

Role of Angiotensin and the Clock System in the Circadian Regulation of Plasminogen Activator Inhibitor-1

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Received 17 November 2008/ Accepted 12 December 2008

Key Words: plasminogen activator inhibitor-1, clock, renin-angiotensin system, Cry, angiotensin type 1 receptor

The clock system and the renin-angiotensin system (RAS) have been reported to play an important role in the circadian expression of plasminogen activator inhibitor-1 (PAI-1) gene. However, the degree of involvement of these systems remains unknown. In the present study, we investigated the roles of the clock system and the RAS in the circadian expression of PAI-1 in the peripheral tissues *in vivo* by using *Cry1/Cry2* double knockout (*Cry1/2*-deficient) and angiotensin type 1 (AT1a) receptor knockout (AT1a-deficient) mice. We observed no significant rhythmicity in the PAI-1 expression in all tissues examined in *Cry1/2*-deficient mice; this suggests that the clock system is indispensable for the circadian expression of PAI-1. In AT1a-deficient mice, apparent circadian oscillation of PAI-1 expression was observed in the lung and liver but not in the kidney, suggesting that AT1a-mediated signaling modulates the circadian expression of PAI-1 in a tissue-specific manner.

The clinical incidence of several cardiovascular events, such as myocardial infarction, and stroke has been reported to exhibit circadian variations, with the highest frequency in the morning (4, 11, 26). Although the mechanisms underlying this variation remain unclear, considerable evidence indicates a role of the endogenous fibrinolytic system: circadian variations in the levels of plasminogen activator inhibitor-1 (PAI-1)—a key regulator of fibrinolysis—have been recognized (2, 18).

In mammals, circadian rhythms are generated by transcriptional-translational feedback loops orchestrated by a set of clock genes (1, 6, 7). The heterodimer of CLOCK and brain and muscle arnt-like protein (BMAL) binds to the E-box sites upstream of period and cryptochrome genes and transactivates these genes. The transactivation of the PER and CRY

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proteins by the CLOCK:BMAL heterodimer is inhibited by the proteins themselves, resulting in the formation of a negative feedback loop. The master oscillator is located in the suprachiasmatic nucleus, and autonomous peripheral oscillators have been defined for most tissues, including the heart, kidney, and liver.

The results of studies performed *in vitro* and in animal models suggest that the circadian clock system may be involved in the regulation of PAI-1 expression. Maemura et al. showed that CLOCK forms a heterodimer with BMAL2 and upregulates the expression of the human PAI-1 gene through E-box elements *in vitro* (9). They also demonstrated the circadian expression of PAI-1 mRNA in the heart and kidneys of mice, which suggests that PAI-1 is controlled by clock genes. Further, data from recent experimental and clinical studies have suggested that the renin-angiotensin system (RAS) plays an important role in the regulation of PAI-1 expression. Angiotensin II (Ang II) causes a dose-dependent increase in the expression of PAI-1 in vascular smooth muscle cells, endothelial cells, and cardiomyocytes (5, 23, 24). In humans, activation of the RAS by either sodium depletion or diuretic use is associated with increased plasma PAI-1 antigen concentrations in the morning, whereas angiotensin type 1 (AT1) receptor antagonism and angiotensin-converting enzyme (ACE) inhibition decrease PAI-1 expression in several animal models and in humans (3, 17, 19).

Previously, we have demonstrated that the circadian expression of PAI-1 mRNA is regulated differently among tissues and suggested that both the circadian system and RAS are involved in the circadian regulation of PAI-1 expression (14). However, the extent of involvement of these systems in the circadian expression of PAI-1 mRNA remains unknown. In the present study, we investigated the circadian expression of the PAI-1 gene in genetically modified mice with either impaired circadian rhythm (8) or AT1a-mediated signaling (21).

MATERIALS AND METHODS

Animals

The experiments were carried out on *Cry1/Cry2* double knockout (*Cry1/2*-deficient) (8), AT1a-deficient (21), and C57/BL6j Jcl mice. Male mice (at 6 weeks postpartum) were exposed to complete light (fluorescent light, 300 lux)-dark (LD) cycles for 2 weeks and then exposed to complete darkness for 2 days as a continuation of the dark phase of the last LD cycle. In the second dark-dark (DD) cycle, the animals were sacrificed every 4 h starting at circadian time (CT) 0 after inducing anesthesia with ether and pentobarbital sodium. 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 Reverse Transcriptase-Polymerase Chain Reaction

RNA was extracted from the heart, lung, kidney, and liver as previously described (10). Total RNA (10 µg) was separated on a formaldehyde/1.1% agarose gel, transferred to a nylon membrane, and hybridized using random-primed ³²P-labeled probes. Probes of *mPer2*, *dbp*, *Bmal1*, and PAI-1 were prepared as previously described (10). The membrane was washed and exposed to an imaging plate, and the fragments were visualized using a BAS 2000 Bio-imaging analyzer (FUJIX, Japan). The signals were normalized to those obtained for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. RT-PCR was performed as previously described (10). The primers 5'-GGGACACCCTCAGCATGTTC-3' and 5'-TCTGATGAGTTCAGCATCCAAGA-3' were used for PAI-1 amplification. The

hybridization probe used was 5'-AGAAAGATGTGCACCTCTCCGCCCTC-3'. PCRs were performed using the TaqMan PCR reagent kit (Applied Biosystems), according to the manufacturer's instructions.

Measurement of Total Plasma PAI-1 Levels

Total plasma PAI-1 levels were measured using Total Murine PAI-1 antigen assay (Molecular Innovations, Inc.), according to the manufacturer's instructions.

Statistical Analysis

The single cosinor method was used for the analysis of circadian rhythm. The rhythm characteristics estimated by this method include the acrophase (time of peak value in the fitted cosine function, expressed as the lag from midnight in hours and minutes), the mesor (middle value of the fitted cosine representing a rhythm-adjusted mean), and the amplitude (half the difference between the minimum and maximum of the fitted cosine function). A p value ≤ 0.01 , as determined from a comparison of residuals before and after cosine curve fitting, indicated the presence of a rhythm.

RESULTS

Expression of Clock Genes

To investigate whether AT1a-mediated signaling influences the expression of clock genes, the expression of mRNAs that encoded the mPer2, Bmal1, and dbp proteins in the heart, kidney, lung, and liver was assessed in wild-type mice, *Cry1/2*-deficient mice, and AT1a-deficient mice by using northern blot analysis (Figure 1). Statistical analysis using the single cosinor method revealed highly significant ($p < 0.01$) 24-h variations in the expression of all clock genes in wild-type and AT1a-deficient mice. In contrast, circadian oscillations in the expression of all clock genes disappeared in *Cry1/2*-deficient mice; this finding is consistent with that of a previous report (10). These results demonstrate that the AT1a receptor pathway did not mediate the circadian variations observed in clock gene expression in the peripheral tissues examined.

Expression of PAI-1 mRNA

We next examined the circadian expression of PAI-1 mRNA in the heart, kidney, lung, and liver in wild-type mice, *Cry1/2*-deficient mice, and AT1a-deficient mice by using northern blot analysis (for the heart, lung, and kidney) or quantitative RT-PCR (for the liver) (Figure 2). In the lung and liver, PAI-1 mRNA expression showed a significant daily rhythm in wild-type mice and AT1a-deficient mice but not in *Cry1/2*-deficient mice. In the kidney, PAI-1 mRNA expression showed a significant circadian rhythm in wild-type mice, whereas no significant rhythmicity was observed in AT1a-deficient mice and *Cry1/2*-deficient mice. In the heart, no significant circadian variation was noted in PAI-1 gene expression in wild-type, AT1a-deficient mice, and *Cry1/2*-deficient mice.

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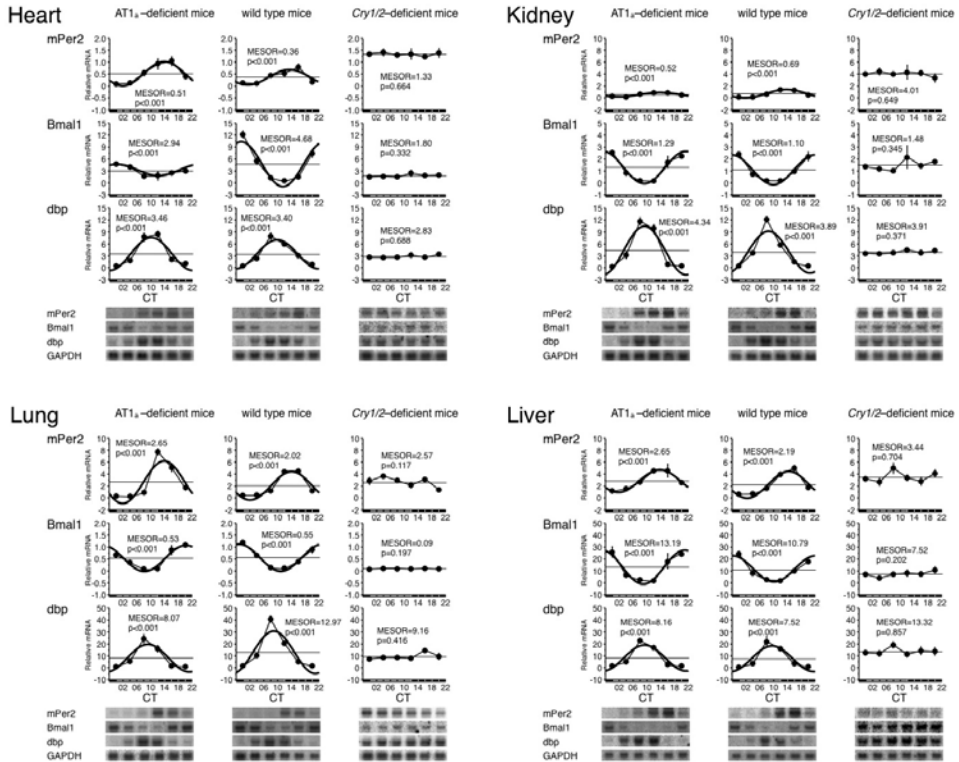
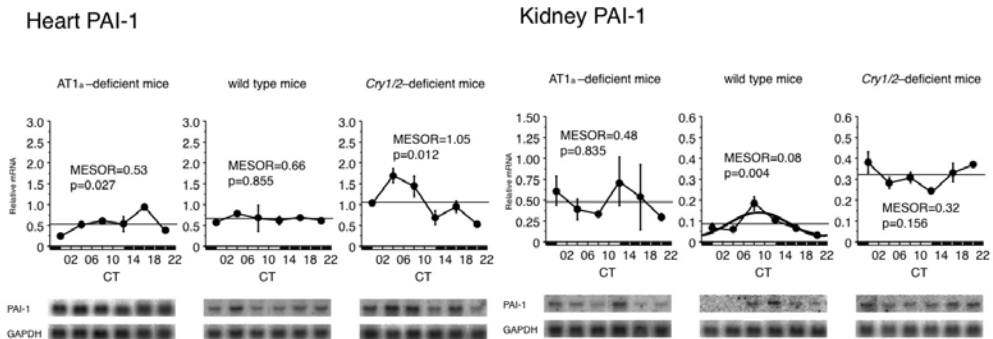


Figure 1 Circadian expression of clock genes in the heart, kidney, lung, and liver of C57/BL6j mice, AT1 α -deficient mice, and *Cry1/2*-deficient mice. mRNA levels were determined by northern blot analysis as described in the materials and methods section. Representative autographs are shown. Values are expressed as the relative ratio of the control samples. CT indicates circadian time. Three to six mice were used in each experiment.



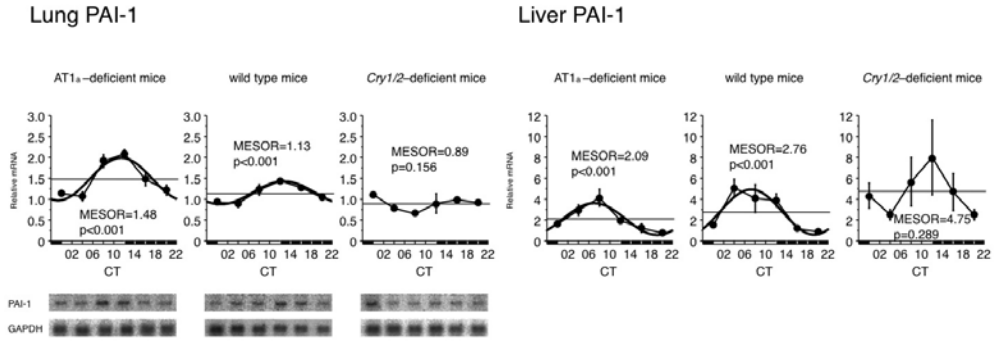


Figure 2 PAI-1 gene expression in the heart, kidney, lung, and liver of C57/BL6j mice, AT1a-deficient mice, and *Cry1/2*-deficient mice. mRNA levels were determined by northern blot analysis (heart, kidney, and lung) or by real-time quantitative RT-PCR (liver) as described in the materials and methods section. Representative autographs are shown. CT indicates circadian time. Three mice were used in each experiment.

Total Plasma PAI-1 Antigen

To determine the role of the RAS and clock system in the circadian variation of PAI-1 levels in circulating plasma, changes in the total plasma PAI-1 levels were examined. Apparent circadian changes in plasma PAI-1 levels were observed in wild-type mice. PAI-1 levels in AT1a-deficient mice and *Cry1/2*-deficient mice exhibited no circadian oscillation. In addition, average concentration of PAI-1 in *Cry1/2*-deficient mice was higher than that in wild-type mice and AT1a-deficient mice.

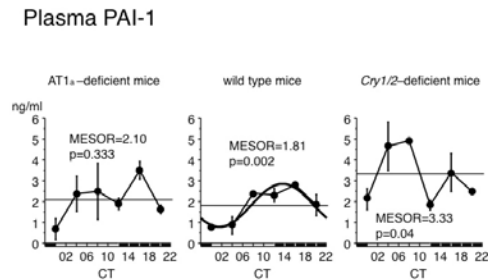


Figure 3 Total plasma PAI-1 levels in C57/BL6j mice, AT1a-deficient mice, and *Cry1/2*-deficient mice. Total plasma PAI-1 levels were measured using Total Murine PAI-1 antigen assay. CT indicates circadian time. Three mice were used in each experiment.

DISCUSSION

In the present study, we examined the roles of the RAS and clock system in the circadian expression of PAI-1 in peripheral tissues by using wild-type and genetically modified mice. We observed no significant rhythmicity in PAI-1 expression in all the tissues examined in the *Cry1/2*-deficient mice; this suggests that the clock system is indispensable for the circadian expression of PAI-1. In AT1a-deficient mice, apparent circadian oscillation of PAI-1 expression was observed in the lung and liver but not in the kidney, suggesting that AT1a-mediated signaling alters the circadian expression of PAI-1 in a tissue-specific manner.

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Considering the fact that PAI-1 performs a variety of biological functions in addition to the regulation of the fibrinolytic cascade, it is important to elucidate the mechanism of PAI-1 regulation in each organ.

Maemura et al. demonstrated that the CLOCK:BMAL2 (cycle-like factor, CLIF) heterodimer is capable of inducing PAI-1 gene expression by binding the E-box element in the PAI-1 promoter (9). They also observed the apparent diurnal variations in the PAI-1 mRNA expression in the heart and kidneys in mice; this finding suggests that the circadian expression of PAI-1 gene mRNA is regulated by the clock system. Schoenhard et al. reported that BMAL1 functions as a direct transcriptional regulator of PAI-1 expression, and this shows that CLOCK:BMAL1 and CLOCK:BMAL2 additively contribute to PAI-1 gene transcription *in vitro* (20). In the present study, we assessed mRNA expression in different organs, including the heart, lung, kidney, and liver, as well as the plasma PAI-1 level, and demonstrated that PAI-1 expression in different tissues is closely controlled by clock genes *in vivo*.

Another important aspect of our findings is that the average expression level of PAI-1 mRNA is greater in the heart, kidney, and liver, and is accompanied by an increase in the plasma PAI-1 level. This suggests that the loss of clock genes may result in increased PAI-1 expression, which could be a contributing risk factor for cardiovascular disease. However, from the data presented here, we cannot exclude the possibility that Cry, a strong negative regulator of clock genes, suppresses PAI-1 mRNA expression independent of its clock function. In fact, Oishi et al. reported that circadian expression of PAI-1 mRNA was low in Clock mutant mice (16). Investigations using other models such as Bmal1-deficient mice models will be useful to elucidate the detailed mechanisms of this process (15, 25). Nevertheless, modulation of clock gene expression may be a novel therapeutic option for the regulation of PAI-1 expression.

Previous studies suggest that the RAS may play an important role in the regulation of plasma and tissue PAI-1 expression (5, 23, 24). *In vitro* studies have demonstrated that Ang II is a potent stimulator of PAI-1 in cultured endothelial cells and smooth muscle cells. ACE inhibitors and AT1 receptor antagonists have been shown to reduce plasma PAI-1 levels *in vivo*; this supports the physiological importance of the RAS in the modulation of PAI-1 levels (3, 17, 19). While these studies provide evidence of the important role played by the RAS in PAI-1 expression, the role of the AT1 receptor in the regulation of PAI-1 expression remains controversial (12, 13, 22). In the present study, we have demonstrated that the AT1a receptor is important for the circadian expression of PAI-1 in the kidney and for plasma PAI-1 levels, but not in the liver and lungs in mice.

In the kidney, circadian expression of the clock genes, mPer2, Bmal1, and dbp, in AT1a-deficient mice is indistinguishable from that in wild-type mice. These results suggest that endogenous Ang II-AT1a signaling does not affect the circadian expression of the clock genes in the kidney. In contrast, circadian expression of PAI-1 in the kidney of AT1a-deficient mice was inhibited. These results indicate that both clock genes and AT1-mediated signaling are required to maintain the circadian expression of PAI-1 in the kidney.

In the present study, we did not observe the circadian expression of PAI-1 mRNA in the heart of wild-type mice and in the kidney of AT1a-deficient mice, which is not consistent with the previous reports (9, 15, 22). We feel these discrepancies may have arisen due to the methods of sample preparation. We kept the mice in the complete darkness for two days before sacrifice to exclude the effects induced by the light (CT: Circadian time) whereas others exposed the mice in LD cycles (ZT: Zeitgeber time). Since light is known to induce

circadian rhythm independent of the biological clock located in the suprachiasmatic nucleus, as a masking effect, we believe evaluation with CT is more appropriate to evaluate the function of endogenous biological clock.

In conclusion, this report supports the view that the circadian clock plays an important role in the diurnal fluctuation of PAI-1 expression, and that the clock genes and the RAS may differentially affect on the circadian expression of PAI-1 in various tissues.

ACKNOWLEDGMENTS

This study was supported in part by Grant-in-Aid for Scientific Research (C) to N.E. and Grant-in-Aid for Scientific Research (C) to H.N. from Japan Society for the Promotion of Science (JSPS).

REFERENCES

1. **Bell-Pedersen, D., Cassone, V.M., Earnest, D.J., Golden, S.S., Hardin, P.E., Thomas, T.L., Zoran, M.J.** 2005. Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nat Rev Genet.* **6**:544-556.
2. **Bremner, W.F., Sothorn, R.B., Kanabrocki, E.L., Ryan, M., McCormick, J.B., Dawson, S., Connors, E.S., Rothschild, R., Third, J.L., Vahed, S., Nemchausky, B.M., Shirazi, P., Olwin, J.H.** 2000. Relation between circadian patterns in levels of circulating lipoprotein(a), fibrinogen, platelets, and related lipid variables in men. *Am Heart J.* **139**:164-173.
3. **Brown, N.J., Agirbasli, M.A., Williams, G.H., Litchfield, W.R., Vaughan, D.E.** 1998. Effect of activation and inhibition of the renin-angiotensin system on plasma PAI-1. *Hypertension.* **32**:965-971.
4. **Casetta, I., Granieri, E., Portaluppi, F., Manfredini, R.** 2002. Circadian variability in hemorrhagic stroke. *JAMA.* **287**:1266-1267.
5. **Feener, E.P., Northrup, J.M., Aiello, L.P., King, G.L.** 1995. Angiotensin II induces plasminogen activator inhibitor-1 and -2 expression in vascular endothelial and smooth muscle cells. *J Clin Invest.* **95**:1353-1362.
6. **Galleo, M., Virshup, D.M.** 2007. Post-translational modifications regulate the ticking of the circadian clock. *Nat Rev Mol Cell Biol.* **8**:139-148.
7. **Levi, F., Schibler, U.** 2007. Circadian rhythms: mechanisms and therapeutic implications. *Annu Rev Pharmacol Toxicol.* **47**:593-628.
8. **Maeda, A., Tsujiya, S., Higashide, T., Toida, K., Todo, T., Ueyama, T., Okamura, H., Sugiyama, K.** 2006. Circadian intraocular pressure rhythm is generated by clock genes. *Invest Ophthalmol Vis Sci.* **47**:4050-4052.
9. **Maemura, K., de la Monte, S.M., Chin, M.T., Layne, M.D., Hsieh, C.M., Yet, S.F., Perrella, M.A., Lee, M.E.** 2000. CLIF, a novel cycle-like factor, regulates the circadian oscillation of plasminogen activator inhibitor-1 gene expression. *J Biol Chem.* **275**:36847-36851.
10. **Mohri, T., Emoto, N., Nonaka, H., Fukuya, H., Yagita, K., Okamura, H., Yokoyama, M.** 2003. Alterations of circadian expressions of clock genes in Dahl salt-sensitive rats fed a high-salt diet. *Hypertension.* **42**:189-194.
11. **Muller, J.E., Toffler, G.H.** 1991. Circadian variation and cardiovascular disease. *N Engl J Med.* **325**:1038-1039.
12. **Naito, Y., Tsujino, T., Fujioka, Y., Ohyanagi, M., Iwasaki, T.** 2002. Augmented diurnal variations of the cardiac renin-angiotensin system in hypertensive rats.

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- Hypertension. **40**:827-833.
13. **Naito, Y., Tsujino, T., Kawasaki, D., Okumura, T., Morimoto, S., Masai, M., Sakoda, T., Fujioka, Y., Ohyanagi, M., Iwasaki, T.** 2003. Circadian gene expression of clock genes and plasminogen activator inhibitor-1 in heart and aorta of spontaneously hypertensive and Wistar-Kyoto rats. *J Hypertens.* **21**:1107-1115.
 14. **Nonaka, H., Emoto, N., Ikeda, K., Fukuya, H., Rohman, M.S., Raharjo, S.B., Yagita, K., Okamura, H., Yokoyama, M.** 2001. Angiotensin II induces circadian gene expression of clock genes in cultured vascular smooth muscle cells. *Circulation.* **104**:1746-1748.
 15. **Ohkura, N., Oishi, K., Fukushima, N., Kasamatsu, M., Atsumi, G.I., Ishida, N., Horie, S., Matsuda, J.** 2006. Circadian clock molecules CLOCK and CRYs modulate fibrinolytic activity by regulating the PAI-1 gene expression. *J Thromb Haemost.* **4**:2478-2485.
 16. **Oishi, K., Ohkura, N., Amagai, N., Ishida, N.** 2005. Involvement of circadian clock gene Clock in diabetes-induced circadian augmentation of plasminogen activator inhibitor-1 (PAI-1) expression in the mouse heart. *FEBS Lett.* **579**:3555-3559.
 17. **Oshima, S., Ogawa, H., Mizuno, Y., Yamashita, S., Noda, K., Saito, T., Sumida, H., Suefuji, H., Kaikita, K., Soejima, H., Yasue, H.** 1997. The effects of the angiotensin-converting enzyme inhibitor imidapril on plasma plasminogen activator inhibitor activity in patients with acute myocardial infarction. *Am Heart J.* **134**:961-966.
 18. **Reilly, D.F., Westgate, E.J., FitzGerald, G.A.** 2007. Peripheral circadian clocks in the vasculature. *Arterioscler Thromb Vasc Biol.* **27**:1694-1705.
 19. **Sawathiparnich, P., Kumar, S., Vaughan, D.E., Brown, N.J.** 2002. Spironolactone abolishes the relationship between aldosterone and plasminogen activator inhibitor-1 in humans. *J Clin Endocrinol Metab.* **87**:448-452.
 20. **Schoenhard, J.A., Smith, L.H., Painter, C.A., Eren, M., Johnson, C.H., Vaughan, D.E.** 2003. Regulation of the PAI-1 promoter by circadian clock components: differential activation by BMAL1 and BMAL2. *J Mol Cell Cardiol.* **35**:473-481.
 21. **Sugaya, T., Nishimatsu, S., Tanimoto, K., Takimoto, E., Yamagishi, T., Imamura, K., Goto, S., Imaizumi, K., Hisada, Y., Otsuka, A., Uchida, H., Sugiura, M., Fukuta, K., Fukamizu, A., Murakami, K.** 1995. Angiotensin II type 1a receptor-deficient mice with hypotension and hyperreninemia. *J Biol Chem.* **270**:18719-18722.
 22. **Tsujino, T., Naito, Y., Kawasaki, D., Okuda, S., Sakoda, T., Fujioka, Y., Sugaya, T., Ohyanagi, M.** 2005. Circadian expression of plasminogen activator inhibitor-1 in angiotensin II type 1a receptor knockout mice. *Clin Exp Hypertens.* **27**:159-168.
 23. **van Leeuwen, R.T., Kol, A., Andreotti, F., Kluft, C., Maseri, A., Sperti, G.** 1994. Angiotensin II increases plasminogen activator inhibitor type 1 and tissue-type plasminogen activator messenger RNA in cultured rat aortic smooth muscle cells. *Circulation.* **90**:362-368.
 24. **Vaughan, D.E., Lazos, S.A., Tong, K.** 1995. Angiotensin II regulates the expression of plasminogen activator inhibitor-1 in cultured endothelial cells. A potential link between the renin-angiotensin system and thrombosis. *J Clin Invest.* **95**:995-1001.
 25. **Westgate, E.J., Cheng, Y., Reilly, D.F., Price, T.S., Walisser, J.A., Bradfield, C.A., FitzGerald, G.A.** 2008. Genetic components of the circadian clock regulate thrombogenesis in vivo. *Circulation.* **117**:2087-2095.
 26. **Willich, S.N., Goldberg, R.J., Maclure, M., Perriello, L., Muller, J.E.** 1992. Increased onset of sudden cardiac death in the first three hours after awakening. *Am J Cardiol.* **70**:65-68.