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Daily Expression of mRNAs for the Mammalian Clock Genes *Per2* and *Clock* in Mouse Suprachiasmatic Nuclei and Liver and Human Peripheral Blood Mononuclear Cells

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ABSTRACT—The mammalian circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus and in most peripheral tissues. Clock genes drive the biological clock. However, circadian expression variations of the human clock genes are still unclear. In this study, we analyzed the daily variations of *mPer2* and *mClock* mRNA expression in both the mouse SCN and liver to evaluate the central and peripheral alterations in the rodent clock genes. We also examined whether there are the daily variations of the clock genes *hPer2* and *hClock* in human peripheral blood mononuclear cells (PBMCs). The daily variation of *mClock* and *mPer2* mRNA expression in mouse SCN and liver were determined at ZT2, ZT6, ZT10, ZT14, ZT18 or ZT22. We isolated PBMCs from 9 healthy volunteers at 9:00 and 21:00 and examined the expression of *hPer2* and *hClock* mRNA by RT-PCR analysis. The animals exhibited a robust daily rhythm in the RNA levels of *mPer2* in the SCN and liver ($P<0.01$, respectively). In humans, *hPer2* mRNA expression also had daily variation, and the *hPer2* mRNA levels at 9:00 were significantly larger than those at 21:00 ($P<0.01$). While, the *Clock* mRNA in both mice and humans exhibited no daily variation. These findings suggest that the variation in *hPer2* mRNA expression may be useful for assessing human peripheral circadian systems.

Keywords: Clock gene, Liver, *Per2*, Peripheral blood mononuclear cell, Suprachiasmatic nucleus

In all living organisms, one of the most indispensable biological functions is the circadian clock (suprachiasmatic nuclei, SCN), which acts like a multifunction timer to regulate homeostatic systems such as sleep and activity, hormone levels, appetite, and other bodily functions with a 24-h cycle (1). Like any timing system, the circadian clock is made up of three components (2 – 6): an input pathway adjusting the time, a central oscillator generating the circadian signal, and an output pathway manifesting itself in circadian physiology and behavior. The daily changes in light intensities are thought to be the major environmental cue involved in circadian entrainment. Light-signals are perceived by photoreceptor cells in the retina and transmitted to neurons of the SCN via the retino-hypothalamic tract (2). Recently, clock genes were identified as the genes that ultimately control a vast array of circadian rhythms in physiology and behavior (7). Three mammalian clock genes (*mPer1*, *mPer2* and *mPer3*) are rhythmically expressed in the SCN. The *mPer1* and *mPer2* are induced in response to light (8). In particular, *mPer1* induction is considered to be an initial event in light-induced resetting and entrainment of the circadian biological clock (2). The transcriptional machinery of the core clockwork regulates a clock-controlled output rhythm (3). Namely, CLOCK-BMAL1 heterodimers act through an E-box enhancer to activate the transcription of *period*, vasopressin and *Dbp* mRNA (3, 4, 9). The PER, TIM and CRY proteins (10, 11) can inhibit this activation.

The expressions of mammalian circadian clock genes are found not only in the central pacemaker of the SCN but also in other peripheral tissues (12 – 15). While the

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mechanism of the clock genes has been examined extensively in rodents that are nocturnally active (13 – 17), there are few reports in diurnally active humans (18, 19). Bjarnason et al. reported that clock genes such as *hClock*, *hTim*, *hPer1*, *hCry1* and *hBmal1* are expressed in human oral mucosa and skin (18). Moreover, *hPer1*, *hCry1* and *hBmal1* display a rhythmic expression profile (18). However, it is difficult to obtain samples; i.e., oral mucosa or skin, from humans, because local anesthesia is necessary for biopsies. If peripheral blood can be used, it would be convenient and easy to evaluate the changes in the expression of the peripheral clock genes. Oishi et al. reported that *rPer2* mRNA expression in rats exhibited circadian oscillations in peripheral blood mononuclear cells (PBMCs) (14). On the other hand, it is unclear whether *hPer2* mRNA in humans has rhythmic expression in PBMCs. In this study, we analyzed the daily variation of *mPer2* and *mClock* mRNA expression in both the SCN and liver of mice to evaluate the central and peripheral alterations of these rodent clock genes. We also hypothesized that one of the human clock genes, *hPer2*, would be expressed in human PBMCs, and we investigated the daily expression of both *hPer2* and *hClock* mRNA expression in PBMCs from healthy volunteers.

MATERIALS AND METHODS

Animals and treatment

Male ICR mice (5-week-old) were purchased from Charles River Japan, Inc. (Kanagawa). The mice were housed 10 per cage in a light-controlled room (ZT0, light on; ZT12, light off; ZT, Zeitgeber time) at a room temperature of 24 ± 1°C and humidity of 60 ± 10% with food and water ad libitum. All mice were exposed to their light-dark cycle for 2 weeks before the experiments. During periods referred to as darkness, a dim red light was used to aid in the treatment of the mice. The 24-h rhythm of *mClock* and *mPer2* mRNA expression in the SCN and liver were determined at ZT2 (9:00), ZT6 (13:00), ZT10 (17:00), ZT14 (21:00), ZT18 (1:00) or ZT22 (5:00).

RT-PCR analysis of mClock and mPer2 mRNA

The brain and liver were quickly removed after cervical dislocation. Coronal hypothalamic slices (500 μm) were prepared through the SCN using rodent brain matrix (RBM-2000C; ASI Instruments, Warren, MI, USA). The SCNs were punched out bilaterally from the hypothalamic slices. The mRNA levels were determined based on a previously described method (20). Total RNA from the liver of individual mice was extracted separately using the Trizol reagent (Gibco BRL, Rockville, MD, USA). In addition, total RNA from the SCN of three mice was extracted in each group to obtain an adequate amount of RNA. A SuperScript One-Step RT-PCR System (Gibco BRL) was used for the reverse transcription of approximately 100 ng of RNA, and *mClock*, *mPer2* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs were amplified by PCR. PCR was performed six times on the different pooled SCN from three mice or the different livers from individual mice. PCR reactions were carried out with *mClock*, *mPer2* and *mGAPDH* primers. Cycling parameters were 94°C for 3 min, 27 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by a final extension step at 72°C for 10 min. The primer pairs used for the amplification of each product were as follows: 5'-AAGATTCTGGGTCTGACAAT-3' and 5'-TTGCAG CTTGAGACATCGCT-3' (*mClock*; 1599 – 1899, 301 bp, AF000998); 5'-ACACCACCCCTTACAAGCTTC-3' and 5'-CGCTGGATGATGTCTGGCTC-3' (*mPer2*; 840 – 1619, 780 bp, AF035830); 5'-GACCTCAACTACATGGTC TACA-3' and 5'-ACTCCACGACATACTCAGCAC-3' (*mGAPDH*; 155 – 332, 178 bp, M32599). The PCR products were run on 3% agarose gels (Nusieve 3:1 agarose; Takara Shuzo Co., Ltd., Ohtsu). The gel was photographed with Polaroid® type film after staining with ethidium-bromide. The intensity of the ethidium-bromide fluorescence in each band was assessed using NIH image software on a Macintosh computer. The ratio of the amplified target to the amplified competitor, calculated by dividing the value of *mClock* or *mPer2* mRNA by that of the internal control, *mGAPDH*, was compared. To calculate the relative RNA values while equalizing differences in the peak of the rhythm in the controls, values were normalized so that the peak value equaled 1.0.

Healthy subjects

Nine clinically healthy volunteers (7 men and 2 women) aged 22 – 42 years (mean ± S.D.; 29.0 ± 6.1 years) were investigated. Although we did not restrict the time schedule and the daily activity in the present study, the volunteers took food between 7:00 and 8:00 at breakfast time, between 12:00 and 14:00 at lunch time, and around 20:00 at night on the average. Moreover, they usually went to sleep around 23:00 and awoke around 7:00. They did not consume alcohol or caffeine and took no medications during or on the day preceding the study. Informed consent was obtained from all participants.

Peripheral blood mononuclear cells preparation

PBMCs were isolated at both 9:00 and 21:00 from 10-ml of heparinized human venous blood, which was layered on a Ficoll-Paque density gradient (Amersham Pharmacia Biotech, Tokyo) and centrifuged for 30 min at 300 × g at room temperature.

RT-PCR analysis of hClock and hPer2 mRNA

Total RNA was extracted from PBMCs using Isogen (Wako, Tokyo) according to the manufacturer's recommendations. Moreover, 20 μg of total RNA was treated with deoxyribonuclease (Wako). First strand cDNA was synthesized according to the protocol of Life Technologies with 5 μg of RNA, 10 μl of 5 × first strand buffer (Gibco BRL), 5 μl of 0.1 M DTT, 5 μl of 10 mM dNTP mix (Takara), 2 μl of random hexamers (Promega, Madison, WI, USA), 1 μl of RNase inhibitor (Wako Chemical, Richmond, VA, USA), 2 μl of M-MLV reverse transcriptase (Gibco BRL), and DEPC-treated H_2O to yield a final volume of 50 μl. The reaction mixture was incubated for 1-h at 37°C, and the mixture was heated to 95°C for 5 min and then cooled to 4°C.

Oligonucleotide primers were selected to amplify specific regions of human *Clock* (18) and *Per2* (Genebank accession number NM003894). Human *GAPDH* (21) primers were used as an internal control. The sequence of the oligonucleotide primers were as follows: *hGAPDH* (253 bp): 5'-GTCATCCATGACAACTTTGGTATCG-3' and 5'-GCAGGTCAGGTCCACCACTG-3'; *hClock* (171 bp): 5'-AAGTTAGGGCTGAAAGACGACG-3' and 5'-GAACTCCGAGAAGAGGCAGAAG-3'; *hPer2* (147 bp): 5'-GCAGGTGAAAGCCAATGAAG-3' and 5'-CACCGCAAACATATCGGCAT-3'.

PCR amplification was then performed with 1 μl of the cDNA supplemented with 4 μl of 10 × PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 0.05 μM of each primer, 1.5 U of Taq polymerase (Applied Biosystems), and then adjusted to 40 μl with sterile water. Cycling parameters were 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 59.7°C for 1 min, and 72°C for 7 min and 1 cycle of 72°C for 7 min. Each primer pair for *hClock* or *hPer2* was co-amplified with *hGAPDH*. The PCR products were separated on 1.5% agarose gels (Wako) stained with ethidium-bromide (Wako) and photographed using a BioDoc-It System (UVP, Inc., Upland, CA, USA). Bands were quantified as a ratio of the target gene to that of the control gene by ATTO Densitograph 4.0 (ATTO Inc., Tokyo).

Statistical analyses

The differences in *mClock* and *mPer2* mRNA expression were examined using ANOVA (Statview; Abacus Concepts, Berkeley, CA, USA). The values of the relative expression of mRNA in both *hPer2* and *hClock* are presented as the mean ± S.D. For the assessment of differences we used the Mann-Whitney U test to compare the ratios of mRNA expression between 9:00 and 21:00. Differences were considered significant at $P<0.05$.

RESULTS

Daily variation of mClock and mPer2 mRNA expression in SCN and liver

To explore the daily variation of *mClock* and *mPer2* mRNA expression in the SCN and liver, male ICR mice were housed in a light-controlled room. The mice exhibited no daily rhythm in the *mClock* mRNA in the SCN (Fig. 1). The relative ratio of *mClock* mRNA in the liver tended to be higher at ZT2 (9:00) (1.00 ± 0.38) than at ZT14 (21:00) (0.63 ± 0.20), but it was not statistically significant.

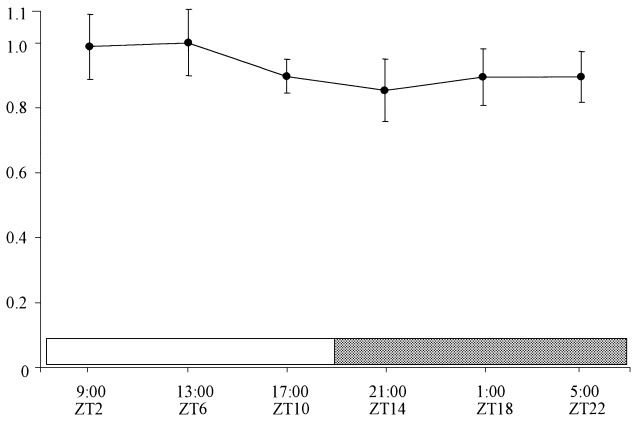

Fig. 1. Relative mRNA levels of *mClock* in the SCN. Each point represents the mean ± S.E.M. in 6 mice. No significant daily variation was found in the expression of *mClock* mRNA in the SCN. The white bar indicates the light period, and the black bar indicates the dark period.

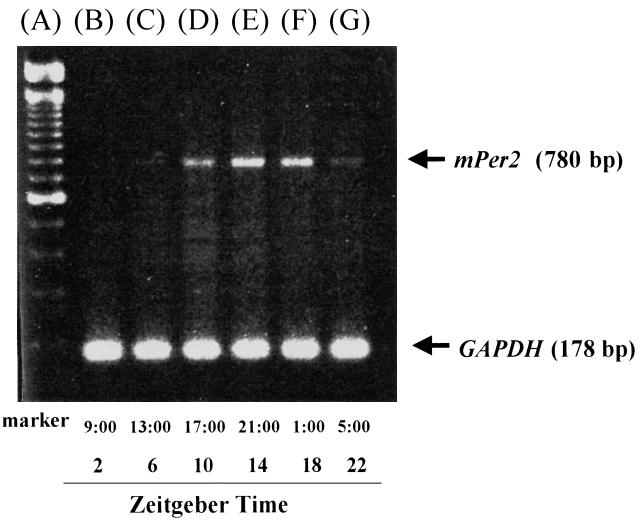

Fig. 2. Examples of RT-PCR analysis of *mPer2* mRNA in the liver of mice. Lane A: 100 base-pair marker, Lane B: 9:00 (ZT2), Lane C: 13:00 (ZT6), Lane D: 17:00 (ZT10), Lane E: 21:00 (ZT14), Lane F: 1:00 (ZT18), Lane G: 5:00 (ZT22).

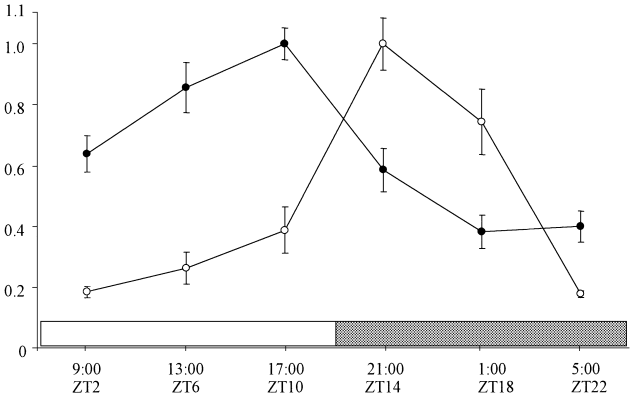

Fig. 3. Relative mRNA levels of *mPer2* in the SCN (closed circle) and in the liver (open circle) of mice. Each point represents the mean ± S.E.M. in 6 mice. The mRNA expressions of *mPer2* in both the SCN and liver show a significant daily variation ($P<0.01$, respectively, ANOVA). The white bar indicates the light period, and the black bar indicates the dark period.

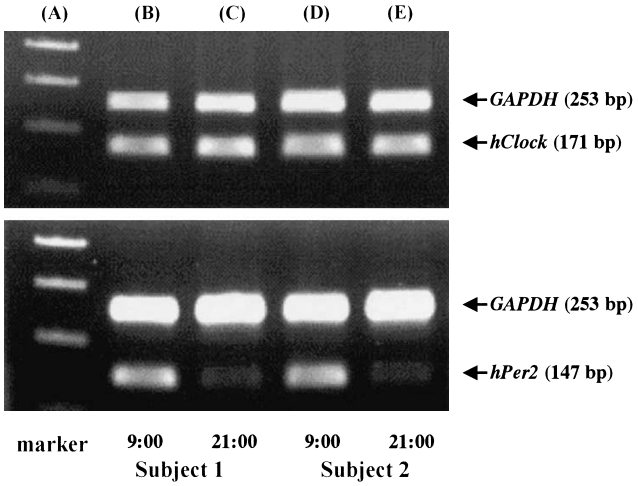

Fig. 4. Examples of duplex RT-PCR analysis of *hClock* and *hPer2* mRNA in 2 healthy subjects. Lane A: 100 base-pair marker, Lane B: subject 1 (9:00), Lane C: subject 1 (21:00), Lane D: subject 2 (9:00), Lane E: subject 2 (21:00).

Figure 2 shows the results of PCR for gene expression in the liver of mice. Primers of *mPer2* were co-amplified with those of *mGAPDH*. As seen in Fig. 2, the expression levels of *mPer2* mRNA in the liver revealed remarkable daily variation.

The animals exhibited a robust daily rhythm in the *mPer2* RNA levels in both the SCN and liver ($P<0.01$, respectively, Fig. 3). The highest and lowest levels of *mPer2* mRNA expression in the SCN were at ZT10 (17:00), and at ZT18 (1:00), respectively. The highest and lowest levels of *mPer2* mRNA expression in the liver were at ZT14 (21:00), and at ZT2 (9:00), respectively. The *mPer2* RNA oscillations in the SCN preceded those in the liver by several hours.

Daily variation of hPer2 and hClock in PBMCs from healthy subjects

Figure 4 shows the results for duplex PCR for gene expression in 2 subjects. Primers of either *hClock* or *hPer2* were co-amplified with those of *hGAPDH*. As seen in Fig. 4, *hClock* mRNA remained almost stable between 9:00 and 21:00. On the other hand, as shown in Fig. 5, the expression levels of *hPer2* mRNA revealed remarkable daily variation. The relative ratio of *hPer2* mRNA expression was significantly higher at 9:00 (1.05 ± 0.70) than at 21:00 (0.19 ± 0.23) ($P<0.01$). However, no significant difference was found in the *hClock* mRNA expression between 9:00 (1.35 ± 0.97) and 21:00 (1.46 ± 0.90).

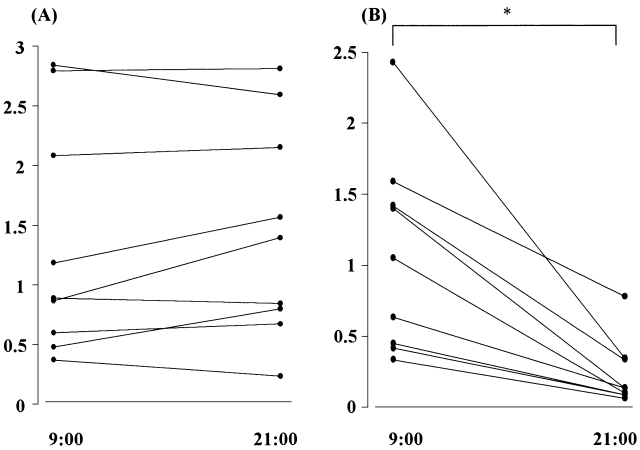

Fig. 5. Relative mRNA levels of *hClock* (A) and *hPer2* (B). The levels are expressed as the ratios relative to those of GAPDH using RT-PCR. *$P<0.01$.

DISCUSSION

The mice exhibited a robust daily rhythm in their RNA levels for *mPer2* mRNA in both the SCN and liver. On the other hand, no significant daily variation of *mClock* mRNA was detected. We have also performed semiquantitative RT-PCR analysis to examine the daily expression in the clock genes *hPer2* and *hClock* in PBMCs from healthy volunteers. We selected *Clock* as a constitutive expression gene and *Per2* as a circadian expression gene to examine in this study. Especially, the levels of *rPer2* mRNA in rat PBMCs reportedly showed clear circadian change (14). We first found that *hPer2* mRNA expression has a signifi-

cant daily variation in human PBMCs. Moreover, we have found that *hClock* mRNA expression exhibits no daily variation between 9:00 and 21:00.

Daily variation of mPer2 in SCN and liver

The mice exhibited no daily rhythm in the *mClock* mRNA in the SCN. The relative ratio of *mClock* mRNA in the liver tended to be higher at ZT2 (9:00) than at ZT14 (21:00), but it was not statistically significant. It is reported that the abundance of Clock is not circadianly regulated in both the SCN and peripheral tissue in mammals (5). On the other hand, some rhythmic change has recently been observed for *mClock* mRNA in mouse liver (22). The difference among experiments may be attributed to the experimental procedure.

In this study, the animals exhibited a robust daily rhythm in the *mPer2* RNA levels in both the SCN and liver. The highest levels of *mPer2* mRNA expression in the SCN were at ZT10 (17:00). The highest levels of *mPer2* mRNA expression in the liver were at ZT14 (21:00). The *mPer2* RNA oscillations in the SCN preceded those in the liver by several hours. These results coincide with the previous results (5). Oishi et al . evaluated the circadian expression of *rPer2* mRNA in both the SCN and peripheral tissues by means of Northern blots. The maximal mRNA expression in rat PBMCs is observed early during the dark phase (ZT14) (14). The expression pattern of *rPer2* in rat PBMCs is similar to that of *mPer2* in liver in the present study. However, the expression of *mPer2* mRNA in mice PBMCs was not determined because of technical difficulty.

The circadian rhythms of physiology and behavior are influenced by various environmental factors such as feeding schedules, genetic factors, and social interactions as well as lighting conditions and several drugs (2, 19, 23, 24). Even when the circadian rhythm of locomotor activity was severely disturbed during the continuous administration of corticosterone, normal rhythmicity was observed for the expression of *mPer1* mRNA in the SCN. A time-restricted feeding schedule can change the rhythmic phase of locomotor activity and physiologic function, including corticosterone and the clock genes in the periphery by up to 12 h while leaving the rhythmic phase of the clock genes in the SCN unaffected (25). The circadian rhythm in the periphery is governed by that in the SCN, since the circadian rhythms in physiologic function and *Per* mRNA expression are abolished in SCN-lesioned rats (13) and *mClock* mutant mice (3). The mechanisms employed by the circadian output pathways from the SCN to the periphery are poorly understood but are likely to involve both nervous and humoral signals (26, 27). We found that the ICR mice exhibited a robust circadian rhythm of *mPer2* mRNA expression in both the SCN and liver. However, the changes in *mPer2* RNA expression in the SCN precede those in the liver by several hours. The present findings suggest that the levels of the clock genes in peripheral tissues are related to those in the central system and this evidence may become a reference rhythm for the circadian timing of medication.

Daily variation of hPer2 and hClock mRNA expression in PBMCs

We find that the expression of *hPer2* mRNA shows significant daily variation, and the relative ratio of the expression of *hPer2* mRNA is significantly higher at 9:00 than at 21:00.

On the other hand, we find that the peak levels of *mPer2* mRNA expression in mice livers are at 21:00 and their trough levels are between 5:00 and 9:00, which is different from the pattern in humans. Moreover, we find that the mRNA levels of *hClock* exhibit no daily variation, which is coincided with the result of Bjarnason et al. (18). Although inter-subject difference in *hPer2* mRNA expression was large (Fig. 5), the results of *hPer2* mRNA expression showed no significant relationship with age. The difference between male and female was not determined because the number of females was small in this study. The reason for each individual difference in the levels of *hPer2* mRNA is still unclear, and further study will be needed to reveal it.

Oishi et al . evaluated the circadian expression of *rPer2* mRNA in both the SCN and peripheral tissues. The maximal mRNA expression in rat PBMCs was observed early during the dark phase (ZT14) (14). This expression pattern of *rPer2* is unique for nocturnally active rodents. A previous study had shown that restricted feeding rapidly entrained the liver independently of the SCN and the light-dark cycle (16, 28). Moreover, restricted-feeding-induced a phase-shift (from nighttime-peak to daytime-peak) of the expression of *mPer1* and *mPer2* mRNA in the cerebral cortex and hippocampus but not in the SCN (29). The pattern of *hPer2* mRNA expression in humans may be different from that in rodents because the feeding and activity of humans are almost opposite those of rodents.

In conclusion, we have found that *hPer2* mRNA expression in PBMCs from healthy volunteers has a significant daily variation, which is different from that in mice. Several drugs can cause alterations in the circadian rhythms of physiologic phenomenon (23, 24). Thus, drug administration at certain times in a day might improve the outcome of pharmacotherapy. Although clinicians have accepted chronotherapy for the treatment of nocturnal asthma (30), most drugs are given without regard to time of day. A new concept of adverse effects due to alterations in the clock function by a drug has recently been reported (17). Ohdo et al. have shown the disruptive effect of IFN-α on the rhythm of locomotor activity, body temperature, and *mPer* gene mRNA expression in the periphery and the SCN. Moreover, the changes in the dosing schedule report-

edly minimized the disruptive effects of IFN-α on clock function (17). Monitoring the rhythmicity of clock genes may be useful for choosing the most appropriate time of day for the administration of drugs, which may increase their therapeutic effects and/or reduce their side effects. Although the gene expression of the peripheral clock genes is not the same as in SCN, the evidence of variation in *hPer2* mRNA expression in PBMCs may be useful for detecting some drug effects on circadian systems in humans. In addition, the difference in the circadian expression of peripheral *Per2* mRNA between humans and rodents should be considered in clinical pharmacology. Further studies will be needed to clarify the efficacy of peripheral clock gene expression.

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