The vascular clock system generates the intrinsic circadian rhythm of vascular contractility

Toshiro Saito*

Division of Molecular Cardiology, Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University, Japan

Abstract

Many of the cardiovascular parameters or incidences of coronary artery diseases display circadian variations. These day/night time variances may be attributable to the diurnal change in vascular contractility. However, the molecular mechanism of the vascular clock system which generates the circadian variation of vascular contractility has remained largely unknown. Recently we found the existence of the intrinsic circadian rhythm in vascular contractility. A clock gene *Rorα* in vascular smooth muscle cells (VSMC) provokes the diurnal oscillatory change in the expression of Rho-associated kinase 2 (ROCK2), which induces the time-of-day-dependent variation in the agonist-induced phosphorylation of myosin light chain (MLC) and myofilament Ca^{2+} sensitization. In this review, we introduce our recent findings with reference to the molecular basis of the biological clock system and the current literature concerning cardiovascular chronobiology.

Introduction

Living organisms on earth have evolved various cellular mechanisms to adapt to environmental fluctuations. The evolutional development of the biological circadian rhythm may not be irrelevant to the earth's rotation period.

In the cardiovascular system, many parameters exhibit circadian variation. The physiological changes in the blood pressure or heart rate have a diurnal rhythm. The incidence of cardiac sudden death or myocardial infarction displays apparent circadian changes, peaking primarily during the morning and secondarily during the evening (1). Countless studies have documented the day/night time variance of sympathetic nerve activity, plasma fibrinolytic activity, platelet aggregability or vascular reactivity (2, 3). It is conceivable that the circadian changes in cardiovascular parameters are attributable to this day/night time variation. However, the

precise mechanism underlying the circadian rhythm of vascular contractility has not yet been fully elucidated.

We recently found the existence of an endogenous circadian rhythm of vascular contractility intrinsic to smooth muscle. A clock gene *Rorα* appears to generate diurnal variation in the ROCK2 expression in VSMC, which in turn induces the oscillatory change in MLC phosphorylation and Ca^{2+} sensitization. Thus, the vascular clock system contributes to day/night time variations in vascular contractility.

In this review, we introduce our finding from the perspective of the basic understanding of the circadian clock system and the current literature concerning cardiovascular chronobiology.

The framework and molecular mechanism of the circadian clock

In mammalian species, multiple organs, excluding testis, possess the circadian clock system which produces the diurnal rhythm. The interplay between the central clock located in the suprachiasmatic nucleus (SCN) and the peripheral clock in multiple tissues determines the diurnal rhythm systemically (4–6). Neurohormonal factors, such as sympathetic nervous activity and glucocorticoid secretion, mediate this interplay (4–6). Recent investigations have uncovered the vital role of this clock system in regulating the cellular homeostasis or organic functionality (7–10). Under certain conditions, the peripheral clock is more important for the organic feature (5, 11). Accordingly, the significance of the peripheral clock in multiple tissues has been intensively investigated as well as that of the central clock.

The molecular mechanism of the biological clock is composed of transcriptional-translational autoregulatory feedback loops according to a set of clock genes, such as *Clock*, *Bmal*, *Cry*, *Per*, *Rev-erb* and *Ror* (4, 6) (Fig. 1). The transcription factors CLOCK and BMAL1 form a heterodimer and activate the transcription of the *Cry* and *Per* genes (Fig. 1). CRY and PER proteins suppress the transactivation of CLOCKBMAL1 and inhibit their own transcription, once their expression levels have reached a high concentration. Additional loops consisting of other clock genes, such as *Rev-erb* and *Ror*, also regulate this central loop (Fig. 1). These products of clock genes work as transcription factors and produce the diurnal rhythmic expression in approximately 10% of genomic genes, which in turn provide the diurnal variation for organic functionality.

Day/nighttime variations in the blood pressure

Since there are many cardiovascular parameters that display time-of-day-dependent variation, the chronobiology has been intensively investigated in this field. With the use of genetically altered mouse models, many features of clock genes in generating the circadian changes in cardiovascular parameters have been uncovered (Table 1).

Blood pressure normally exhibits a diurnal variation, with a peak during daytime and a nadir during night, correlating with the circadian change in the sympathetic nervous activity. It is well-known that non-dipper patients, whose blood pressure does not decrease at night, have an increased risk of cardiovascular disease.

In human study, Shea et al. unveiled the existence of an endogenous circadian blood pressure rhythm (12). To eliminate the effect of neurohormonal factors or other external cues on the blood pressure, 28 normotensive adults were assessed for circadian changes in the blood pressure across 3 complementary, multiday, in-laboratory protocols performed in dim light, throughout which behavioral and environmental influences were controlled. As a result, the authors found that there exists a robust endogenous circadian rhythm in the blood pressure unrelated to that in the cortisol, catecholamines, cardiac vagal modulation, heart rate or urine flow. The highest blood pressure occurs at the circadian time corresponding to around 9:00 pm, suggesting that
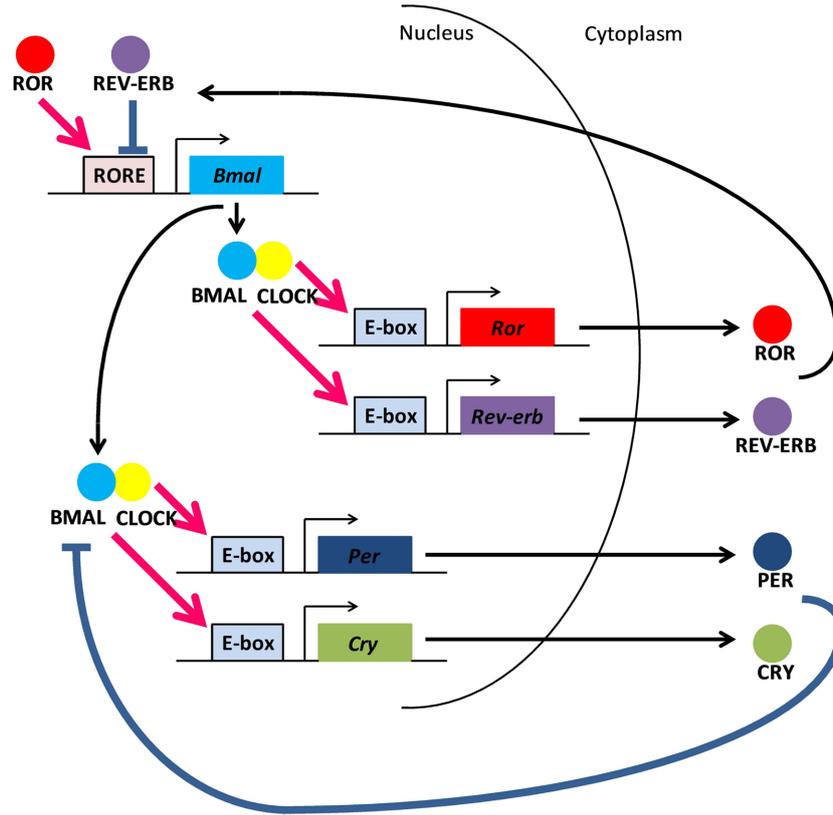
Fig. 1. A network of the circadian clock system consists of transcriptional-translational autoregulatory feedback loops.

Table 1. Cardiovascular Phenotypes in Genetic Models of Dysfunctional Circadian Clock

Clock Gene	Phenotype in Genetically Altered Mice	Reference
Clock	Enhanced vascular injury in the ligated carotid artery of mutant mice	(7)
	Changes in fatty acid metabolism in the heart of mutant mice	(25)
	Loss of diurnal variation in thrombomodulin in the lung of mutant mice	(26)
Bmal1	Loss of diurnal variation in blood pressure in systemic KO mice	(27)
	Enhanced vascular injury in the ligated carotid artery in systemic KO mice	(7)
	Development of arteriosclerosis in the transplanted aortic grafts of systemic KO mice to WT mice	(28)
	Impairment of endothelium-dependent vaso-relaxation, up-regulation of superoxide in aorta and uncoupling of eNOS in systemic KO mice	(29)
	Loss of diurnal variation in blood pressure and vascular contractility in smooth-muscle-specific KO mice	(11)
	Dilated cardiomyopathy and abnormality in mitochondrial metabolism in the heart of cardio-myocyte-specific KO mice	(30)
	Dilated cardiomyopathy in cardiomyocyte-specific KO mice	(31)
Per2	Development of arteriosclerosis in the transplanted aortic grafts of *Per2/3* double systemic KO mice to WT mice	(28)
	Impairment of diurnal change in endothelium-dependent vaso-relaxation in mutant mice	(32)
	Disruption in fatty acid metabolism during ischemia in the heart of systemic KO mice	(33)
	Larger infarct size during ischemia in the heart of systemic KO mice	(17)
Cry1, Cry2	Loss of diurnal variation in blood pressure and up-regulation of baroreflex sensitivity in *Cry1/2* double systemic KO mice	(13)

this blood pressure rhythm is unlikely to underlie the primary morning peak in adverse cardiovascular events. However, this timing appears to coincide with the period in which nocturnal hypertension can be observed in riser/non-dipper patients. The dysregulation of the endogenous circadian rhythm of blood pressure may underlie the pathology in nocturnal riser/non-dipper or extreme-dipper patients.

In nocturnal animals, studies agree that the mean arterial pressure itself is higher in the early dark phase than in the early light phase. Conversely, acute development in the mean arterial pressure induced by the administration of phenylephrine or angiotensin II is greater in the early light phase than in the early dark phase (11, 13, 14). Considering that an acute increase in the arterial pressure by phenylephrine or angiotensin II is primarily determined by vasoconstriction in the peripheral vessels, there may be a circadian change in vascular contractility which peaks during the early light phase in nocturnal animals.

Generally vascular contractility is considered to be one of the determinants of the blood pressure. These results suggest that there may be diurnal variation in vascular contractility, which peaks in the early light phase in nocturnal animals, thus corresponding to the early dark phase in diurnal human.

The pathophysiological role of the clock system in the heart

Two studies have documented that the infarct size after acute myocardial infarction has a circadian dependence on the time of the onset of ischemia (15, 16). The clock gene *Per2* has been reported to generate the time-of-day-dependent variation in ischemic tolerance after myocardial ischemia in mice (17). This cardioprotective effect of *Per2* is through the transcriptional regulation of glycolysis and cardiac metabolism during ischemia. However, phase difference may exist in the rhythmic expression of the clock gene between diurnal human and nocturnal rodent, and thus, the authors in this study discussed that the cardioprotective effect of *Per2* in mice cannot simply be extrapolated to the circadian rhythmicity of ischemic tolerance in humans (17).

Sudden cardiac death from ventricular arrhythmias is the main cause of mortality in the population with heart disease. The incidence of sudden cardiac death shows diurnal variation, increasing sharply within a few hours of rising in the morning (18). A common mechanism that underlies susceptibility to ventricular arrhythmias is abnormalities in the duration or pattern of myocardial repolarization. The clock gene *Bmal1* has been identified as the responsible molecule generating the rhythmic QTc variation in mice (19). *Bmal1* regulates the rhythmic expression of transcription factor *Klf15*, which in turn generates the circadian rhythmicity in the expression of Kv channel-interacting protein 2 (KChIP2), a critical subunit for the transient outward potassium current.

Circadian changes in the response to MLC phosphorylation in VSMC

Based upon these observations, we speculated that there might be circadian oscillation in vascular contractility intrinsic to the smooth muscle (20). We initiated research employing the use of VSMC in culture to exclude any influence from the central clock or external cues. Since the phosphorylation of MLC is an essential step in smooth muscle contraction, we examined the diurnal variation of the phosphorylation level of MLC with two different methods. One is the Phos-tag SDS-PAGE method (21). The other is the conventional SDS-PAGE method with a phospho-specific antibody against P-MLC (Ser19) or PP-MLC (Thr18 and Ser19). Phos-tag is a compound that specifically binds to phosphorylated protein, and the Phos-tag containing SDS-PAGE separates the phosphorylated form of proteins from the non-phosphorylated form.

The anti-MLC antibody recognizes the non-, mono-, or di-phosphorylated form of MLC distinctly in the

lysate of VSMC on Phos-tag SDS-PAGE (Fig. 2A). This method allows us to evaluate MLC phosphorylation stoichiometrically in a self-contained manner.

To evaluate the circadian variation of MLC phosphorylation, dexamethasone pulse treatment was used to induce the synchronized circadian rhythm in cultured VSMC (5). After synchronization, the level of MLC phosphorylation analyzed at 2 min after thrombin stimulation at the indicated times, but not the resting condition, displayed an oscillatory change with peaks at 36 h and 60 h and a nadir at 48 h (Fig. 2B). In the conventional SDS-PAGE method, PP-MLC (Thr18 and Ser19) analyzed at 2 min after thrombin stimulation at each indicated time, but not P-MLC (Ser19), exhibited a similar oscillatory change (Fig. 2C). These results suggested that VSMC possess an intrinsic diurnal rhythm in the response to MLC phosphorylation, and that the di-phosphorylated form of MLC plays a major role in this rhythmic variation.

Role of ROCK2 in the circadian oscillation of MLC phosphorylation in VSMC

The kinase activity of ROCK2, which was evaluated by the phosphorylation of MYPT1 at Thr853, displayed an oscillatory change with the same phase as that observed in MLC phosphorylation (Fig. 3A). The protein level of ROCK2 also showed a rhythmic variation with the same phase as that observed in the phosphorylation of MLC or MYPT1 at Thr853, suggesting that the circadian rhythm of the ROCK2 expression regulates the diurnal variation of ROCK2 activity and MLC phosphorylation (Fig. 3B left). The mRNA level of ROCK2 showed an oscillatory change with the phase at 4 hours prior to the protein expression, suggesting the involvement of transcriptional regulation by clock gene products (Fig. 3B right).

Role of RORα in the circadian changes in the transcription of ROCK2 in VSMC

Among the regulatory elements for the known clock gene products, two ROR response elements (ROREs) are preserved in the promoter region of the *Rock2* gene across various mammalian species. The forced expression of RORα and RORγ, but not of other RORE-binding transcriptional factors, increased the promoter activity in the human *Rock2* gene more than 5-fold the level observed with control vectors in VSMC (Fig. 3C). The knockdown of RORα abolished the rhythmic expression of ROCK2 (Fig. 3D). These results strongly suggested that clock gene RORα generates the circadian rhythm of the ROCK2 expression, presumably through its direct transcription.

Circadian changes in the myofilament sensitivity to Ca^{2+} and MLC phosphorylation in the mouse aorta

Consistent with the result obtained in VSMC, the expression of ROCK2 protein in the aortas of wild-type mice kept under a 12-hour dark-light cycle exhibited a circadian oscillation with a peak at Zeitgeber time (ZT)0/24, which corresponds to approximately 36 hours after dexamethasone pulse treatment in cultured cells (Figs. 4A and B). The expression of RORα also showed a circadian change, with a phase peak 4 hours prior to that of ROCK2 (Figs. 4A and B). In the aortas of *staggerer* mice (1), which lack a functional RORα, the ROCK2 expression did not show any apparent circadian changes (Figs. 4A and B). The protein level of ROCK2 in the aortas of *staggerer* mice was similar with that seen at the nadir in those of wild-type mice (Fig. 4C).
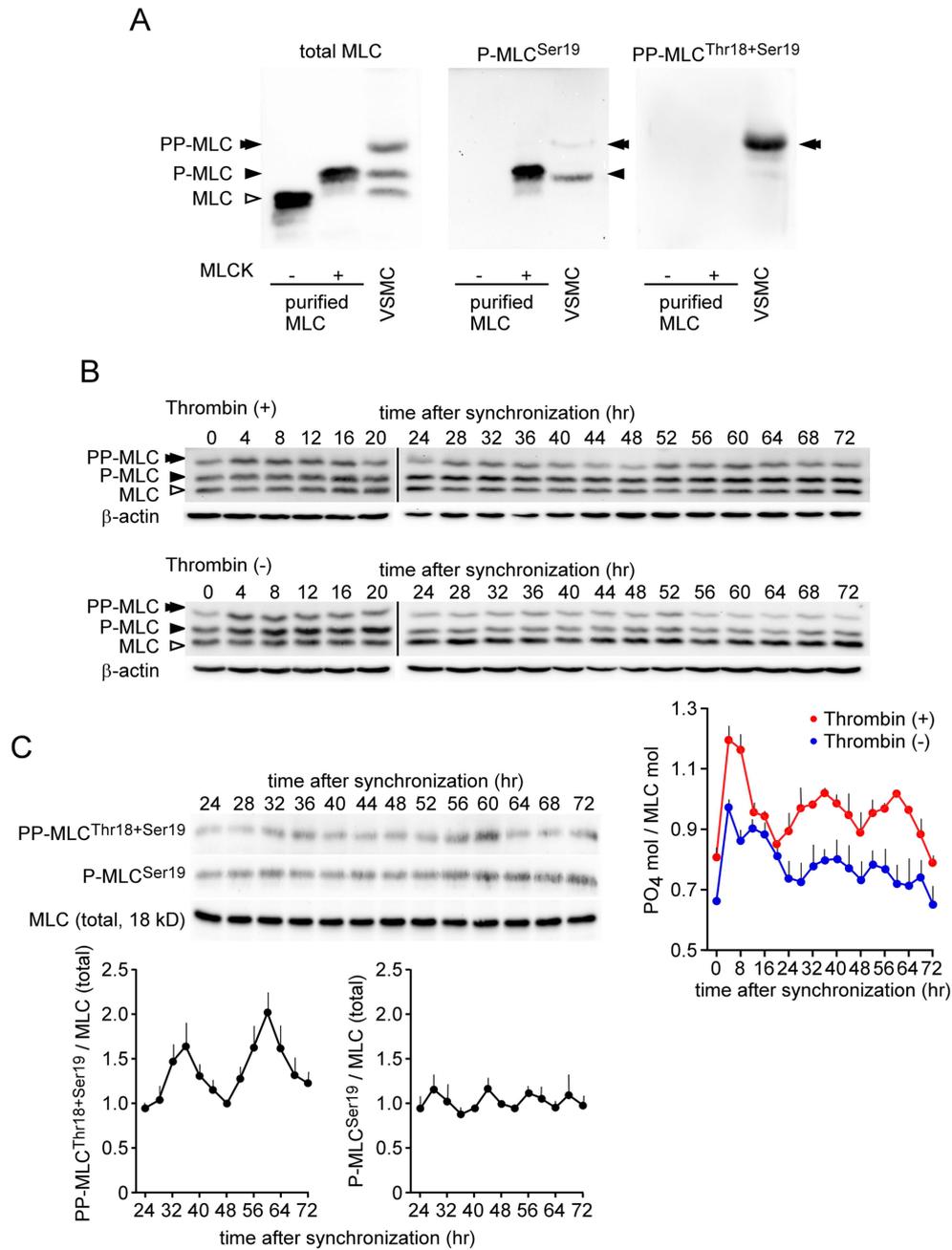
Fig. 2. The circadian changes in the thrombin-induced phosphorylation of MLC in porcine coronary artery smooth muscle cells.

(A) Immunoblot detection of the purified MLC with and without phosphorylation by MLC kinase (MLCK) and the lysates of porcine coronary artery smooth muscle cells (VSMC) with the anti-MLC antibody (total MLC) and the antibodies specific to the monophosphorylated MLC (P-MLC[Ser19]) and diphosphorylated MLC (PP-MLC[Thr18+Ser19]), after Phos-tag SDS-PAGE. (B, C) The representative immunoblots and summaries of the circadian changes in the MLC phosphorylation as analyzed by Phos-tag SDS-PAGE (B; n=3 for Thrombin (+), n=5 for Thrombin (-)) and conventional SDS-PAGE (C: n=3). A maximum of 13 samples could be analyzed in one gel. The vertical bars between immunoblot images indicate the border between different gels. The samples were obtained before (B) and 2 min after the stimulation with 1 unit/mL thrombin (B, C) at the indicated times after synchronization of the circadian rhythm. The immunoblot detection was performed with the anti-MLC antibody in B or antibodies specific for the MLC mono-phosphorylated (P-MLC[Ser19]) and the MLC di-phosphorylated (PP-MLC[Thr18+Ser19]) in C. In B, immunoblot images of β-actin are shown below those for MLC detection. In C, the level of phosphorylation, as normalized to the level of total MLC, was expressed as a relative value to that obtained at 48 h. All data are expressed as the means ± SEM [modified from ref. (20)].
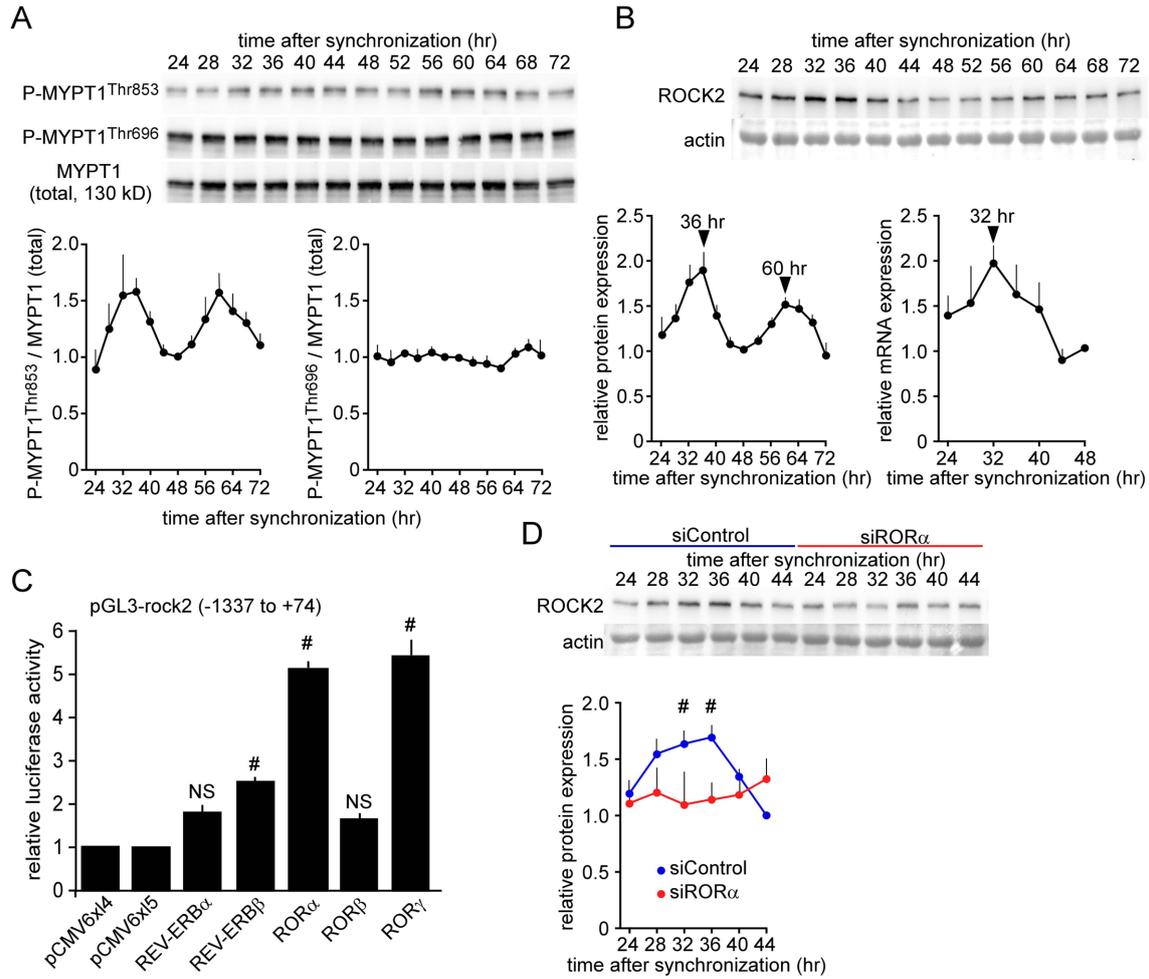
Fig. 3. The circadian changes in the activity and the expression of ROCK2 under the regulation of RORα in porcine coronary artery smooth muscle cells.
(A) The circadian changes in the level of MYPT1 phosphorylation at residues corresponding to Thr853 (n=5) and Thr696 (n=4) in human MYPT1 obtained 2 min after the stimulation with 1 unit/ml thrombin. (B) The representative immunoblot detection of ROCK2 and summaries of circadian changes in the level of ROCK2 protein (left; n=5) and mRNA (right; n=4). (C) The effects of REV-ERBα, REV-ERBβ, RORα, RORβ, and RORγ on the activity of the human *Rock2* promoter, as evaluated by the luciferase activity. pCMV6xl4 was used as a vector for REV-ERBα and RORα, while pCMV6xl5 was used for REV-ERBβ, RORβ and RORγ (n=3). The data are expressed as the relative values to those obtained with the corresponding empty vectors. (D) The effects of the knockdown of RORα on the circadian changes in the expression of the ROCK2 protein (n=4). The data are expressed as the relative values to those obtained at 48 h (A, B) and 44 h of control siRNA (D). All data are expressed as the means ± SEM. [#]$P<0.05$ vs. 48 h (Steel's test) (A, B left). [#]$P<0.05$ (Student's *t*-test) (B right, C, D) [modified from ref. (20)].

ROCK2 plays a pivotal role in regulating the myofilament Ca^{2+} sensitivity. To investigate the functional relevance of the circadian expression of ROCK2, the myofilament Ca^{2+} sensitivity in aortas of mice was examined at ZT0 and ZT12, respectively. α-toxin-permeabilized preparations of the aortas enabled us to evaluate the myofilament Ca^{2+} sensitivity by measuring the contraction at a fixed concentration of Ca^{2+} (31). Ca^{2+} sensitization was induced by GTPγS, a non-hydrolyzable GTP analog.

At first, we examined Ca^{2+}-dependent contraction by increasing the Ca^{2+} concentration in a stepwise manner without inducing Ca^{2+} sensitization by GTPγS (Fig. 4D). The pCa^{2+}-tension relationship did not differ between ZT0 and ZT12 in both wild-type and *staggerer* mice. The pCa^{2+}-tension relationship seen in *staggerer*
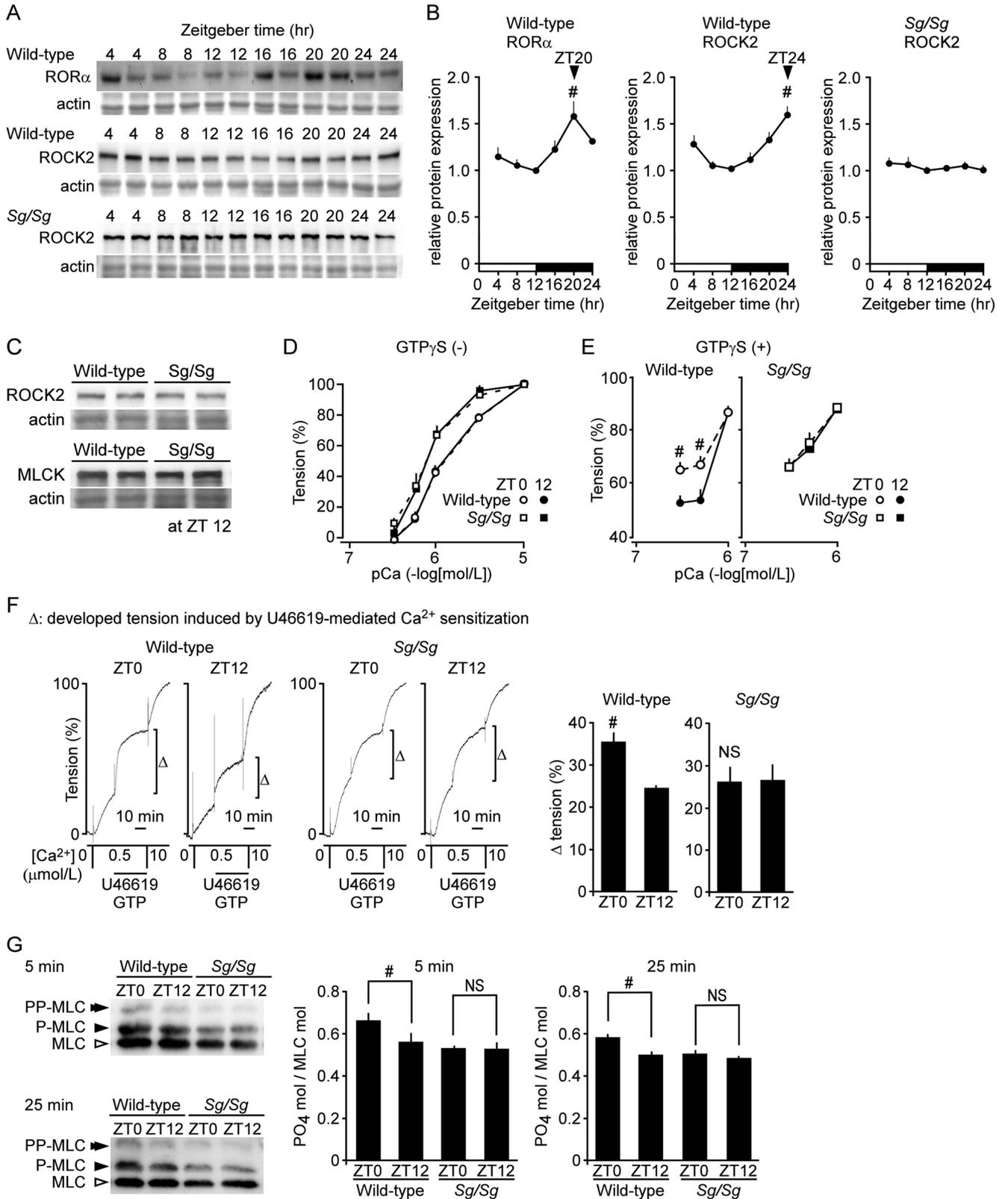
Fig. 4. See figure legend on next page.

mice shifted to the left of that seen in wild-type mice. However, the protein level of MLCK was similar between wild-type and *staggerer* mice (Fig. 4C). The reason for the shift to left in the pCa^{2+}-tension relationship in *staggerer* mice remained uncertain.

The level of tension obtained with 10 µmol/l of GTPγS and either 0.3 or 0.5 µmol/l of Ca^{2+} at ZT0 was significantly greater than that of the corresponding contraction observed at ZT12 in the wild-type mice (Fig. 4E). The circadian change in the myofilament Ca^{2+} sensitivity was consistently observed for receptor-mediated contraction. The contraction induced by 1 µmol/l of U46619 in the presence of 10 µmol/l of GTP during the 0.5-µmol/l Ca^{2+}-induced contraction at ZT0 was significantly greater than that observed at ZT12 in wild-type mice (Fig. 4F). In accordance with the diurnal variation in the Ca^{2+} sensitization, the U46619-induced MLC phosphorylation displayed a circadian change. MLC phosphorylation 5 and 25 min after the application of U46619 and GTP at ZT0 was significantly greater than that observed at ZT12 in wild-type mice (Fig. 4G). In contrast, these circadian changes in the myofilament Ca^{2+} sensitivity and MLC phosphorylation were abolished in *staggerer* mice (Figs. 4E–G). These results indicated that the RORα-mediated circadian rhythm of the ROCK2 expression generates the diurnal change in the MLC phosphorylation and myofilament Ca^{2+} sensitivity in the aortas of mice (Fig. 5).

Conclusion

Our findings demonstrated, for the first time, the existence of the intrinsic circadian rhythm of vascular contractility with a peak at the beginning of the light phase in rodents. The rhythmic expression of a clock gene, *Rorα*, provokes the oscillatory change in ROCK2 transcription, which in turn produces the circadian rhythm of MLC phosphorylation and myofilament Ca^{2+} sensitivity in response to contractile stimuli (Fig. 5). The intrinsic rhythm of the myofilament Ca^{2+} sensitivity peaks at the beginning of the light phase in rodent, which corresponds to the early dark phase in human, when the endogenous circadian blood pressure rhythm also peaks. This intrinsic rhythm of the myofilament Ca^{2+} sensitivity may underlie the endogenous circadian

Fig. 4. The absence of the circadian changes in the expression of ROCK2 and Ca^{2+} sensitivity of the contractile apparatus in the aortas of *staggerer* mice.

(A, B) The representative immunoblots (A) and summaries (B) showing the circadian patterns of the expression of the RORα and ROCK2 proteins in the aortas of wild-type and *staggerer* (*Sg/Sg*) mice that were housed under 12-h light (open bar) and 12-h dark (closed bar) cycle conditions. The expression levels are expressed as the relative values to those obtained at Zeitgeber time 12. All data are expressed as the means ± SEM. (n=5–6 for RORα in wild-type; n=6–7 for ROCK2 in wild-type; n=6–7 for ROCK2 in *Sg/Sg*). #$P<0.05$, ##$P<0.01$ (Student's *t*-test). (C) The expression of ROCK2 and MLCK proteins in the aortas of wild-type and *Sg/Sg* mice at Zeitgeber time 12 (ZT12) was directly compared on the same gel.

(D, E) The pCa^{2+}-tension curves of the contractions induced by stepwise increases in the Ca^{2+} concentrations in the absence of GTPγS (GTPγS (-) in D) and the contractions induced by 10 µmol/l GTPγS and 0.3, 0.5 or 1 µmol/l Ca^{2+} (GTPγS (+) in E), in the wild-type (n=4–5) and *staggerer* (*Sg/Sg*; n=4–5) mice at Zeitgeber time (ZT) 0 and 12. The level of tension obtained with different Ca^{2+} concentrations in either the presence or absence of GTPγS was expressed as a percentage, while the levels of tension obtained in Ca^{2+}-free solution and with 10 µmol/l Ca^{2+} were assigned a value of 0% and 100%, respectively. All data are expressed as the means ± SEM. #$P<0.05$ vs. ZT12.

(F, G) Representative recordings and summaries of the contractions (F) and MLC phosphorylation (G) induced by 1 µmol/l U46619 in the presence of 10 µmol/l GTP during the 0.5 µmol/l Ca^{2+}-induced contractions in wild-type (n=4) and *staggerer* (n=4) mice at Zeitgeber time (ZT) 0 and 12. The increase in tension induced by U46619 (Δ) was expressed as a percentage of the tension obtained with 10 µmol/l Ca^{2+}. MLC phosphorylation was analyzed using Phos-tag SDS-PAGE. All data are expressed as the means ± SEM. #$P<0.05$ and NS, not significantly different vs. ZT12 [modified from ref. (20)].
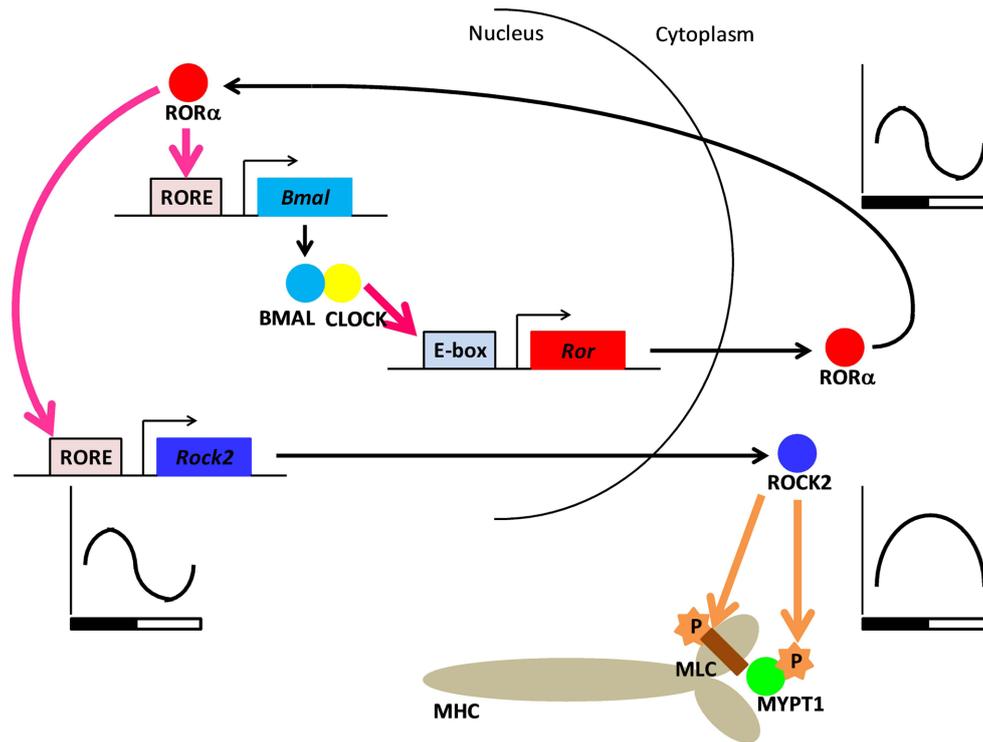
Fig. 5. A proposed model of the vascular clock system which generates the intrinsic circadian rhythm of vascular contractility in mice.

blood pressure rhythm. Some relationship may therefore exist between the intrinsic rhythm of the myofilament Ca^{2+} sensitivity and the pathogenesis of coronary artery disease or nocturnal hypertension, however, the precise pathological role of the vascular clock mechanism remains to be investigated.

Acknowledgments

Conflict of interest

The author declares that he has no conflict of interest.

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