Augmented Diurnal Variations of the Cardiac Renin-Angiotensin System in Hypertensive Rats

Yoshiro Naito, Takeshi Tsujino, Yoshio Fujioka, Mitsumasa Ohyanagi, Tadaaki Iwasaki

Abstract—There are several controversies concerning the enhanced gene expression of cardiac renin-angiotensin system components in spontaneously hypertensive rats (SHR) compared with their normotensive control Wistar-Kyoto (WKY) rats. We hypothesized that these discrepancies arise from circadian fluctuations in gene expression. We examined the circadian mRNA expression of renin, angiotensinogen, ACE, and angiotensin type 1a (AT1a) and type 2 (AT2) receptors in the hearts of SHR and WKY rats by real-time quantitative reverse transcription–polymerase chain reaction. The cardiac mRNA expression of the renin-angiotensin system components showed circadian oscillations in both SHR and WKY rats. The amplitudes of these circadian fluctuations were greater in the SHR than in the WKY rats. The mRNA levels of the renin-angiotensin system components were also increased in the SHR compared with the WKY rats at many time points (especially during the dark phase). However, the levels of ACE, AT1a receptor, and AT2 receptor mRNA in the SHR and WKY rats were almost the same during the late light phase. In contrast to mRNA expression, ACE activity was similar both at the time of maximum and minimum mRNA expression. The AT1 receptor antagonist candesartan upregulated AT1a receptor mRNA and downregulated ACE mRNA at specific time points only in the SHR group. Our findings of differential diurnal expression of cardiac renin-angiotensin system genes in SHR and WKY rats appear to explain the discrepancies between prior studies. However, the physiological relevance of the differential circadian mRNA expression of the renin-angiotensin system components remains to be elucidated. (Hypertension. 2002;40:827-833.)

Key Words: circadian rhythm ■ renin-angiotensin system ■ renin ■ angiotensinogen ■ angiotensin-converting enzyme ■ receptors, angiotensin II ■ rats, spontaneously hypertensive

Many studies in the last 3 decades have found that the cardiac renin-angiotensin system (RAS) is independently regulated from the systemic RAS and plays important pathophysiologic roles in cardiac hypertrophy and remodeling.1–3 Many studies have examined the differences in the cardiac RAS in spontaneously hypertensive rats (SHR) and their normotensive counterpart, the Wistar-Kyoto (WKY) rat; these studies have shown some discrepancies in the expression of RAS component genes in SHR and WKY rats4–8 (Table 1). Some data have indicated an increased mRNA expression of angiotensinogen, ACE, and angiotensin type 1a (AT1a) receptors in SHR compared with WKY rats, whereas other studies have found no differences between the two strains.

Many physiological processes are under the control of a biologic clock. In mammals, the central circadian pacemaker is located in the suprachiasmatic nucleus of the brain, whose phase is directly light-entrained by the retina.9 Recent experiments have shown that peripheral tissues also have oscillatory systems that contain a transcriptional-translational feedback loop of clock genes.9–11 The heart also contains fully functional internal clocks, and several cardiac genes exhibit circadian oscillation in their expression.12–14 We hypothesized that differential circadian expression of the RAS in SHR and WKY rats may explain the discrepancies between previous reports.

In the present study, we investigated the circadian mRNA expression of the RAS components in the hearts of SHR and WKY rats. We found augmented diurnal variations in mRNA expression of cardiac RAS in SHR hearts. We also examined the effect of the AT1a receptor antagonist candesartan on the circadian expression of cardiac RAS genes to better understand the regulation to cardiac RAS from angiotensin II.

Methods

Materials

Candesartan (CV-11974) was a gift from Takeda Pharmaceutical Co. Other materials and chemicals were obtained from commercial sources.

Animal Preparation

The experiments were carried out in 72 male SHR and 72 age-matched male WKY rats. We used the descendants of SHR and WKY rats provided by Prof Kozo Okamoto (Kinki University, Osaka-Sayama, Japan).15,16 They were kept in a separate...
The left ventricle was isolated from other parts of the heart, snap-frozen in liquid nitrogen, and stored at −80°C. One animal was killed at each time point on 6 separate days for each group to ensure the reproducibility of the gene expression cycle.

RNA Extraction and Real-Time Quantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from the heart by using TRIzol (Invitrogen) according to the manufacturer’s protocol. The principle of the real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) has been described by Heid et al.17 We performed it by using the TaqMan One-Step RT-PCR Master Mix Reagents Kit with an ABI Prism 7900 HT Detection System (Applied Biosystems) according to the manufacturer’s protocol. Oligonucleotide probes and primers were designed based on the cDNA sequences reported in the GenBank database. The sequences of the probes and primers are listed in Table 2. The same amount of reagents, primers, and probes were used for every reaction.

To obtain a calibration curve, serial dilutions of stock standard RNA (total RNA extracted from the WKY heart at ZT4; 100, 20, 10, 5, and 2.5 ng) were used. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold value above baseline. The target message in the unknown samples is quantified by measuring the Ct and by using a calibration curve to determine the starting target message quantity. The Ct ranged from 25 to 28 in the assays for angiotensinogen, ACE, AT1aR, AT2R, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), whereas the Ct ranged from 31 to 34 for renin. The relative amount of each mRNA was normalized to the housekeeping gene GAPDH mRNA. We confirmed that the level of GAPDH mRNA expression in the heart was similar in the SHR and WKY rats and remained constant throughout the day by Northern analysis (data not shown).

The amplitude of the circadian mRNA oscillations is expressed as the peak-to-trough ratio for mRNA expression (P/T).

ACE Activity

ACE activity in left ventricle was measured as previously described.18

<table>
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<th>TABLE 1. Previous Studies of the Differential Gene Expression of Cardiac RAS in SHR and WKY Rats</th>
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The age of rats at the time of examination is indicated in parentheses. ATNG indicates angiotensinogen; ACE, angiotensin-converting enzyme; AT1aR, angiotensin type 1a receptor; AT2R, angiotensin type 2 receptor.

*SHR>WKY means mRNA expression was greater in SHR than in WKY rats.
†SHR=WKY means mRNA expression was equal in SHR and WKY rats.

<table>
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<th>TABLE 2. Primer and Probe Sequences Used in Real-Time Quantitative RT-PCR Analysis</th>
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GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase.
Statistical Analysis
Values are reported as mean±SEM. Statistical analysis was performed with ANOVA followed by the unpaired Student t test (Statview version 4, Abacus Concepts). Differences were considered significant when the probability value was <0.05.

Results
Effect of Drug Treatment on Blood Pressure
In the control group, the SBP was significantly higher in the SHR compared with the WKY rats at 13 weeks of age (SHR versus WKY; 210.3±8.6 versus 148.4±7.7 mm Hg; P<0.001). In both groups, candesartan (0.5 mg/kg per day) lowered the SBP after 1 week of treatment (SHR versus WKY; 153.2±1.2 versus 113.4±1.7 mm Hg; P<0.001).

Circadian mRNA Expression of RAS in Hearts of SHR and WKY Rats
All components of the cardiac RAS (renin, angiotensinogen, ACE, AT1a receptor, and AT2 receptor) exhibited rhythmic variations in their gene expression. Renin expression peaked at ZT0 and ZT12 and was at a trough value at ZT8 and ZT16 in the WKY rats (Figure 1A). In the SHR group, renin mRNA peaked at ZT0 and was at a trough value at ZT8 and ZT20. The cardiac renin mRNA levels in the SHR hearts were significantly greater than in the WKY rats at ZT4, ZT12, and ZT16, and there was a trend toward increased expression at ZT0, ZT8, and ZT20 (Figure 1A). When the amplitude of oscillation was evaluated based on the P/T ratio, the P/T in the SHR and WKY rats was similar (SHR versus WKY; 2.5 versus 2.1).

Angiotensinogen expression peaked at ZT8 and was at a trough value at ZT4 in the WKY rats (Figure 1B). In the SHR, angiotensinogen mRNA peaked at ZT8 and was at a trough value at ZT0. Cardiac angiotensinogen expression in the SHR group increased throughout the day compared with the WKY rats. When the amplitude of oscillations was evaluated based on the P/T ratio, the P/T in the SHR and WKY rats was similar (SHR versus WKY; 1.7 versus 1.4).

ACE mRNA expression peaked at ZT16 and reached a trough at ZT8 in the WKY rats (Figure 1C). However, in the SHR group, ACE mRNA expression peaked at ZT0 and was at a trough value at ZT8. Furthermore, P/T was greater in
SHR than in WKY rats (SHR versus WKY; 2.4 versus 1.6). The cardiac ACE mRNA levels in the SHR group were significantly greater than in the WKY rats at ZT0, and there was a trend toward increased expression at ZT4, ZT12, ZT16, and ZT20 (Figure 1C). However, the cardiac ACE mRNA levels of the SHR and WKY rats were almost the same at ZT8.

The circadian expression patterns of the cardiac angiotensin AT1a and AT2 receptor mRNA expression were similar to that of ACE. AT1a receptor expression peaked at ZT12 and reached a trough at ZT4 in the WKY rats (Figure 1D). The AT1a receptor mRNA level in the SHR group was significantly greater than in the WKY group, except at ZT8. The P/T for the AT1a receptor mRNA in the SHR group was greater than that in the WKY group (SHR versus WKY; 2.3 versus 1.3). AT2 receptor mRNA expression showed only weak oscillations in the WKY rats (Figure 1E). AT2 receptor expression peaked at both ZT12 and ZT0 and had a trough at ZT8 in the SHR group. The P/T for AT2 receptor expression in the SHR group was greater than in the WKY group (SHR versus WKY; 2.8 versus 1.4). AT2 receptor mRNA expression was significantly greater in the SHR group than in the WKY rats, except at ZT8 and ZT 20.

Effects of an AT1a Receptor Antagonist on Circadian mRNA Expression of RAS in Hearts From SHR and WKY Rats

Two-week treatment with candesartan had little effect on diurnal mRNA expression patterns of cardiac RAS in WKY rats (Figure 2). The changes in cardiac mRNA expression of renin and angiotensinogen by candesartan treatment also did not reach statistical significance in SHR (Figures 3A and 3B). In contrast, chronic candesartan treatment downregulated ACE mRNA and upregulated AT1a receptor mRNA in the SHR group at specific time points (Figures 3C and 3D). ACE mRNA expression in the candesartan-treated SHR group was significantly lower than in control SHR at ZT0, and there was a trend toward decreased expression at ZT4, ZT12, and ZT16 (Figure 3C). ACE mRNA levels in the control group and the candesartan-treated group were almost the same at ZT8 and ZT20 for SHR. AT1a receptor mRNA expression in candesartan-treated SHR was significantly greater than in
control SHR at ZT16, and a trend toward increased expression at ZT4, ZT8, ZT12, and ZT20 (Figure 3D) was seen. AT1a receptor mRNA levels in the control group and the candesartan-treated group were similar at ZT0 in SHR. The differences in cardiac mRNA expression of AT2 receptor between control and candesartan-treated SHR did not reach statistical significance except at ZT12 (Figure 3E).

**ACE Activity in Hearts of SHR and WKY Rats**

To determine whether protein expression tracked mRNA expression, we measured ACE activity in hearts of SHR and WKY rats at ZT0 (time of peak mRNA expression) and at ZT8 (time of trough mRNA expression). Despite differences in mRNA expression, ACE activity was almost the same at ZT0 and ZT8 in both strains (Table 3). Moreover, ACE activity was greater in WKY hearts than in SHR, even though the mRNA expression was greater in SHR.

**Discussion**

The diurnal variations in plasma renin activity, angiotensin II, and the pressor response to angiotensin II infusion have been reported in humans. We demonstrated, for the first time, circadian variations in the expression of the RAS components and observed marked differences in these variations between SHR and WKY hearts. The expression of ACE, AT1a receptor, and AT2 receptor are modestly upregulated during the dark (active) phase in WKY rats. In contrast to WKY rats, these genes are dramatically upregulated during the dark phase and early light phase in SHR, but they are downregulated to the same levels as in WKY rats at ZT8. Our finding of differential circadian expression patterns of the cardiac RAS in SHR and WKY rats appears to explain some of the discrepancies between previously published observations (Table 1).

**TABLE 3. ACE Activity at ZT0 and ZT8 in SHR and WKY Rats**

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR</th>
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<tbody>
<tr>
<td>ZT0</td>
<td>1.7±0.1</td>
<td>0.9±0.1*</td>
</tr>
<tr>
<td>ZT8</td>
<td>1.6±0.1</td>
<td>0.9±0.1*</td>
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Values reported as nmol/mg wet wt/hr. Data represent the mean±SEM for 6 independent experiments in SHR and WKY rats. ZT indicates Zeitgeber time.

*P<0.05 vs WKY.
To examine the regulation of cardiac RAS by angiotensin II and to elucidate the mechanisms responsible for differential circadian variations in the cardiac RAS between SHR and WKY rats, we investigated the effect of an AT1a receptor antagonist on the circadian expression of cardiac RAS. Interestingly, candesartan treatment downregulated ACE mRNA in SHR at specific time points and almost equalized its circadian expression pattern to that of WKY. This finding suggests that the presence of an abnormality in the regulation by angiotensin II in SHR may account for the enhanced expression of ACE mRNA. Because aldosterone induces ACE gene expression in cultured neonatal rat cardiocytes,22 angiotensin II may upregulate ACE by increasing aldosterone production in SHR. However, because candesartan treatment failed to normalize the circadian expression of other components of the RAS in SHR to that of WKY rats, the increased effect of angiotensin II on gene expression could not solely explain the differences between SHR and WKY rats. The mechanisms responsible for different circadian variations of cardiac RAS expression between SHR and WKY rats remain largely unknown.

Candesartan upregulated cardiac AT1a receptor expression at specific time points in SHR, whereas it had no effect in WKY rats. Regulations of AT1a receptor mRNA expression by angiotensin II varies with the organ studied and the animal model used.23 The level of cardiac AT1a receptor mRNA in canbesartan-treated SHR was the same as in the control group at ZT0, whereas it was significantly greater than in the control SHR at ZT16. This finding is partially consistent with a previous study that showed that the ACE inhibitor delapril increases AT1a receptor mRNA in SHR hearts.24 Our finding of differential effects of drug treatment at different time points underscores the importance of specifying the time of evaluation in in vivo experiments.

Alterations in the circadian gene expression of the cardiac RAS may participate in the development of hypertension. However, circadian fluctuations in the mRNA level do not necessarily correspond to circadian oscillations in the protein level. In fact, ACE activity was almost the same at the time of peak and trough mRNA expression. However, the effect of an AT1 receptor antagonist on the cardiac gene expression of ACE and the AT1a receptor varied temporally in SHR. This finding suggests that the effect of angiotensin II on cardiac gene expression fluctuates diurnally, even though it is not known whether the variation is caused by the cardiac or circulating RAS. However, the physiological relevance of the differential circadian mRNA expression of the RAS components will not be known until the circadian protein expression of the components of the cardiac RAS is determined. Moreover, our findings should be applied carefully to the understanding and treatment of human hypertension because rats are nocturnal while humans are diurnal.

ACE activity was greater in WKY hearts than in SHR hearts, which is consistent with previous studies.25,26 We showed, for the first time, simultaneous increased mRNA but decreased enzymatic activity for ACE in SHR hearts. Further studies are necessary to elucidate the responsible mechanisms. In conclusion, diurnal variations of the cardiac RAS are augmented in SHR compared with WKY rats. The gene expression of the cardiac RAS is increased in SHR at specific time points. Regulation of cardiac ACE and AT1a receptor mRNA by angiotensin II was observed only in SHR. Discrepancies between former studies with respect to cardiac RAS expression in SHR and WKY rats could be largely explained by this phenomenon. However, the physiological relevance of the differential circadian mRNA expression of the RAS components remains to be elucidated.

**Perspectives**

If there are abnormal diurnal variations in RAS function in the hypertensive heart, effective dosing of ACE inhibitors or AT1 receptor antagonists at specific time points may provide better treatment for cardiac hypertrophy and heart failure.

**References**

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