

Pivotal Role of Rho-Associated Kinase 2 in Generating the Intrinsic Circadian Rhythm of Vascular Contractility

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Background—The circadian variation in the incidence of cardiovascular events may be attributable to the circadian changes in vascular contractility. The circadian rhythm of vascular contractility is determined by the interplay between the central and peripheral clocks. However, the molecular mechanism of the vascular intrinsic clock that generates the circadian rhythm of vascular contractility still remains largely unknown.

Methods and Results—The agonist-induced phosphorylation of myosin light chain in cultured smooth muscle cells synchronized by dexamethasone pulse treatment exhibited an apparent circadian oscillation, with a 25.4-hour cycle length. The pharmacological inhibition and knockdown of Rho-associated kinase 2 (ROCK2) abolished the circadian rhythm of myosin light chain phosphorylation. The expression and activity of ROCK2 exhibited a circadian rhythm in phase with that of myosin light chain phosphorylation. A clock gene, *RORα*, activated the promoter of the ROCK2 gene, whereas its knockdown abolished the rhythmic expression of ROCK2. In the mouse aorta, ROCK2 expression exhibited the circadian oscillation, with a peak at Zeitgeber time 0/24 and a nadir at Zeitgeber time 12. The myofilament Ca^{2+} sensitization induced by $\text{GTP}\gamma\text{S}$ and U46619, a thromboxane A2 analog, at Zeitgeber time 0/24 was greater than that seen at Zeitgeber time 12. The circadian rhythm of ROCK2 expression and myofilament Ca^{2+} sensitivity was abolished in *staggerer* mutant mice, which lack a functional *RORα*.

Conclusions—ROCK2 plays a pivotal role in generating the intrinsic circadian rhythm of vascular contractility by receiving a cue from *RORα*. The ROCK2-mediated intrinsic rhythm of vascular contractility may underlie the diurnal variation of the incidence of cardiovascular diseases. (*Circulation*. 2013;126:104-114.)

Key Words: circadian rhythm ■ muscle, smooth ■ myosins ■ vasoconstriction

The cardiovascular system displays circadian rhythms in some physiological parameters, including blood pressure. In addition, the occurrence of coronary artery events, such as myocardial infarction and angina pectoris, has a circadian variation with a peak during the morning.^{1,2} These changes may be attributable to the diurnal variation of sympathetic nerve activity, plasma fibrinolytic activity, platelet aggregability, or vascular contractility.^{3,4} The circadian changes in the vascular tone and reactivity to adrenergic receptor agonists have been well documented.⁵ However, the precise mechanism underlying the circadian rhythm of vascular contractility has not been fully elucidated.

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In mammals, the diurnal rhythm of biological processes is driven by the biological clock system. The molecular mechanism of the biological clock is based on the transcriptional-translational autoregulatory feedback loops composed of

a set of clock genes.⁶ The transcription factors CLOCK and BMAL1 work as a heterodimer to activate the transcription of the *Cry* and *Per* genes.⁶ Once CRY and PER have reached a critical concentration, they repress the transactivation of CLOCK-BMAL1 and inhibit their own transcription.⁶ Additional loops involving other clock genes, such as *Rev-erb* and *Ror*, interact with and modulate this central loop.⁶ The circadian oscillation of the biological clock is then dictated by the rhythmic expression of genes, which generate the rhythm of physiological processes. The circadian rhythm of peripheral tissues is determined by the interplay between the central and peripheral clock mechanisms. The central clock influences the rhythm of the peripheral tissues by generating the circadian rhythm of neurohumoral cues.⁶ Recently, the peripheral clock system has been shown to dominate in regulating the expression of several important genes in different organs under certain conditions.^{7,8} However, the role of the vascular intrinsic clock in the regulation of vascular contractility and its molecular mechanisms still remain elusive.

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The present study thus aimed to clarify whether there is any intrinsic diurnal oscillation in the vascular contractility and, if so, to elucidate the underlying molecular mechanism. The importance of the endothelium in regulating the physiological vascular tone is well recognized; however, the smooth muscle also plays a fundamental role in determining the vascular contractility.^{9,10} Our investigations were initiated with cultured vascular smooth muscle cells to exclude any influence of the central clock or external cues. Because the phosphorylation of myosin light chain (MLC) plays a central role in the regulation of smooth muscle contraction,¹¹ the existence of circadian oscillation of MLC phosphorylation was first investigated. Accordingly, a clock gene *RORα* and its regulation of the expression of Rho-associated kinase 2 (ROCK2) were found to generate the circadian oscillation of MLC phosphorylation. The physiological significance of this clock mechanism in the regulation of vascular contractility was then evaluated using *staggerer* mutant mice, which lack a functional *RORα*.¹² As a result, the present study elucidated, for the first time, a pivotal role of ROCK2 in generating the intrinsic circadian rhythm of vascular contractility.

Methods

An expanded Methods sections is available in the online-only Data Supplement.

Cell Culture and Dexamethasone Pulse Treatment

The porcine coronary artery smooth muscle cells (PCSMCs) and porcine aortic smooth muscle cells were cultured in DMEM containing 10% FBS until semiconfluence for 3 to 4 days before experimental use. The cells at semiconfluence were incubated with 100 nmol/L of dexamethasone in growth medium for 2 hours to induce a synchronized circadian rhythm.

Animals

The original *staggerer* mutant mice, B6C3Fe-*a/a-Rora*^{sg} mice (Jackson Laboratory, Bar Harbor, ME), were adjusted by crossing these mice with C57BL/6J inbred mice (CLEA Japan, Tokyo, Japan) for >10 generations.¹³ The study protocol was approved by the animal care and use committee of Kyushu University. The animals were treated in accordance with the guidelines stipulated by the committee.

Real-Time Polymerase Chain Reaction Analysis

The total RNA was extracted from cultured cells and subjected to a real-time polymerase chain reaction analysis with FastStart SYBR Green Master kit using a LightCycler (Roche, Basel, Switzerland). The expression level of each mRNA was normalized to the level of β-actin obtained from the corresponding reverse transcription product.

Western Blot Analysis

The proteins from cultured smooth muscle cells or mice aortas were extracted in the lysis buffer and subjected to Western blot analysis. The immune complex was detected with an ECL plus detection kit (GE Healthcare, Buckinghamshire, United Kingdom). The light emission was detected and analyzed with a ChemiDoc XRS-J instrument and the computer program Quantity One (BioRad). The density of the immunoreactive band was normalized to that of the corresponding actin band to adjust for any possible variations in sample loading.

Phos-tag SDS-PAGE Analysis of the MLC Phosphorylation

MLC phosphorylation was evaluated with Phos-tag SDS-PAGE analysis, as described previously.¹⁴ The level of MLC phosphorylation (PO₄ mol/MLC mol) was calculated as follows:

$$\text{MLC phosphorylation} = (\text{P-MLC} + \text{PP-MLC} \times 2) / (\text{MLC} + \text{P-MLC} + \text{PP-MLC})$$

where MLC, P-MLC, and PP-MLC indicate the optical density of the non-, mono-, and di-phosphorylated forms of MLC.

Transfection of Small Interfering RNA

Small interfering RNAs (siRNAs) were synthesized with a 3'-UU overhang by Dharmacon (Lafayette, CO). The siRNAs were transfected with the HVJ Envelope Vector Kit GenomONE (Ishihara Sangyo, Osaka, Japan).

Pull-Down Assay for the GTP-Bound Forms of RhoA

The GTP-bound form of RhoA in the cell extract was recovered using a RhoA-binding domain of ROCK2 as a (His)₆-tagged pull-down probe and Ni²⁺-nitrilotriacetic acid resin. Equal amount of the resin eluates and cell lysates were subjected to an immunoblot analysis with an anti-RhoA antibody, as described above.

Luciferase Assay

The activity of the *rock2* promoter (−1337 to +74) was evaluated in PCSMCs 32 hours after transfection, using the dual-luciferase reporter assay system (Promega). The activity of firefly luciferase was normalized to that of Renilla luciferase, and the value obtained with the empty vector was considered to be 1.

Tension Measurement in the α-Toxin-Permeabilized Preparations of Mouse Aortas

The aortic rings were permeabilized with 5000 U/mL of staphylococcal α-toxin (Sigma, St. Louis, MO),¹⁴ and subjected to the studies. The entire procedure, from euthanization to tension measurement, was completed within 1.5 to 2.0 hours.

Data Analysis

The data are expressed as the mean±SEM of the indicated number of experiments or mice. Either the Steel test or Student *t* test was used to determine the statistical significance of the differences among groups or between 2 groups, respectively, as indicated in the Figure legends. A value of *P*<0.05 was considered to be statistically significant.

Results

Circadian Changes in the Response of MLC Phosphorylation in Vascular Smooth Muscle Cells

The circadian rhythms of PCSMCs were synchronized by 2-hour pulse treatment with dexamethasone. The mRNA expression levels of the *bmal1*, *rev-erba*, *per1*, and *cry1* genes exhibited oscillatory changes that were consistent with those described in previous reports, thus indicating the successful synchronization of the circadian rhythm (Figure S1, available in the online-only Data Supplement).^{6,15}

The lysate of PCSMCs exhibited 3 immunoreactive bands on Phos-tag SDS-PAGE (Figure 1A). The lowest band corresponded with the purified unphosphorylated MLC. The middle band corresponded with the purified monophosphorylated MLC, both of which were detected

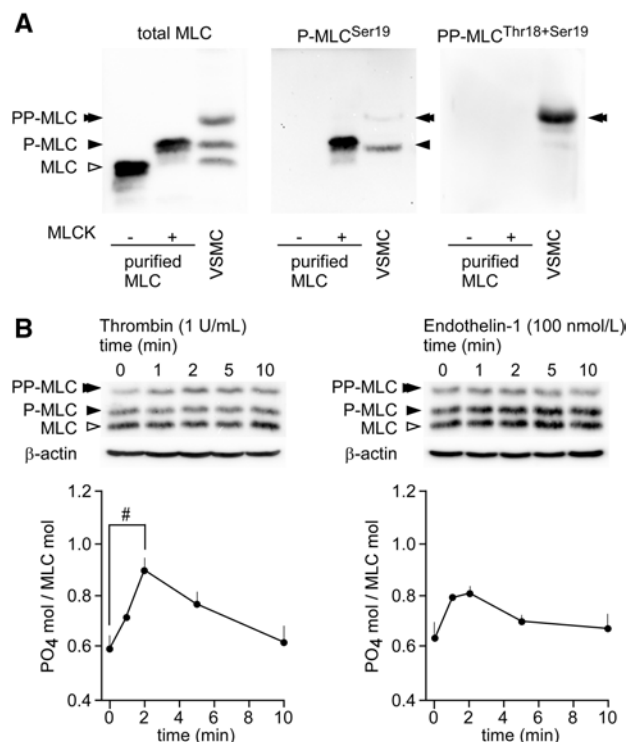


Figure 1. The evaluation of myosin light chain (MLC) phosphorylation with Phos-tag SDS-PAGE. **A**, Immunoblot detection of the purified MLC with and without phosphorylation by MLC kinase (MLCK) and the lysates of porcine coronary artery smooth muscle cells (VSMC) with the anti-MLC antibody (total MLC) and the antibodies specific to the monophosphorylated MLC (P-MLC^{Ser19}) and diphosphorylated MLC (PP-MLC^{Thr18+Ser19}), after Phos-tag SDS-PAGE. The cell lysates were obtained 2 minutes after the stimulation of cells with 1 U/mL of thrombin. **B**, The time courses of MLC phosphorylation induced by 1 U/mL of thrombin (n=3) and 100 nmol/L of endothelin 1 (n=3) were analyzed with Phos-tag SDS-PAGE, followed by immunoblot detection with the anti-MLC antibody. Representative immunoblot images of β -actin are shown below those for MLC detection. All of the data are expressed as the mean \pm SEM. #*P*<0.05 (Student *t* test).

by an antibody specific for P-MLC^{Ser19} (Figure 1A). The uppermost band was detected by an antibody specific for PP-MLC^{Thr18+Ser19} (Figure 1A). Thrombin and endothelin 1 were used as contractile stimuli in the present study, because they have been suggested to play a pathological role in cardiovascular diseases.^{16,17} Thrombin (1 U/mL) and endothelin 1 (100 nmol/L) transiently increased MLC phosphorylation with a peak at 2 minutes after the stimulation (Figure 1B).

After the dexamethasone pulse treatment, the levels of MLC phosphorylation both at rest and 2 minutes after thrombin stimulation transiently increased with a peak at 4 to 8 hours and then returned within 20 to 24 hours to levels seen at 0 hours (Figure 2A). The level of MLC phosphorylation recorded 2 minutes after thrombin stimulation, but not the resting level, exhibited an oscillatory change, with peaks at 36 hours and 60 hours and a nadir at 48 hours (Figure 2A). The frequency analysis by an autoregressive model revealed that it had a 25.4-hour cycle length (Figure 2B). An analysis using the antibodies specific for PP-MLC^{Thr18+Ser19} and P-MLC^{Ser19} revealed a similar oscillatory change in the levels of PP-MLC^{Thr18+Ser19} but not P-MLC^{Ser19} (Figure 2C).

Role of ROCK2 in the Circadian Oscillation of the MLC Phosphorylation in Vascular Smooth Muscle Cells

MLC kinase (MLCK), protein kinase C (PKC), ROCK, and Zipper-interacting kinase (ZIPK) play a major role in the regulation of MLC phosphorylation.^{10,18} The inhibitors of MLCK (10 μ mol/L of ML-9), PKC (1 μ mol/L of GF109203), and ROCK (3 μ mol/L of Y27632) were applied 10 minutes before and during thrombin stimulation. The treatment with these inhibitors had no effect on the cell viability (Table S1, available in the online-only Data Supplement). All 3 of the inhibitors suppressed the thrombin-induced MLC phosphorylation to a similar level (≈ 0.5 PO₄ mol/MLC mol) at 24 hours after synchronization (Figure 3A). Only ROCK inhibitor abolished the circadian oscillation of MLC phosphorylation (Figure 3A). In line with these observations, the knockdown of ROCK2, but not MLCK or ZIPK, abolished the circadian oscillation of the MLC phosphorylation (Figure 3B). The level of expression of ROCK2, MLCK, and ZIPK after siRNA-mediated knockdown was $55.4 \pm 6.3\%$ (n=7), $50.9 \pm 5.1\%$ (n=6), and $64.9 \pm 4.3\%$ (n=9), respectively, of that seen with the control siRNA (Figure 3B).

ROCK phosphorylates MYPT1, a noncatalytic subunit of MLC phosphatase, at Thr696 and Thr853 in human MYPT1, whereas other kinases, including ZIPK, phosphorylate MYPT1 at Thr696.^{18,19} The phosphorylation of Thr853 thus reflects the circadian oscillation of ROCK activity. The phosphorylation of Thr853 induced by thrombin exhibited circadian oscillation with peaks at 36 and 60 hours and a nadir at 48 hours (Figure 4A). This circadian pattern was similar to that seen with the thrombin-induced MLC phosphorylation (Figure 4A). The phosphorylation of Thr696 exhibited no apparent circadian oscillation (Figure 4A).

The level of expression of ROCK2 mRNA and protein exhibited circadian changes (Figure 4B). The pattern of the change in the level of ROCK2 protein was similar to that seen with the phosphorylation of MLC and MYPT1 (Thr853). The level of *rock2* mRNA oscillated with a phase that had a peak ≈ 4 hours earlier than the protein expression (Figure 4B). The expression of the ROCK2 protein also exhibited similar circadian changes in porcine aortic smooth muscle cell, with peaks at 32 to 36 hours and 60 hours (Figure S2, available in the online-only Data Supplement). In contrast, the level of the protein expression of MLCK, PKC α , and ZIPK exhibited no apparent circadian oscillation (Figure 4C). The level of the GTP-bound form of RhoA seen after thrombin stimulation also remained constant (Figure 4D).

Circadian Changes in the Phosphorylation of MLC and MYPT1 After Endothelin 1 Stimulation in Vascular Smooth Muscle Cells

The levels of PP-MLC^{Thr18+Ser19} and P-MYPT1^{Thr853} seen 2 minutes after stimulation with 100 nmol/L of endothelin 1 exhibited a similar circadian change to those seen with thrombin stimulation, with peaks at 36 and 60 hours and a nadir at 48 hours (Figure S3A and S3B, available in the online-only Data Supplement). The levels of P-MLC^{Ser19} and P-MYPT1^{Thr696} exhibited no apparent circadian changes (Figure S3A and S3B, available in the online-only Data Supplement). These

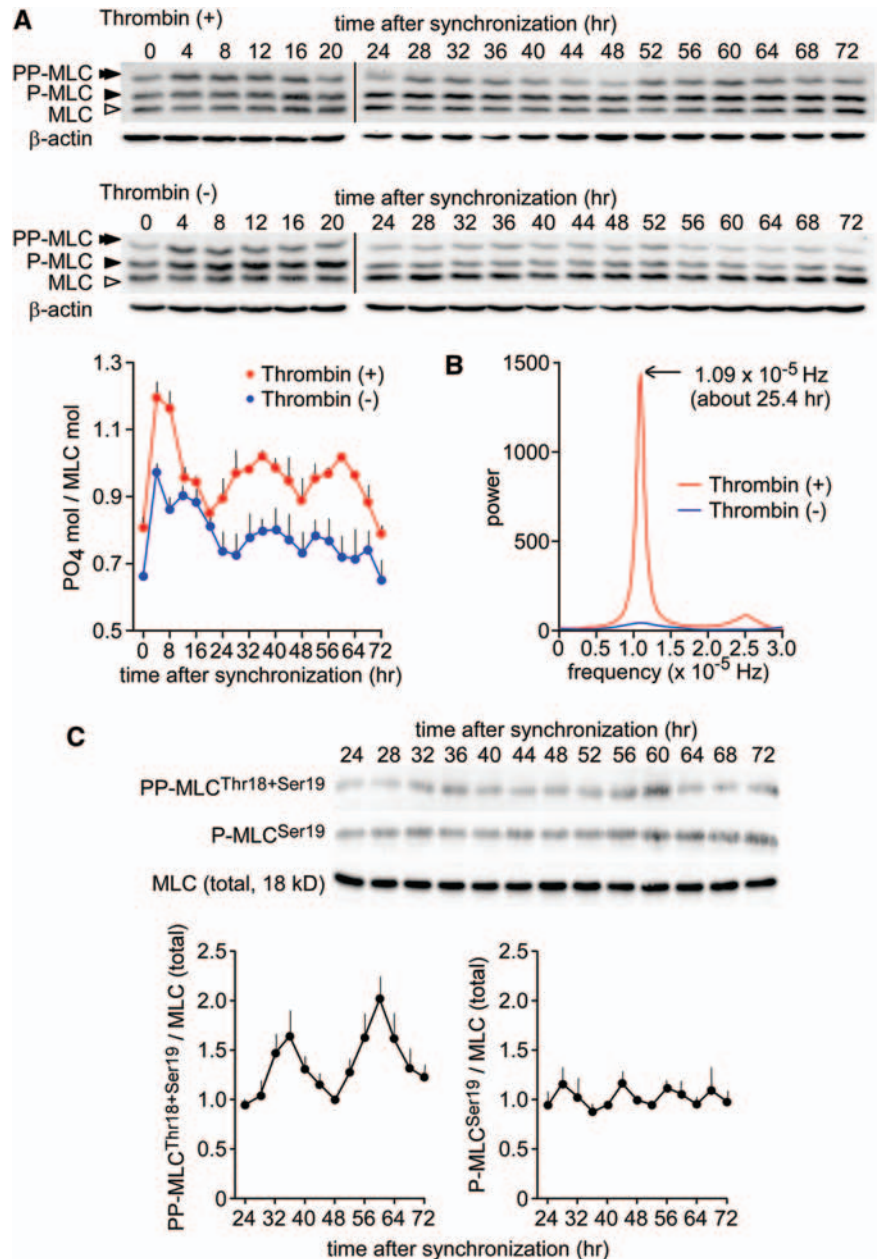


Figure 2. The circadian changes in the thrombin-induced phosphorylation of myosin light chain (MLC) in porcine coronary artery smooth muscle cells. **A** and **C**, The representative immunoblots and summaries of the circadian changes in the MLC phosphorylation as analyzed by Phos-tag SDS-PAGE (**A**; $n=3$ for thrombin [+], $n=5$ for thrombin [-]) and conventional SDS-PAGE (**C**; $n=3$). A maximum of 13 samples could be analyzed in 1 gel. The vertical bars between immunoblot images indicate the border between different gels. **B**, The mean values of MLC phosphorylation in **A** were subjected to an analysis of frequency with an autoregressive model. The samples were obtained before (**A**) and 2 minutes after the stimulation with 1 U/mL of thrombin (**A** and **C**) at the indicated times after synchronization of the circadian rhythm. The immunoblot detection was performed with the anti-MLC antibody in **A** or antibodies specific for the MLC monophosphorylated (P-MLC^{Ser19}) and the MLC diphosphorylated (PP-MLC^{Thr18+Ser19}) in **C**. In **A**, 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 hours. All of the data are expressed as the mean \pm SEM.

observations seen with endothelin 1 stimulation were similar to those seen with thrombin stimulation.

Role of ROR α in the Circadian Changes in the Transcription of ROCK2 in Vascular Smooth Muscle Cells

Among the regulatory elements for the known clock gene products, 2 ROR response elements (ROREs) separated by 102 to 108 nucleotides are preserved in the promoter region of the *rock2* gene among various mammalian species (Table S2, available in the online-only Data Supplement). REV-ERB α , REV-ERB β , ROR α , ROR β , and ROR γ are all capable of binding to RORE.^{20,21} The luciferase promoter assay with the -1337 to +74 nucleotide region of the human *rock2* gene showed that ROR α and ROR γ , but not ROR β , REV-ERB α or REV-ERB β , increased the promoter activity >5-fold the level seen

with control vectors in PCSMCs (Figure 5A). The endogenous expression of ROR α exhibited circadian changes, with a phase that peaked \approx 4 hours earlier than the ROCK2 protein expression (Figure 5B versus Figure 4B), whereas the expression of ROR γ showed no apparent circadian oscillation (Figure 5B). The suppression of the ROR α expression by siRNA abolished the circadian rhythm of the ROCK2 expression, whereas a control siRNA had no effect (Figure 5C).

ROR α -Mediated Regulation of Circadian Changes in ROCK2 Expression and Myofilament Sensitivity to Ca²⁺ in the Mouse Aorta

The expression of ROCK2 protein (Figure 6A and 6B) but not ROCK1 protein (Figure S4, available in the online-only Data Supplement) in the aortas of wild-type mice kept under a 12-hour dark-light cycle exhibited a circadian oscillation,

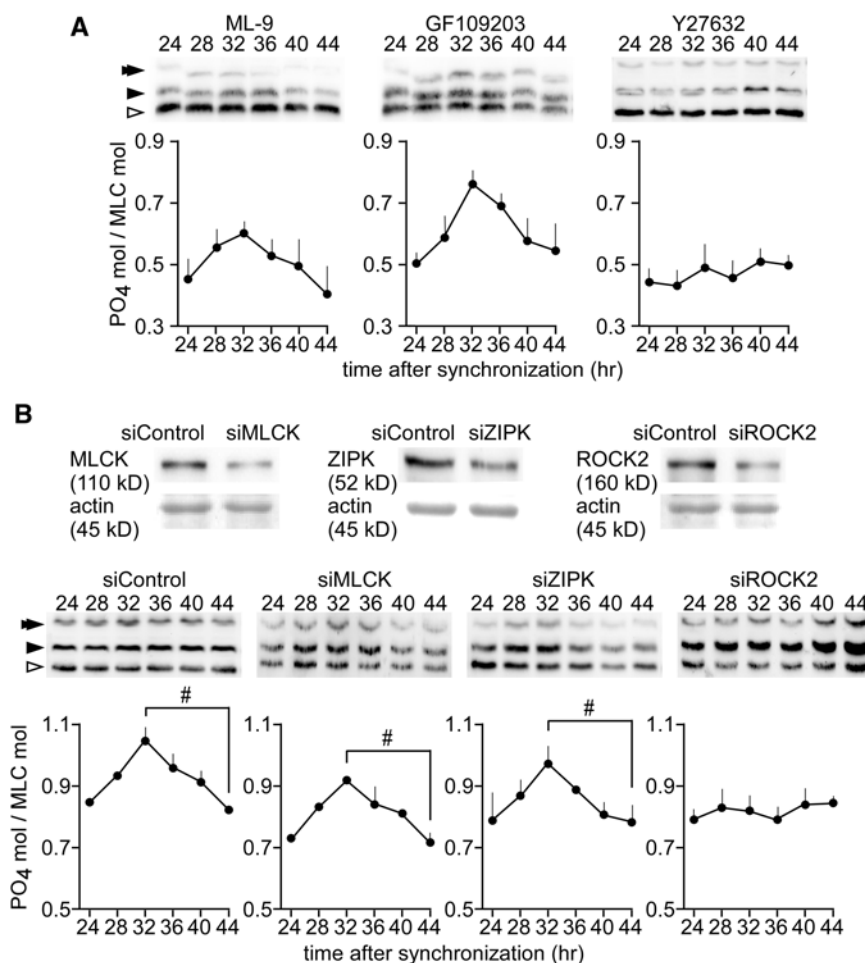


Figure 3. The involvement of Rho-associated kinase 2 (ROCK2) in the circadian changes in the thrombin-induced myosin light chain (MLC) phosphorylation in porcine coronary artery smooth muscle cells. **A**, The circadian changes in the MLC phosphorylation induced by 1 U/mL of thrombin in the presence of 10 μ mol/L of ML-9, 1 μ mol/L of GF109203, and 3 μ mol/L of Y27632 ($n=3$). The cells were treated with inhibitors for 10 minutes before and during thrombin stimulation at the indicated times after synchronization of the circadian rhythm. **B**, Representative immunoblots of MLC kinase (MLCK), Zipper-interacting kinase (ZIPK), and ROCK2 in the cells transfected with control small interfering RNA (siRNA) or targeted siRNA and the Phos-tag SDS-PAGE analysis of the effects of the knockdown of MLCK, ZIPK, and ROCK2 on the circadian changes in the MLC phosphorylation induced by 1 U/mL of thrombin ($n=3-4$). The open, single, and double arrowheads indicate non-, mono-, and di-phosphorylated MLC, respectively. All of the data are expressed as the mean \pm SEM; # $P<0.05$ (Student t test).

with a peak at Zeitgeber time (ZT) 0/24, which corresponds to ≈ 36 hours after dexamethasone pulse treatment in the cultured cells.^{6,15} The expression of ROR α also exhibited a circadian change, with a phase peak 4 hours earlier than that of ROCK2 (Figure 6A and 6B). In the aortas of *staggerer* mice, which lack a functional ROR α , the ROCK2 expression did not show any apparent circadian changes (Figure 6A and 6B). Instead, the level of ROCK2 protein in *staggerer* mice was similar to that seen at the nadir (ZT12) in the aortas of wild-type mice (Figure 6C). The level of MLCK was also similar between wild-type and *staggerer* mice at ZT12 (Figure 6C).

ROCK plays an important role in modulating the myofilament Ca^{2+} sensitivity.^{11,18} The functional relevance of the ROR α -mediated circadian expression of ROCK2 was evaluated using the α -toxin-permeabilized preparations, which allowed us to directly evaluate the myofilament Ca^{2+} sensitivity by examining the contraction at a fixed concentration of Ca^{2+} . GTP γ S, a nonhydrolyzable GTP analog, was used to induce the myofilament Ca^{2+} sensitization.¹⁴

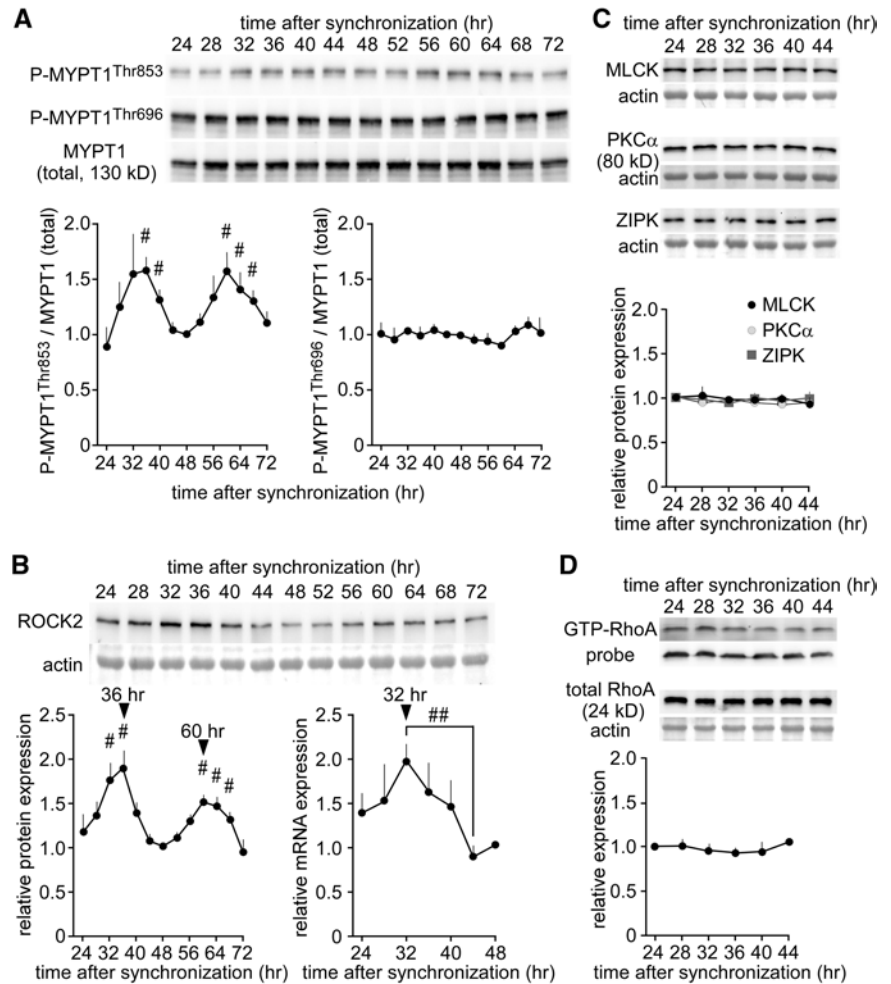
First, the Ca^{2+} -dependent contraction was examined by increasing the Ca^{2+} concentrations in a stepwise manner in the absence of any simulation of Ca^{2+} sensitivity. The p Ca^{2+} -tension relationship of this contraction did not differ between ZT0 and ZT12 in both wild-type and *staggerer* mice (Figure 7A, GTP γ S [–]). However, the p Ca^{2+} -tension relationship seen in *staggerer* mice shifted to the left of that seen in the

wild-type mice (Figure 7A, GTP γ S [–]). The diurnal change in the Ca^{2+} -induced contraction became prominent in the presence of GTP γ S in the wild-type mice (Figure 7A, GTP γ S [+]). The aortic ring preparations were first contracted with 0.3, 0.5, or 1.0 μ mol/L of Ca^{2+} and then stimulated with 10 μ mol/L of GTP γ S (Figure 7B). The level of tension obtained with 10 μ mol/L of GTP γ S and either 0.3 or 0.5 μ mol/L of Ca^{2+} at ZT0 was significantly greater than that of the corresponding contraction seen at ZT12 in the wild-type mice (Figure 7A, GTP γ S [+]). However, the contraction seen with 10 μ mol/L of GTP γ S and 1 μ mol/L of Ca^{2+} , which reached closer to the maximal level of contraction, was similar at ZT0 and ZT12. In contrast, there was no significant difference in the GTP γ S-induced contraction between ZT0 and ZT12 in the *staggerer* mice (Figure 7A, GTP γ S [+]).

Circadian Changes in Myofilament Sensitivity to Ca^{2+} and MLC Phosphorylation Induced by U46619 in the Mouse Aorta

The present study examined whether the circadian change in myofilament Ca^{2+} sensitivity was observed for the receptor-mediated contraction. The Ca^{2+} -sensitizing effect of a thromboxane A2 analog, U46619, was examined at ZT0 and ZT12 in the α -toxin permeabilized aortic ring preparations. The contraction was induced by 1 μ mol/L of U46619 in the presence of 10 μ mol/L of GTP during the 0.5- μ mol/L

Figure 4. The circadian changes in the activity and the expression of Rho-associated kinase 2 (ROCK2) in porcine coronary artery smooth muscle cells. **A**, The circadian changes in the level of MYPT1 phosphorylation at residues corresponding with Thr853 (n=5) and Thr696 (n=4) in human MYPT1 obtained 2 minutes after the stimulation with 1 U/mL of 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 circadian patterns of the protein levels of MLC kinase (MLCK), protein kinase C (PKC)- α , and Zipper-interacting kinase (ZIPK; n=3). **D**, The circadian pattern of the activity of RhoA as evaluated by the level of the GTP-bound form of RhoA in the total RhoA (GTP-RhoA/total RhoA) obtained 2 minutes after the stimulation with 1 U/mL of thrombin (n=4). The values of GTP-RhoA/total RhoA were obtained after normalizing the levels of GTP-RhoA and total RhoA to those of the pull down probe and actin, respectively. The data are expressed as the relative values to those obtained at 48 hours (A and B) and 24 hours (C and D). All of the data are expressed as the mean \pm SEM; # P <0.05 vs 48 hours (Steel test; A and B, left). ## P <0.01 (Student t test; B, right).



Ca²⁺-induced contraction, as reported previously (Figure 8A).²² The extent of the contraction seen at ZT0 was significantly greater than that seen at ZT12 in wild-type mice (Figure 8A). In contrast, there was no significant difference in the U46619-induced contraction between ZT0 and ZT12 in the *staggerer* mice (Figure 8A).

In accordance with the diurnal change in the Ca²⁺-sensitizing effect of U46619, the U46619-induced MLC phosphorylation also exhibited a circadian change (Figure 8B). MLC phosphorylation was analyzed 5 and 25 minutes after the stimulation with 1 μ mol/L of U46619 in the presence of 10 μ mol/L of GTP during the 0.5- μ mol/L Ca²⁺-induced contractions. The MLC phosphorylation at ZT0 at both 5 and 25 minutes was significantly greater than that seen at ZT12 in the wild-type mice (Figure 8B). In contrast, there was no significant difference in the U46619-induced MLC phosphorylation between ZT0 and ZT12 in the *staggerer* mice (Figure 8B).

Discussion

The present study elucidated, for the first time, the existence of the vascular clock mechanism intrinsic to the smooth muscle that generates the circadian oscillation of the myofilament Ca²⁺ sensitivity with a peak at the beginning of the light phase, in a manner independent of external cues. The present study further delineated the signaling pathway from

the clock gene to the circadian rhythm of the myofilament Ca²⁺ sensitivity. The circadian oscillation of the expression of a clock gene, *ROR α* , appears to be translated to the oscillation of ROCK2 transcription, which in turn generates the oscillation of MLC phosphorylation in response to contractile stimuli. ROCK2-mediated potentiation of MLC phosphorylation is an important mechanism underlying myofilament Ca²⁺ sensitivity.^{23,24} Myofilament Ca²⁺ sensitivity plays a critical role in determining the extent of the vascular response to contractile stimuli and vascular contractility.^{18,24} As a result, the present study suggests ROCK2 to be a key oscillator generating the circadian rhythm of myofilament Ca²⁺ sensitivity and vascular contractility. The circadian oscillation of the MLC phosphorylation induced by thrombin and endothelin 1 was consistently observed by the 2 different analyses. The Phos-tag SDS-PAGE analysis allowed us to perform a stoichiometric evaluation of MLC phosphorylation for each time point in a self-contained manner, thus minimizing the influence of the variations in the amount of proteins loaded. The analysis with phosphor-specific antibodies further revealed that the circadian oscillation of the agonist-induced MLC phosphorylation was mainly attributed to PP-MLC^{Thr18+Ser19}. In the Phos-tag SDS-PAGE analysis, both the resting and stimulated levels of MLC phosphorylation transiently increased during the 4 to 8 hours after the dexamethasone pulse treatment. Pulse treatment

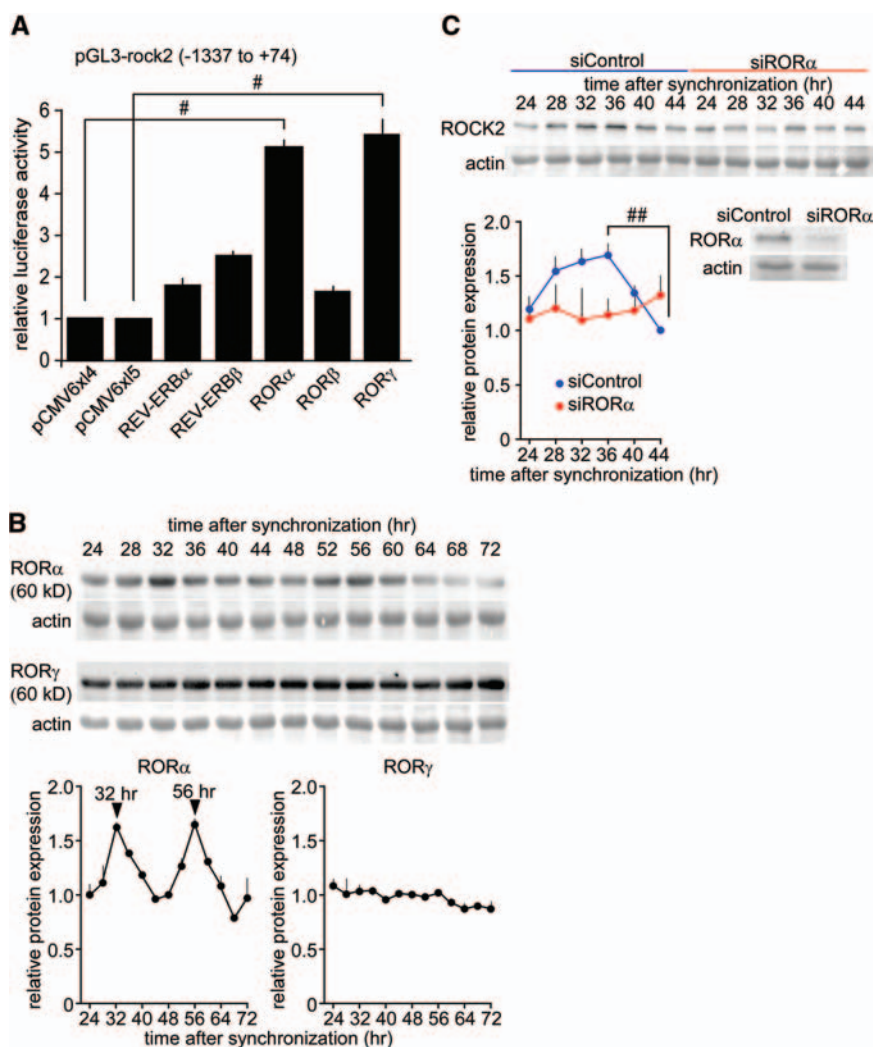


Figure 5. ROR α regulation of the circadian expression of Rho-associated kinase 2 (ROCK2) in porcine coronary artery smooth muscle cells. **A**, The effects of REV-ERB α , REV-ERB β , ROR α , ROR β , and ROR γ on the activity of the human *rock2* promoter, as evaluated by the luciferase activity. pCMV6x4 was used as a vector for REV-ERB α and ROR α , whereas pCMV6x5 was used for REV-ERB β , ROR β , and ROR γ (n=3). The data are expressed as the relative values to those obtained with the corresponding empty vectors. **B**, The circadian changes of the endogenous expression of ROR α and ROR γ (n=3). The data were expressed as relative values to those obtained at 48 hours. **C**, The effects of the knockdown of ROR α on the circadian changes in the expression of the ROCK2 protein (n=4). The data are expressed as the relative values to those obtained with a control small interfering RNAs (siRNAs) at 44 hours. All of the data are expressed as the mean \pm SEM; #*P*<0.05, ##*P*<0.01 (Student *t* test).

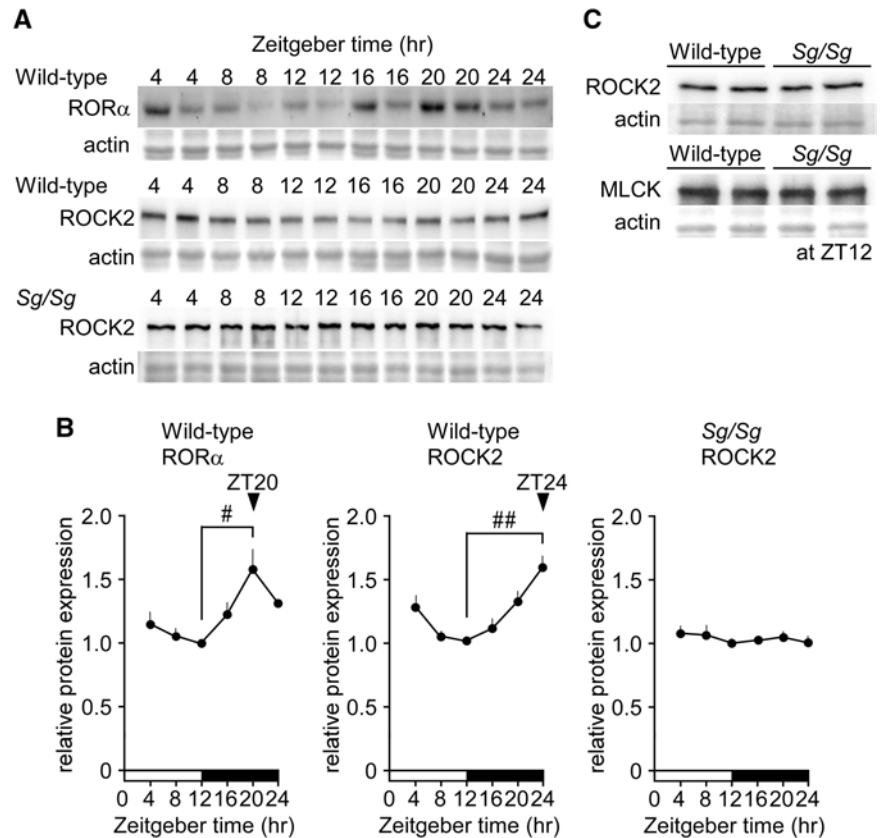
with dexamethasone has been shown to induce an early surge of the expression of various genes, including not only clock genes, such as *per1*, but also *c-fos* or β -actin, which do not show any oscillation.^{25,26} Therefore, the transient increase in MLC phosphorylation may be attributable to the expression of not only clock-controlled genes but also unrelated genes that can affect the MLC phosphorylation. As a result, most of the analyses of circadian rhythm were performed starting from 24 hours after the dexamethasone pulse treatment.

Because the circadian oscillation of MLC phosphorylation was observed with both thrombin and endothelin 1, the mechanisms regulating MLC phosphorylation common to both agonists are likely responsible for the oscillation. MLCK is a major kinase that phosphorylates MLC in a Ca²⁺-dependent manner,²⁷ whereas ROCK, ZIPK, and integrin-linked kinase phosphorylate MLC in a Ca²⁺-independent manner.¹⁸ Any of these kinases could induce PP-MLC^{Thr18+Ser19}.^{18,28} On the other hand, the dephosphorylation of MLC is mainly catalyzed by a type 1 phosphatase consisting of 3 subunits.²⁹ The activity of this MLC phosphatase is suppressed either when MYPT1 is phosphorylated by ROCK, ZIPK, or integrin-linked kinase or when an inhibitor protein, CPI-17, is phosphorylated by ROCK or PKC.^{18,29} The pharmacological inhibitors of MLCK, PKC, and ROCK suppressed the thrombin-induced MLC

phosphorylation to a similar level. However, only the ROCK inhibitor abolished the circadian oscillation of the MLC phosphorylation. The knockdown of MLCK, ZIPK, and ROCK2 suppressed the thrombin-induced MLC phosphorylation to a similar level at a nadir, whereas only ROCK2 knockdown abolished the circadian oscillation. It should be noted that an \approx 50% reduction of ROCK2 expression was sufficient to abolish the circadian rhythm of MLC phosphorylation. The specificity of this phenomenon was supported in that only ROCK2 knockdown was effective in suppressing the circadian rhythm of MLC phosphorylation, whereas the degree of knockdown was similar among the 3 kinases.

There are 2 isoforms of ROCK, ROCK1 and ROCK2, which share 65% overall homology at the amino acid level.³⁰ ROCK2 is the major isoform in gizzard smooth muscle, and ROCK2 plays a predominant role in the regulation of vascular smooth muscle contraction.^{28,31} An in silico analysis revealed that the promoter regions of the mammalian *rock1* genes lack any regulatory element for the known clock genes. Indeed, no obvious circadian rhythm was observed for the ROCK1 protein expression in the aorta of wild-type mice (Figure S4, available in the online-only Data Supplement). Therefore, ROCK2 is suggested to play a key role in the circadian rhythm of MLC phosphorylation.

Figure 6. The absence of the circadian changes in the expression of Rho-associated kinase 2 (ROCK2) in the aortas of *staggerer* mice. **A** and **B**, The representative immunoblots (**A**) and summaries (**B**) showing the circadian patterns of the expression of the ROR α and ROCK2 proteins in the aortas of wild-type and *staggerer* (*Sg/Sg*) mice that were housed under 12-hour light (open bar) and 12-hour dark (closed bar) cycle conditions. The expression levels are expressed as the relative values to those obtained at Zeitgeber time 12 (ZT12). All of the data are expressed as the mean \pm SEM ($n=5-6$ for ROR α 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 t test). **C**, The expression of ROCK2 and myosin light chain (MLC) kinase (MLCK) proteins in the aortas of wild-type and *Sg/Sg* mice at ZT12 was directly compared on the same gel.



The present study further demonstrates that the circadian oscillation of the expression and activity of ROCK2 correlate with the circadian rhythm of the MLC phosphorylation. ROCK is known to phosphorylate MYPT1 at both Thr696 and Thr853 in humans, with a 3-fold preference for Thr853 over Thr696.¹⁹ Thr696 is also phosphorylated by other kinases, including ZIPK and integrin-linked kinase.^{18,19} Therefore, the phosphorylation of Thr853 more accurately indicates the activity of ROCK. The observations of the present study thus indicated that the rhythm of ROCK2 expression was correlated with the rhythm of activity (the phosphorylation of Thr853). The circadian oscillation of Thr853 phosphorylation and the in-phase oscillation of MLC phosphorylation were similarly observed after stimulation with thrombin and endothelin 1. A key role of ROCK2 in generating the circadian rhythm of the MLC phosphorylation is thus consistent as a mechanism common to both agonists. ROCK can modulate MLC phosphorylation either by inhibiting MLC phosphatase activity via the phosphorylation of MYPT1 or CPI-17 or by directly phosphorylating MLC.^{18,29} However, the basal level of MLC phosphorylation did not show any apparent circadian oscillation. The circadian oscillation of MLC phosphorylation was apparently attributed to the agonist stimulation. Furthermore, the substrate specificity of ROCK for MYPT1 (Michaelis constant value, 0.1–0.2 μ mol/L) was higher than that for MLC (2.5–5.0 μ mol/L).³¹ It is therefore conceivable that ROCK2 generates the circadian oscillation of MLC phosphorylation mainly through the inhibition of the MLC phosphatase activity.

We concluded that the oscillation of ROCK2 expression was regulated by a clock gene, ROR α , based on the following

observations: the luciferase promoter assay demonstrated that the human *rock2* promoter was responsive to ROR α and ROR γ . The expression of ROR α , but not ROR γ , exhibited circadian oscillation in phase with that of ROCK2 mRNA. The rhythmic expression of ROCK2 was abolished by knocking down the expression of ROR α . The 2 ROREs, which are separated by a 102- to 108-nucleotide interval, are well preserved in the promoter regions of the mammalian *rock2* genes (Table S2, available in the online-only Data Supplement). RORE is known to be responsible for gene expression during the dark phase.²⁰ This role of RORE is consistent with the observation that the expression of ROCK2 protein peaked at 36 and 60 hours after the dexamethasone pulse treatment, both of which correspond with the transition from the dark phase to the light phase.^{6,15} There are 3 isoforms of ROR, and the rhythmic expression of each isoform shows tissue specificity.^{12,21} ROR α expression is rhythmic in white adipose tissue but not in brown adipose tissue, liver, or muscle, whereas ROR γ expression is rhythmic specifically in brown adipose tissue and liver.²¹ ROR α thus appears to play a major role in the transcriptional regulation of the circadian rhythm of ROCK2 expression in vascular smooth muscle.

The physiological significance of the observations in the cultured smooth muscle cells was demonstrated by using the aortas of *staggerer* mice, which lack a functional ROR α .¹² The use of the aorta is supported by the observation that the circadian oscillation of ROCK2 expression was similarly observed in both PCSMCs and porcine aortic smooth muscle cells. As a result, the ROR α -mediated circadian expression of ROCK2 was demonstrated to occur both in vivo and in culture.

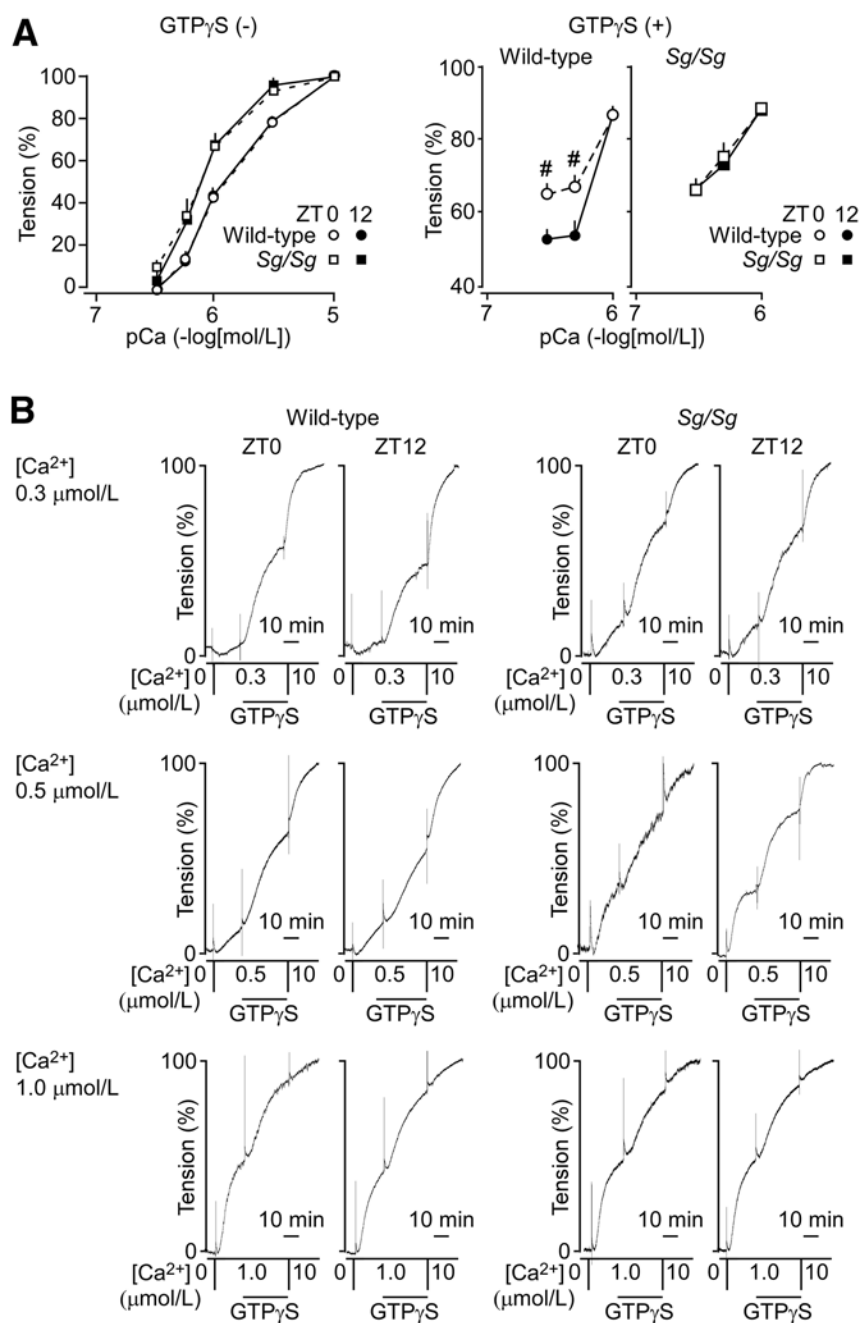


Figure 7. The circadian changes in the Ca $^{2+}$ sensitivity of the contractile apparatus in the α -toxin-permeabilized preparations of aortas of wild-type and *staggerer* mice. **A**, The pCa $^{2+}$ -tension curves of the contractions induced by stepwise increases in the Ca $^{2+}$ concentrations in the absence of GTP γ S (GTP γ S [-]) and the contractions induced by 10 μ mol/L of GTP γ S and 0.3, 0.5, or 1.0 μ mol/L of Ca $^{2+}$ (GTP γ S [+]), in the wild-type ($n=4-5$) and *staggerer* (Sg/Sg; $n=4-5$) mice at Zeitgeber time (ZT) 0 and 12. **B**, Representative recordings showing the experimental protocol used to examine the contractions induced by 10 μ mol/L of GTP γ S and 0.3, 0.5, or 1.0 μ mol/L of Ca $^{2+}$. The level of tension obtained with different Ca $^{2+}$ concentrations in either the presence or absence of GTP γ S was expressed as a percentage, whereas the levels of tension obtained in Ca $^{2+}$ -free solution and with 10 μ mol/L of Ca $^{2+}$ were assigned a value of 0% and 100%, respectively. All of the data are expressed as the mean \pm SEM; # $P<0.05$ vs ZT12.

The functional significance of the oscillation of ROCK2 expression in the regulation of smooth muscle contraction was demonstrated by using α -toxin-permeabilized preparations. The contraction of smooth muscle is regulated by Ca $^{2+}$ signaling and the change in the myofilament Ca $^{2+}$ sensitivity.¹¹ ROCK plays an important role in modulating the myofilament Ca $^{2+}$ sensitivity.^{11,18} The use of permeabilized preparations allowed the focused investigation on the myofilament Ca $^{2+}$ sensitivity and thereby enabled the successful detection of the diurnal changes in the myofilament Ca $^{2+}$ sensitivity as a consequence of the circadian oscillation of ROCK2 expression. The results indicated that the myofilament Ca $^{2+}$ sensitivity increased in association with an increase in MLC phosphorylation when the ROCK2 expression reached a peak (ZT0/24) and decreased when the ROCK2 expression reached

a nadir (ZT12). These diurnal changes were abolished in the *staggerer* mice. In contrast, there were no significant diurnal changes in the Ca $^{2+}$ -dependent contractile mechanism. These findings thus suggest that the ROR α -mediated circadian oscillation of ROCK2 expression is translated specifically to the oscillation of the myofilament Ca $^{2+}$ sensitivity by modulating the MLC phosphorylation.

It was noticed that the Ca $^{2+}$ -tension relationship of the Ca $^{2+}$ -induced contraction in the *staggerer* mice shifted to the left of that obtained in the wild-type mice. The precontractions induced by Ca $^{2+}$ before the application of GTP γ S or U46619 in the *staggerer* mice were higher than those seen in the wild-type mice. These observations suggest that the activity of some Ca $^{2+}$ -dependent contractile mechanisms was enhanced in *staggerer* mice. However, the level of MLCK expression

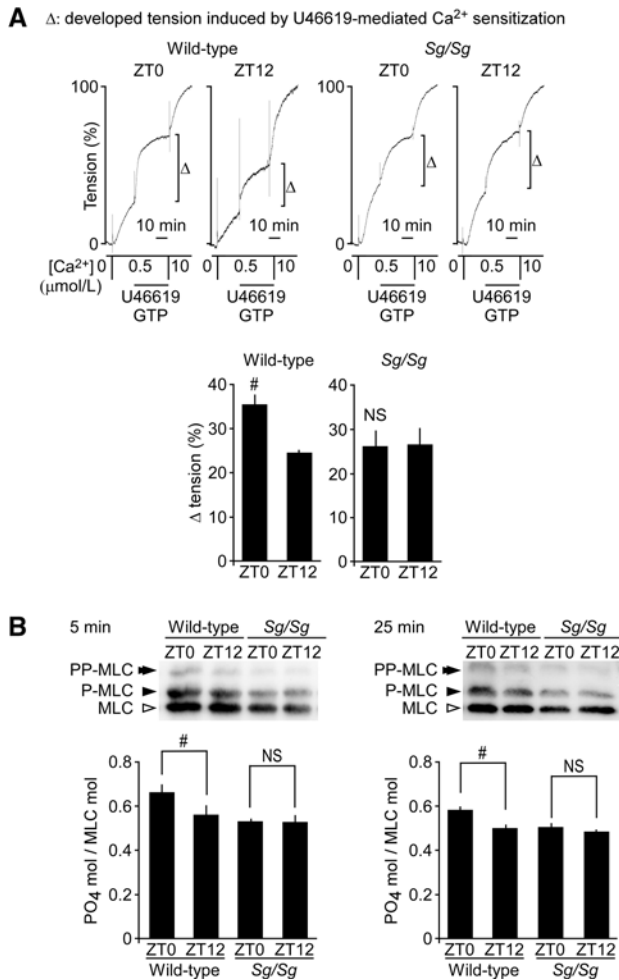


Figure 8. The circadian changes in the Ca^{2+} sensitivity and myosin light chain (MLC) phosphorylation induced by U46619 in the α -toxin-permeabilized preparations of aortas of wild-type and *staggerer* mice. Representative recordings and summaries of the contractions (**A**) and MLC phosphorylation (**B**) induced by 1 $\mu\text{mol/L}$ of U46619 in the presence of 10 $\mu\text{mol/L}$ of GTP during the 0.5- $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ of Ca^{2+} . MLC phosphorylation was analyzed using Phos-tag SDS-PAGE. All of the data are expressed as the mean \pm SEM. # $P<0.05$; NS, not significantly different vs ZT12.

in the *staggerer* mice was similar to that seen in the wild-type mice. The precise mechanism underlying this enhancement of the Ca^{2+} -induced contraction in *staggerer* mice remains to be investigated.

In conclusion, the present study revealed that the vascular intrinsic clock mechanism involving ROR α generates the circadian rhythm of myofilament Ca^{2+} sensitivity. ROCK2 was identified as a key oscillator generating the circadian rhythm of the response of MLC phosphorylation and myofilament Ca^{2+} sensitivity. It is conceivable that the interplay between the central and the peripheral clocks plays an important role in determining the circadian rhythm of vascular contractility. How this vascular intrinsic rhythm of myofilament Ca^{2+} sensitivity interplays with the external cues from the central clock mechanism remains to be elucidated. Furthermore, the

intrinsic rhythm of the myofilament Ca^{2+} sensitivity peaks at the beginning of the light phase, when the occurrence of cardiovascular diseases also peaks. The increased ROCK activity is suggested to play an important role in the pathogenesis of coronary vasospasm in both animal models and patients with vasospastic angina.^{10,32,33} The ROCK-mediated oscillation of myofilament Ca^{2+} sensitivity is therefore suggested to underlie the onset of cardiovascular events. However, such a pathological role of the vascular clock mechanism remains to be investigated.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The cardiovascular system displays circadian rhythms in some physiological parameters, including blood pressure. The occurrence of pathological events, such as myocardial infarction and angina pectoris, also exhibits circadian variation. The circadian changes in vascular contractility underlie these physiological and pathological circadian events. The present study elucidated the details of the vascular intrinsic clock mechanism that regulate vascular contractility. The most prominent achievement of the present study is the identification of ROCK2 as a clock-regulated gene. The circadian oscillation of the expression of a clock gene, *ROR α* , is translated to the oscillatory expression of ROCK2, which in turn generates the oscillation of myofilament Ca^{2+} sensitivity and vascular contractility. ROCK2 plays an important role in the regulation of smooth muscle contraction, especially under pathological setting. Furthermore, ROCK2 regulates smooth muscle growth and contributes to the development of vascular lesions. Therefore, it remains to be investigated how this intrinsic vascular clock is, if at all, modified under pathological conditions and how it contributes to the pathogenesis and pathophysiology of cardiovascular diseases, such as hypertension, coronary vasospasm, or atherosclerosis. In addition, vascular function is regulated by the interplay between the central and peripheral clocks. How these 2 clock systems cross-talk to each other and how they regulate vascular function as an integrated system remain to be elucidated. The present study thus provides a novel conceptual insight into vascular biology regarding the circadian regulation of vascular contractility and thereby contributes to understanding the pathogenesis of cardiovascular disease and developing new therapeutic strategies.

Pivotal Role of Rho-Associated Kinase 2 in Generating the Intrinsic Circadian Rhythm of Vascular Contractility

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SUPPLEMENTAL MATERIAL

Expanded Methods

Cell culture and dexamethasone pulse treatment

The cultured porcine coronary artery and aortic smooth muscle cells were prepared as described previously.¹ The cells were grown in Dulbecco's Modified Eagle Medium (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum, penicillin and streptomycin in a 37°C incubator maintained at 5% CO₂. The cells were cultured until semi-confluence for 3-4 days. The cells at semi-confluence were incubated with 100 nmol/L dexamethasone in growth media for 2 hr to induce a synchronized circadian rhythm.

Animals

Staggerer mice carry an intragenic deletion in the coding region of the *rora* gene, which causes a frame shift in the coding region, and leads to the expression of the dominant negative ROR α , thereby leading to a functional knockout phenotype.² The original genetic background of the B6C3Fe-a/a-Rora^{sg} mice obtained from Jackson Laboratory (Bar Harbor, ME, U.S.A) was adjusted by crossing these mice with C57BL/6J inbred mice purchased from CLEA Japan (Tokyo, Japan) for more than 10 generations, as described previously.³ Homozygous *staggerer* mice were obtained by crossing heterozygous male and female breeders. The identification of homozygotes was carried out by PCR genotyping as described by Jackson Laboratory. The C57BL/6J (CLEA Japan) mice were used as wild-type controls. The male wild-type and homozygous *staggerer* mice (6-7 weeks old) were housed in a temperature-controlled room under a 12 hour light/12-hour dark cycle with *ad libitum* access to food and water. Zeitgeber times (ZT) 0 and 12 were designated as lights on and off, respectively. The study protocol was approved by the Animal Care and Use Committee of Kyushu University. The animals were treated in accordance with the guidelines stipulated by the committee.

Real-time PCR analysis

The total RNA was extracted from cultured cells using the RNeasy kit (Qiagen, Hilden, Germany) and cDNA was synthesized using 2 μ g total RNA, a random primer, and ReverTra-plus reverse transcriptase (Toyobo, Osaka, Japan). Five microliters of reverse transcription product was then subjected to a real-time PCR analysis with FastStart SYBR Green Master kit using a LightCycler (Roche, Basel, Switzerland). The sequences of primers used in PCR reactions were as follows; ATT TCC CCT CCA CCT gCT C (forward primer

for *bmal1*), CgT TgT CTg gTT CgT TgT CTT C (reverse primer for *bmal1*), ggC AgC ggT TAC gAT TgA (forward primer for *rev-erba*), AAg CAT CCA gCA gAA CAT CC (reverse primer for *rev-erba*), gCT CAT CgC AgA gCg TAT CC (forward primer for *per1*), gTg TgC CgT gTg gTg AAg A (reverse primer for *per1*), gTC CTT CTg CCC ATT TTg CTA (forward primer for *cry1*), CAC AgC AgC AAC AAA TAA TCC AC (reverse primer for *cry1*), CAT gTA TgA AgA Tgg ATg AAA CAg g (forward primer for *rock2*), AAA CAC CCA CAg ACC ACC AA (reverse primer for *rock2*), gTg Cgg gAC ATC AAg gAg AA (forward primer for β -actin) and TgT CCA CgT CgC ACT TCA T (reverse primer for β -actin). The expression level of each mRNA was normalized to the level of β -actin obtained from the corresponding RT product.

Western blot analysis

The proteins from cultured smooth muscle cells or mice aortas were extracted in the lysis buffer (50 mmol/L Tris-HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl, 10 mmol/L MgCl₂, 10 μ g/mL leupeptin, 10 μ g/mL aprotinin, 10 μ mol/L 4-aminidophenylmethane sulfonyl fluoride, 5 μ mol/L microcystin, 20 μ mol/L NaF, 1 mmol/L Na₃VO₄). The 5-20 μ g protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, U.S.A.). After blocking with either 5% non-fat dry milk (Wako Pure Chemical, Tokyo, Japan), blocking oneP (Nacalai Tesque, Kyoto, Japan) or 7% blockace (DS phamabiomedical, Osaka, Japan), the membranes were incubated with the indicated primary antibodies and appropriate secondary antibodies conjugated with horseradish peroxidase. The primary antibodies used were generated against MLC (sc-15370; Santa Cruz, Santa Cruz, CA, U.S.A.), phosphor-MLC at Ser19 (#3671; Cell signaling, Beverly, MA, U.S.A.), phosphor-MLC at Thr18 and Ser19 (#3674; Cell signaling), MYPT1 (612164; BD Transduction, San Jose, CA, U.S.A.), phosphor-MYPT1 at Thr696 (#07-251; Upstate; Lake Placid, NY, U.S.A.), phosphor-MYPT1 at Thr853 (#4563; Cell signaling), ROCK2 (610624; BD Transduction), ROCK1 (#4035; Cell signaling), MLCK (ab76092; Abcam, Cambridge, UK), ZIPK (#2928; Cell signaling), PKC α (sc-8393; Santa Cruz), ROR α (sc-28612; Santa Cruz) and ROR γ (sc-28559; Santa Cruz). The immune complex was detected with an ECL plus detection kit (GE Healthcare, Buckinghamshire, UK). The light emission was detected and analyzed with a ChemiDoc XRS-J instrument and the computer program Quantity One (BioRad). The corresponding actin bands were detected with naphthol blue black staining in most of the analyses. In Phos-tag SDS-PAGE analysis, they were immunologically detected using a primary antibody for β -actin (Sigma) and an appropriate secondary antibody conjugated with

horseradish peroxidase, because the amount of β -actin in the protein samples used for the Phos-tag SDS-PAGE analysis was too small to be quantitatively detected by naphthol blue black staining. The density of the immunoreactive band was normalized to the density of the corresponding actin band to adjust for any possible variations in sample loading. The image capture time was adjusted to keep the intensity of the imaging below the maximum limit of the system, and therefore within a linear range.

Phos-tag SDS-PAGE analysis of the MLC phosphorylation

The extracts of cultured smooth muscle cells were prepared as described above. The samples for the analysis of the α -toxin-permeabilized aortic ring preparations were obtained during the measurement of tension, as previously described.⁴ The bathing buffer was promptly changed to 90% (v/v) acetone, 10% (w/v) trichloroacetic acid, and 10 mmol/L dithiothreitol (DTT) prechilled at -80°C to stop the reaction, at the indicated time points. The specimens were then transferred into microcentrifuge tubes, and were then extensively washed and stored in acetone containing 10 mmol/L DTT at -80°C. The specimens were air-dried to remove acetone, and each ring preparation was extracted in 25 μ L of the sample buffer (50 mmol/L Trishydroxymethyl aminomethane, 2% (w/v) SDS, 5% (v/v) glycerol, 0.01% (w/v) NaN₃, 0.01% (w/v) bromophenol blue, and 5% (v/v) β -mercaptoethanol). The supernatant was heated to 100°C for 5 min before electrophoresis. The protein samples were then separated by SDS-PAGE on 12.5% polyacrylamide gels containing 30 μ mol/L Phos-tagTM (NARD Institute, Amagasaki, Japan) and 60 μ mol/L MnCl₂. Five μ g protein samples were used for the analysis of the cultured cells. However, the protein concentrations of the samples of aortic ring preparations were not determined, because of the interference by the composition of the sample buffer. Therefore, an equal volume of the sample (5 μ L) was loaded into the gels. After electrophoresis, the gel was soaked in transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, 10% methanol) containing 2 mM EDTA to remove Mn²⁺ for 40 min, and then in transfer buffer without EDTA for 20 min. Proteins were then transferred to polyvinylidene difluoride membranes (Bio-Rad). After blocking with 5% non-fat dry milk, all forms of MLC were detected using an anti-MLC antibody (sc-15370; Santa Cruz) and a horseradish peroxidase-conjugated secondary antibody. The detection and analysis of the enhanced chemiluminescence signal were performed as described above. The level of MLC phosphorylation (PO₄ mol/MLC mol) was calculated as follows:

$$\text{MLC phosphorylation} = (\text{P-MLC} + \text{PP-MLC} \times 2) / (\text{MLC} + \text{P-MLC} + \text{PP-MLC})$$

where MLC, P-MLC, PP-MLC indicate the optical density of the non-, mono- and di-phosphorylated forms of MLC.

Transfection of small interfering RNA (siRNA)

The partial nucleotide sequences for the porcine ROCK2 and ZIPK were determined using RT-PCR products of porcine coronary artery smooth muscle cells, and they have been deposited to the DDBJ/EMBL/GenBank under accession numbers: AB671755 (a catalytic domain of ROCK2), AB671756 (coiled-coil domain of ROCK2), AB671757 (RhoA-binding domain of ROCK2), AB671758 (PH domain of ROCK2) and AB671759 (ZIPK). Otherwise, the sequences on the database were used to design the siRNAs. These siRNAs were synthesized with a 3' UU overhang against the following target sequences: GCA gAC AAg AAA CgA AAU UUg (ROCK2), ggg CCA CAg AUA Aug AAA AAA (MLCK), CAA ggA gUA CAC UAU CAA AUC (ZIPK), and CgA gAA gAU ggA AUA CUA A (ROR α), by Dharmacon (Lafayette, CO, U.S.A.). The control siRNA with a sense sequence AAg CUC UUC UAC gUg CUU CUA AUA A, was purchased from Invitrogen (Carlsbad, CA, U.S.A.). The siRNAs were transfected with the HVJ Envelope Vector Kit GenomONE (Ishihara Sangyo, Osaka, Japan) on the next day after plating the cells after being incorporated into the HVJ-envelope vector according to the manufacturer's instructions. The cells were exposed to 100 nmol/L siRNA incorporated in the vector for 2 hr. Thereafter, the cells were cultured for 3 days before subjecting them to dexamethasone pulse treatment.

Pull-down assay for the GTP-bound forms of RhoA

The cell extract was prepared in buffer consisting of 50 mmol/L Tris-HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mmol/L NaCl, 10 mmol/L MgCl₂, 10 μ g/mL leupeptin, 10 μ g/mL aprotinin, and 10 μ mol/L 4-aminidophenylmethane sulfonyl fluoride. The 500 μ g protein samples were incubated with 30 μ g of a pull-down probe for 45 min at 4°C. The RhoA-binding domain (RB; residues 941–1075) of ROCK2 tagged with a (His)₆ tag was used as a pull-down probe, as previously described.⁵ The mixture was then incubated with Ni²⁺-nitrilotriacetic acid resin (Qiagen) at 4°C for 1 h. The resin was collected by a brief centrifugation and thoroughly washed in the resin wash buffer (50 mmol/L Tris-HCl, 1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L MgCl₂, 10 μ g/mL leupeptin, 10 μ g/mL aprotinin, 10 μ mol/L 4-aminidophenylmethane sulfonyl fluoride, pH 7.2), and the bound protein was then eluted in SDS-sample buffer (50 mmol/L Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 5% β -mercaptoethanol, 0.01% NaN₃, 0.01% bromophenol blue) by heating at 100°C for 5 min. Equal volumes (20 μ l) of the resin eluates and 5 μ g cell lysates were subjected to SDS-PAGE and an immunoblot analysis with an anti-RhoA antibody (sc-418; Santa Cruz). The chemiluminescence detection of RhoA was as described above. The bands

of the pull-down probe and actin were detected with naphthol blue black staining after immunoblot detection. The densities of RhoA in the resin eluates and the cell lysates were normalized to those of the pull-down probe and actin, respectively, and then were used to obtain the GTP-bound RhoA/total RhoA ratios.

Construction of the luciferase reporter plasmid for the human rock2 promoter assay

The promoter region of the human *rock2* gene was obtained by screening a λ Dash II human genomic library (Agilent Technology, Tokyo, Japan) with a probe obtained by PCR amplification of a 1734-bp fragment of the human *rock2* gene from human leukocyte genomic DNA. The PCR primers were designed based on the sequences in the NCBI database (Genomic contig NT_005334.15). A promoter region of the *rock2* gene in the genomic clone was then amplified by PCR and subcloned into a luciferase reporter plasmid pGL3-basic (Promega, Tokyo, Japan). The sequences of the insert were confirmed to be identical to those of the clone of the genomic library. The reporter plasmid was thus pGL3-rock2 (−1337 to +74). The number in parentheses indicates the residue numbers at the 5′ - and 3′ ends of the promoter region, respectively, while the first base of the first exon was assigned to be +1.

Luciferase assay

The cells were transfected 24 hr after the initial plating, with 2 μ g pGL3-rock2 (−1337 to +74), 1 μ g expression vector of clock gene (Origene, Rockville, MD, U.S.A.) and 0.5 μ g phRL-TK (Promega) in 2 ml DMEM containing 10 μ l lipofectamine (Invitrogen). The culture medium was replaced 6 hr after transfection, and the promoter activity was evaluated 32 hr after transfection. The clock genes examined were pCMV-XL4-REV-ERB α , pCMV-XL4-ROR α , pCMV-XL5-REV-ERB β , pCMV-XL5-ROR β , and pCMV-XL5-ROR γ . The cells were washed in PBS twice, and then were lysed in passive lysis buffer (Promega). The lysates were kept frozen at −80°C until use. After thawing and clarification of the lysates by a brief centrifugation on a tabletop microcentrifuge (13,000 \times g, 30 sec, room temperature), the luciferase activity was determined using the dual-luciferase reporter assay system (Promega) and a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). The activity of firefly luciferase was normalized to that of renilla luciferase, and the value obtained with the empty vector, pCMV-XL4 or pCMV-XL5, was considered to be 1.

Tension measurement in the α -toxin-permeabilized preparations of mouse aortas

At the indicated Zeitgeber times, mice were euthanized under pentobarbital anesthesia. The ascending aorta was then immediately excised and cut into ring preparations. Next, the aortic rings were permeabilized with 5,000 units/mL staphylococcal α -toxin (Sigma, St. Louis, MO, USA) in cytosolic substitution solution (CSS) for 60 min at 30°C, as previously described.^{4,6} These permeabilized preparations were then mounted between two tungsten wires and were stretched to 1.5-fold their resting length. After obtaining complete relaxation in Ca^{2+} -free CSS, the experimental protocol was started, and the tension development was recorded at 30°C. Each ring preparation was used to examine the contractile response to either a step-wise increase in the Ca^{2+} concentration, 10 $\mu\text{mol/L}$ GTP γ S (Sigma) or 1 $\mu\text{mol/L}$ U46619 (Sigma) at each Zeitgeber time point. The response to GTP γ S was examined during the precontraction induced by 0.3, 0.5 or 1 $\mu\text{mol/L}$ Ca^{2+} . The response to U46619 was examined in the presence of 10 $\mu\text{mol/L}$ GTP during the contraction induced by 0.5 $\mu\text{mol/L}$ Ca^{2+} . The entire procedure, from euthanization to tension measurement, was completed within 1.5-2 hr. The composition of the Ca^{2+} -free CSS was 100 mmol/L potassium methanesulphonate, 2.2 mmol/L Na_2ATP , 3.38 mmol/L MgCl_2 , 10 mmol/L EGTA, 10 mmol/L creatine phosphate, and 20 mmol/L Tris-malate (pH 6.8). CSS containing the indicated concentrations of free Ca^{2+} was prepared by adding an appropriate amount of CaCl_2 , while assuming the Ca^{2+} -EGTA binding constant to be 10^6 L/mol.^{4,6}

Trypan blue exclusion test

A standard Trypan blue exclusion test was used to evaluate the viability of the porcine coronary artery smooth muscle cells after exposure to the pharmacological inhibitors (ML-9, GF109203 and Y27632). The cells cultured in a 35-mm dish were harvested 12-min exposure to the inhibitors by a brief treatment with trypsin in 0.5 mL buffer. The cell suspensions were then immediately mixed with 0.5 mL PBS containing 3 mg/ml Trypan blue, and observed in the hemocytometer under a phase-contrast microscope (Olympus, Tokyo, Japan). The number of trypan blue positive cells and the total cells were counted. The percentage of the trypan blue positive cells in the total cells was calculated to indicate the death rate.

Other materials

Thrombin and endothelin-1 were purchased from Sigma and the Peptide Institute (Osaka, Japan), respectively. ML-9, GF109203 and Y27632 were purchased from Calbiochem (La Jolla, CA, USA).

Data analysis

The data are expressed as the means \pm SEM of the indicated number of experiments or mice. Either Steel's test or Student's *t*-test was used to determine the statistical significance of the differences among groups or between two groups, respectively, as indicated in the figure legends. A value of $P < 0.05$ was considered to be statistically significant.

Supplementary Table 1. Trypan blue exclusion test of porcine coronary artery smooth muscle cells after 12-min exposure to H₂O₂ (10 mmol/L), ML-9 (10 μmol/L), GF109203 (1 μmol/L) or Y27632 (3 μmol/L). The percentage of the trypan blue positive cells out of the total cells was calculated to indicate the death rate.

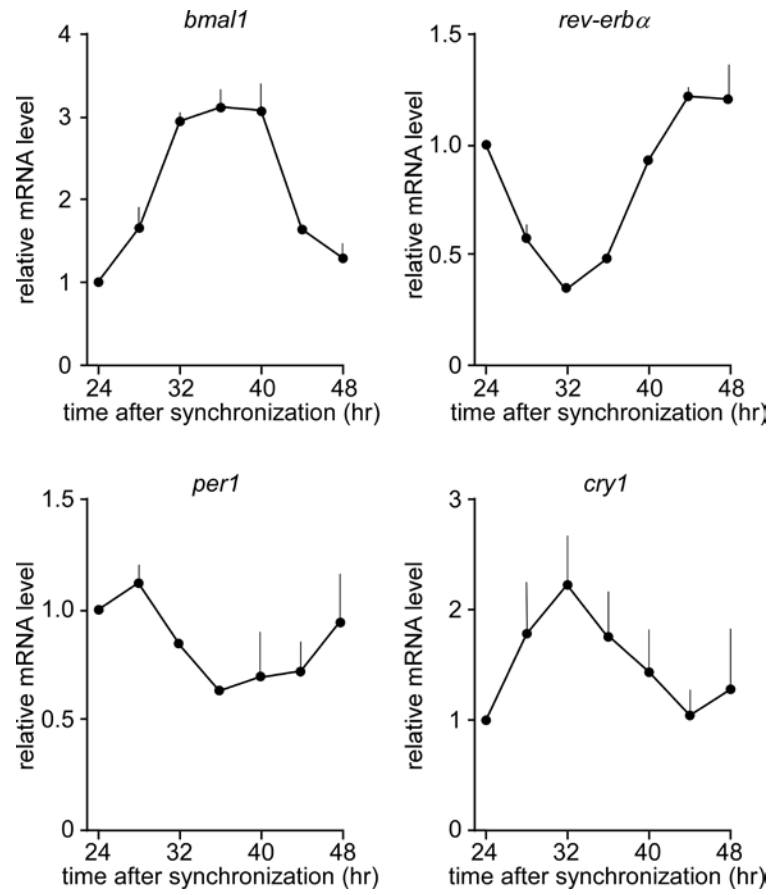
	Numbers of trypan blue positive cell	Numbers of total cell	Death rate (%)
No treatment	10	778	1.29
H ₂ O ₂	411	452	90.9
ML-9	7	487	1.44
GF109203	5	580	0.86
Y27632	9	576	1.56

Supplementary Table 2. A comparison of the ROR response elements in the promoter region of the mammalian *rock2* genes.

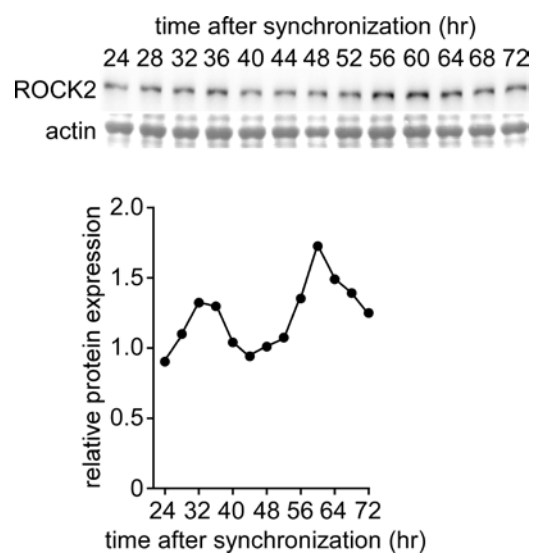
species	distal element	proximal element	Numbers of nucleotide between two elements
<i>Homo sapiens</i>	-1028 GG TTT TGGTCA	-913 TT ACTGGGTCA	104 nt
<i>Mus musculus</i>	-1039 GG TTT TGGTCA	-920 TT ACTGGGTCA	108 nt
<i>Rattus norvegicus</i>	-1026 GGC TTT TGGTCA	-907 TT ACTGGGTCA	108 nt
<i>Macaca mulatta</i>	-1030 GG TTT TGGTCA	-915 TT ACTGGGTCA	104 nt
<i>Bos taurus</i>	-470 GG TTT TGGTCA	-357 TT ACTGGGTCA	102 nt
<i>Pan troglodytes</i>	-2496 GG TTT TGGTCA	-2381 TT ACTGGGTCA	104 nt

The transcription start site is assigned +1. The residues corresponding to the consensus sequences of ROR response element ⁷ are highlighted in red.

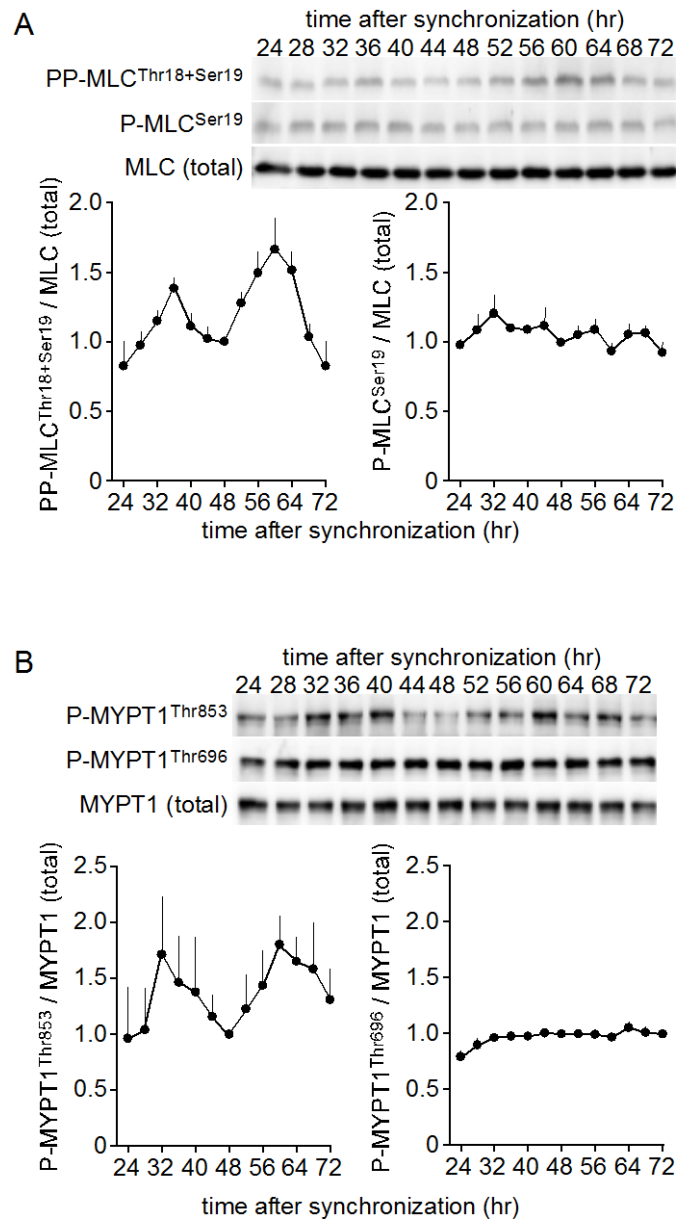
Supplementary Figure 1



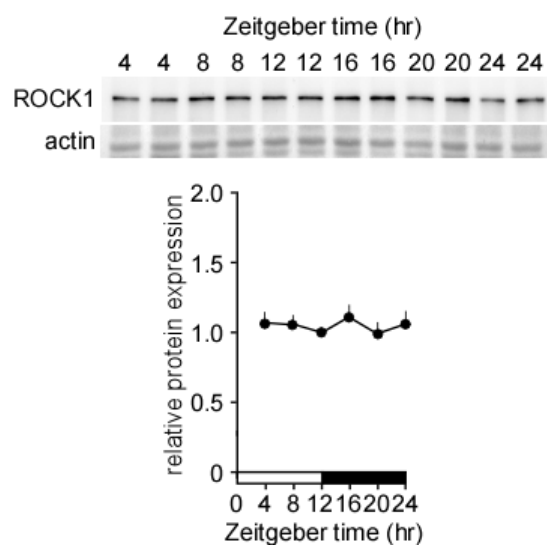
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure Legends

Supplementary Figure 1. The circadian changes in the expression of clock genes in porcine coronary artery smooth muscle cells.

The mRNA levels of canonical clock genes at the indicated times after synchronization of the circadian rhythm were analyzed by quantitative PCR (n=3). The levels were expressed as the relative values to those obtained at 24 hr. All data are expressed as the means \pm SEM.

Supplementary Figure 2. The circadian changes in the expression of ROCK2 in porcine aortic smooth muscle cells.

The circadian changes in the level of the ROCK2 protein at the indicated times after synchronization (n=2). The data are expressed as relative values to those obtained at 48 hr.

Supplementary Figure 3. The circadian changes in the phosphorylation of myosin light chain (MLC) and MYPT1 induced by endothelin-1 in porcine coronary artery smooth muscle cells.

(A, B) The circadian changes in the phosphorylation of MLC at Thr18 and Ser19 (A; n=3), and the phosphorylation of MYPT1 at the residues corresponding to Thr696 and Thr853 in human MYPT1 (B; n=3) obtained 2 min after the stimulation with 100 nmol/L endothelin-1 at the indicated times after synchronization (n=3). The levels of phosphorylation, as normalized to the level of total MLC (A) and total MYPT1 (B), were expressed as relative values to those obtained at 48 hr. All data are expressed as the means \pm SEM.

Supplementary Figure 4. The absence of a circadian rhythm in the expression of ROCK1 in the aortas of wild-type mice.

A representative immunoblot and summary showing the circadian pattern of the expression of ROCK1 protein in the aortas of wild-type mice. The mice were housed under 12-hr light (open bar) and 12-hr 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 \pm SEM (n=4).

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