Angiotensin II Induces Circadian Gene Expression of Clock Genes in Cultured Vascular Smooth Muscle Cells

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Background—Daily rhythms of mammalian physiology and endocrinology are regulated by circadian pacemakers. The master circadian pacemaker resides in the suprachiasmatic nucleus, which is located in the hypothalamus of the brain, but circadian oscillators also exist in peripheral tissues. Because many studies have demonstrated apparent circadian variations in the frequency of cardiovascular disorders, it is of great interest to investigate a possible relation between circadian gene expression and cardiovascular function. We examined whether a circadian oscillation system exists in the aorta and/or in cultured vascular smooth muscle cells (VSMCs).

Methods and Results—The mRNA levels of clock genes were assayed by northern blot analysis. The mouse aorta showed a clear circadian oscillation in the expression of mPer2, dbp, and Bmal1. Brief treatment of VSMCs with angiotensin II induced a robust increase in mPer2 gene expression, followed by a marked reduction in mPer2 mRNA levels and subsequent synchronous cycling of mPer2, dbp, and Bmal1 mRNAs. The induction of mPer2 in VSMCs by angiotensin II was completely abolished by treatment with CV11947, a specific angiotensin II type1 receptor antagonist.

Conclusions—The present results demonstrate that the aorta and VSMCs possess a circadian oscillation system which is comparable to that of the suprachiasmatic nucleus and that the circadian gene expression in VSMCs is induced by angiotensin II through the angiotensin II type1 receptor. Our in vitro system will provide a useful tool to further analyze the physiological significance of the peripheral clock in cardiovascular function. (Circulation. 2001;104:1746-1748.)

Key Words: angiotensin ■ circadian rhythm ■ molecular biology ■ muscle, smooth

In mammals, behavioral and physiological processes display ≈24-hour rhythms that are regulated by circadian pacemakers. On the basis of surgical ablation and transplantation experiments, the central circadian pacemaker is thought to reside in the hypothalamic suprachiasmatic nucleus. However, several lines of evidence indicate that peripheral tissues and immortalized cells also contain circadian oscillators; the molecular mechanisms of these oscillators are virtually identical to those in the suprachiasmatic nucleus. The molecular mechanism of this circadian oscillator is based on interacting transcriptional-translational autoregulatory feedback loops. The feedback loop involves 3 homologs of the Drosophila gene period (mPer1, mPer2, and mPer3) and 2 cryptochrome genes (mCry1 and mCry2). The rhythmic transcription of the mPer and mCry genes is driven by the transcription activator genes Clock and Bmal1.

Various cardiovascular functions, including blood pressure, heart rate, and coagulation parameters, are known to show a diurnal variation. In addition, several cardiovascular events, such as myocardial infarction, sudden cardiac death, and stroke, show well-defined patterns in their occurrence throughout the day. Although such diurnal variations are widely known, the underlying molecular mechanisms have not yet been clarified. Research into the molecular mechanisms for diurnal variations of cardiovascular regulation may lead to a better understanding of the pathogenesis of cardiovascular disorders and, hence, the emergence of novel therapeutic strategies for these diseases.

Our long-term goal is to elucidate the molecular mechanisms underlying the association between diurnal variations of cardiovascular function and biological clocks. In the present study, we initially investigated whether the aorta in vivo and vascular smooth muscle cells (VSMCs) in vitro possess circadian oscillators. As a trigger of the oscillators for VSMCs, we used angiotensin II, a key molecule for cardiovascular regulation, because it has been shown to activate the mitogen-activated protein kinase (MAPK) cascade. We demonstrated that both the aorta and VSMCs possess circadian oscillator systems and that angiotensin II induces the circadian expression of clock genes in VSMCs.
Methods

Materials
Angiotensin II was purchased from the Peptide Institute, Inc. PD123,319 was purchased from Sigma. CV11974 was a gift from Takeda Pharmaceutical Co, Ltd, Osaka, Japan. Other materials and chemicals used were obtained commercially.

Animals
Male BALB/c mice (Japan Animal Company, Osaka, Japan) purchased 5 weeks postpartum were exposed to 2 weeks of complete light (fluorescent light, 300lux)/dark cycles and then kept in complete darkness for 2 days as a continuation of the dark phase of the last cycle. The expression profiles of clock gene mRNA were examined in the second dark-dark cycle every 4 hours, starting at the beginning of the light cycle. CT indicates circadian time. The care and use of the animals strictly followed the guidelines of the Animal Research Committee of Kobe University Graduate School of Medicine.

Cell Culture
VSMCs were isolated from the rat thoracic aorta by enzymatic dissociation, as described previously. Before stimulation, we incubated the cells with 5% DMEM for 72 hours. The cells were stimulated by brief treatment with a medium containing angiotensin II, after which medium was replaced by serum-free DMEM.

Northern Blot Analysis
Northern blot analysis was performed as described previously. Probes of mPer1, mPer2, dbp, and Bmal1 were prepared as previously described.

Results
First, we examined whether a circadian oscillation system exists in the aorta in vivo. We killed mice at the indicated times, and total RNA extracted from the descending aortas was subjected to northern blot analysis. As shown in Figure 1, the expression of mPer2, one of the important oscillators, has a peak at CT12 and a trough at CT0. The circadian rhythm of dbp, a clock-controlled gene that is directly regulated by circadian feedback loops and, therefore, is a most suitable marker to evaluate the circadian oscillators, is very similar to that of mPer2, with peak at CT8/CT12 and a trough at CT20/24. Bmal1 has an antiphase rhythm, with peaks at CT0 and a trough at CT8. These observations indicate that the aorta has a circadian oscillation system.

We then investigated whether VSMCs, which represent the most abundant cell type in vessel walls, possess circadian oscillators. Brief treatment (2 hours) of VSMCs with angiotensin II (final concentration, 100 nmol/L) resulted in the rhythmic expression of mPer2, dbp, and Bmal1 for at least 3 circadian cycles, but the control GAPDH gene did not show a circadian rhythm (Figure 2A). After an initial short-term increase, mPer2 mRNA was repressed and began to show a rhythm lasting ∼24 hours, which consisted of peaks after 24 to 28 hours and 48 hours and troughs at 12 hours, 36 to 40 hours, and 60 hours. Robust cycling of Bmal1 mRNA was observed, with mRNA levels accumulating antiphase to mPer2 and dbp mRNA cycles.

Levels of dbp mRNA decreased until 8 to 12 hours, then peaked at 16 to 20 hours and 44 to 48 hours, with a trough at 32 hours and 56 hours. The peaks and troughs of RNA
accumulation were synchronous, which is consistent with the findings observed in rat fibroblasts treated with serum.2

Finally, we examined the initial state of mPer2 mRNAs in VSMCs after treatment with angiotensin II. In the initial step of circadian gene expression, a marked transient induction of mPer2 was observed in VSMCs stimulated with angiotensin II (Figure 2B). This effect was completely abolished by 1 hour of preincubation with CV11974 (100 nmol/L), a highly specific and selective angiotensin II type 1 receptor antagonist. In contrast, 1 hour of preincubation with PD123,319, a specific angiotensin II type 2 receptor antagonist, had no effect on the induction of mPer2 mRNA on VSMCs. These observations indicate that angiotensin II induces circadian gene expression via the angiotensin II type 1 receptor.

Discussion
In the present study, we demonstrated that molecular oscillators exist in VSMCs and that angiotensin II, a multifunctional molecule regulating cardiovascular function,9 induces the rhythmic expression of clock genes in VSMCs. Our observations that the aorta shows circadian expressions of clock genes further support the physiological relevancy of our in vitro system. The present study is the first to demonstrate that angiotensin II type 1 receptor activation triggers circadian gene expression in VSMCs. VSMCs are responsible for the structural and functional characteristics of vessel walls, including contraction/relaxation, growth, development, remodeling, and repair.15 In addition, VSMCs are involved in the pathogenesis of a variety of cardiovascular diseases, such as atherosclerosis, restenosis, and hypertension. Our present findings should have a major impact on future studies concerning the possible roles of peripheral clocks in the physiology and the pathophysiology of the cardiovascular system.

The circadian system is organized in a hierarchical fashion: the central pacemaker is located in the suprachiasmatic nucleus, whose phase is directly light-entrained by the optic nerves,16 and this is thought to synchronize or direct circadian gene expression in peripheral cell types by neuronal and/or hormonal factors. However, recent studies revealed that restricted feeding or the administration of dexamethasone, a glucocorticoid hormone analogue, changes the phase of circadian gene expression in peripheral tissues without affecting the phase of cyclic gene expression in the suprachiasmatic nucleus.3,4 Thus, the peripheral tissues without affecting the phase of cyclic gene expression in its target organs, such as the aorta, the kidney, and the heart. Alternations of the expressions of clock genes in VSMCs by angiotensin II could be a response to the changes in environment. Alternatively, these changes may lead to functional abnormalities in the underlying vascular pathological processes of cardiovascular disorders.

Whether the circadian oscillators in VSMCs are important for the function of VSMCs is still unknown. To further elucidate the physiological function of circadian oscillators in VSMCs, it is important to find the target genes whose expressions are under the control of the clock genes or clock-controlled genes. A differential hybridization screening using our in vitro system may provide a feasible strategy to identify molecularly such clock-controlled genes in VSMCs. In addition, an analysis of VSMCs isolated from mice carrying targeted mutations in mCry genes that are known to abolish the biological clock in the peripheral tissues5 should be another plausible strategy to further investigate the physiological relevancy of the biological clocks in VSMCs.

Note Added in Proof
During the review process for this manuscript, McNamara et al proposed a molecular mechanism for the hormonal control of clock gene expression in the vasculature (McNamara P, Seo S, Rudic RD, et al. Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock. Cell. 2001;105:877–889).

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