Physical and Inflammatory Stressors Elevate Circadian Clock Gene mPer1 mRNA Levels in the Paraventricular Nucleus of the Mouse

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Stress induces secretion of corticosterone through activation of the hypothalamic-pituitary-adrenal axis. This corticosterone secretion is thought to be controlled by a circadian clock in the suprachiasmatic nucleus (SCN). The hypothalamic paraventricular nucleus (PVN) receives convergent information from both stress and the circadian clock. Recent reports demonstrate that mammalian orthologs (Per1, Per2, and Per3) of the Drosophila clock gene Period are expressed in the SCN, PVN, and peripheral tissues. In this experiment, we examined the effect of physical and inflammatory stressors on mPer gene expression in the SCN, PVN, and liver. Forced swimming, immobilization, and lipopolysaccharide injection elevated mPer1 gene expression in the PVN but not in the SCN or liver. A stress-induced increase in mPer1 expression was observed in the corticotropin-releasing factor-positive cells of the PVN; however, the stressors used in this study did not affect mPer2 expression in the PVN, SCN, or liver. The present study suggests that a stress-induced disturbance of circadian corticosterone secretion may be associated with the stress-induced expression of mPer1 mRNA in the PVN. (Endocrinology 142: 4910–4917, 2001)

NOT ONLY PHYSICAL stress from forced swimming or immobilization but also inflammatory stress from the injection of lipopolysaccharide (LPS) have been shown to activate the hypothalamic-pituitary-adrenal (HPA) axis (1, 2). Stress-induced secretion of corticotropin-releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus triggers the synthesis and release of ACTH and, ultimately, glucocorticoids. Thus, CRF participates in the mediation of behavioral responses to stress in mammals (3). In immobilized rats or those receiving LPS injection, a great induction of fos-like immunoreactivity in CRF positive cells of the PVN was observed (4, 5), indicating that CRF in the PVN functions as an important neuropeptide mediating stress-induced responses. Several lines of evidence have suggested that the PVN acts as an important relay site for transferring information from the suprachiasmatic nucleus (SCN), a center for the circadian clock, to the pineal body (6). Actually, the PVN receives many different kinds of input from the SCN in the form of vasoressin, glutamate, and ɑ-aminoxyliceric acid neurotransmitters, which are thought to convey circadian information (7–9). Interestingly, destruction of the SCN attenuates behavioral rhythms as well as the corticosterone secretion rhythm (10). The results from the above-mentioned studies strongly suggest that the PVN is an important brain site that receives stress-related information as well as information from the circadian clock.

Concerning stress-related information, reports show that basal levels of corticosterone are elevated not only from repeated stress (11, 12) but also from acute, inescapable stress (13). This increase in basal values was especially evident at the diurnal trough of the circadian rhythm and persisted for several days after the stressor was removed. Further, Honma et al. (14) reported that destruction of PVN catecholamines suppresses a restricted feeding-associated advance of the corticosterone rhythm in rats. Thus, daily fixed restricted feeding, a form of mild stress, entrains corticosterone rhythm through change in the PVN function.

As for the circadian clock, a recent molecular approach led to the discovery of clock genes such as Per, Clock, Bmal1, and Cry that provide a mechanism for the regulation of circadian and seasonal rhythms in mammals (15–17). Although a high expression of mPer1, mPer2, and mPer3 (mouse orthologs of the Dorsophila period gene) have been observed in the SCN, moderate expression of these genes was also demonstrated in other brain regions such as the cerebral cortex, hippocampus, and PVN and in peripheral tissues such as the liver, heart, and skeletal muscle. To determine whether stress affects circadian activity of the HPA axis through change in the expression of mPer1 and/or mPer2 mRNA, we examined the effect of stressors such as forced swimming, immobilization, and LPS on expression of these genes in the PVN and SCN of mice.

In addition, a recent paper reported that glucocorticoid could reset the circadian clock in peripheral tissue such as the liver (18). Therefore, we were further interested in studying how stress can affect liver mPer gene expression through corticosterone secretion.

Materials and Methods

Animals

In all experiments, we used 4- to 6-wk-old male ddY mice (Takasugi, Saitama, Japan) housed in a 12-h light/12-h dark cycle. All animals were allowed free access to food and water before the start of food-restricted experiments.
Forced swim test and immobility test procedures

The forced swim protocol described by Porsolt et al. (19) was modified for use in the mice experiments. Mice were subjected to a 15-min swim in a cylindrical Plexiglass tank (20 cm in diameter) containing water (25 C) 30 cm in depth and then returned to their home cages. These same mice were then killed 45 or 165 min following the test. Mice in the immobilization group were placed in metal mesh restrainers. After 60 min of immobilization, mice were either immediately killed or returned to their home cages and decapitated 120 min later. Control mice kept in their home cages were killed at the same 60-min time point because mPer1 expression in the SCN reaches a maximal increase 60 min after light exposure (20).

Telemetry temperature measurement

Under pentobarbital anesthesia, a total of eight mice (three for saline injection, five for LPS injection) were surgically implanted with radiotelemetry transmitters (DSI, St. Paul, MN), which permitted continuous monitoring of core body temperature and activity. After 1 month of recovery from surgery, these animals were injected with the specified drug while the core temperature was monitored.

Sample preparation

Mice were deeply anesthetized with ether and intracardially perfused with saline followed by 0.1 M phosphate buffer (PB) (pH = 7.4) containing 4% paraformaldehyde (PFA). Brains were removed, postfixed in 0.1 M PB containing 4% PFA for 24 h at 4 C, and transferred into 20% sucrose in PB for 72 h at 4 C. Brain slices (40 mm thick) including the PVN and SCN were made using a cryostat (HM505E, Microm, Walldorf, Germany) and were placed in 2X saline sodium citrate until they were processed for hybridization.

In situ hybridization

In situ hybridization was applied to quantify mPer1 and mPer2 mRNA expression in the various brain areas. Slices were treated with 1 mg/ml proteinase K in 10 mm Tris-HCl buffer (pH 7.5) containing 10 mm EDTA for 10 min at 37 C, followed by 0.25% acetic anhydride in 0.1 M triethanolamine and 0.9% NaCl for 10 min. The slices were then incubated in the hybridization buffer (60% formamide, 10% dextran sulfate, 10 mm Tris-HCl, pH 7.4, 1 mm EDTA, 0.6 m NaCl, 1X Denhardt’s solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA), 0.2 mg/ml tRNA, 0.25% SDS) containing 32P-labeled cRNA probes for 16 h at 60 C. Radioisotope (50 mCi/µl [PerkinElmer Life Sciences, Inc., Boston, MA]-labeled antisense cRNA probes [mPer1 (538–1752), mPer2 (1–638)] (20) were made from restriction enzyme-linearized cDNA templates obtained from Dr. Okamura (Kobe University). After a high-stringency posthybridization wash with 2X saline sodium citrate/50% formamide, slices were treated with RNaseA (10 µg/ml) at 30 min at 37 C. To confirm that the signal intensities fell within the linear range of the detection system, a preliminary experiment was conducted to determine the adequate concentration of each radioisotopelabeled antisense cRNA probe.

The radioactivity of each slice on BioMax MR film (Kodak, New Haven, CT) was analyzed using a microcomputer interface to an image analysis system (MCID, Imaging Research, Inc., Ontario, Canada) after conversion into optical density by 32P-c Autoradiographic microscales (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). For data analysis, we subtracted the intensities of the optical density in the corpus callosum from those in the SCN and PVN of each section and regarded this value as the net intensity in the SCN and PVN. The intensity values of the sections from the rostral to the caudal part of the SCN and PVN (three to five sections per mouse brain) were then summed. The sum was considered to be a measure of the amount of mPer1 or mPer2 mRNA in this region.

Emulsion autoradiography for mPer1 and immunohistochemistry for CRF peptide

After fixation with 4% PFA, slices including the SCN and PVN were processed for immunohistochemistry according to the avidin-biotin-peroxidase complex method. Primary antibody (anti-CRF) (Cambridge Research Biochemical, Cleveland, OH) was diluted to a concentration of 1:5000 in 0.1 M phosphate buffer containing 1% normal goat serum in 0.3% Triton X-100.

For emulsion autoradiography, the same slices were dipped into emulsion (NTB2, Kodak) after hybridization with an mPer1 probe, after which they were air dried for 3 h and stored in light-tight slide boxes at 4 C for 2 wk. The slides were developed with a D19 developer (Kodak) and then fixed with Fuji fix (Fujifilm, Tokyo, Japan). The subnuclear silver grain distribution of each brain slice was examined using an optical microscope.

Statistics

Results are expressed as the mean ± sem. The significance of differences between groups was determined by a one-way ANOVA followed by Dunnett’s test or the t test.

Results

Forced swimming-induced mPer1 expression in CRF-positive PVN cells

Fig. 1 shows the emulsion autoradiogram of mPer1 and CRF-positive cells in the SCN and PVN. During the day, the SCN exhibits high mPer1 expression in the SCN but not in the PVN (Fig. 1, A and B). Sixty minutes after forced swimming, the expression of mPer1 increased in the PVN but not in the SCN. To identify the phenotype of mPer1 mRNA-positive cells in the PVN, we examined mPer1 induction and CRF expression using the same PVN slices. In this experiment, we used animals pretreated with colchicine (10 mg/kg) 24 h before the forced swim. Pretreatment with colchicine intensified the expression of CRF-positive cells in the SCN (Fig. 1G). If the number of mPer1 mRNA-labeled dots was at least 2 times higher than that seen in the background anterior hypothalamic area, this cell was identified as being doubly labeled. Interestingly, mPer1 mRNA and CRF immunoreactivity were colocализed to the same PVN cells (Figs. 1, H and I). Approximately 75% of examined CRF-positive cells coexpressed mPer1 mRNA using complete series (n = 3) of sections (four to six slices). The mPer1 signal was not restricted to the CRF-rich zone of the PVN and labeling was seen in other parvocellular compartments.

Forced swimming- and immobilization-induced mPer1 but not mPer2 expression in the PVN

The basal level of mPer1 and mPer2 expression was high in the SCN, and forced swimming or immobilization failed to affect mPer1 or mPer2 expression in the SCN (Fig. 2, A and B). On the other hand, 60 min after the stressors were presented, there was a strong induction of mPer1 but not mPer2 in the PVN, in which basal levels of mPer1 and mPer2 were low (Fig. 2, A and B). This increase of mPer1 in the PVN returned to basal level 3 h after stressor application. Sense control probes for mPer1 and mPer2 were used to examine nonspecific binding of these probes to the brain tissue. However, there were no signs of nonspecific binding data (data not shown).

Day/night differences in forced swimming-induced mPer1 expression

To test the hypothesis that the lack of stress-induced mPer1 expression in the SCN relates to the high level of mPer1 basal activity, we examined the effect of a stressor on the mPer1 and
expression during the nighttime, a time during which mPer mRNA was low in the SCN (Fig. 3). Results indicated that the basal level of mPer gene expression was low in the SCN but high in the PVN during the night (Fig. 3A). The daily rhythm of mPer1 expression was diurnal in the SCN and nocturnal in the PVN (Fig. 3A). Forced swimming failed to affect mPer1 or mPer2 expression in the SCN at ZT17 and ZT7. Figure 3 confirms that forced swimming elevated mPer1 expression in the PVN during the day (ZT7), but mPer1 or mPer2 expression showed no response in any of the brain regions tested during the nighttime (ZT17) (Fig. 3B).

There is a clear day/night difference in the expression of D site-binding protein mRNA in the SCN; however, such a difference was not observed in the PVN (Fig. 3B). The forced swim did not cause any change in DBP mRNA expression in the PVN or SCN during either the daytime or nighttime.

**Effect of LPS injection on mPer1 and mPer2 expression in the PVN and SCN**

Because physical stress caused an increase in mPer1 expression in the PVN, we examined whether inflammatory stress via LPS injection affected mPer1 expression in the PVN. Injection of LPS (50 μg/kg) at ZT24 significantly increased body temperature 1, 3, and 24 h after injection (Fig. 4C). Because this body temperature increase lasted for 24 h, we examined mPer1 and mPer2 expression in the SCN and PVN 1, 3, and 24 h after LPS injection. One hour after LPS injection, mPer1 expression in the PVN was significantly increased, but this increment returned to the level of the control saline-injection group (Fig. 4, A and B). Similar to physical stress, LPS injection did not affect mPer2 expression in the PVN or mPer1 and mPer2 expression in the SCN.
Effect of stressor application on mPer expression in the liver

Day/night differences in mPer1 and mPer2 expression in the liver were observed (Fig. 5A). The peak expression of both mPer1 and mPer2 mRNAs occurred at ZT11, and the profile was similar to that of the PVN but not the SCN. Administration of similar doses of LPS did not affect liver mPer1 or mPer2 expression (Fig. 5B). In the next experiment, we examined the effect of forced swimming on mPer1 and mPer2 expression in the liver and obtained negative data (Fig. 5C).

Discussion

The present results clearly demonstrate that forced swimming, immobilization, and LPS injection caused a rapid induction of mPer1 but not mPer2 mRNA in the PVN. This increase in mPer1 returned to the basal level 3 h after stress application. Thus, the present results suggest that mPer1 gene expression in the PVN is associated with stress-induced responses. The present results also demonstrate a nocturnal expression of mPer1 and mPer2 in the PVN and liver tissue and a diurnal expression in the SCN.

It is well known that there is a robust circadian rhythm for corticosterone release thought to be primarily controlled by the SCN because lesion of the SCN abolished this rhythm (10). In intact animals, the circadian rhythm of corticosterone release may be controlled by the circadian oscillation of mPer1 and/or mPer2 in the PVN under the influence of the SCN. The following lines of evidence support this conjecture: A recent report (21) demonstrated that the SCN regulates corticosterone release via the HPA and sympathetic pathway through the spinal cord. Because the light-induced rapid increase of mPer1 in the SCN causes behavioral phase shifts (20, 22), it is important to know how mPer1 may be involved in regulating the expression and release of CRF or other relevant factors such as vasopressin by parvocellular neurosecretory neurons. Furthermore, daily restricted feeding,
which causes a kind of chronic mild stress, is known to reset not only circadian corticosterone release (14) but also the PVN mPer1 expression rhythm (23).

The present results clearly reveal that not all stressors affect mPer1 and mPer2 expression in the SCN, suggesting an independence of the main circadian clock from stressful cir-
cumstances, which is also supported by our previous finding (23, 24) that oscillation of SCN mPer1 and mPer2 expression was unaffected by daily restricted feeding.

In this experiment, stress-induced mPer1 expression was observed with only daytime treatment. Consistent with our results, Kelliher et al. (25) demonstrated that rats experienced less stress when placed in a test situation during the active (dark period) phase of their circadian cycle during the assessment of neurochemical and neuroendocrine indices of stress. Notably, there was no stress-induced mPer1 expression in the PVN at night when the basal level of mPer1 expression was elevated. Plasma corticosterone levels were elevated 15–30 min after the onset of forced swimming (26), suggesting this hormonal response was not regulated by the expression of mPer1 or mPer2 mRNA. Thus, stress affects ACTH and/or corticosterone release and mPer1 and mPer2 expression independently. Balsalobre et al. (18) recently reported that the circadian clock in peripheral tissues was reset by glucocorticoid signaling. It is well known that forced swimming and LPS injection produce a corticosterone secretion (2), and we observed the effect of these two stressors on mPer1 expression in the liver. However, mPer1 and mPer2 expression in the liver demonstrated no response to these stressors, possibly because they were too weak to produce mPer1 expression in the liver but of sufficient strength to cause mPer1 expression in the PVN. When we examined the effect of LPS on body temperature, injection of LPS exhibited a long-lasting effect of up to 24 h after injection. LPS-induced PVN mPer1 expression, on the other hand, occurred 1 h after injection and returned to the control level 3 or 24 h later.

DBP, a member of the PAR leucine zipper transcription factor family, is reportedly a clock-controlled gene as well as a factor related to the promotion of the mPer1 gene (27). Because acute stress did not affect DBP mRNA in the present study, it was ascertained that DBP failed to respond to environmental stimuli.

Although both forced swimming and immobilization caused strong increase in the mPer1 mRNA in the PVN, these stressors only slightly enhanced CRF-immunolabeling cells...
in the PVN (data not shown). In the following experiment, therefore, mice were treated with colchicine to enhance CRF immunolabeling before they were subjected to stress. Colchicine is itself a stressor and would have been sufficient to induce \textit{mPer1} expression in its own right. In fact, our preliminary experiment demonstrated that colchicine application alone moderately induced \textit{mPer1} expression in the PVN (data not shown). Thus, the present results demonstrate that \textit{mPer1} responds in this instance to both the drug and the stressors.

Acute stress elevated both CRF mRNA in the PVN (28) and \textit{fos} immunoreactivity in CRF-positive cells of the PVN (29). Thus, the \textit{mPer1} response to acute stress closely resembled that of \textit{fos}, suggesting that \textit{mPer1} behaves as a kind of immediate early gene. Reportedly, the rapid expression of \textit{mPer1} mRNA does not require any translational steps because serum-induced expression of these genes is not blocked by anisomycin (22, 29).

The mechanism of rapid increase in \textit{mPer1} mRNA remains unknown at present. Several researchers have reported that \textit{mPer1} mRNA was induced by a high concentration of serum (29), forskolin (PKA activator), and phorbolester (PKC activator) (30, 31). Activation of PKA and PKC followed by cAMP responsive element binding protein phosphorylation may be an important step in producing the \textit{mPer1} expression.

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Neural inputs to the PVN from monoaminergic systems may
be important regulators for the induction of CRF and mPer1 genes. The PVN is innervated by the serotonergic and noradrenergic systems, both of which are stimulated in response to stress (32–35) and have been implicated in the regulation of CRF release (36, 37). Therefore, such transmitters may facilitate mPer1 expression, although further experiments are required for their identification. Recently, Takekida et al. (38) demonstrated that an adenraline receptor agonist, isoprenaline, elevates mPer1 and mPer2 expression in the pineal body, suggesting such a possibility.

In summary, the present results indicate that upregulation of the mPer1 gene in the PVN CRF neurons is suggestive of a mechanism in which stress signals affect corticosterone secretion.

Acknowledgments

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