Differential Entrainment of Peripheral Clocks in the Rat by Glucocorticoid and Feeding

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The suprachiasmatic nucleus is the master circadian clock and resets the peripheral clocks via various pathways. Glucocorticoids and daily feeding are major time cues for entraining most peripheral clocks. However, recent studies have suggested that the dominant timing factor differs among organs and tissues. In our current study, we reveal differences in the entrainment properties of the peripheral clocks in the liver, kidney, and lung through restricted feeding (RF) and antiphasic corticosterone (CORT) injections in adrenalectomized rats. The peripheral clocks in the kidney and lung were found to be entrained by a daily stimulus from CORT administration, irrespective of the meal time. In contrast, the liver clock was observed to be entrained by an RF regimen, even if daily CORT injections were given at antiphase. These results indicate that glucocorticoids are a strong zeitgeber that overcomes other entrainment factors regulating the peripheral oscillators in the kidney and lung and that RF is a dominant mediator of the entrainment ability of the circadian clock in the liver. **(*Endocrinology* 153: 2277–2286, 2012)**

Most living organisms have developed internal clock mechanisms that generate precise rhythms around a 24-h cycle. One such system, termed the circadian clock, governs daily variations in physiology and behavior. In mammals, the suprachiasmatic nucleus (SCN) is the center of the circadian clock and resides in the hypothalamus (1). The molecular oscillator in the SCN consists of interacting positive and negative transcription/translation feedback loops (2–4). The transcriptional activators CLOCK and BMAL1 form heterodimers and stimulate the transcription of other clock genes, such as the *Period* (*Per*) genes (*Per1*, *Per2*, and *Per3*), the *Cryptochrome*s (*Cry*) (*Cry1* and *Cry2*), retinoid-related orphan receptors (*ROR*) (*RORα*, *RORβ*, and *RORγ*) and *Rev-erbes* (*Rev-erbα* and *Rev-erbβ*) that bind to the E-box response elements in the promoter regions of these genes. Accumulated PER and CRY proteins form a complex that represses the transcriptional activity of the CLOCK/BMAL1 heterodimer. The ROR transcriptional activators and REV-ERB repressors control the transcriptional regulation of *Clock* and *Bmal1* through their binding to the REV response element (RRE). This autoregulatory loop generates gene expression oscillations of approximately 24 h. In addition, the mammalian SCN can adapt to environmental changes in day/night cycles. Light information from the retinas is delivered to the SCN via the retino-hypothalamic tract and is the most effective time cue for the central clock. Nocturnal light induces the *Per1* and *Per2* genes, which leads to a resetting of the circadian clock in the SCN (5).

The molecular circadian clock operates not only in the SCN but also in peripheral organs and tissues (6, 7). The peripheral clocks are entrained by the central circadian clock in the SCN and express overt circadian rhythms during physiological events (8, 9). Hence, it is commonly assumed that the mammalian circadian system is a complex hierarchical structure headed by the SCN. The SCN relays phase information to the peripheral clocks through various pathways, such as autonomic nervous systems, endocrine signals, and body temperature rhythms (10–15). Furthermore, daily rest-activity cycles governed by the SCN generate feeding-fasting rhythms, a strong entrainment factor for peripheral oscillators (16). These time

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Abbreviations: ADX, Adrenalectomy; CORT, corticosterone; *Cry*, *Cryptochrome*; DMSO, dimethylsulfoxide; FAA, food anticipatory activity; FEO, food-entrainable oscillator; GC, glucocorticoid; L/D, 12-h light, 12-h dark; LSS, least squares spectrum; *Per*, *Period*; PRC, phase-response curve; RF, restricted feeding; *ROR*, retinoid-related orphan receptor; RRE, REV response element; SCN, suprachiasmatic nucleus; ZT, zeitgeber time.

cues are complementary in terms of entrainment mechanisms, but many reports have suggested that the dominant timing factor differs among various organs and tissues. In actuality, this mechanism has not been fully elucidated in whole organisms because of the complexity involved in the simultaneous functioning of multiple factors.

The glucocorticoids (GC), such as cortisol and corticosterone (CORT), are known factors that entrain peripheral clocks, and the concentration of GC circulating in the blood displays a dynamic circadian rhythm (17). Daily fluctuations of GC secretion are regulated by the SCN through the hypothalamus-pituitary-adrenal axis (11, 18, 19). GC also induce *Per1*, a process which is regarded as an initial genetic event required to shift the circadian clock. A GC-responsive element is present in the genomic upstream region of the *Per1* gene and plays a role in activating *Per1* expression (20, 21). The administration of GC agonists causes a strong phase resetting of the circadian oscillator in fibroblasts (6, 22). *In vivo*, a single injection of a GC agonist also induces a phase shift of the peripheral clock in the liver and kidney (6). Moreover, daily inhalation of a GC agonist determines the phase of the peripheral clock in the lung (23).

The daily feeding pattern is another major time cue for the peripheral oscillators. Nocturnal rodents mainly eat during the night, but if food is only available during the day, various physiological and metabolic functions are entrained to adjust to this feeding time (24, 25). In most organs and tissues, the peripheral clock also can be entrained to the timing of meals (16). Under time restricted feeding (RF) conditions, an animal anticipates the upcoming feeding event and displays food anticipatory activity (FAA) for several hours up to the point of daily food presentation. Many studies have now demonstrated that FAA is an output of a food-entrainable oscillator (FEO) and that the FEO is independent of the molecular oscillator constructed from known clock genes (26, 27). The mechanism by which the peripheral clock entrains to the feeding time is still unclear. However, the feeding cue is a powerful entrainment factor that overcomes signals from the SCN.

Under ordinary circumstances, it seems that a conflict between entraining factors does not occur, because the SCN functions to synergize these factors. The phase of rhythmic GC secretion and feeding behavior are determined by the SCN, and both of these factors bring the peripheral oscillators to the same phase. Hence, it is not known whether GC or feeding are the dominant entraining factor. In our present study, we analyzed the competing effects of GC injections and RF in the rat by modifying the time of administration to evaluate which is the dominant entrainment factor in different organs. To eliminate the effects of endogenous GC, we adrenalectomized (ADX) the subject rats. As a result, a daily CORT injection entrained the phase of kidney and lung clock but did not entrain the liver clock. In contrast, the phase of the liver clock was found to be fixed by the meal timing in spite of a high-dose administration of GC.

Materials and Methods

Animals

Eight-week-old male Wistar rats purchased from Japan Lab Animals Co., Ltd. (Osaka, Japan) were used in this study. All animals were housed in plastic cages (345 × 403 × 177 mm; CLEA, Tokyo, Japan) under a 12-h light, 12-h dark (L/D) cycle [light on 0900 h, considered zeitgeber time (ZT) 0; 300 lux] under constant temperature (23 C). Each cage was equipped with a water bottle and rodent laboratory chow pellets (CE-2; CLEA). A dim red light was used for the treatment of animals during the dark period. All experiments were approved by Committee of Animal Care and Use of Kinki University School of Medicine, and all experimental procedures were conducted in accordance with institutional guidelines for use of experimental animals.

Surgical procedures

Rats underwent a bilateral ADX under anesthesia using pentobarbital (0.6 ml/kg body weight, ip, Somnopentyl; Merck, Whitehouse Station, NJ). A control group underwent a sham operation, in which bilateral incisions were made in the skin and immediately closed under anesthesia. Both the ADX and sham-operated rats were allowed to recover for 1 wk after the operation. Confirmation of a successful ADX was determined based on the CORT blood concentration (<10 ng/ml in serum at ZT 11). All ADX animals had free access to a 1% NaCl solution during the experiment.

Serum collection and CORT measurements

Blood samples were collected from the rat tails and centrifuged for serum extraction. The serum CORT levels were then assessed using an ELISA kit (minimum sensitivity, 40 pg/ml, AssayMax; Assaypro, St. Charles, MO) and microplate reader (Model 680; Bio-Rad, Hercules, CA). The intraassay coefficient of variation was less than 9%.

Drug administration

CORT (Tokyokasei, Tokyo, Japan) was dissolved in dimethylsulfoxide (DMSO) at 10 or 90 mg/ml and delivered ip into the subject animals. A control group of ADX rats received a dose of 0.3 ml/kg DMSO ip.

Experimental protocols

Study 1. Effects of an ADX on the expression of peripheral clock genes in the rat

We investigated the influence of endogenous GC deprivation upon the peripheral oscillators in the rats. ADX and sham-operated control rats were killed at ZT 4, 8, 12, 16, 20, and 24 (0). Sampling at ZT 12 was performed before light-off and at ZT 24 was performed before light-on. Before killing the animals, spon-

taneous locomotor activity in 23 ADX and eight control rats was recorded for 24 h. The daily fluctuations of clock gene expression in the peripheral organs were measured using a quantitative PCR method (at each time point, n = 3–4 for ADX, n = 3 for sham-operated rats). The daily expression rhythms of rat Per1 (*rPer1*) and *rPer2* in the SCN were also measured using an *in situ* hybridization method (n = 3 for each group at each time point).

*Study 2. Effects of CORT injections on r*Per1 *expression in ADX rats*

To confirm that ip injections of CORT into ADX rats had measurable effects on peripheral oscillators, the induction of *Per1* was assessed in the liver and kidney. Three ADX rats of a baseline control group were killed at ZT 0.5. Other ADX rats were injected with 3 or 27 mg/kg CORT or DMSO at ZT 0.5, and sample tissues were collected at 60 and 180 min after injection (n = 3 for each group at each time point). Next, four ADX rats of a baseline group were killed at ZT 11.5, at which stage other ADX rats were injected with 27 mg/kg CORT or DMSO, and sample tissues were collected at 60 min (n = 3 for each group) and 180 min (n = 3 for CORT, n = 4 for DMSO) after injection. To assay for increases in the serum CORT levels after the injections, serum was collected from the intact rats at ZT 4, 8, 12, 16, 20, and 24 (n = 5 for each time point) to evaluate the normal concentration of CORT and was collected from three ADX rats at 0.5, 1, 2, 4, 8, 12, and 24 h after injection with 3 or 27 mg/kg CORT at ZT 0.5.

Study 3. Effects of daily desynchronous CORT administration in ADX rats

To assess whether daily CORT injections entrain the circadian rhythm of the peripheral tissues of ADX rats, the animals were injected with 3 or 27 mg/kg CORT, or with a vehicle (DMSO) at ZT 0.5 for seven consecutive days. Tissues were then sampled at 4-h intervals beginning at ZT 4 for 24 h on d 7 (Fig. 1A). In our present study, the time of administration was determined based on previous reports that the fixed point indicated by the phase-response curve (PRC) for GC was at around ZT 12 in the peripheral clock (20, 23). The PRC illustrates the relationship between the timing and the effects of a stimulus, and the fixed point on the PRC is the time at which the phase of the circadian clock is fixed by the daily pulsatile phase-shifting stimulation (28). Hence, if CORT was administrated at ZT 0.5, it would be expected that the circadian rhythms of the peripheral clock will show antiphasic rhythms compared with control animals. The daily activities of some rats (n = 12 for CORT, n = 11 for DMSO injected) were recorded on d 6. The daily fluctuations in clock gene expression were measured using quantitative PCR for the peripheral organs (n = 3–4 for 3 mg/kg CORT, n = 3–4 for 27 mg/kg CORT, n = 3–8 for DMSO injections; each time point). In the 27 mg/kg CORT ip experiment, the clock gene expression pattern of the lung was examined in addition to that of the liver and kidney. The daily expression rhythms of *Per1* and *Per2* in the SCN were measured using *in situ* hybridization (n = 4–6 for 3 mg/kg CORT, n = 3–4 for 27 mg/kg CORT, n = 7–9 for DMSO injections; each time point).

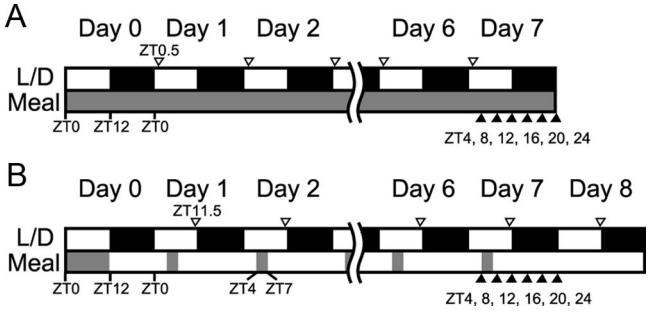

FIG. 1. Protocols used in study 3 (A) and study 4 (B). The *panels* indicate the lighting conditions, feeding conditions, drug administration, and sampling time points. The L/D cycle *bar* indicates the light and dark period. Food was given at the time indicated by the *gray area*. An *open triangle* indicates the drug administration time, and a *closed triangle* indicates each sampling time point.

Study 4. Effects of CORT and food intake on the peripheral clocks of ADX rats

Because food intake is a potent zeitgeber for the peripheral clocks, we examined in ADX rats whether the peripheral clocks in the liver, kidney, and lung are entrained to GC or to feeding. Food was removed from the animal cages at ZT 12 on the day before commencement of the RF period, and the ADX rats were then allowed access to food for 3 h from ZT 4 to 7 for seven consecutive days (Fig. 1B). Under RF conditions, rats received an injection of 27 mg/kg CORT or vehicle at ZT 11.5 for eight consecutive days. The injections were also started on d 1 and carried out in parallel to the RF treatments. The locomotor activity of some rats was recorded during the experimental period to analyze the FAA (n = 15 for each group on d 0–6; n = 4 for each group on d 7–8). In our current study, the FAA was defined as the activity levels during ZT 2–4, as described in previous reports (24, 25). Rats were killed at 4-h intervals beginning at ZT 4 for 24 h on d 7, apart from a group of animals that was retained for activity analysis. The rats killed at ZT 12 were not administered CORT on d 7. The rats were deprived of food throughout d 8 to observe the FAA. The daily fluctuations of clock gene expression were measured using quantitative PCR for the peripheral organs (n = 3 for CORT, n = 3–4 for DMSO; each time point). The daily expression rhythms of *Per1* and *Per2* in the SCN were measured using *in situ* hybridization (n = 3 for each group and each time point) to examine the effects of RF on the central circadian rhythm.

Sampling

All rats were anesthetized with pentobarbital and intracardially perfused with 40 ml of saline. The liver, kidney and lung were then quickly dissected and preserved in RNA later (Ambion, Austin, TX). The rats were subsequently perfused with 40 ml of a fixative containing 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brains were postfixed in the same fixative at 4 C and then soaked in 0.1 M PBS (pH 7.4), containing 20% sucrose for 48 h.

Analysis of mRNA levels

The mRNA expression levels were assayed using quantitative PCR and *in situ* hybridization using previously described methods with some modifications (29). The details of these procedures are outlined in the Supplemental Materials and Methods, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Activity recording

Rats were individually housed in plastic cages. Each cage was equipped with an infrared area motion sensor (NaPiOn; Panasonic, Osaka, Japan), which detects any spontaneous movements of the animal. Locomotor activity data were recorded on an IBM-PC, and the data were analyzed using a locomotor activity analysis program (Clock Lab; Actimetrics, Wilmette, IL).

Statistical analysis

To estimate the phase of cycling genes, the least squares spectrum (LSS) method was used (30). This technique estimates the cosine curve with a 24-h periodicity that best fits the time series data for mRNA levels using the least square and provides the probability of no circadian rhythmicity (*P* value) from the error value of this least square fitting (31). When the *P* value was smaller than 0.05, we regarded the profiles as rhythmic and considered the phase of the estimated cosine curve to represent the phase of the cycling genes. The acrophase of the r*Per1* gene expression rhythms in the CORT-injected rats was not used in these estimations, because acute but not rhythmic induction of *Per1* by CORT ip seemed to mask the circadian expression rhythms. Two-way ANOVA was used to compare two groups of time series data. The statistical significance of anticipation activity development was analyzed using Dunnett's multiple comparisons with a control. The acute induction of the r*Per1* gene by CORT ip was analyzed with the Tukey-Kramer multiple comparisons test. $P < 0.05$ was considered statistically significant.

Results

Study 1. Effects of an ADX on the expression of peripheral clock genes in the rat

ADX had no significant effects on the r*Per1* and r*Per2* gene expression rhythms in the SCN under L/D cycles (Supplemental Fig. 1A). The distribution of daily locomotor activity was also found to be unaltered by this treatment (Supplemental Fig. 2A). The clock gene expression patterns in the livers of the ADX rats showed a diurnal oscillation, but the r*Per1* expression levels decreased compared with the sham-operated rats ($P < 0.001$) (Fig. 2). In the kidney of the ADX rats, r*Per1* gene expression decreased drastically and showed no significant daily rhythm (Fig. 2). In contrast, r*Per2*, r*Cry1*, and r*Bmal1* expression showed significant daily rhythms, although at small amplitudes compared with the control rats ($P = 0.0253$, r*Cry1*; $P < 0.001$, r*Bmal1*). The acrophase of the clock gene expression rhythms in the liver and kidney of the ADX rats was then obtained by LSS analysis (Supplemental Table 1), but large differences from the control rats were not observed.

Study 2. Effects of CORT injections on r*Per1* expression in ADX rats

Induction of the r*Per1* gene after CORT ip was observed in the liver and kidney of ADX rats (Fig. 3). Doses

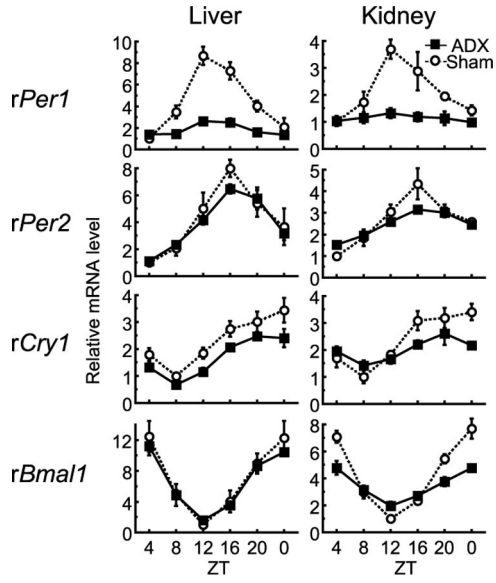

FIG. 2. Daily rhythms of clock gene expression in the liver and kidney of both ADX and sham-operated rats. Daily fluctuations in the clock gene mRNA levels are expressed as the mean ± SEM from ADX (n = 3 for ZT 8, n = 4 for other time points) or sham-operated rats (n = 3 for each time point). The *solid and dotted lines* represent the ADX and sham-operated group, respectively. The *ordinate* indicates the relative mRNA levels normalized to the corresponding *GAPDH* transcript levels. The basal values for the sham-operated rats were assigned a value of 1.

of 3 or 27 mg/kg CORT at ZT 0.5 strongly induced r*Per1* gene expression after 60 min in both the liver and kidney (Fig. 3A). After 180 min, the elevation in r*Per1* mRNA in the 3 mg/kg CORT-injected rats was reduced. In the 27 mg/kg-injected animals, however, r*Per1* transcripts were maintained at a high level after 180 min. In addition, the 27 mg/kg CORT dose at ZT 11.5 in ADX rats induced r*Per1* gene expression in both the liver and kidney, similar to the effects seen at ZT 0.5 (Fig. 3B). Figure 4 shows the calculated time course of the serum CORT levels after a single ip injection in ADX rats. The serum CORT levels steadily decreased after the injection. For the 3 mg/kg dosage, the serum CORT concentration exceeded 200 ng/ml, which was found to be the peak value for endogenous serum CORT rhythms in adrenal-intact rats (Fig. 4C), and this concentration was maintained for 1 h (Fig. 4A). For the 27 mg/kg dosage, the serum CORT reached levels above 200 ng/ml for over 4 h (Fig. 4B). No residual CORT was detectable after 24 h.

Study 3. Effects of daily desynchronous CORT administration in ADX rats

Daily CORT administration did not affect the r*Per1* and r*Per2* gene expression rhythms in the SCN between CORT-injected and control rats (Supplemental Fig. 1B). In addition, the daily locomotor activity profiles did not show significant differences between these animal groups (Supplemental Fig. 2B). In contrast, the circadian profiles

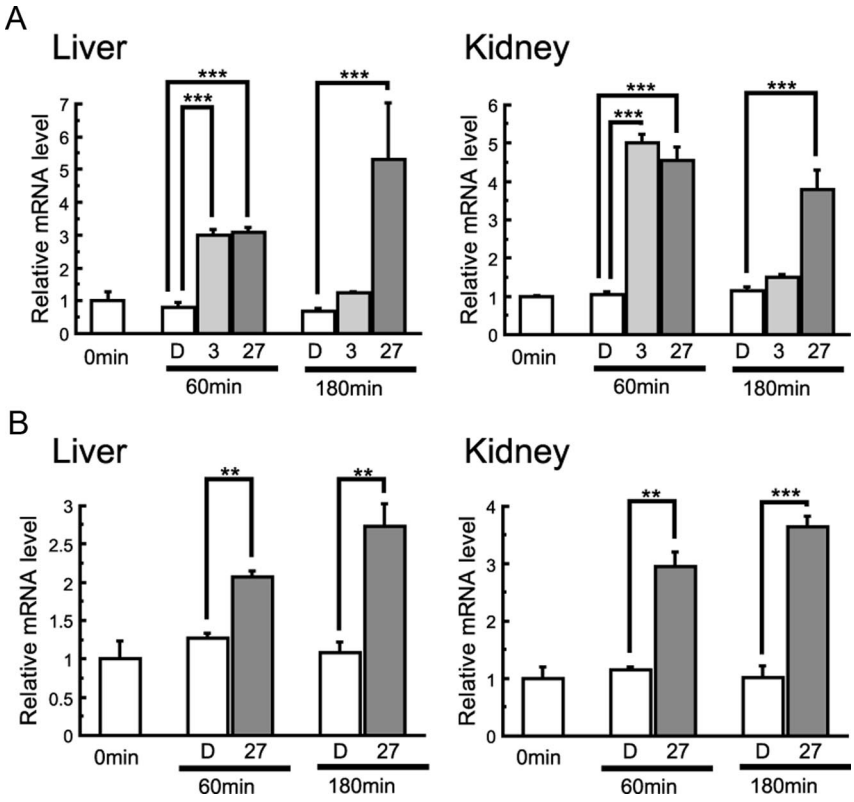

FIG. 3. Induction of r*Per1* gene expression by CORT injection into the liver and kidney of ADX rats. A and B, Significant induction of the r*Per1* gene after CORT administration at ZT 0.5 and ZT 11.5, respectively. The *ordinate* indicates the relative mRNA levels normalized to the corresponding *GAPDH* transcript levels. The mRNA expression levels before injection (0 min) were assigned a value of 1. D, Vehicle (DMSO); 3, 3 mg/kg CORT; 27, 27 mg/kg CORT. Each data series represents the mean ± SEM; **, $P < 0.01$; ***, $P < 0.001$ *vs.* vehicle control.

of the clock genes, r*Per2*, r*Cry1*, and r*Bmal1*, showed obvious differences among the liver, kidney, and lung (Fig. 5). In the kidney, the r*Per2* and r*Bmal1* expression rhythms were entrained to a daily administration of 3 mg/kg CORT at ZT 0.5 (Fig. 5 and Supplemental Table 1). However, the r*Cry1* gene did not demonstrate significant circadian rhythm. In addition, the liver circadian rhythm was found not to be entrained by a 3 mg/kg CORT stimulus. The daily CORT administration regimen was observed to affect the temporal profiles of clock gene expression in the liver. For example, the circadian profiles of the r*Cry1* and r*Bmal1* genes were altered significantly in the CORT injected group ($P < 0.001$, r*Cry1*; $P = 0.0245$, r*Bmal1*). However, the acrophase of the injected rats was the same as that of the control rats (Fig. 5 and Supplemental Table 1).

The high-dose (27 mg/kg) CORT-administered group showed a strong induction of r*Per1* expression at ZT 4 in all measured organs (Fig. 5). In the kidney and lung, the r*Per2*, r*Cry1*, and r*Bmal1* expression rhythms displayed a dynamic oscillation pattern, which was found to be entrained to the daily administration of 27 mg/kg CORT at ZT 0.5 (Fig. 5 and Supplemental Table 1). Surprisingly, however, although the prominent induction of r*Per1* gene expression was observed in the liver of these high-dose subjects, the phases of the r*Per2*, r*Cry1*, and r*Bmal1* expression rhythms were barely altered (Fig. 5 and Supplemental Table 1). The peak level of r*Per2* gene expression was also found to have declined in comparison with the control rats ($P = 0.0297$).

Study 4. Effects of CORT and food intake on the peripheral clocks of ADX rats

There were no significant differences found between the r*Per1* and r*Per2* gene expression rhythms in the SCN between the CORT injected and control rats under RF conditions (Supplemental Fig. 1C). In the control rats that received RF and vehicle injections, peripheral oscillators

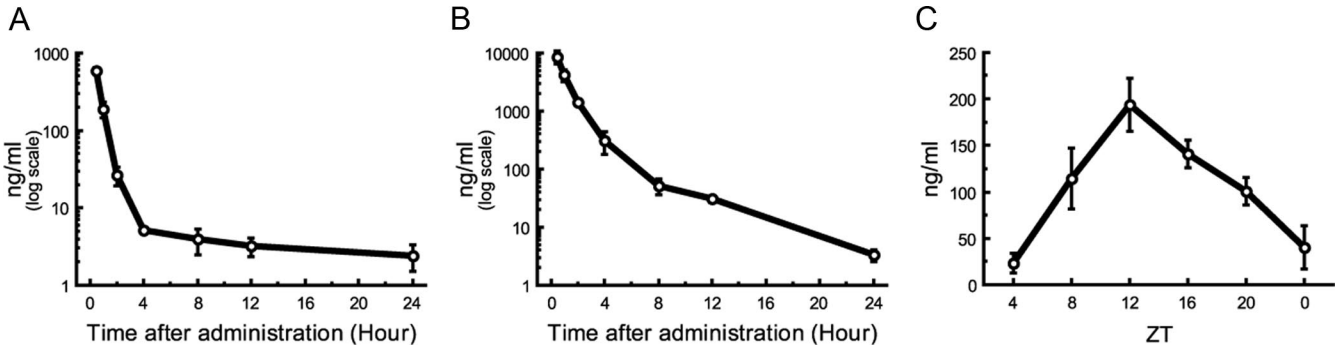

FIG. 4. Transition curves of the serum CORT levels after injection of this compound into ADX rats and of the endogenous daily rhythm of the serum CORT concentration in adrenal-intact rats. Transition curve for 3 mg/kg CORT injections (A) and that for 27 mg/kg-injected rats (B). The *vertical axis* is on a log scale. C, Daily fluctuation in the serum CORT concentration in adrenal-intact rats. Each data series represents the mean ± SEM.

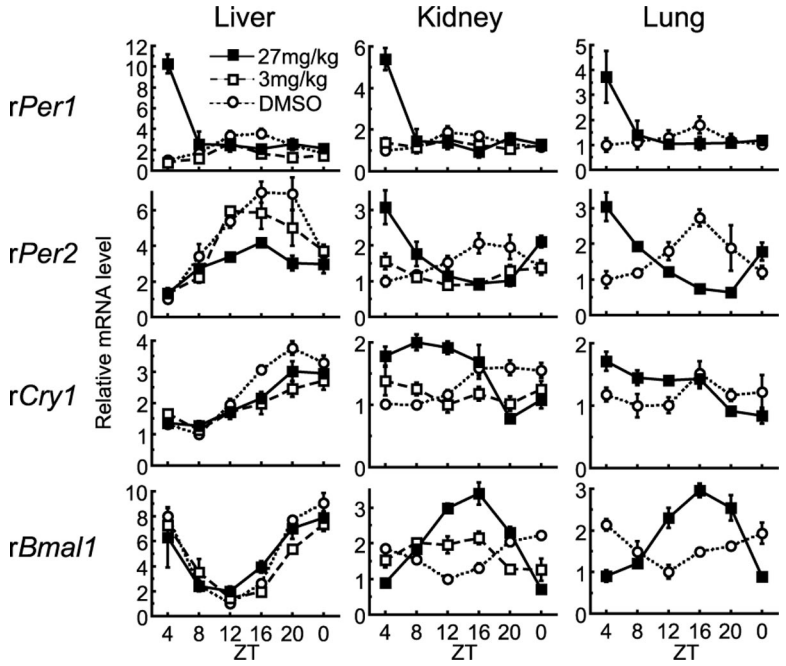

FIG. 5. Time-dependent variation in the expression of clock gene mRNA in the peripheral tissues of 3 or 27 mg/kg CORT-injected rats. Daily fluctuations in clock gene transcripts are expressed as the mean ± SEM. The 27 mg/kg CORT ip group is represented by a *solid line* (n = 4 for ZT 20, n = 3 for other time points in the liver and kidney; n = 3 for each time point in the lung). A *dashed line* denotes the 3 mg/kg CORT ip group (n = 4 for ZT 8 and 16, n = 3 for other time points). A *dotted line* represents the vehicle ip group (n = 6, 7, 6, 8, 7, and 6 for ZT 4–24, respectively, in the liver and kidney; n = 4 for ZT 16, n = 3 for other time points in the lung). These data were normalized to the *GAPDH* expression levels. The basal values for the vehicle-injected rats were set at 1.

in the liver, kidney, and lung were found to be entrained by RF (Fig. 6). In the liver of the control animals, robust rhythms were observed in the r*Per1*, r*Per2*, r*Cry1*, and r*Bmal1* gene expression patterns. These rhythms were also clearly entrained to the RF (Fig. 6 and Supplemental Table 1). In the kidney and lung also, the r*Bmal1* gene expression rhythm was found to be entrained by the fixed meal time. The daily expression of the r*Per1*, r*Per2*, and r*Cry1* genes in both organs tended to synchronize to the meal time in the controls but did not show significant daily rhythms. In contrast, differences were observed between the liver and other organs in the CORT-injected rats (Fig. 6). In the kidney and lung, robust daily rhythms were detected for r*Per2*, r*Cry1*, and r*Bmal1* gene expression and were entrained by the CORT injection (Fig. 6 and Supplemental Table 1). In contrast, the liver clock was found to be entrained to the RF regardless of the daily asynchronous administration of CORT. Although the expression of the r*Per2* gene at ZT 20 was slightly elevated by CORT, the results of LSS analysis demonstrated that the r*Per2* expression rhythm was synchronized to the periodic meal time. We further found that the phase of *rCry1* expression rhythm was delayed and its acrophase was ZT 15.6.

We next tested whether the daily administration of CORT had any effect on the formation of FAA in ADX rats. In the experiments, the FAA was formed by RF over seven consecutive days and was detected on d 8 under fasting conditions in both the CORT-injected and control group (Fig. 7). During d 2–6, no significant differences in the amount of FAA were detected between the CORT- and vehicle-injected groups (Fig. 7B). However, the time course of the development of FAA was found to differ between the two groups. Clear FAA, which was significant in comparison with baseline activity (defined as the amount of activity from ZT 2 to 4 on the d 0), appeared on and after d 3 in the control rats. The CORT-injected rats did not exhibit any significant FAA until d 5, but by d 6, the FAA was significant and equivalent to that in the control rats.

Discussion

Although many reports have suggested that humoral factors secreted from the adrenal gland fix the phase of the peripheral clocks, ADX produced few effects on the phase of the circadian rhythm of clock gene oscillation in our current study in rats. Pathways other than humoral factors synthesized in the adrenal glands must therefore play a role in entraining these rhythms. Previous studies have suggested that the autonomic nervous system also delivers timing cues from the SCN to the peripheral organs (10, 11, 18). Food intake is also a strong stimulus for the peripheral oscillators, but the precise pathways that transmit the timing cues to the periphery have not yet been delineated. The nocturnal characteristics of the rat in which food intake occurs mostly during the early night enable the use of this animal model to entrain the peripheral clocks after ADX (ADX rats) (32).

Interestingly, our present results showed that the r*Per1* gene expression levels were significantly reduced in the liver and kidney of the ADX rats. The *per1* gene has a GC-responsive element within its own promoter region, and the daily expression of *per1* in the peripheral clock was found to be similar to that of the endogenous CORT level. Thus, *Per1* rhythmic expression in the periphery may be greatly dependent on GC. In support of this, it has

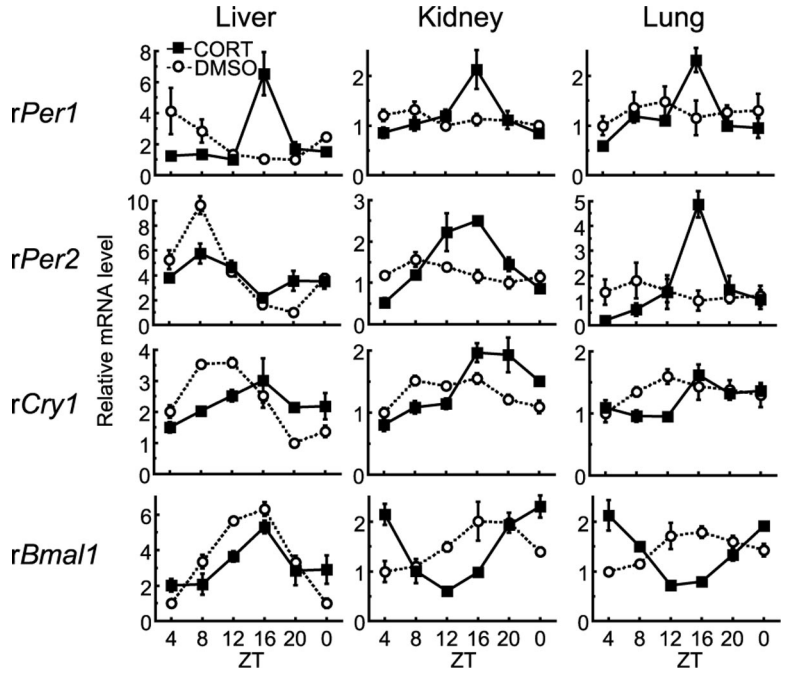

FIG. 6. Circadian clock gene expression patterns in the peripheral tissues of daily CORT-injected rats under RF. Daily profiles of the clock gene mRNA are expressed as the mean ± SEM. A *solid line* denotes the 27 mg/kg CORT ip group (n = 3 for each time point). A *dotted line* represents the vehicle ip group (n = 4 for ZT 4 and 8, n = 3 for other time points in the liver and kidney; n = 3 for each time point in the lung). Data were normalized to the *GAPDH* expression levels. The basal values for the vehicle-injected rats were set at 1.

been previously reported that SCN-transplanted animals that lack recovery of GC rhythm (33) also show decreased amplitude of their *Per1* circadian rhythm in peripheral tissues (29), suggesting an involvement of GC in maintaining the normal circadian oscillation of the peripheral clocks.

In contrast, our current analyses show that the circadian rhythm in the SCN is not affected by GC deprivation in the ADX rats. The mechanism underlying the distinct profiles of *Per1* expression in the SCN and in the peripheral tissues is open to speculation only at present. One possibility is that there may be a difference in the transcriptional properties of *Per1* and that some additional integrated transcriptional feedback loop operates to maintain the stable circadian rhythm in the SCN. Another possibility is that the strong cell-to-cell interactions that work among oscillating neurons in the SCN (34) may contribute to the maintenance of rhythmic *Per1* expression.

In adrenal-intact rats, the peak time of endogenous CORT secretion is at nearly ZT 12, and the fixed point indicated by the PRC of the peripheral clock after GC administration was also found previously to be at around ZT 12 (20, 23). In our present study, this phase relationship between the GC peak and circadian clock gene oscillations was observed to be maintained between the time of CORT injection and the circadian rhythm of the kidney and lung. We thus concluded that CORT had fully shifted the circadian clock in the kidney and lung, suggesting that it functions to fix the phase of the circadian rhythm in peripheral organs. GC have been also shown to entrain the circadian gene expression in fibroblasts and peripheral organs (6, 22, 23), and our current findings are consistent with this.

In contrast to the data we obtained in the kidney and lung, the peripheral clock in the rat liver was found to be entrained by feeding in spite of a desynchronous high-dose CORT administration. Although the feeding conditions in study 3 involved an *ad libitum* food intake, it is possible that the liver circadian clock was entrained to the daily feeding, which occurred mostly during the early night. The mechanism underlying the difference in the entrainment properties of the peripheral clock between the liver and other organs is thus a question of some interest. In the liver, CORT administration prominently induces r*Per1*, which may be the primary step in phase resetting (5). Balsalobre *et al.* (6) have previously reported that an ip injection of GC agonist induces phase shifts of less than 4 h in the albumin promoter D-site binding protein (*DBP*) and in the *Rev-erbα* gene expression rhythms in the mouse liver. Furthermore, in SCN-ablated mice, a GC agonist has been shown to entrain clock gene oscillation in the liver (35). It is thus probable that the liver clock has the ability to be entrained by GC. However, our present data suggest that food intake is a stronger zeitgeber for the liver clock than daily rhythms of endogenous GC secretion or injection. Food intake has been shown in earlier reports to result in changes to the cellular metabolic state, and it has been further demonstrated that various metabolites affect the molecular feedback loop of the peripheral clock (36–41). The liver is a metabolically important organ, so it is quite possible that the liver clock is readily entrained to RF by these metabolites. Furthermore, it is possible that the feedback pathway from the peripheral organs to the brain plays significant roles in this process. The liver is linked to the hypothalamus, which is a central regulator of energy homeostasis via the autonomic nervous system, and sympathetic input has been shown to entrain the liver clock (10). It is probable, therefore, that these autonomic pathways are involved in the phase determination of the liver clock via feeding-related stimuli.

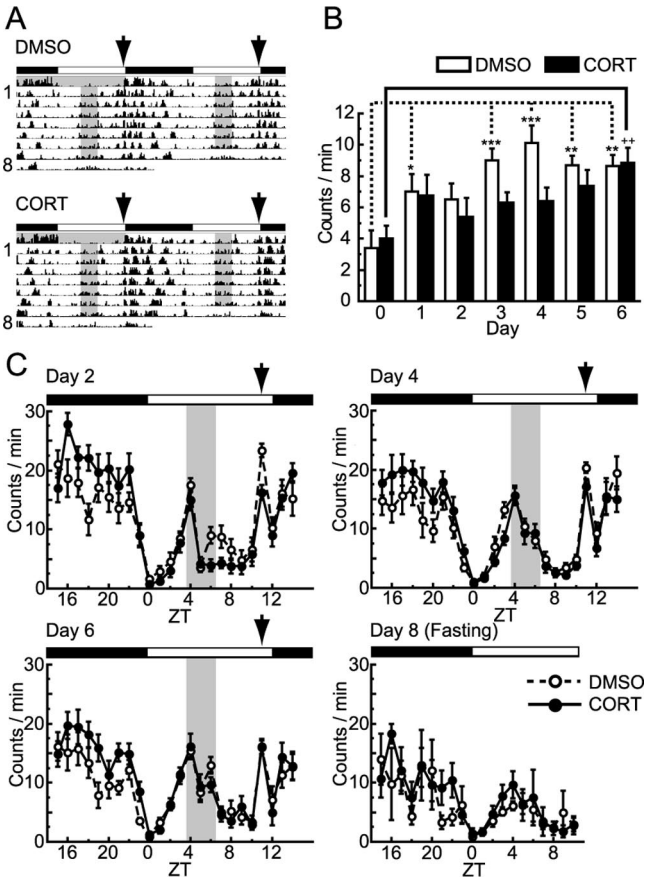

FIG. 7. Effects of CORT administration on the FAA of ADX rats under RF. A, Representative double-plot actograms of CORT- or vehicle-injected rats under RF conditions. The first day of the RF treatment was set as d 1, and the RF day number is indicated on the *left* of the actogram. B, Time-course analysis of the development of FAA. Shown are the activity counts allocated to a 2-h time interval, ZT 2–4. C, Daily distribution of locomotor activity on d 2, 4, 6, and 8. Each data point represents the activity counts per minute averaged across a 1-h bin. The *top bar* indicates the light and dark period, and the time of administration is indicated by an *arrow*. Food was given at the time indicated by the *translucent gray area*. Each data series represents the mean ± SEM; *, $P < 0.05$; ** and $^{++}$, $P < 0.01$; ***, $P < 0.001$ *vs.* the baseline value at d 0.

The phase of the liver clock is therefore probably determined by the interplay between these mechanisms.

Per1 induction has been deemed an initial transcriptional event during the onset of a phase shift (5). In spite of *Per1* mRNA induction by CORT administration, the liver did not show a significant phase shift in our present experiments. The molecular mechanism causing the inhibition of the phase shift in this case has not been delineated, but our results in study 3 suggest that *Per1* induction modulates the expression levels of some circadian feedback loop components but only causes partial effects so that the eventual phase shift is blocked somewhere in the feedback loop. The transcription of genes under the control of an E-box, such as *Per2* and *Cry1*, was also found to be decreased, although the expression profiles of *Bmal1* were largely unaffected. *Bmal1* transcription is regulated by an RRE, and we speculate that feeding-related stimuli, such as those exerted by metabolites and inputs from the autonomic nervous system, may dominate RRE-dependent transcription and that the phase-shifting perturbation was nullified in the liver. Further studies are needed to elucidate this mechanisms, by which the impacts of external stimuli can be circumvented in this system.

The effects of different CORT injection doses manifested mainly in terms of the amplitude of clock gene circadian oscillations. The larger dose of CORT resulted in not only a higher initial serum CORT concentration but also a longer temporal duration. It is interesting in this regard that the peak levels of *Per1* induction were similar between the 27 and 3 mg/kg CORT-injected animals, but the duration of the *Per1* increase was longer at the higher dose. It is possible that the longer period of *Per1* expression reflects the duration of a high serum CORT concentration and that this underlies the higher amplitude of the clock gene oscillations that result from the higher CORT dose.

The daily CORT concentration patterns generated by injection of this compound appeared to differ from the endogenous CORT secretion pattern. Unlike the endogenous circadian CORT rhythm, the serum CORT concentration in the ADX rats showed its highest value immediately after injection and then decreased rapidly. Hence, it is possible that the entrainment properties of the peripheral clock in relation to the endogenous GC rhythm differ from those found in our present study. Further studies using a CORT administration protocol that faithfully reflects the endogenous CORT secretion pattern are thus required.

It is known that RF conditions not only develop clear FAA but also cause a phase shift in the peripheral clocks and endocrine rhythms, including those of the GC (24, 25). The interactions between the FEO and clock genes remain unclear, but recent reports have suggested that FEO does not require the traditional circadian clock mechanisms (26), although some clock gene knockout mice do show an irregular FAA pattern (42, 43). The ADX rats in our current study formed FAA after a feeding restriction from ZT 4 to 7, and daily CORT administrations at ZT 11.5 did not prevent this. This finding suggests that RF establishes FAA even without a phase shift in the GC rhythms or peripheral clocks in the kidney and lung. In contrast, the development of FAA in the CORT-injected group tended to be delayed compared with the control group. These findings suggest that either or both GC and other humoral factors secreted asynchronously from the peripheral oscillators affect the developmental process underlying FAA.

In summary, we show from our current data that the peripheral clocks in the rat kidney and lung are entrained to the daily stimuli that result from CORT administration, irrespective of the meal time. In contrast, the liver clock in the rat is entrained to the time of feeding, even if daily CORT injections are performed in antiphase. These results indicate that GC are a strong zeitgeber for the peripheral oscillators, which is impervious to the influences of other entrainment factors, at least in the kidney and lung, but that the rat liver is dominated by an unknown entrainment factor which is linked to feeding.

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