Alterations of Circadian Expressions of Clock Genes in Dahl Salt-Sensitive Rats Fed a High-Salt Diet

Takao Mohri, Noriaki Emoto, Hideaki Nonaka, Hiroyuki Fukuya, Kazuhiro Yagita, Hitoshi Okamura, Mitsuhiro Yokoyama

Abstract—In mammals, behavioral and physiologic processes display 24-hour rhythms that are regulated by a circadian system consisting of central and peripheral oscillators. Because various cardiovascular functions show diurnal variations and abnormal patterns of circadian blood pressure variation carry a high risk of cardiovascular complications, we investigated whether the expression of clock genes is altered in an animal model of hypertension. In Dahl salt-sensitive rats fed a high-salt (4% NaCl) diet for 6 weeks (DS-H), radiotelemetry monitoring showed increased amplitude of circadian variations in blood pressure. The ratio of heart weight to body weight and the ratio of kidney weight to body weight were higher in DS-H. Echocardiographic data showed that the wall thickness of the left ventricle was greater in DS-H. Northern blot analysis and single cosinor analysis revealed that the amplitudes of circadian expression changes of the clock genes (mPer2, Bmal1, and dbp) in the heart, liver, and kidney were significantly decreased in DS-H rats compared with a normal-salt-diet group, except for Bmal1 in the liver. The circadian expression changes of plasminogen activator inhibitor-1, a clock-regulated gene, were attenuated in the hearts of DS-H. The present results demonstrate that DS-H show altered circadian expression of peripheral clock genes. Detailed analyses of the relation between circadian expression of clock genes and blood pressure regulation might reveal a role for chronologic therapy of cardiovascular disease. (Hypertension. 2003;42:189-194.)

Key Words: circadian rhythm • hypertension, experimental • hypertrophy • molecular biology • rats, Dahl

The circadian clock system is responsible for the daily timing of physiologic processes.1–4 Several genes essential for mammalian circadian clock function have been identified. These include homologues of the Drosophila gene period (mPer1, mPer2, and mPer3), cryptochrome isoforms (mCry1 and mCry2), and PAS helix-loop-helix transcription factors (Clock and Bmal1). The products of these genes constitute a model of a molecular oscillator, which is based on interconnected positive and negative transcriptional-translational feedback loops. Clock-controlled genes, including dbp, whose products show circadian accumulation but are not required for clock oscillation, can be regulated by the same feedback loops.

The mammalian central clock is located in the suprachiasmatic nucleus (SCN) within the hypothalamus in the brain. However, circadian expression of the clock and clock-controlled genes is observed not only in the SCN but also in most peripheral tissues. Recent evidence suggests that the circadian clock system has a hierarchical organization: a central pacemaker located in the SCN directs peripheral oscillators that are present in most tissues. Although central and peripheral clocks share important features in their molecular make-up, only the central clock is self-sustained. This suggests that SCN-derived signals synchronize peripheral oscillations to prevent the dampening of circadian gene expression. It appears that multiple signaling pathways consisting of hormonal and/or neuronal factors are used for entraining peripheral oscillators.

Cardiovascular or hemodynamic parameters, such as heart rate (HR) and blood pressure (BP), exhibit variations consistent with circadian rhythm.5 In addition, several kinds of acute pathologic cardiac events exhibit diurnal variations,6 and abnormal circadian BP patterns carry a high risk of cardiovascular complications.7 For instance, the degree of nocturnal BP elevation in patients with systemic hypertension is correlated with the severity of end-organ damage.8 These findings strongly suggest that there is a functional correlation between the circadian clock system and cardiovascular physiology as well as pathology. These considerations led us to hypothesize that cardiovascular disorders might alter the expression changes of clock genes in peripheral tissues. Therefore, in the present study, we set out to investigate the circadian expression changes of clock genes in peripheral tissues in Dahl salt-sensitive rats (DS). We found that expression of clock genes and a clock-controlled gene in the peripheral tissues was altered in this hypertensive rat strain.
Also, we observed that the circadian expression of plasminogen activator inhibitor-1 (PAI-1), a clock-regulated gene, was attenuated in the heart in DS fed a high-salt diet (DS-H). In contrast, we found that no significant circadian expression pattern of the PAI-1 gene was observed in the liver, suggesting that expression of the clock-regulated gene is differentially regulated among tissues.

Methods

Animals and Procedures

Male DS (Seac Yoshitomi, Ltd, Fukuoka, Japan) at 4 weeks of age underwent long-term implantation of a device that telemetrically monitors BP, HR, and motor activity. The telemetry system consists of a radiofrequency transmitter (TA11PA-C40), a receiver panel, and an acquisition system. Under anesthesia with halothane (4% induction; 1% to 1.5% maintenance in oxygen-enriched air), the catheter of the transducer was implanted into the abdominal aorta just below the bifurcation of the renal arteries, and the sensor itself was fixed to the peritoneum. After recovery, the rats were returned to their cages and maintained on a normal-salt diet (0.3% NaCl) for a 1-week period (“before loading”). They were then fed either a high-salt diet (4% NaCl; DS-H, n = 3) or a normal-salt diet (0.3%; DS-N, n = 3) for 6 weeks (“after loading”) and were exposed to complete 12-hour light/dark cycles under controlled environmental conditions at an ambient temperature of 23°C ± 1°C. They were allowed free access to water and food. Measurements were taken every 1 minute with use of a Dataquest system (Data Sciences). Ten-minute averages from 3 consecutive measurements were calculated for further analysis.

Transthoracic echocardiography was performed with an HP Sonos 100 (Hewlett-Packard Co) with a 10-MHz imaging transducer. End-diastolic left ventricular internal diameter (EDD), end-systolic left ventricular internal diameter (ESD), and left ventricular posterior wall thickness were measured. Percent fractional shortening (%FS) was calculated as %FS = [(EDD − ESD)/EDD] × 100.

To examine the expression level of clock and clock-controlled genes, we prepared 72 male DS rats at 4 weeks of age. Rats were kept in 12-hour light/dark cycles (lights on at 9 AM, lights off at 9 PM). All of them were fed a normal-salt diet (0.3% NaCl). One week later, they were divided into 2 groups, which received either a high-salt diet (4% NaCl; DS-H) or a normal-salt diet (0.3% NaCl; DS-N) for 6 weeks. Rats were then kept in complete darkness for 2 days to evaluate the circadian rhythm generated by the internal pacemaker. On the day of the experiment, rats were killed every 4 hours (n = 6, each time point). To ensure the reproducibility of the gene cycle, 3 DS-H rats and 3 DS-N rats were killed at each time point on 2 separate days. The care and use of the animals strictly followed the guidelines of the Animal Research Committee of Kobe University Graduate School of Medicine.

Northern Blot Analysis and Real-Time, Quantitative RT-PCR

RNA was extracted from the heart, kidney, and liver as described previously. Total RNA (10 μg) was separated on a formaldehyde/1.1% agarose gel, transferred to a nylon membrane, and hybridized to random-primed, 32P-labeled probes. Probes of mPer2, dbp, Bmal1, and PAI-1 were prepared as previously described. The membrane was washed and exposed on an imaging plate, and the fragments were visualized with a BAS 2000 Bio-imaging analyzer (FUJIX Japan). mRNA was densitometrically quantified with software supplied by FUJIX BAS 2000. The signals were normalized to those obtained for glyceraldehyde 3-phosphate dehydrogenase mRNA. Glyceraldehyde 3-phosphate dehydrogenase mRNA is not subject to circadian regulation (data not shown). To compare the expression level of clock genes among different tissues, mRNA levels of clock genes were expressed as relative amounts, with the heart sample of circadian time 20 in DS-N rats as a standard. Real-time, quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) was performed as described previously. The primers 5’-GATGGCTCAGAACAAGCTTCAA-3’ and 5’-GGCAGTTCCAGGTGTCGACT-3’ were used for rat PAI-1 amplification. The hybridization probe was 5’-TACACTGAGTTCCACCATCCGATGGG-3’. The PCRs were carried out with the Taqman PCR reagent (Applied Biosystems) as recommended by the manufacturer.

Data Analysis and Statistics

The single cosinor method was used for analysis of circadian rhythm. The rhythm characteristics estimated by this method include the mesor (middle value of the fitted cosine representing a rhythm-adjusted mean), the amplitude (half the difference between the minimum and maximum of the fitted cosine function), and the acrophase (time of peak value in the fitted cosine function, expressed as the lag in hours and minutes from midnight). A probability value determined from comparison of residuals, before and after cosine curve fitting, of ≤0.05 indicates detection of a rhythm. Rhythm characteristics (mesor, amplitude, and acrophase) for each variable were compared between groups by unpaired Student t test.

Results

Radiotelemetric Measurement of BP and HR in Awake, Freely Moving Rats on a Continuous Basis Continuous monitoring by a radiotelemetry system was used for measurements of BP and HR in awake, free moving rats. Figure 1 shows telemetric data of systolic blood pressure (SBP) collected over a 3-day period. Cosinor analysis revealed that SBP, diastolic blood pressure (DBP), and HR in DS exhibited significant circadian rhythm (P < 0.001) (Table 1). The normal-salt diet fed for 6 weeks resulted in a slight increase in a rhythm-adjusted 24-hour mean (mesor) in SBP and DBP without a significant change in their circadian amplitudes. A high-salt diet caused a marked increase in mesors and circadian amplitudes in SBP and DBP. Circadian amplitudes in SBP and DBP were significantly higher in DS-H than in DS-N (DS-H, 9.8 ± 1.2 vs DS-N, 100.0 ± 2.5).
The kidney weight/body weight was 17% greater in DS-H than in DS-N (4.95 ± 0.00 vs. 4.39 ± 0.00 mg/g, respectively). The kidney weight/body weight was 17% greater in DS-H than in DS-N (4.95 ± 0.00 vs. 4.39 ± 0.00 mg/g, respectively).

No significant difference was detected in body weight between the 2 groups. The ratio of heart weight to body weight was 17% greater in DS-H than in DS-N (3.92 ± 0.29 vs. 3.35 ± 0.29, respectively). The kidney weight/body weight was 17% greater in DS-H than in DS-N (4.95 ± 0.00 vs. 4.39 ± 0.00 mg/g, respectively). The kidney weight/body weight was 17% greater in DS-H than in DS-N (4.95 ± 0.00 vs. 4.39 ± 0.00 mg/g, respectively).

The expression of mRNAs encoding *mPer2*, *Bmal1*, and *dbp* in the heart, kidney, and liver was investigated in DS-H and DS-N by Northern blot analysis. Statistical analysis with the single cosinor method revealed that highly significant (*P* < 0.001) 24-hour variations in the expression of all clock genes examined were observed in DS-H and DS-N (Figure 2). Table 3 shows that the amplitudes of the variations in *mPer2* and *dbp* were significantly lower in DS-H than DS-N in all 3 tissues examined. In the heart and kidney, the amplitude of circadian expression of *Bmal1* of DS-H was significantly lower than that in DS-N. In the liver, the amplitude of circadian expression of *Bmal1* of DS-H was slightly but not significantly lower than that of DS-N. Mesors of *mPer2* (kidney and liver), *Bmal1* (kidney), and *dbp* (heart, kidney, and liver) were also lower in DS-H compared with DS-N. There were no significant differences in acrophases for clock gene expressions between the 2 groups.

### Attenuation of Diurnal Variation of Clock Genes in DS-H

The expression of mRNAs encoding *mPer2*, *Bmal1*, and *dbp* in the heart, kidney, and liver was investigated in DS-H and DS-N by Northern blot analysis. Statistical analysis with the single cosinor method revealed that highly significant (*P* < 0.001) 24-hour variations in the expression of all clock genes examined were observed in DS-H and DS-N (Figure 2). Table 3 shows that the amplitudes of the variations in *mPer2* and *dbp* were significantly lower in DS-H than DS-N in all 3 tissues examined. In the heart and kidney, the amplitude of circadian expression of *Bmal1* of DS-H was significantly lower than that in DS-N. In the liver, the amplitude of circadian expression of *Bmal1* of DS-H was slightly but not significantly lower than that of DS-N. Mesors of *mPer2* (kidney and liver), *Bmal1* (kidney), and *dbp* (heart, kidney, and liver) were also lower in DS-H compared with DS-N. There were no significant differences in acrophases for clock gene expressions between the 2 groups.

### Decreased Amplitude of Variations in PAI-1 Expression in the Heart in DS-H

To investigate the functional consequence of the decreases in amplitude of clock gene circadian expression changes, we examined the expression of PAI-1, a well-characterized clock-regulated gene,8 by Northern blot analysis (heart and kidney) or quantitative RT-PCR (liver) (Figure 3). In the heart, highly significant circadian rhythms in the expression of PAI-1 were observed in both groups (*P* < 0.001). Mesors and acrophases in the expression of PAI-1 were not affected by a high-salt diet. However, the amplitude of PAI-1 expression was significantly lower in DS-H than in DS-N (0.23 ± 0.01 vs. 0.35 ± 0.01, respectively; *P* < 0.01). In the kidney, although no significant rhythmicity in the expression of PAI-1 could be found in DS-N, a significant circadian expression of PAI-1 was observed in DS-H (*P* < 0.01). In the liver, cosinor analysis revealed that there were no significant circadian variations in PAI-1 gene expression in both DS-H and DS-N.

### Discussion

We have recently shown that the aorta and vascular smooth muscle cells possess a circadian oscillation system and that angiotensin II is capable of inducing an oscillation of clock genes in cultured vascular smooth muscle cells.11 However, the circadian regulation of clock genes in peripheral tissues and hence, their physiologic functions, is still unclear. Because several genes that trigger circadian oscillation in tissue-culture cells also affect cardiovascular function (see following paragraphs),11–14 we hypothesized that the cardiovascular abnormality might change the expression of peripheral clock genes. Thus, the aim of the present study was to investigate whether the expression changes of clock genes in peripheral tissues are altered in an animal model of a cardiovascular disorder. We showed that the amplitudes of circadian expressions of clock genes in peripheral tissues are decreased in DS-H. We also found that circadian expression...
of PAI-1, a clock-regulated gene, is impaired in the heart. Although lack of establishment of a cause-effect relation represents a limitation to this study, this is the first to show the exaggerated amplitude of BP in the DS-H with the blunting of amplitude in the circadian expression of peripheral clock genes. In the present model, the decreases in amplitude of circadian gene expressions are relatively modest. Thus, it is possible that the clock components are still “in excess” to perform their cellular functions. Because alterations of peripheral clock genes might be amplified beyond critical thresholds during a prolonged period of uncontrolled hypertension and the subsequent congestive heart failure, long-term studies are required to show the functional consequences of the changes in clock gene expression.

It has been reported that BP failed to fall during the night (nondipper) in patients with sodium-sensitive essential hypertension and that a nondipper response is associated with more serious end-organ damage. Therefore, we initially thought that a DS model would be a useful one in which to investigate whether the loss of a diurnal variation in BP affects the circadian expression of clock genes in peripheral tissues. However, continuous monitoring with a radiotelemetry system revealed that DS-H rats showed higher-amplitude circadian changes in BP than did DS-N, indicating that this rat is not suitable as a salt-sensitive hypertension model for our original purpose. Nevertheless, the present results demonstrated that the circadian expression pattern of peripheral clock genes is not identical to the circadian variation of BP in this model. Whether a compensatory mechanism exists between the amplitudes of clock gene expression and diurnal variation in BP remains to be elucidated.

One important finding of this study is that the amplitudes of circadian changes in expression of peripheral clock genes are decreased in DS-H. In this model, systemic hypertension induces left ventricular hypertrophy and renal hypertrophy with glomerulosclerosis, which is consistent with our

Figure 2. Circadian expression of clock genes in the heart (A), kidney (B), and liver (C) in DS-H (left) and DS-N (right). Levels of mRNA were determined by Northern blot analysis as described in Methods. Autoradiographs from representative Northern blots are shown. Values are expressed as a relative ratio of the heart sample of circadian time (CT) 20 in DS-N. We used 3 rats in each experiment, and these experiments were each performed twice.
observations. Previously, Young et al demonstrated that pressure-overload hypertrophy in the heart impairs the circadian rhythms of clock-controlled genes, including dbp, hex, and reh, without altering the circadian rhythm of clock genes. In addition, in the hypertrophied state of DS-H, several neurohormonal factors are activated, such as angiotensin II and endothelin-1, which were shown to trigger circadian gene expression in tissue-culture cells. It is tempting to speculate that myocardial remodeling and renal remodeling secondary to hypertension-induced hypertrophy in DS-H result in alteration of the expression of clock genes.

However, we think it unlikely that this is the case for the liver, because it is not a target organ for hypertension and we did not detect liver congestion due to congestive heart failure in the current sets of experiments. One possible explanation is that high BP might affect sympathetic and/or parasympathetic nervous activity via an arterial baroreceptor reflex, resulting in the altered expression of peripheral clock genes, because the autonomic nervous system appears to participate in the pathways that connect central and peripheral clocks.

Another possibility is that the attenuated amplitude of clock genes in peripheral organs is due to the altered expression of central clock genes. A high-salt diet and/or high BP might alter the expression of clock genes in the SCN, affecting the input signaling pathways for peripheral oscillators. Quantitative analysis of the expression levels of clock genes in the SCN is necessary to address this issue. Obviously, further studies with additional animal models of hypertension, as well as treatments with antihypertensive agents, will be required to dissect the molecular mechanisms of altered expression of peripheral clock genes.

What are the functional consequences of decreases in the amplitude of circadian expression changes in peripheral clock genes? Because PAI-1 is regulated by clock genes in endothelial cells and the heart, our observation that the amplitude of expression of PAI-1 is decreased in the hearts of DS-H suggests the physiologic relevance of the altered expression of clock genes in the heart. However, it is still unclear whether clock genes regulate the expression of the PAI-1 gene directly or indirectly, for instance, via the renin-angiotensin system. To clarify this point, a conditional knockout study will be required. In contrast, we failed to observe clear circadian expression changes of PAI-1 in the liver, suggesting that the modes of regulation in the expression of clock-

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### Table 3. Circadian Expression of Clock Genes in the Heart, Kidney, and Liver in DS-H and DS-N

<table>
<thead>
<tr>
<th>Organ</th>
<th>Mesor</th>
<th>Amplitude</th>
<th>Acrophase, CT (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS-H</td>
<td>0.99 ± 0.05</td>
<td>0.73 ± 0.1*</td>
<td>13.4 ± 0.3</td>
</tr>
<tr>
<td>mPer2</td>
<td>1.18 ± 0.08</td>
<td>1.05 ± 0.1</td>
<td>14.9 ± 0.6</td>
</tr>
<tr>
<td>Bmal1</td>
<td>0.60 ± 0.08</td>
<td>0.84 ± 0.1</td>
<td>23.6 ± 0.4</td>
</tr>
<tr>
<td>dbp</td>
<td>12.5 ± 0.72</td>
<td>15.27 ± 1.0</td>
<td>9.6 ± 0.4</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS-H</td>
<td>1.83 ± 0.1*</td>
<td>1.40 ± 0.1*</td>
<td>15.2 ± 0.4</td>
</tr>
<tr>
<td>mPer2</td>
<td>5.34 ± 0.24*</td>
<td>4.67 ± 0.3</td>
<td>23.6 ± 1.0</td>
</tr>
<tr>
<td>dbp</td>
<td>210.9 ± 18.45*</td>
<td>212.03 ± 26.1*</td>
<td>11.2 ± 0.4</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS-H</td>
<td>2.74 ± 0.14</td>
<td>1.99 ± 0.2</td>
<td>14.4 ± 0.4</td>
</tr>
<tr>
<td>mPer2</td>
<td>8.54 ± 0.33</td>
<td>7.47 ± 0.5</td>
<td>20.2 ± 0.2</td>
</tr>
<tr>
<td>dbp</td>
<td>235.7 ± 19.25</td>
<td>246.83 ± 27.2</td>
<td>10.5 ± 0.4</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS-H</td>
<td>5.18 ± 0.3*</td>
<td>4.21 ± 0.4</td>
<td>15.9 ± 0.6</td>
</tr>
<tr>
<td>mPer2</td>
<td>4.01 ± 0.31</td>
<td>3.36 ± 0.4</td>
<td>24.0 ± 0.2</td>
</tr>
<tr>
<td>dbp</td>
<td>65.2 ± 10.23*</td>
<td>96.34 ± 14.5*</td>
<td>11.0 ± 0.1</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS-H</td>
<td>8.82 ± 0.83</td>
<td>10.33 ± 1.2</td>
<td>16.2 ± 0.5</td>
</tr>
<tr>
<td>mPer2</td>
<td>4.09 ± 0.27</td>
<td>3.93 ± 0.4</td>
<td>21.9 ± 0.6</td>
</tr>
<tr>
<td>dbp</td>
<td>91.6 ± 14.79</td>
<td>135.24 ± 20.9</td>
<td>11.0 ± 0.2</td>
</tr>
</tbody>
</table>

*P < 0.05 vs DS-N.
regulated genes are tissue specific. This notion is supported by recent DNA microarray data demonstrating that circadian gene expression changes differ between the liver and heart; the distribution of circadian phases in the 2 tissues are markedly different, and very few genes show circadian regulation in both tissues. Therefore, we suggest that PAI-1 is not an appropriate clock-regulated gene to investigate the biologic function of the clock in the liver. In the kidney, a clear circadian rhythm of PAI-1 expression was observed in DS-H, whereas no significant rhythm was detected in DS-N. This suggests that when the rats are exposed to a high-salt diet and/or hypertension, the effects of clock genes on the expression of PAI-1 in the kidney might be stronger than other factors, such as angiotensin II. Taken together, identification and characterization of clock-regulated genes specific to each organ are important in elucidating the physiologic function of peripheral clocks.

Perspectives
It is still unclear how the loss of circadian control contributes to disease status at the organ and systemic levels. Several lines of in vivo evidence indicate that the circadian clock might play a role in growth control. In addition, a recent gene-targeting study demonstrated that mice deficient in the mPer2 gene are cancer prone, suggesting that the mPer2 gene is involved in cell cycle regulation and tumor suppression. Although the biologic significance of the altered expression of clock genes in DS-H is still unknown, our present findings suggest the value of future studies of the possible roles of the circadian clock in cardiovascular pathology, such as cardiac hypertrophy and unregulated proliferation of vascular smooth muscle cells. An understanding of the functional relation between cardiovascular diseases and the expression of clock genes will be fundamental for the development of chronologic therapy of cardiovascular diseases, as well as for circadian biology.

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