

Alteration of Intrinsic Biological Rhythms during Interferon Treatment and Its Possible Mechanism

SATORU KOYANAGI¹ and SHIGEHIRO OHDO

Clinical Pharmacokinetics, Division of Clinical Pharmacy, Department of Medico-Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan

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ABSTRACT

One of the most indispensable biological functions for all living organisms is the circadian clock, which acts like a multifunctional timer to regulate the homeostatic system, including sleep and wakefulness, hormonal secretions, and various other bodily functions with a 24-h cycle. We reported previously that interferon (IFN) has the ability to modulate the biological clock system at the genetic level. In the present study, this mechanism was investigated further by evaluating the effects of IFN- α on circadian output function. Treatment of cultured hepatic cells (HepG2) with IFN- α significantly decreased the protein levels of CLOCK and BMAL1, which are positive regulators of circadian output rhythm, then their mRNA levels. Aurintricarboxylic acid, a ligand inhibitor of IFN- α , dose dependently

inhibited the IFN- α -induced phosphorylation of the signal transducer and activator of transcription 1 (STAT1) protein in HepG2 cells, accompanied by the restoration of *Clock* and *Bmal1* mRNA levels. The continuous administration of IFN- α significantly decreased CLOCK and BMAL1 protein levels in the suprachiasmatic nucleus and liver of mice, thereby preventing oscillations in the expression of clock and clock-controlled output genes. These results reveal a possible pharmacological action by IFN- α on the core circadian oscillation mechanism and indicate that the disruptive effect of IFN- α on circadian output function is the underlying cause of its adverse effects on 24-h rhythms in physiology and behavior.

Most living organisms exhibit behavioral and physiological rhythms with a period length of about 24 h. During the past 50 years, it has been clearly established that such 24-h rhythms were not simply a response to the daily rotation of the Earth but instead were caused by an internal time-keeping system called the circadian clock. The circadian clock system is made up of three components: an input pathway adjusting the time, a central oscillator generating the circadian signal, and an output pathway manifesting itself in circadian physiology and behavior (Kalsbeek et al., 1996; Shigeyoshi et al., 1997; Jin et al., 1999). Daily variations in light intensity are the major environmental cues setting the

circadian clock. In mammals, the central oscillator resides in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Moore and Eichler, 1972), and daily light-dark cycles strongly entrain the self-oscillating circadian signal generated within the SCN.

Recently, several clock genes have been identified as ultimately controlling a vast array of circadian rhythms in physiology and behavior. Three mammalian homologs of the *Drosophila melanogaster* clock gene *period* (*Per1*, *Per2*, and *Per3*) are rhythmically expressed in the SCN; two of them, *Per1* and *Per2*, are induced in response to light (Albrecht et al., 1997; Shigeyoshi et al., 1997). Notably, the *Per1* induction is considered the initial event in light-induced resetting and entrainment of the circadian biological clock (Akiyama et al., 1999). The core circadian oscillator is composed of interacting positive and negative transcription-translation feedback loops. The currently held view of the mammalian feedback loop holds that transcription of the *Per* and *Cryptochrome* (*Cry1* and *Cry2*) genes is activated by the binding of CLOCK:BMAL1 heterodimers to the E-box enhancer element, and subsequently suppressed by complexes of *Per* and *Cry* proteins (Gekakis et al., 1998; Kume et al., 1999). At the same time, these negative limbs act as positive regulator for ex-

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¹ Present address; Department of Biochemistry, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan.

ABBREVIATIONS: IFN, interferon; SCN, suprachiasmatic nucleus; ATA, aurintricarboxylic acid; ZT, zeitgeber time; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; JAK, Janus tyrosine kinase; dbp, albumin D-element binding protein; STAT, signal transducer and activator of transcription.

pression of *Bmal1* mRNA, leading to the formation of CLOCK:BMAL1 heterodimers which drive *Per* and *Cry* transcription and restart the oscillation (Shearman et al., 2000; Preitner et al., 2002; Yu et al., 2002). The transcriptional machinery of the core circadian clockwork also regulates clock-controlled output rhythms. Namely, CLOCK:BMAL1 heterodimers act through an E-box enhancer to activate the transcription of vasopressin preproressophysin, albumin D-element binding protein and prokineticin 2 mRNAs, showing a specific circadian output function (Jin et al., 1999; Ripperger et al., 2000; Cheng et al., 2002).

The circadian clock system is a fundamental mechanism for adapting to daily variations in environmental conditions. The altered homeostatic regulation, including the disturbance of the 24-h rhythm, is implicated in various physiological as well as psychiatric disorders (Winget et al., 1984; Cho et al., 2000). Several drugs can alter the 24-h rhythms of biochemical, physiological, and behavioral processes (Duncan, 1996). The change of rhythmicity is sometimes associated with therapeutic effects (such as with antidepressant drugs) but in other cases may lead to illness and altered homeostatic regulation.

Interferons (IFNs), a group of cytokines, have been widely used therapeutically as antiviral and antitumor agents in humans. However, IFNs have various adverse effects on the central nervous system, such as depression and neurosis, and are even reported to sometimes cause suicidal tendencies (Dafny et al., 1985; Baron et al., 1991). We reported previously that IFN- α has the ability to modulate the biological clock system at the genetic level, because the rhythmicity of clock genes and the photic induction of the *Per1* gene in the SCN are disturbed by repetitive administration of IFN- α (Ohdo et al., 2001). Although the study indicated that the disruptive effect of IFN- α on the photic induction of *Per1* gene is associated with an altered rhythmicity of clock gene expression, the effects of IFN- α on the generation of circadian output signals remain to be clarified. To address this issue, the influence of IFN- α on circadian output signals driven by central clock function was investigated. Here, we demonstrated a possible pharmacological action by IFN- α on the core circadian oscillation mechanism. The present findings suggest that the disruptive effects of IFN- α on the generation of circadian output signals are the underlying cause of its adverse effects on 24-h rhythms in physiology and behavior.

Materials and Methods

Materials

IFN- α was purchased from PeproTech EC Ltd. (London, UK), dissolved in sterilized saline to a concentration of 10 MIU/ml, and stored frozen in aliquots at -20°C . Aurintricarboxylic acid (ATA) was purchased from Sigma Chemical (St. Louis, MO) and solubilized in dimethyl sulfoxide.

Cells and Animals

Human hepatoma (HepG2) cells were purchased from Sanko Junyaku Co., Ltd. (Tokyo, Japan) and cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. Male ICR mice (5 weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan). They were housed three to six per cage under a standardized light/dark cycle at a room temper-

ature of $24 \pm 1^{\circ}\text{C}$ and humidity of $60 \pm 10\%$ with food and water available ad libitum. The animals were treated in accordance with the Law (No. 105) and Notification (No. 6) of the Japanese Government. Under the light/dark cycle, zeitgeber time (ZT) 0 was designated as lights on and ZT12 as lights off. During the dark period, a dim red light was used to aid treatment of the mice.

Experimental Design

To study the effect of IFN- α on clock gene expression in cultured cells, confluent cultures of HepG2 cells in 175-cm² culture flasks were incubated for 72 h in 20 ml of serum-starved (0.5% serum) medium containing various concentrations of IFN- α . After the incubation, the cells were washed twice and collected in ice-cold PBS. Half of the cells were used to extract total RNA for the analysis of *Clock* and *Bmal1* mRNA levels, and half were used to analyze for CLOCK and BMAL1 protein levels. To investigate the role of the JAK-STAT signaling pathway in the action of IFN- α on clock gene expression, HepG2 cells were treated for 72 h with IFN- α (50 IU/ml) and a given concentration of ATA, a competitive ligand inhibitor of IFN- α . The cell nuclear fraction was extracted and the levels of phosphorylated STAT1 protein were analyzed by immunoblotting. Total RNA was also extracted to analyze *Clock* and *Bmal1* mRNA levels by quantitative RT-PCR. To study the influence of IFN- α on CLOCK and BMAL1 protein levels in the SCN and liver, groups of three to four mice were continuously administered IFN- α (14 kIU/h) or saline as a control, using an osmotic minipump (model 2001, ALZET; Palo Alto, CA) for 7 days. This dosage of IFN- α is reported to exert biological activity within the SCN of mice (Ohdo et al., 2001). CLOCK and BMAL1 protein levels were examined at two time points (ZT6 and ZT18) on day 7 after initiation of the drug treatment. Protein levels in the SCN and liver were determined by immunohistochemical and immunoblot analysis, respectively. To study the influence of IFN- α on the expression of clock (*Per1*, *Per2*, and *Cry1*) and clock-controlled output genes (for arginine-vasopressin preproressophysin and albumin D-element binding protein) in the SCN or liver, groups of four to six mice were subcutaneously administered IFN- α or saline as described above. The rhythms of the mRNA expression of circadian clock genes in the SCN and liver were assessed at ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22 on day 7 after initiation of the drug treatment. To examine the influence of IFN- α on the SCN oscillatory function, groups of four to six mice were continuously administered IFN- α or saline as described above. The SCN oscillatory function was evaluated by measuring vasopressin levels at each of the six times outlined above. To study the influence of IFN- α on the 24-h rhythm of locomotor activity, groups of three mice were continuously administered IFN- α or saline as described above. Their locomotor activity was measured automatically using electric digital counters.

Quantitative RT-PCR Analysis.

RNA Extractions. Total RNA from HepG2 cells was extracted using TRIzol reagent (Invitrogen). Total RNA from mouse tissues were extracted as follows: the brain and liver were quickly removed at each of the six times outlined above. 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. Total RNA from the liver of individual mice was extracted separately by using the TRIzol reagent. Total RNA from the SCN of three mice in each group was extracted to obtain an adequate amount of RNA.

RT-PCR. The cDNA of human *Clock* (GenBank accession number AB002332), human *Bmal1* (GenBank accession AB000813), *Per1* (GenBank accession AF022992), *Per2* (GenBank accession AF035830), *Cry1* (GenBank accession AB000777), vasopressin preprorepressophysin (*vasopressin*) (GenBank accession M88354), albumin D-element binding protein (*dbp*) (GenBank accession U29762) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (human; GenBank accession M33197, mouse; GenBank accession M32599) were synthesized and amplified using a superscript one-step RT-PCR system (Invitrogen). To quantify mRNAs, the kinetic analysis of amplified products, ensuring that signals are derived only from exponential phase of amplifications, was performed in each sample as follows: after the first 25 cycles of amplification, an aliquot of 5 μ l was drawn for electrophoresis and the tubes were submitted to one more cycle of PCR. This procedure was repeated until a total of 30 cycles was reached. The PCR products were run on 3% agarose gel. The gel was photographed with Polaroid type film after staining with ethidium bromide. The density of each band was analyzed using NIH image software on a Macintosh computer (Apple, Cupertino, CA).

The exponential phase of *GAPDH* amplification in all experimental conditions was located between cycles 26 and the 28, and the exponential phases of all target genes (clock and clock-controlled output gene) were located between cycles 27 and 30. The amplified efficiency of *GAPDH* and clock or clock-controlled output genes was comparable. Therefore, the amplified products were quantified at cycle 27 or 28. The ratio of the amplified target to the amplified internal control (calculated by dividing the value of each *Clock*, *Bmal1*, *Per*, *Cry*, *vasopressin*, or *dbp* by that of *GAPDH*) was compared among the groups.

Immunoblot Analysis.

Protein Extractions. Cellular nuclear fractions were prepared as follows: collected cells were incubated in cell lysis buffer (0.35% Tween 20 and 200 μ M $\text{Na}_2\text{V}_2\text{O}_5$ in PBS) on ice for 5 min, and the lysates were centrifuged for 10 min at 3,000g. After removal of the soluble fraction, the obtained pellets were resuspended in nuclear extraction buffer (20 mM Tris-HCl, pH 7.8, 400 mM NaCl, and 14 mM 2-mercaptoethanol) at 4°C for 30 min. The resuspensions were centrifuged for 10 min at 12,000g, and the resulting supernatants were used as cellular nuclear fractions. Nuclear fractions from mouse liver were prepared as follows: mice were deeply anesthetized with ether. Liver was perfused with 0.01M PBS and homogenized with ice-cold lysis buffer (20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin/ml, and 10 μ g/ml antipain). After removal of the soluble fraction by centrifugation at 1,000g for 10 min, the pellet was washed three times with PBS. After a final centrifugation, the pellets were resuspended in nuclear extraction buffer at 4°C for 30 min, and then nuclear fractions were obtained by centrifugation for 10 min at 12,000g. Protein concentrations were determined using a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA).

Immunoblot. Lysates containing 20 μ g of total protein were resolved on SDS-polyacrylamide gel by electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was reacted with antibodies against CLOCK or BMAL1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The immunocomplexes were further reacted with peroxidase-conjugated secondary antibodies and made visible with 4-chloro-1-naphthol as the peroxidase substrate. The phosphorylated signal transducer and activator of transcription 1 (pSTAT1) protein in nuclear fractions of HepG2 cells were also analyzed using antibodies against phosphorylated Tyr-701-STAT1 (Santa Cruz Biotechnology, Inc.) as described above.

Immunohistochemical Analysis

Immediately after preparation of the liver samples, the brain was quickly removed, and coronal brain slices were prepared as described above. The brain slices were fixed for 4 h with 4% paraformaldehyde in 0.1 M phosphate buffer and immersed in 30% sucrose buffer. They were then frozen and sectioned at 30 μ m in the coronal plane. To reduce background staining, sections were placed in a 0.3% hydrogen-peroxide solution for 30 min, and then incubated with blocking buffer containing 1.5% goat serum for 4 h. After incubation with blocking buffer, the sections were incubated with anti-CLOCK or anti-BMAL1 antibodies (Santa Cruz Biotechnology, Inc.). The immunoreaction was visualized with avidin-biotin/peroxidase in conjunction with 3',3'-diaminobenzidine. Microscopic images of the anterior hypothalamic area were captured under bright-field illumination to count the number of immunoreactive nuclei (as defined by immunoreaction above a threshold background) in the whole SCN. An observer was blinded to reduce the bias such as subjective interpretation of results.

Measurement of Vasopressin Levels in the SCN

The brain was quickly removed at each of the six times outlined above. The SCN was punched out bilaterally from prepared brain slices and homogenized in 500 μ l of 0.5 M HClO_4 solution. The homogenate was dried up by SpeedVac concentrator (Thermo Savant, Holbrook, NY), and the dried sample was reconstituted with 150 μ l of assay buffer. Half of the reconstituted sample was used to determine the total protein concentration. The amount of vasopressin in the other half was determined by EIA (Assay Designs, Inc., Ann Arbor, MI). The amount of vasopressin in the SCN was expressed as nanograms per milligram of protein.

Monitoring of Locomotor Activity Rhythm

Locomotor activity was measured using the Scanet SV-10 test system (Toyo Sangyo Co. Ltd., Toyama, Japan). Groups of three mice were kept in cages equipped with 144 pairs of infra-red beam emitters, and their locomotor activity was defined as the number of infra-red beam breaks per mouse per unit of time. The activity records are double-plotted so that each day's activity is shown both to the right and below that of the previous day's.

Statistical Analysis

The significance of the 24-h variation in each parameter was tested by analysis of variance. The statistical significance of differences among groups was evaluated by analysis of variance and the Dunnett or Tukey multiple comparison test. A 5% level of probability was considered significant.

Results

The Effects of IFN- α on CLOCK and BMAL1 Expression in Cultured Hepatic Cells. Recent genetic and biochemical evidence has demonstrated that CLOCK:BMAL1 heterodimers are responsible for the generation of circadian output rhythms (Jin et al., 1999; Kume et al., 1999; Bunger et al., 2000; Ripperger et al., 2000). As shown in Fig. 1A, treatment of HepG2 cells with IFN- α for 72 h caused a significant reduction in *Clock* and *Bmal1* mRNA levels in a concentration-dependent manner. A significant reduction in *Clock* mRNA was observed at a concentration of 10 IU/ml, whereas

that in *Bmal1* mRNA occurred at 25 IU/ml. A similar concentration-dependent reduction was observed in CLOCK and BMAL1 protein levels (Fig. 1B). IFN- α significantly decreased the immunoreactivities of both proteins at a concentration of 50 IU/ml.

Next, the possible role of the JAK-STAT signaling pathway in the disruptive effect of IFN- α on clock gene expression was investigated (Fig. 2). The treatment of HepG2 cells with IFN- α resulted in an extensive amount of phosphorylated-STAT1 protein compared with the control level. The competitive ligand inhibitor of IFN- α , ATA, dose dependently inhibited the IFN- α -induced phosphorylation of STAT1 protein, accompanied by the restoration of *Clock* and *Bmal1* mRNA levels. During these experiments, there were no significant influences of IFN- α on cell viability as determined by tetrazolium bromide [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] assay (data not shown).

Influence of IFN- α on CLOCK and BMAL1 Protein Levels in SCN and Liver of Mice. In mammals, the master circadian pacemaker controlling the rhythmicity of physi-

ology and behavior resides in the SCN of the anterior hypothalamus (Sakamoto et al., 1998; Yamazaki et al., 2000). We thus examined whether IFN- α affected CLOCK and BMAL1 protein levels in the SCN of mice. The CLOCK immunoreactive cells in the SCN of control mice were not significantly different between ZT6 and ZT18, whereas BMAL1 immunoreactive cells were significantly greater at ZT18 than at ZT6 ($p < 0.05$; Fig. 3A). The continuous administration of IFN- α significantly decreased CLOCK immunoreactive cells in the SCN at both time points ($p < 0.05$, respectively). The treatment also caused a significant reduction in BMAL1 immunoreactivity in the SCN at ZT18 ($p < 0.05$), but not at ZT6.

Because peripheral tissues also express CLOCK and BMAL1 proteins, we investigated the influence of IFN- α on CLOCK and BMAL1 protein levels in the liver of mice. Neither CLOCK nor BMAL1 immunoreactivity in the liver of control mice differed significantly between ZT6 and ZT18 (Fig. 3B). The continuous administration of IFN- α significantly decreased CLOCK immunoreactivity in the liver at both time points ($p < 0.05$, respectively). Similarly, the treatment with IFN- α also caused a significant reduction in BMAL1 immunoreactivity in the liver at both time points ($p < 0.01$ for ZT6 and $p < 0.05$ for ZT18).

Influence of IFN- α on mRNA Expression of Clock and Clock-Controlled Output Genes. We next tested whether decreased protein levels of CLOCK and BMAL1 in IFN- α -treated mice were associated with altered rhythms of mRNA expression of clock and clock-controlled output genes. There were significant 24-h rhythms of *Per1*, *Per2*, *Cry1*, and vasopressin mRNA expression in the SCN of control mice, with a peak level at ZT2 for *Per1*, at ZT10 for *Per2*, at ZT14 for *Cry1* and at ZT6 for *vasopressin* ($p < 0.01$, respectively; Fig. 4A). In contrast to the control, the continuous administration of IFN- α severely blunted the rhythms of clock and clock-controlled output gene expression in the SCN. Trough levels for each gene expression did not differ between control and IFN- α -treated mice, but the peaks of expression decreased significantly in the treated mice compared with the control mice ($p < 0.01$, respectively). In addition, the treatment with IFN- α also caused phase shift of *Per1* mRNA rhythm.

In the liver of control mice, there were also significant 24-h rhythms of *Per1* and *dbp* mRNA expression, with a peak at ZT14 for *Per1* and at ZT10 for *dbp* ($p < 0.01$, respectively, Fig. 4B). In contrast, the continuous administration of IFN- α severely blunted the rhythms of *Per1* and *dbp* mRNA

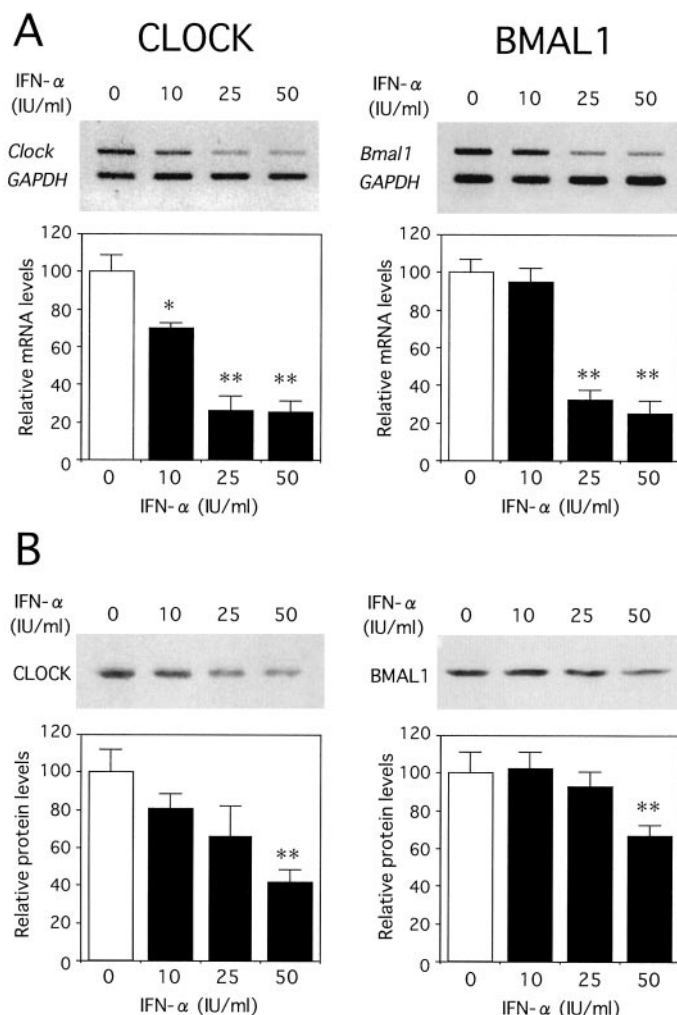


Fig. 1. The effects of IFN- α on the expression of CLOCK and BMAL1 mRNA (A) and protein (B) in HepG2 cells. Cells were treated with the indicated concentration of IFN- α for 72 h. Total RNA and a cell nuclear fraction were prepared and analyzed by RT-PCR and by immunoblotting, respectively. For plots of intensity, the mean value of the untreated group is set at 100. Each value represents the mean \pm S.E.M. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$ compared with the untreated group (Dunnett test).

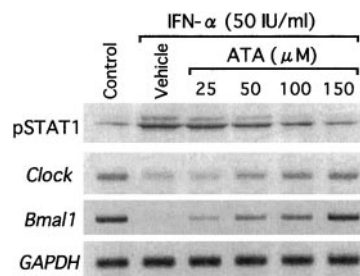


Fig. 2. The disruptive effect of IFN- α on clock gene expression is inhibited by a competitive ligand inhibitor, ATA. Cells were treated for 72 h with 50 IU/ml IFN- α in the presence or absence (vehicle) of ATA at the indicated concentrations. A cell nuclear fraction was prepared and analyzed for phosphorylated STAT1 protein (pSTAT1) by immunoblotting. Total RNA was also extracted and analyzed for *Clock* and *Bmal1* mRNA by RT-PCR analysis.

expression in the liver. Trough levels for each mRNA expression did not differ between control and IFN- α -treated mice, but peak levels decreased significantly in the treated mice compared with the control mice ($p < 0.01$, respectively).

Influence of IFN- α on Daily Rhythms of SCN Vasopressin Expression and Locomotor Activity. Finally, we investigated how IFN- α affected overt rhythms in physiology and behavior. The amount of vasopressin in the SCN of control mice exhibited a significant 24-h rhythm ($p < 0.01$; Fig. 5A), with a peak at ZT6 and nadir at ZT18. In marked contrast, the continuous administration of IFN- α completely abolished the vasopressin rhythm: the amount of vasopressin remained at trough levels of control. In the behavioral study,

control mice clearly exhibited an entrained locomotor activity rhythm, and thus hyperactivity was observed during the dark phase (Fig. 5B). In contrast, continuous administration of IFN- α substantially altered the entrained locomotor rhythm. The locomotor activity bands were sparsely distributed, and the active phase was obscured after the decrease in total counts of daily activity.

Discussion

Recent molecular dissections of the circadian biological clock system have revealed that oscillation in the transcription of specific clock genes plays a central role in the generation of circadian rhythms. CLOCK:BMAL1 heterodimers are recognized as positive regulators for the oscillatory gene

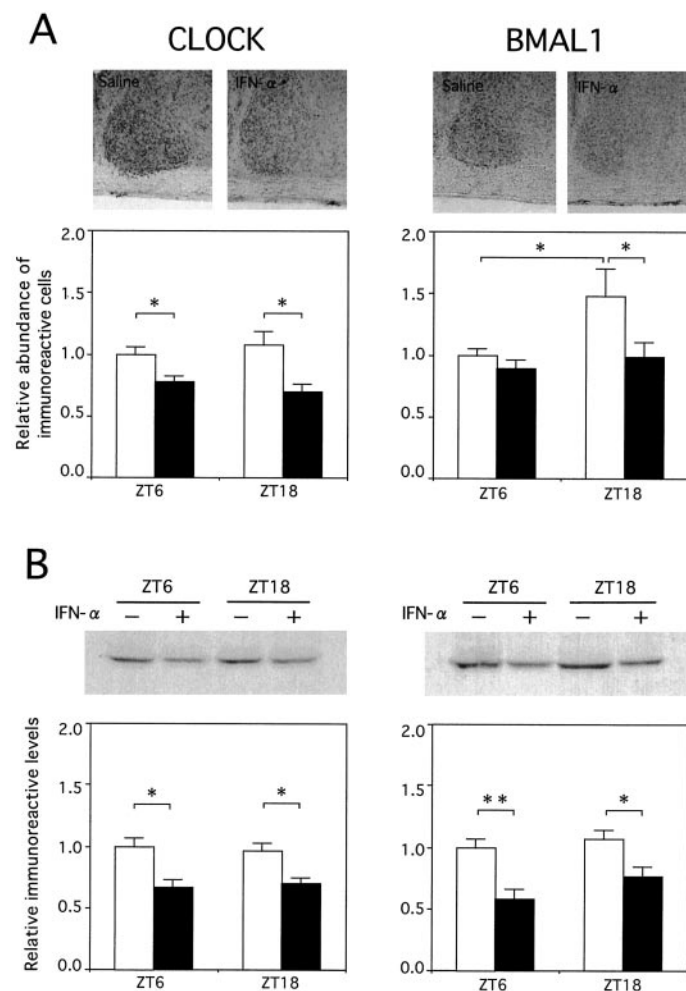


Fig. 3. CLOCK and BMAL1 immunoreactivity in the SCN and liver of IFN- α -treated mice. A, temporal profiles of CLOCK (left) and BMAL1 (right) immunoreactivity at ZT6 or ZT18 in the SCN of mice continuously administered IFN- α (14kIU/h, s.c., ■) or saline (□) using an osmotic minipump for 7 days. For plots of abundance, the mean value of immunoreactive cells in the SCN of control group at ZT6 is set at 1.0. Each value represents the mean \pm S.E.M. ($n = 3-4$). *, $p < 0.05$ compared between the two groups (Tukey's test). The photomicrographs (top) show immunoreactivity of CLOCK and BMAL1 at ZT18 in the SCN of saline or IFN- α -treated mice. B, temporal profiles of CLOCK (left) and BMAL1 (right) immunoreactivity at ZT6 or ZT18 in the liver of mice continuously administered IFN- α (14kIU/h, s.c., ■) or saline (□) using osmotic minipumps for 7 days. For plots of intensity, the mean value of the control group at ZT6 is set at 1.0. Each value represents the mean \pm S.E.M. ($n = 3-4$). **, $p < 0.01$; *, $p < 0.05$ compared between the two groups (Tukey's test). Top, immunoblots of CLOCK and BMAL1 proteins at ZT6 or ZT18 in the liver of saline or IFN- α -treated mice.

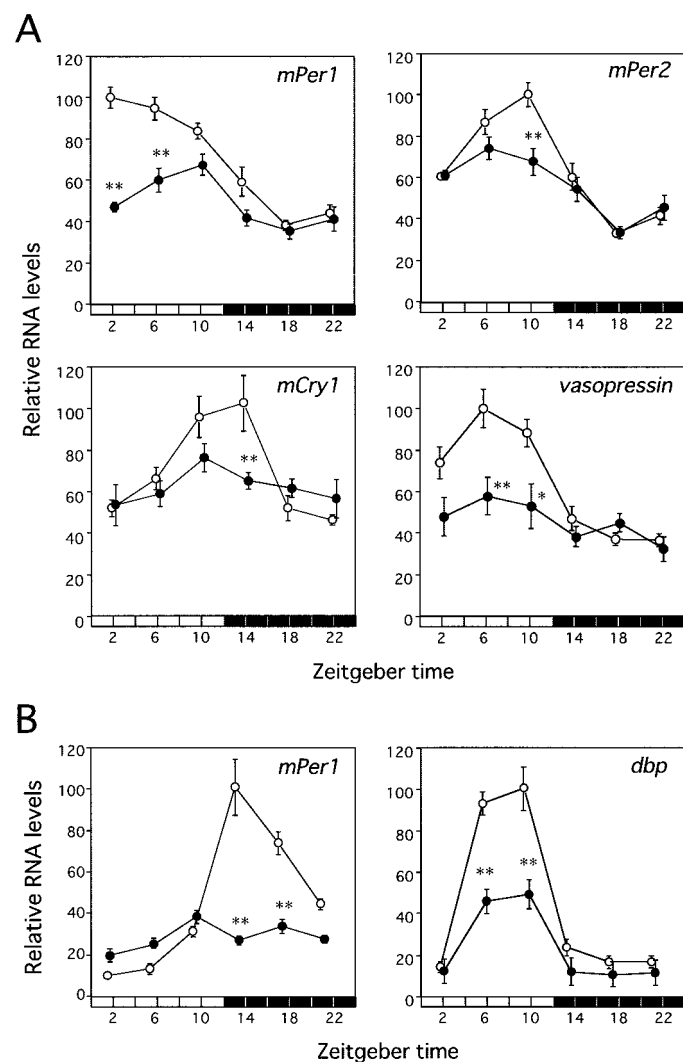


Fig. 4. Influence of IFN- α on mRNA expression of clock or clock-controlled genes. A, 24-h rhythm of mRNA expression for the *Per1*, *Per2*, *Cry1*, and *vasopressin* gene in the SCN of mice continuously administered IFN- α (14kIU/h, s.c., ●) or saline (○) using osmotic minipumps for 7 days. B, 24-h rhythm of mRNA expression for *mPer1* and *dbp* in the liver of mice continuously administered IFN- α (14kIU/h, s.c., ●) or saline (○) using osmotic minipumps for 7 days. For plots of RNA, the mean peak value of the control group for *Per1*, *Per2*, *Cry1*, *vasopressin*, and *dbp* is set at 100. Each point represents the mean \pm S.E.M. ($n = 4-6$). **, $p < 0.01$; *, $p < 0.05$ compared with the value for the saline group at the corresponding zeitgeber times (Tukey's test). The horizontal bar at the bottom indicates the light and dark cycle.

expression, thereby rhythmically controlling the circadian properties of physiology and behavior (Kume et al., 1999; Bunge et al., 2000; Bae et al., 2001). We demonstrated here

that IFN- α caused a significant reduction in CLOCK and BMAL1 protein levels in cultured hepatic cells. This decrease in protein seemed to be reflected by the ability of IFN- α to inhibit the expressions of the mRNA. The biological effects of IFNs are elicited by the binding of a specific receptor on the cell surface and are mediated through activation of the JAK-STAT signaling pathway (Darnell, 1997). IFN-inducible phosphorylated STAT1 and STAT2 associate with a 48-kDa protein to form the transcription factor IFN-stimulated gene factor-3 complex (Qureshi et al., 1995). The protein complex positively and negatively regulates the expression of various genes (Der et al., 1998; Ramana et al., 2000). Here, the phosphorylation of STAT1 proteins induced by IFN- α was negatively correlated with the expression of clock-genes in HepG2 cells. The inhibitory action of IFN- α on clock-gene expression was blocked by treatment with ATA. ATA is a triphenylmethane derivative that competes with the binding of IFN- α to its specific receptor, thereby preventing the phosphorylation of STAT1 and STAT2 proteins (Gan et al., 1990; Grimley et al., 1998). Therefore, the present results indicate that the inhibitory effect of IFN- α on the mRNA expression of *Clock* and *Bmal1* is elicited by activation of the JAK-STAT signaling pathway; thus, there may be a link between the downstream of IFN- α signal transduction and the circadian biological clock system.

Consistent with its inhibitory effects on the expression of clock genes in cultured hepatic cells, the continuous administration of IFN- α significantly decreased CLOCK and BMAL1 protein levels in the SCN and liver of mice. The treatment also severely blunted the 24-h rhythms of the mRNA expression of clock and clock-controlled output genes. These results are consistent with previous findings on the disruptive effects of IFN- α on the rhythmicity of clock-gene expression (Ohdo et al., 2001). CLOCK and BMAL1 form heterodimers that drive the expression of several genes through E-box enhancer elements. The genes include negative regulators of the feedback loop as well as output genes. Therefore, the altered rhythms of *Per* and *Cry* mRNA expression in IFN- α -treated mice may be due to an attenuation of transcriptional activity caused by decreases in CLOCK and BMAL1 protein levels.

The rhythmic expression of output genes seems to be caused by direct transcriptional control by a circadian feedback loop mechanism; CLOCK:BMAL1 heterodimers act through an E-box enhancer element in the *vasopressin* or *dbp* gene to activate transcription, and the activation is inhibited by the PER and CRY proteins (Jin et al., 1999; Ripperger et al., 2000; Yamaguchi et al., 2000). Although *dbp* is considered to be involved in the central feedback loop mechanism as an auxiliary transcription factor of *Per1* (Yamaguchi et al., 2000), it also plays a role in the regulation of several circadian output pathways including locomotor activity, sleep distribution, and liver gene expression (Lopez-Molina et al., 1997; Lavery et al., 1999; Franken et al., 2000). The output genes that are responsible for circadian changes of physiological activity can be directly linked to central clock mechanisms; thus, a functional disorder of central clock oscillation may lead to an altered rhythmicity of physiology and behavior. In support of this view, 24-h rhythms of SCN vasopressin expression and locomotor activity were severely altered by the continuous administration of IFN- α . The vasopressin of SCN origin is considered to transmit circadian output signals

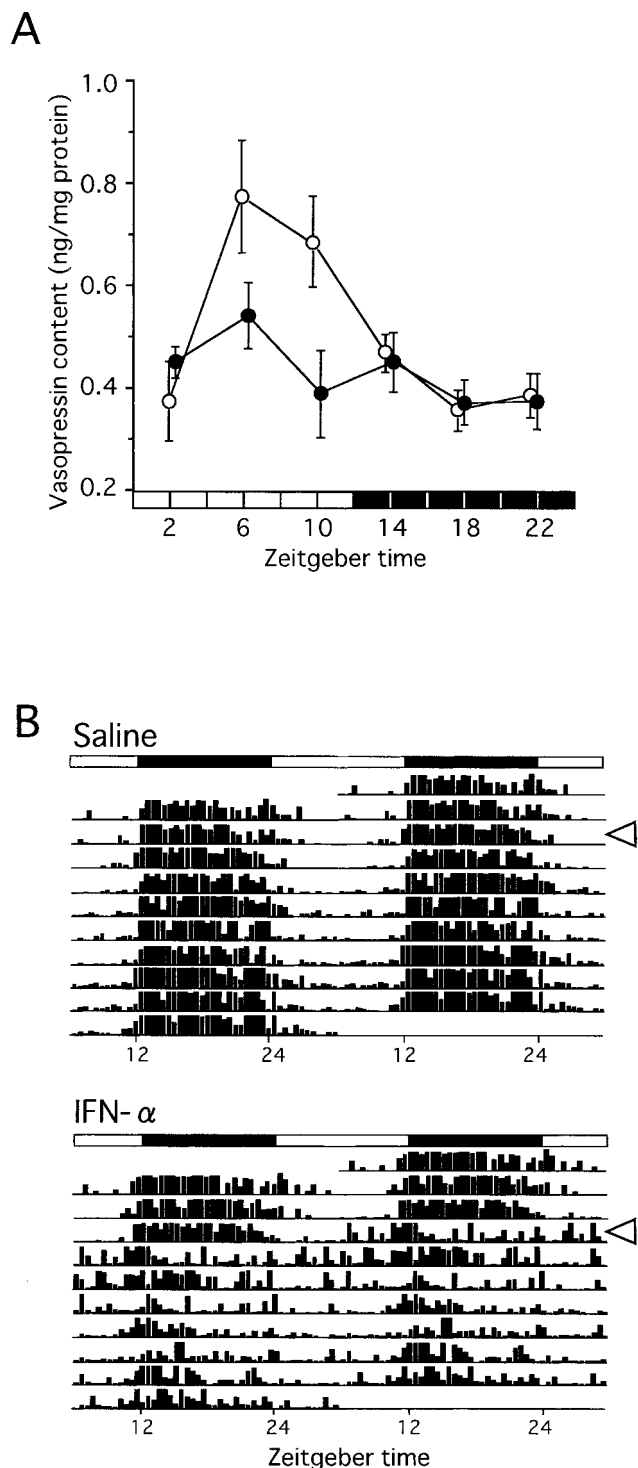


Fig. 5. Influence of IFN- α on the 24-h rhythm of SCN vasopressin contents or locomotor activity. **A**, 24-h rhythm of vasopressin expression in the SCN of mice continuously administered IFN- α (14 kIU/h, s.c., ●) or saline (○) using osmotic minipumps for 7 days. Each point represents the mean \pm S.E.M. ($n = 4-6$). The horizontal bar at the bottom indicates light and dark cycle. **B**, representative locomotor activity records of mice continuously administered IFN- α (14 kIU/h, s.c.) or saline using osmotic minipumps for 7 days. Open arrows, initiation of the drug administration. The horizontal bar at the top indicates the light and dark cycle.

to other parts of the brain (Card et al., 1988; Daikoku et al., 1992). The impaired rhythm of vasopressin production in the SCN of IFN- α -treated mice would reflect the attenuation of circadian output signals driven by SCN oscillatory functions; thus, such attenuated output signals from the central SCN pacemaker may contribute to the altered rhythm of locomotor activity during IFN- α treatment.

Although the continuous administration of IFN- α substantially altered the rhythmicity of locomotor activity, the change in behavioral rhythm was accompanied by a decrease in total activity levels. Indeed, a single administration of IFN- α directly decreases spontaneous motor activity in mice (Crnic and Segall; 1992). According to the currently held model, the mammalian circadian clock system is hierarchically organized, with self-sustained oscillators in the SCN governing the peripheral oscillations (Sakamoto et al., 1998; Yamazaki et al., 2000). Because IFN- α affected both the SCN and periphery, it is difficult to clarify whether the effects of IFN- α on SCN clock genes are secondarily related to those on the rhythmicity of locomotor activity. However, IFN- α directly acts on the SCN neurons (Ohdo et al., 2001) and actually altered the rhythmicity of the expression of clock and clock-controlled output genes in the SCN. Therefore, the disruptive effects of IFN- α on clock-gene function in the SCN may be responsible for not all but some of the adverse behavioral and physiological effects.

The present study suggests that the disruptive effect of IFN- α on circadian output function is the underlying cause of its adverse effect on 24-h rhythms in physiology and behavior. The disruptive effect on circadian output function seems to be elicited by activation of the JAK-STAT signaling pathway. Abnormality in 24-h rhythms such as the sleep-wake cycle and hormonal secretions is implicated in various neurological disorders (Duncan 1996). One should pay attention to changes in clock function during IFN- α treatment, because the altered homeostatic regulation including disturbance of the 24-h rhythm may be a symptom of the various neurological impairments that are observed in patients in IFN- α therapy.

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Address correspondence to: Shigehiro Ohdo, Ph.D., Clinical Pharmacokinetics, Division of Clinical Pharmacy, Department of Medico-Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail: ohdo@phar.kyushu-u.ac.jp