 0.05$ compared with the value between the two times.

Changing the Dosing Schedule Minimizes the Disruptive Effect of PSL on Circadian Clock Function

Because continuous administration of PSL into mice modulated not only the circadian gene expression in peripheral tissues but also rhythmicity in locomotor activity and body temperature [Fig. 4 and supplemental Fig. S2 (published as supplemental data on The Endocrine Society's Journals Online web sit at http://mend.endojournals.org)], we examined whether the alteration of clock function induced by PSL could be overcome by optimizing the dosing schedule. To this end, we subcutaneously administrated a single dose of PSL (5 mg/kg, sc), or vehicle [0.5% dimethylsulfoxide (DMSO) in saline] as a control, at *Zeitgeber* time (ZT)0 or ZT12 daily for 7 d. The expression rhythms of mRNAs of clock genes in liver were assessed on d 7 after initiation of the drug treatment. Although the repetitive administration of PSL at ZT0 substantially altered the mRNA rhythms of *Per1, Per2*, *Rev-erbα,* and *Bmal1* in liver, the administration of PSL at ZT12 had no significant effect on the rhythms of clock genes (Fig. 5).

Consistent with its dosing schedule-dependent effect on the circadian clock gene expression, the repetitive administration of PSL at ZT0 modulated the daily rhythm of locomotor activity and body temperature (Fig. 6). However, these overt rhythms were not significantly altered by the administration of PSL at ZT12. These results suggest that the chronic treatment with exogenous glucocorticoids does not always affect the molecular clock function in peripheral tissues. The alteration of clock function induced by PSL could be avoided by optimizing the dosing schedule.

DISCUSSION

In this study, we showed that chronic treatment with synthetic glucocorticoid PSL modulated circadian clock function at molecular levels. Chronic treatment of cultured hepatic cells with PSL resulted in a significant induction of *Per1* expression and a modulation of

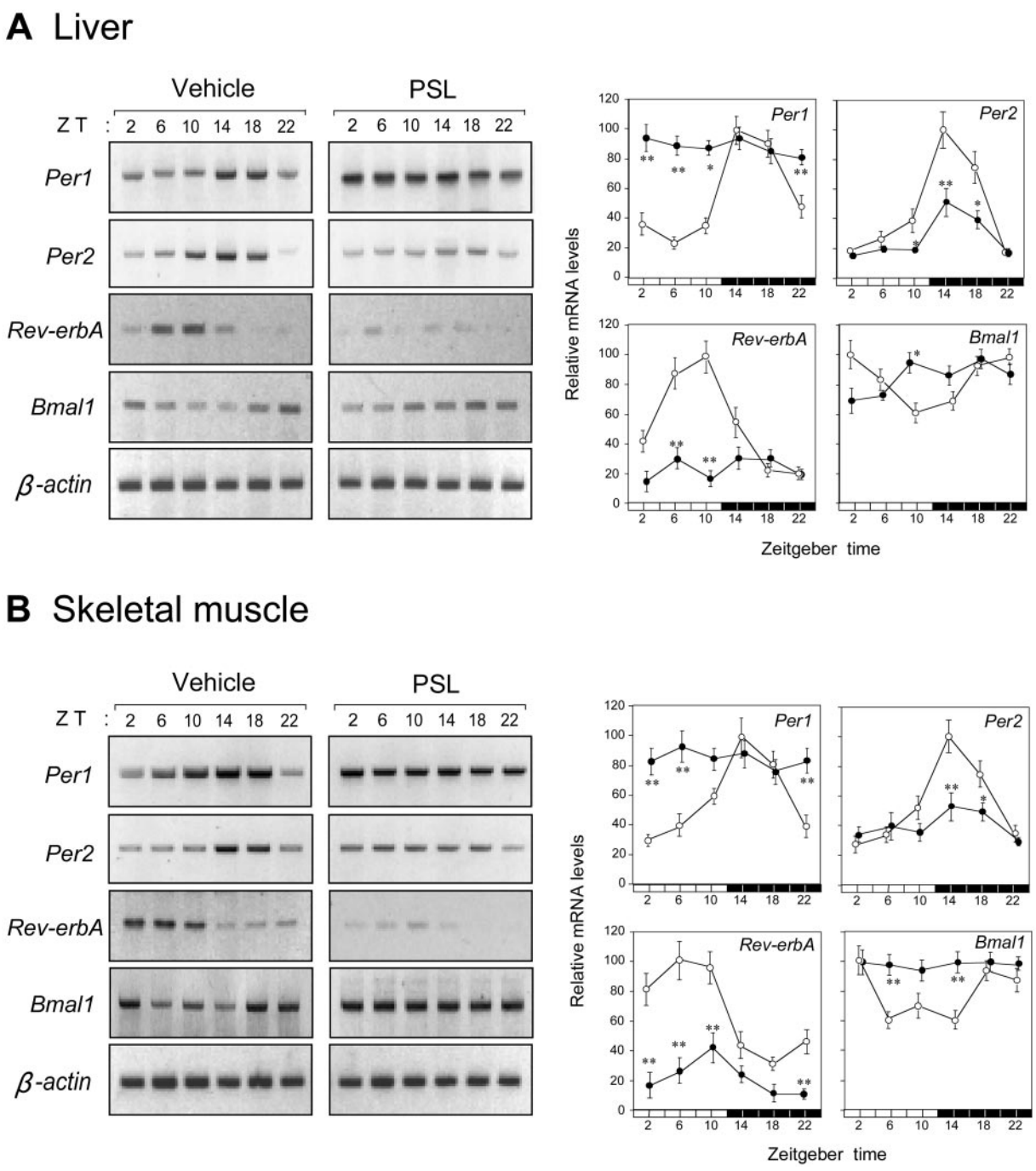

Fig. 4. Influence of PSL on mRNA Rhythm of Clock Genes in Liver (A) and Skeletal Muscle (B)

Mice were continuously infused with PSL (10 μg/h, sc ●) or vehicle (○) using osmotic minipump. The mRNA levels of *Per1*, *Per2*, *Rev-erb*α, and *Bmal1* were determined on d 7 after initiation of the drug treatment. Each point represents the mean ± SEM (n = 4–6). **, $P < 0.01$; *, $P < 0.05$ compared with the value of saline group at corresponding ZTs. The *horizontal bar at the bottom of the panels* indicates light and dark cycle. The *left panels* show representative electrophoretic image of RT-PCR products of clock genes in the liver and skeletal muscle. β-Actin mRNA was used as internal controls for transcripts the expression of which was constant throughout the day.

the mRNA levels of *Per2*, *Cry1*, *Dbp*, *Rev-erb*α, and *Bmal1.* Similar modulation of mRNA levels of those clock genes was also observed when the cells were transfected with PER1 expression constructs. The modulation of mRNA levels of clock gene appears to occur at transcriptional level rather than as a result of altered mRNA stability. The results of luciferase reporter gene analysis revealed that PSL directly acts on GRE in *Per1* gene, thereby activating transcription of its mRNA. The transactivation of *Per1* mRNA by PSL

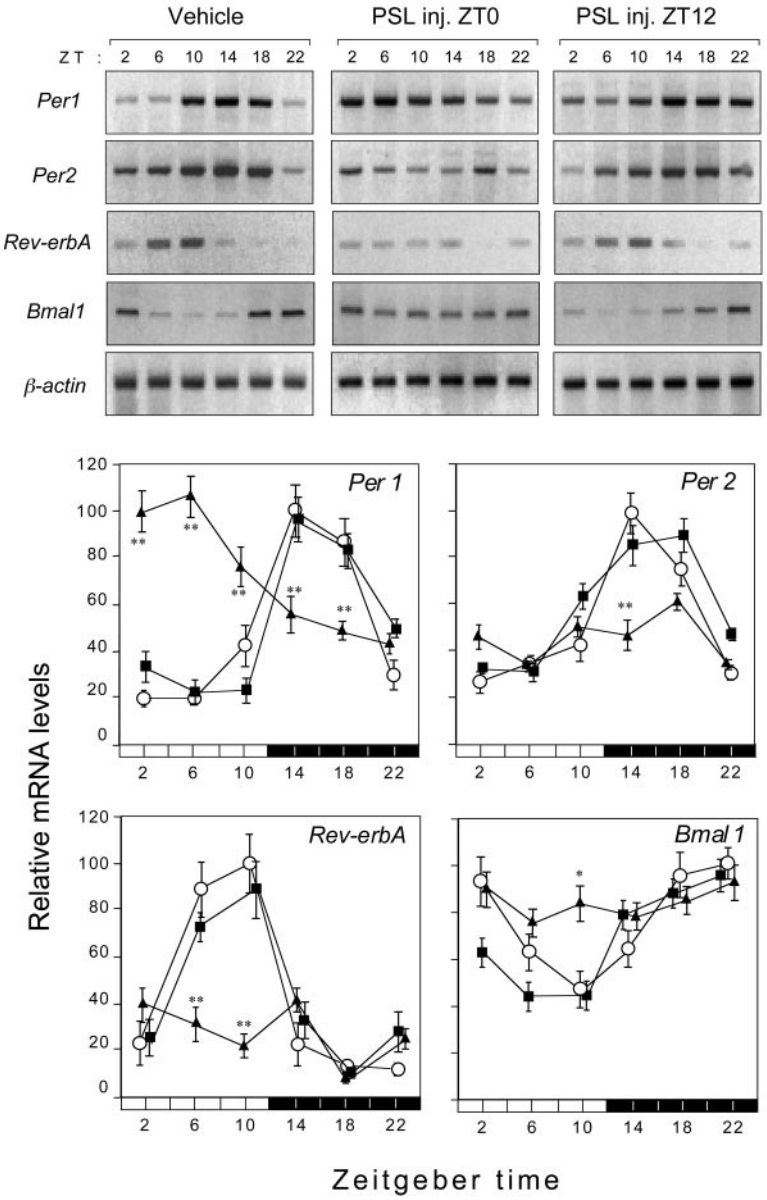

Fig. 5. Influence of PSL Dosing Schedule on mRNA Rhythms of *Per1*, *Per2, Rev-erbα*, and *Bmal1* in Liver

The mRNA levels for each of gene expression were determined on d 7 after initiation of a single daily dose of PSL (10 μg/h, sc) at ZT0 (▲), ZT12 (■), or vehicle (○). Each point represents the mean ± SEM (n = 4–6). **, $P < 0.01$; *, $P < 0.05$ compared with the value of saline group at corresponding ZTs. The *horizontal bar at the bottom of the panels* indicates light and dark cycle. The *upper panels* show representative electrophoretic image of RT-PCR products of clock genes in the liver. β-Actin mRNA was used as internal controls for transcripts the expression of which was constant throughout the day. inj., Injection.

seems to result in the accumulation of its protein. Because the regulation of clock gene transcription is also regulated by other clock gene products (20, 21), the accumulation of PER1 protein is likely to influence the expression of other clock genes. This notion is also supported by the present findings that the modulation of mRNA levels of *Per2*, *Cry1*, *Dbp*, *Rev-erbα,* and *Bmal1* induced by PSL was nullified by pretreating the cells with *Per1* antisense ODNs.

Chronic treatment with PSL also repressed the oscillation in the expression of *Per1, Per2*, *Rev-erbα,* and *Bmal1* in serum-shocked HepG2 cells. The circadian regulations of the transcription of *Per2*, *Rev-erbα,* and *Bmal1* genes are dependent on the functional clock-controlled elements. A noncanonical E box (CACGTT) in the *Per2* gene, canonical E boxes (CACGTG) in the *Rev-erbα* gene, and REV-ERB/retinoid-related orphan receptor-responsive elements in the *Bmal1* gene are suggested to be responsible for rhythmic expression of their mRNAs (8, 9, 20–23). CLOCK:BMAL1 heterodimers activate the transcription of *Per2* and *Rev-erbα* through an E box element, and the activation is

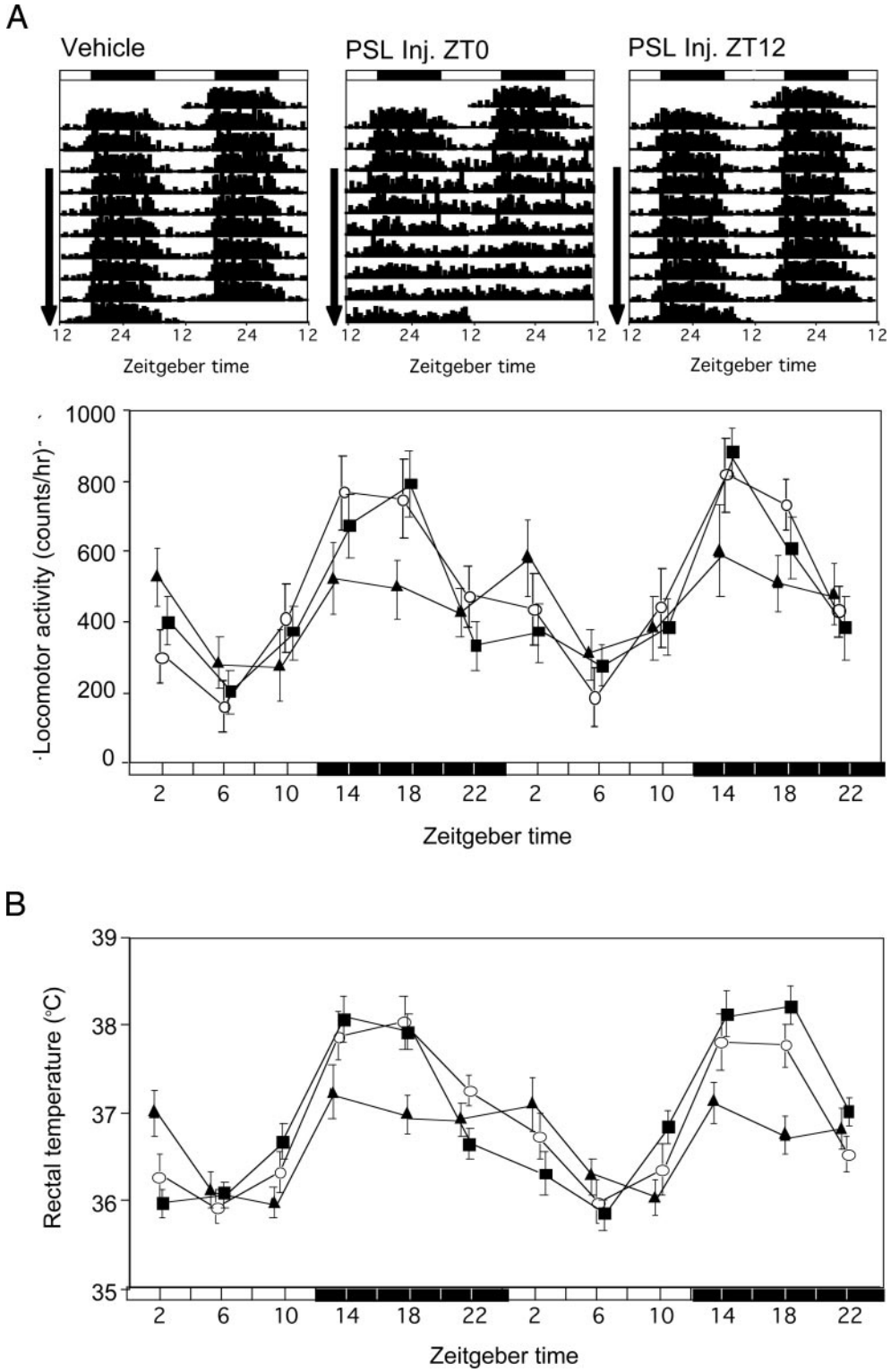

Fig. 6. Influence of PSL Dosing Schedule on Daily Rhythms of Locomotor Activity and Body Temperature

A single daily dose of PSL (5 mg/kg; ▲, ZT0; ■, ZT12) or vehicle (○) was administered sc for 7 d. Temporal profiles of locomotor activity (A) and rectal temperature (B) were determined on d 6 and d 7 after initiation of the drug treatment. Each point represents the mean ± SEM (n = 4–6). Each point represents the mean ± SEM. (n = 4–6). The *horizontal bar at the bottom of the panels* indicates light and dark cycle. *Closed arrows in upper panels* of Fig. 6A indicate the drug administration. Inj., Injection.

inhibited by PER and CRY proteins (21–23). The REV-ERBα protein also acts through retinoid-related orphan receptor-responsive elements to suppress the transcription of *Bmal1*, thereby regulating the rhythmic expression of its mRNA (8, 9). Therefore, PSL-induced accumulation of PER1 may result in repression of CLOCK/BMAL1-induced transactivation of *Per2* and *Rev-erb*α, which, in turn, leads to an increase in the levels of *Bmal1* mRNA. This mechanistic interpretation may also be applicable to previous findings that glucocorticoids are involved in the circadian regulation of *Rev-erb*α expression in mouse liver (24).

Consistent with its modulatory effect of PSL on the expression of clock genes in cultured hepatic cells, the continuous administration of PSL into mice prevented the oscillation in the expression of clock genes in the peripheral tissues. However, the treatment did not affect the mRNA rhythms of clock genes in the SCN. The cause may be that glucocorticoid receptor mRNAs do not accumulate in sufficient amounts to affect mouse SCN neurons (13). On a currently held model, mammalian circadian clock system is hierarchically organized: the master pacemaker in the SCN governs subsidiary oscillators in other brain regions and many peripheral tissues (25, 26). These subsidiary oscillators coordinate a variety of biological processes, producing overt rhythms in physiology and behavior (14, 15). Therefore, the direct action of PSL on molecular clock function of peripheral cells may lead to alteration of intrinsic rhythms of body temperature and locomotor activity.

Although the rhythmic expression of mRNA of clock genes in peripheral cells was changed in response to glucocorticoid stimuli, our present findings indicate that the peripheral oscillators would be less susceptible to exogenous glucocorticoid stimuli, when the glucocorticoid levels are endogenously increased. In nocturnally active rodents, the secretion of glucocorticoid hormones peaks from the late light phase to the early dark phase (14). The cyclic elevation of circulating glucocorticoid levels is thought to synchronize the oscillation in the expression of clock genes in peripheral cells. Therefore, the exogenous administration of PSL into mice at ZT0 may have impaired the endogenous nature of glucocorticoid rhythm and may have altered clock gene expression in their peripheral tissues. This may account for the fact that the disruptive effect of PSL on molecular clock function in peripheral cells can be lessened by choosing the optimal time for giving the dose.

Our results suggest that alteration of molecular clock function by PSL is the basis for adverse effects of glucocorticoid treatment on daily rhythms in physiology and behavior. The alteration of clock function seems to be elicited by the direct action of PSL on the *Per1* gene. Altered homeostatic regulation, including the disturbance of the 24-h rhythms, is often observed in patients in chronic glucocorticoid therapy (27–30). Our present finding may extend to understanding how chronic treatment with glucocorticoids modulates the molecular organization of circadian clockwork.

MATERIALS AND METHODS

Materials

PSL sodium succinate was purchased from Sigma Chemical Co. (St. Louis, MO), dissolved in sterilized saline containing 5% dextrose for treatment. The plasmids containing the mouse *Per1* (GenBank accession no. AB002108) were constructed as described previously (26). The coding region was ligated into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). The mouse *Per1* antisense ODN was designed targeting to the region of the mRNA containing the initiation ATG. The sequences of the mouse *Per1* antisense ODNs and random ODN were 5′-TAG GGG ACC ACT CAT GTC T-3′ and 5′-CCG TTA GTA CTG AGC TGA C-3′, respectively. The random ODN contained an equivalent GC content as the antisense ODNs of *Per1*. All ODNs were purified by HPLC to reduce the possible toxicity of phosphothioate ODNs.

Cell Culture and Transfection

HepG2 cells were maintained in DMEM supplemented with 10% fetal bovine serum. One day before transfection, the cells were seeded (1×10^5 per well) in six-well plates containing DMEM supplemented with 10% fetal bovine serum. Cells were transfected with 1.0 μg of PER1 expression constructs using LipofectAmine-Plus reagent (Invitrogen) according to the manufacturer's instructions. To examine the influence of overexpression of *Per1* on the mRNA levels of clock genes, total RNA from HepG2 cells was extracted at 72 h after transfection using TRIzol reagent (Invitrogen). *Per1* antisense or random ODN was also transfected into the cells using LipofectAmine-Plus reagent. Twenty four hours after transfection, 0.5 μM PSL or vehicle (0.5% DMSO in saline) was directly added in a medium of *Per1* antisense or random ODN-transfected cells. Total RNA was extracted after adding PSL for 48 h. To investigate the influence of PSL on the rhythmic expression of clock genes in PER1-down-regulated cells, Per1 antisense or random ODN was also transfected into the cells. Twenty four hours after transfection, *Per1* antisense or random ODN-transfected cells were treated with 50% horse serum in medium for 2 h and subsequently incubated in the presence or absence of 0.5 μM PSL. After serum treatment, total RNA was extracted at 4-h intervals for 2 d.

Luciferase Reporter Gene Analysis

The promoter or enhancer regions of *Per1* (−2207 to +24), *Per2* (−118 to +10), and *Bmal1* (−580 to +90) were amplified by PCR from mouse genomic DNA. pGL3-*Rev-erb*α-luciferase reporter vectors (−3942 to +1) were kindly provided by Dr. Shimba (Nihon University, Chiba, Japan). Glucocorticoid responsive element (GRE) in the 5′-flanking region of mouse *Per1* gene was mutated by using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). HepG2 cells were transfected with 10 ng of reporter constructs and 1.0 (total) of expression vectors using LipofectAmine-Plus reagent. To correct for variations in transfection efficiency, 0.1 ng of pRL-TK vector (Promega Corp., Madison, WI) was cotransfected in all experiments. The total amount of DNA per well was adjusted by adding pcDNA 3.1 vector (Invitrogen). The ratio of firefly luciferase activity (expressed from reporter construct) to *Renilla* luciferase activity (expressed from pRL-TK) in each sample served as a measurement of normalized luciferase activity.

Quantitative RT-PCR Analysis

The cDNA of human or mouse *Per1*, *Per2*, *Cry1*, *Dbp*, *Rev-erbα,* and *Bmal1,* and β-actin were synthesized and amplified by using a superscript one-step RT-PCR system (Invitrogen). To evaluate the quantitative reliability of RT-PCR, a kinetic analysis of amplified products was done to ensure that signals were derived only from the exponential phase of amplifications (31). The exponential phase of β-actin amplification in all experimental conditions occurred between the 26th and the 28th cycles, and the exponential phases of all target genes (clock genes) occurred between the 27th and the 30th cycles. The amplification efficiencies of β-actin and clock gene were comparable. Therefore, the amplification products were collected and quantified at the 27th or 28th cycle. The ratio of the amplified target to the amplified internal control (calculated by dividing the value of each *Per*, *Cry*, *Dbp, Rev-erbα*, or *Bmal1* by that of β-actin) was compared among groups.

Determination of Half-Lives of Clock Gene's mRNA

Actinomycin D (10 μg/ml) was added to HepG2 cells after 48-h incubation with or without 0.5 μM PSL. Cells were harvested at indicated times, and total RNA was extracted. RT-PCR analysis was performed as described above.

Western Blot Analysis

Nuclear fractions from HepG2 cells were prepared as described previously (31). The lysates containing 30 μg of total protein were loaded on SDS-PAGE, and the proteins were transferred to a polyvinylidene difluoride membrane. The membrane was reacted with antibodies against PER1 (Alpha Diagnostic, International, San Antonio, TX) or β-ACTIN (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Specific antigen/antibody complexes were visualized by using horseradish peroxidase-conjugated secondary antibodies and Super Signal Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL).

Animals and Treatment

Male ICR mice (5 wk of age) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). They were housed under a standardized light-dark cycle at room temperature of 24 ± 1 C and a humidity of 60 ± 10% with food and water *ad libitum*. The animals were adapted to the light-dark cycle for 2 wk before the experiments. During the dark period, a dim red light was used to aid treatment of the mice. An osmotic minipump (model 2001, ALZET; Alza Corp., Palo Alto, CA) was implanted under the skin of mice, and was used for continuous administration of PSL (10 μg/h) or vehicle (5% DMSO in saline). Total RNA from mouse tissues was extracted as follows: The brain, liver, and skeletal muscle were quickly removed at ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22. Coronal brain slices (500 μm) were prepared using a rodent brain matrix (RBM-2000C; ASI Instruments, Inc., Warren, MI), and the SCN was punched out bilaterally from the brain slices. To obtain an adequate amount of RNA from the SCN, the SCN from three mice in each group were combined and extracted. Extraction of total RNA from the liver and skeletal muscle of each individual mouse was carried out separately by using the Trizol reagent.

Determination of Locomotor Activity

Locomotor activity was measured using a photobeam activity monitoring system (Muromachi Kikai, Co. Ltd., Tokyo, Japan), and activity count (number of movements) was recorded at 0.5-h intervals. The activity records were double plotted so that each day's activity is shown both to the right and below that of the previous day. For visualization of locomotor activity rhythm, hourly activity counts were calculated using a moving average with a 4-h window.

Determination of Body Temperature

A lubricated thermocouple was inserted 1.5 cm into the rectum of mice, and the rectal temperature was determined using a digital thermometer (BAT-12; Bio Research Center, Tokyo, Japan).

Statistical Analysis

The significance of the 24-h variation in each parameter was tested by ANOVA. The statistical significance of differences among groups was analyzed by ANOVA and the Tukey multiple comparison test. A 5% level of probability was considered to be significant.

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