Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

Circadian Variation of Rho-Kinase Activity in Circulating Leukocytes of Patients With Vasospastic Angina

Taro Nihei, MD; Jun Takahashi, MD, PhD; Ryuji Tsuburaya, MD, PhD; Yoshitaka Ito, MD, PhD; Takashi Shiroto, MD, PhD; Kiyotaka Hao, MD, PhD; Yusuke Takagi, MD, PhD; Yasuharu Matsumoto, MD, PhD; Masaharu Nakayama, MD, PhD; Satoshi Miyata, PhD; Yasuhiko Sakata, MD, PhD; Kenta Ito, MD, PhD; Hiroaki Shimokawa, MD, PhD

Background: Vasospastic angina (VSA) is known to exhibit circadian variation with an early morning peak. We examined whether Rho-kinase activity in circulating leukocytes, which is a useful biomarker for disease activity assessment of VSA, exhibits circadian variation in patients with VSA.

Methods and Results: In consecutive 31 VSA patients (M/F 23/8, 57±13 [SD] years) and 18 non-VSA patients (M/F 8/10, 57±14 years), we measured Rho-kinase activity in circulating leukocytes at 6:00, 12:00 and 21:00. We also examined the relationship between the Rho-kinase activity and coronary vasomotor responses during provocation test. Rho-kinase activity was significantly higher in VSA patients than in non-VSA patients at 6:00 (1.17±0.17 vs. 0.92±0.22, P<0.001), and showed a significant circadian variation with a peak at 6:00 (1.00±0.15 at 21:00, 1.17±0.17 at 6:00 and 1.12±0.22 at 12:00, P<0.001) in VSA patients, whereas no such variation was noted in non-VSA patients. Importantly, Rho-kinase activity at spasm provocation test was significantly correlated with basal coronary tone defined by vasodilating responses to intracoronary nitrate (r=0.40, P<0.05) and coronary vasoconstricting responses to acetylcholine (r=0.44, P<0.05) in VSA patients. Furthermore, their Rho-kinase activity at 6:00 was positively correlated with nocturnal parasympathetic activity as evaluated by heart rate variability in Holter monitoring (r=0.48, P<0.05).

Conclusions: Rho-kinase activity exhibits distinct circadian variation associated with alterations in coronary vasomotor responses and autonomic activity in VSA patients. (*Circ J* 2014; **78:** 1183–1190)

Key Words: Circadian variation; Rho-kinase activity; Vasospastic angina

Chronobiological rhythms have been observed for many physiological parameters in humans, such as sleep–wakefulness cycle, blood pressure, body temperature, metabolic activity and hormone levels.[1] In the clinical setting, it is also widely known that there is a distinct circadian variation in the occurrence of cardiovascular events, such as myocardial infarction, stroke and sudden cardiac death.[2] Coronary artery spasm, which often occurs at rest from midnight to early morning, is one of the important functional abnormalities of the coronary artery and plays an important role in the pathogenesis of various ischemic heart diseases and arrhythmic events.[3,4] A significant circadian variation of ischemic attacks with a peak incidence from midnight to early morning is also one of the most important clinical features of vasospastic angina (VSA).[5] Although it has been proposed that circadian variation of ischemic episodes in VSA patients may be associated with alterations in autonomic nervous system[6,7] and endothelial function,[8] the precise mechanisms remain to be fully elucidated.

Rho-kinase is an important molecular switch that controls contraction and relaxation of vascular smooth muscle cells (VSMC) independently of intracellular Ca^{2+} concentration.[9] Activated Rho-kinase enhances myosin light chain phosphorylation through inhibition of myosin-binding subunit (MBS) of myosin phosphatase, leading to hyperconstriction of VSMC.[10,11] We have previously demonstrated that the Rho-kinase pathway

Received November 27, 2013; revised manuscript received January 3, 2014; accepted January 8, 2014; released online March 27, 2014
Time for primary review: 12 days
Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan
The Guest Editor for this article was Ken-ichi Hirata, MD.
Mailing address: Hiroaki Shimokawa, MD, PhD, Professor and Chairman, Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan. E-mail: shimo@cardio.med.tohoku.ac.jp
ISSN-1346-9843 doi:10.1253/circj.CJ-13-1458

plays a central role in the molecular mechanism of coronary artery spasm.[12–14] Furthermore, we have recently demonstrated that Rho-kinase activity in circulating leukocytes is a useful biomarker for the diagnosis and disease activity assessment of VSA.[15,16] However, it is unclear whether Rho-kinase activity in circulating leukocytes has a circadian variation in VSA patients.

In the present study, we thus examined whether Rho-kinase activity in circulating leukocytes has a circadian variation in VSA patients and if so, whether such circadian variation in Rho-kinase activity is associated with alterations in coronary vasomotor responses and autonomic nervous activity.

Methods

The protocol of the present study was approved by the Ethical Committees of Tohoku University (No. 2008-470) and all patients provided written informed consent before study entry.

Study Population and Spasm Provocation Test

From December 2011 to June 2013, a total of 506 patients underwent diagnostic cardiac catheterization for evaluation of chest pain and/or electrocardiogram (ECG) abnormalities at Tohoku University Hospital. Of those, 153 patients had no significant coronary stenosis (luminal narrowing >75%) on control angiography of the major coronary arteries, and underwent acetylcholine (ACh) provocation test for coronary artery spasm. All the provocation tests were performed in the morning (9:00 to 12:00 noon). Patients with acute coronary syndrome, acute/chronic heart failure, cardiomyopathy, myocarditis, chronic renal failure, collagen disease and the history of coronary stent implantation were excluded from this study. We also excluded the patients who needed continuous intravenous coronary vasodilators or those with medical treatments including calcium channel blockers before the cardiac catheterization due to the instability of angina. Finally, we enrolled 49 patients and divided them into 2 groups depending on their response to ACh provocation test: the VSA group (n=31) and the non-VSA group (n=18). The positive provocation test was defined as >90% coronary vasoconstriction in response to ACh.[5] Prior to the provocation test, the information on the circadian variation of angina attacks was obtained from all patients. ACh provocation test was performed as reported previously.[15] Briefly, ACh was administered into the coronary artery in an incremental manner (20, 50 and 100 μg) with careful monitoring of arterial pressure and 12-lead ECG and serial coronary angiograms at 1-min intervals. To determine whether multivessel spasm could develop, we routinely performed ACh provocation test for the left coronary artery (LCA) first, where we administered incremental doses of ACh (20, 50 and 100 μg) into the LCA. If the test for the LCA was negative or the ACh-induced spasm in the LCA resolved spontaneously, we then administered ACh into the right coronary artery (RCA) in a step-wise manner (20 and 50 μg). When coronary spasm associated with anginal pain and/or ischemic ECG change was demonstrated, isosorbide dinitrate (ISDN, 2 mg) was injected into the vessel with the spasm. The diagnosis of VSA was made based on the "Guidelines for Diagnosis and Treatment of Patients With Vasospastic Angina" of the Japanese Circulation Society.[5]

Blood Sampling and Measurement of Rho-Kinase Activity in Circulating Leukocytes

All vasodilator drugs, including long-acting nitrates and calcium channel blockers except for sublingual nitroglycerin, were discontinued at least 24 h before the blood sampling, whereas angiotensin-converting enzyme inhibitors, angiotensin receptor blockers and statins were continued. After remaining in the supine position for at least 30 min, venous blood sampling with a heparinized tube was serially performed at 12:00, 21:00, 6:00 and just before the provocation test in the morning. Following the in-hospital rule of wake-up and sleep times, we performed the earliest and latest blood sampling at 6:00 and 21:00, respectively, not to disturb patients' sleep and hence their autonomic nervous system. We also measured Rho-kinase activity in the daytime just before lunch (at 12:00) to minimize the influence of feeding on the Rho-kinase activity. Circulating leukocytes were isolated from venous blood samples as previously reported.[15,17] To measure Rho-kinase activity, Western blot analysis was performed by an independent physician who was blinded to the clinical patient information as previously described.[15,17,18] NIH 3T3 cell lysates were used as a positive control and to standardize the results of Western blot analysis from several membranes. Finally, Rho-kinase activity was determined by the ratio of phosphorylated/total form of myosin-binding subunit (MBS), a Rho-kinase substrate.[15,17,18] Our previous study showed that the interobserver and intraobserver reproducibilities for the present Western blot analysis were 97% and 98%, respectively.[15] Serum levels of high-sensitivity C-reactive protein (hsCRP) were measured using a highly sensitive latex aggregation immunoassay with a biochemical analyzer and serum levels of noradrenaline were also measured by high-performance liquid chromatography (SRL Laboratories, Tokyo, Japan).

Evaluation of Coronary Vasomotor Responses

To evaluate coronary vasomotor responses, quantitative coronary angiography (QCA) with the validated densitometric analysis system (CAAS, Pie Medical Imaging, Maastricht, Netherlands) was performed by 2 independent observers who were blinded to the diagnosis of patients. Our previous studies showed that the present QCA analysis has a high intraobserver (99%) and interobserver reproducibility (98%).[19] Coronary segment assessed by QCA included the middle portion of the left anterior descending coronary artery (LAD), corresponding to the segment 7 according to the American Heart Association guideline classification (**Figure S1**). In the present study, we assessed coronary vasodilating responses to intracoronary isosorbide dinitrate (ISDN, 2 mg) from the baseline diameter as an index of basal coronary tone[20] and percent change in luminal diameter to intracoronary ACh (20 μg) compared with that after intracoronary ISDN as an index of coronary vasoconstricting response.[19]

Heart Rate Variability Analysis

Two-channel 24-hour ambulatory ECG recording (SCM-6000, Fukuda Denshi Co Ltd, Tokyo, Japan) was performed to evaluate heart rate variability (HRV) in 37 patients during hospitalization. We excluded 6 patients with atrial fibrillation or extrasytoles >1,000 beats/day, and the remaining 31 patients (19 VSA patients and 12 non-VSA patients) were finally evaluated. All recordings were analyzed by 2 independent observers who were blinded to the diagnosis of patients. Traditional parameters of HRV were analyzed with the MemCalc system (MemCalc/Chiram3, Suwa Trust, Tokyo, Japan), according to the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology.[21] Time domain analysis of HRV included the mean duration of all normal-to-normal (NN) intervals (mean RR), standard deviation of all NN intervals (SDNN). Spectral analysis was performed with the maximum entropy method.[21] Two frequency bands of in-

terest were considered; low frequency (LF, 0.04–0.15 Hz) that reflects modulation of sympathetic or parasympathetic tone and high frequency (HF, 0.15–0.40 Hz) that reflects modulation of vagal tone. LF/HF ratio was used as an index of sympathovagal balance.[21]

Statistical Analysis

Continuous variables are expressed as mean±standard deviation (SD) and categorical variables as percentages. Unpaired student t test for normal distribution and Mann-Whitney U tests for asymmetric distribution were used to analyze differences in continuous variables. Chi-square test or Fisher's exact test were used for categorical variables. Comparison of Rho-kinase activity at 3 time points was performed with one-way analysis of variance (ANOVA) with repeated measures followed by Tukey test. Furthermore, to compare the circadian variation of Rho-kinase activity between the 2 groups, two-way ANOVA with repeated measures was used. Relations between variables were determined by Pearson or Spearman coefficient analysis depending on the distribution. P values <0.05 were considered to be

Table 1. Baseline Patients Characteristics

Variables	Non-VSA (n=18)	VSA (n=31)	P-value
Age (years)	57±14	57±13	0.97
Male/Female	8/10	23/8	0.04
BMI (kg/m^2)	23.6±3.8	24.3±2.8	0.48
HT	10 (56)	16 (52)	0.79
DM	4 (22)	5 (16)	0.43
Dyslipidemia	6 (33)	18 (58)	0.10
Current smoking	5 (28)	13 (42)	0.32
Number of coronary risk factors	1.4±1.0	1.7±1.2	0.49
LVEF (%)	71.5±4.4	70.7±6.8	0.64
Statin use	4 (22)	15 (48)	0.07
β-blocker use	1 (6)	3 (10)	0.53

Results are expressed as mean±SD or n (%).
BMI, body mass index; DM, diabetes mellitus; HT, hypertension; LVEF, left ventricular ejection fraction; VSA, vasospastic angina.

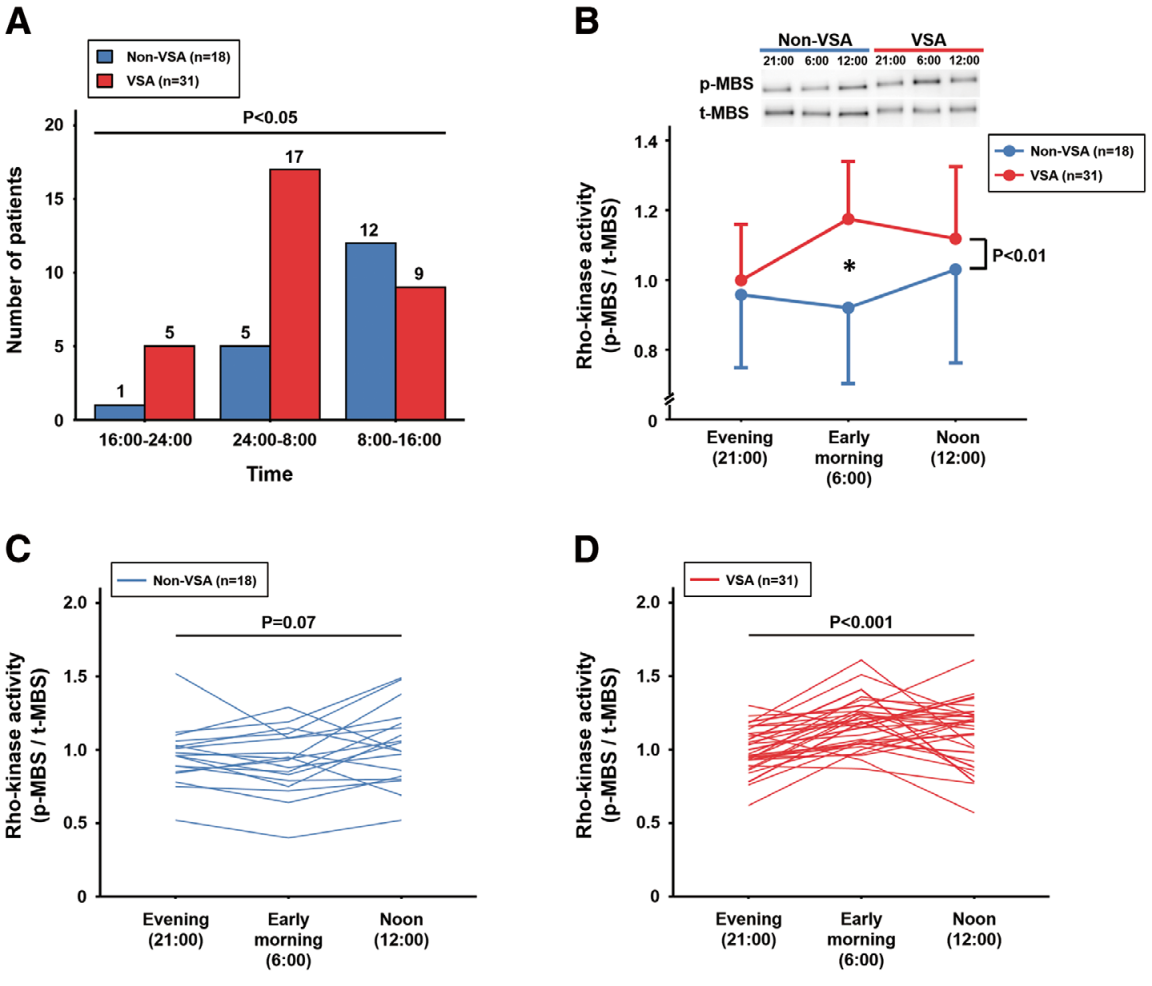

Figure 1. Circadian variation of spontaneous attacks and Rho-kinase activity in circulating leukocytes. (**A**) Circadian variation of spontaneous attacks in all subjects. (**B**) Diurnal fluctuation of Rho-kinase activity in circulating leukocytes. Results are expressed as mean±standard deviation. (**C**,**D**) Individual circadian variation of Rho-kinase activity in non-VSA patients (**C**) and VSA patients (**D**). *P<0.001 for the differences in Rho-kinase activity between the non-VSA and the VSA groups. VSA, vasospastic angina. MBS, myosin-binding subunit; p-/t-, phosphorylated/total form.

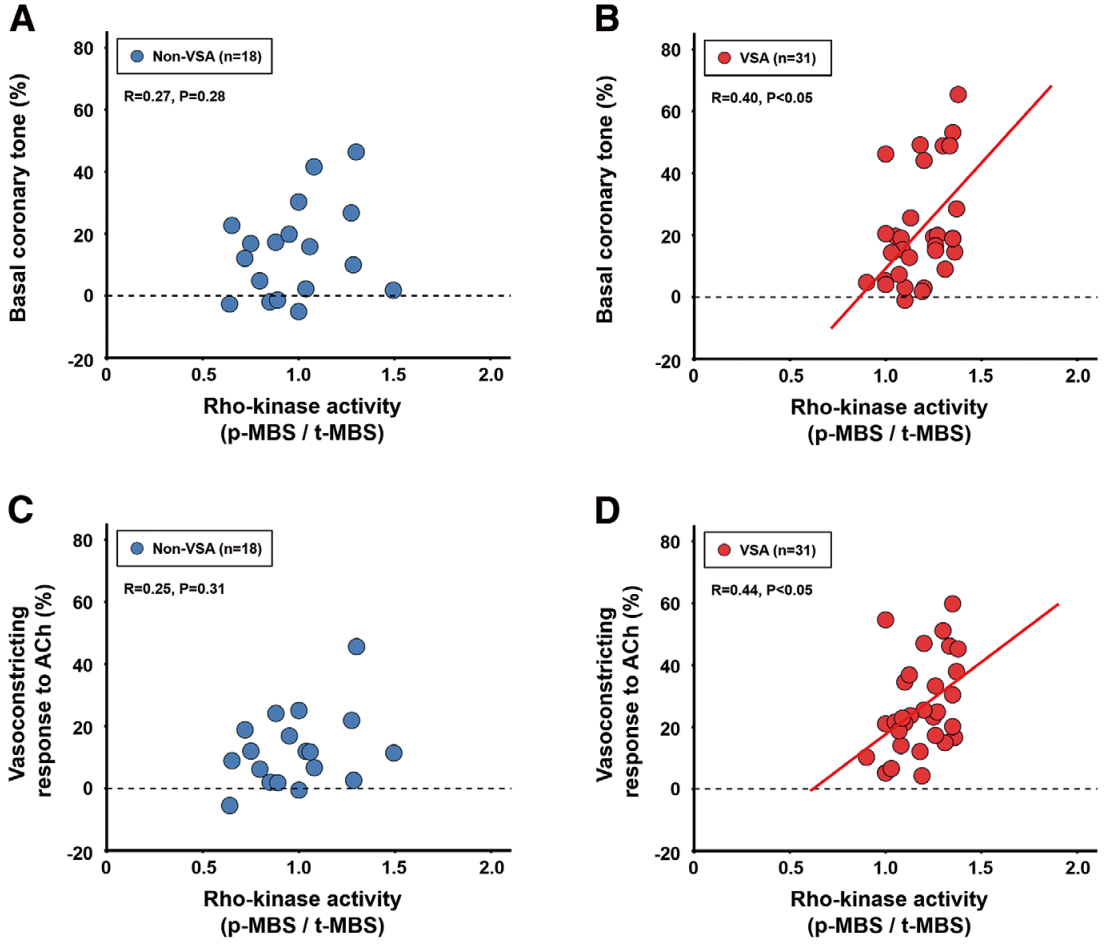

Figure 2. Relationship between Rho-kinase activity in circulating leukocytes and basal coronary tone or coronary vasoconstricting responses. (**A**,**B**) Correlations between basal coronary tone and Rho-kinase activity in circulating leukocytes of non-VSA (**A**) and VSA patients (**B**). (**C**,**D**) Correlations between coronary vasoconstricting responses to ACh and Rho-kinase activity in circulating leukocytes of non-VSA (**C**) and VSA patients (**D**). ACh, acetylcholine; MBS, myosin-binding subunit; p-/t-, phosphorylated/total form; VSA, vasospastic angina.

statistically significant. The statistical analysis was performed with SPSS statistics 21 (IBM Corp, Armonk, NY, USA).

Results

Baseline Clinical Characteristics of Patients

In the VSA group (n=31), coronary spasm was induced at 63±36 μg ACh in the LAD (n=29), left circumflex coronary artery (LCX, n=13) and RCA (n=3), including 14 patients with multivessel spasm. Since ISDN administration was needed to resolve LCA spasm in 28 VSA patients, the provocation test for RCA was finally performed in the remaining 3 patients. All the 13 patients with LCX spasm showed multivessel spasm (LAD+LCX spasms). Also, the type of coronary spasm to ACh was determined as focal (n=4), diffuse (n=21), and both mixed type (n=6). Baseline clinical characteristics of the patients are shown in **Table 1**. The prevalence of female patients was significantly higher in the non-VSA group than in the VSA group. The mean number of coronary risk factors was comparable between the 2 groups (non-VSA 1.4±1.0 vs. VSA 1.7±1.2) and mean left ventricular ejection fraction was well-preserved in both groups. The prevalence of statin use tended to be higher in VSA patients than in non-VSA patients.

Circadian Variation of Rho-Kinase Activity

Distribution of onset times of spontaneous angina attacks, which varied significantly between VSA patients and non-VSA patients (P<0.05 by Fisher's exact test), is shown in **Figure 1A**. Spontaneous attacks occurred most frequently from midnight to morning in VSA patients, whereas the frequency of attacks was the highest during the day in non-VSA patients. Rho-kinase activity in circulating leukocytes was significantly higher in VSA patients than in non-VSA patients at 6:00 (non-VSA 0.92±0.22 vs. VSA 1.17±0.17, P<0.001; **Figure 1B**). It also showed a significant circadian variation with a peak at 6:00 (1.00±0.15 at 21:00, 1.17±0.17 at 6:00, and 1.12±0.22 at 12:00, P<0.001) in VSA patients, whereas no such variation was noted in non-VSA patients (0.96±0.20 at 21:00, 0.92±0.22 at 6:00, and 1.03±0.26 at 12:00, P=0.07; **Figure 1C**,**D**). Moreover, the circadian fluctuation in Rho-kinase activity was significantly different between the 2 groups (P<0.01; **Figure 1B**). Since the prevalence of female patients was significantly higher in the

Table 2. Hemodynamic and Laboratory Data

	Non-VSA (n=18)			VSA (n=31)		
	21:00	6:00	12:00	21:00	6:00	12:00
Heart rate (beats/min)	76.7±11.6	70.4±9.1	78.9±12.8*	72.6±10.3†	66.8±10.5	69.8±10.5
Mean blood pressure (mmHg)	90.1±11.5	90.6±11.6	88.9±10.4	88.3±11.4	90.3±11.3	88.4±13.6
Rho-kinase activity in circulating leukocytes (p-MBS/t-MBS)	0.96±0.20	0.92±0.22	1.03±0.26	1.00±0.15†‡	1.17±0.17	1.12±0.22
hsCRP (mg/dl)	0.056 (0.021–0.290)	0.053 (0.019–0.325)	0.064 (0.021–0.316)	0.034 (0.019–0.068)	0.051 (0.020–0.079)	0.043 (0.019–0.075)
Noradrenaline (pg/ml)	163.0 (126.0–325.8)	181.0 (113.0–328.5)	293.5 (162.5–388.8)	179.5 (131.5–317.3)	203.0 (140.0–277.5)	218.0 (166.5–415.0)

Results are expressed as mean±SD or median (25th–75th percentile). *P<0.05 vs. early morning (6:00) in non-VSA patients; †P<0.01 vs. early morning (6:00) in VSA patients; ‡P<0.05 vs. noon (12:00) in VSA patients.
hsCRP, high-sensitivity C-reactive protein; MBS, myosin-binding subunit; p-/t-, phosphorylated/total form. Other abbreviation as in Table 1.

Table 3. Angiographical Findings

Variables	Non-VSA (n=18)	VSA (n=31)	P-value
Baseline diameter	2.7±0.5	2.5±0.7	0.23
Diameter after ACh 20 μg administration (mm)	2.7±0.5	2.2±0.7	0.01
Diameter after ISDN 2 mg administration (mm)	3.1±0.7	2.9±0.6	0.43
Basal coronary tone (%)	14.3±15.1	21.7±17.8	0.15
Coronary vasoconstricting response (%)	12.3±12.0	26.0±15.3	0.002

Results are expressed as mean ± SD.
ACh, acetylcholine; ISDN, isosorbide dinitrate. Other abbreviation as in Table 1.

non-VSA group, we performed additional analyses for gender. In both the VSA and non-VSA groups, there was no significant difference in the circadian fluctuation of Rho-kinase activity between male and female patients (non-VSA group, P=0.09; VSA group, P=0.53; Figure S2).

There were no significant circadian fluctuations in serum hsCRP or noradrenaline levels between the 2 groups (Table 2). Furthermore, in VSA patients, serum levels of both hsCRP and noradrenaline showed no significant correlation with Rho-kinase activity in circulating leukocytes at each sampling time (Figure S3).

Correlation Between Rho-Kinase Activity and Coronary Vasomotor Responses

QCA findings of the middle segment of LAD during the spasm provocation test are shown in Table 3. Although coronary diameters at baseline and those after intracoronary ISDN were comparable between the 2 groups, basal coronary tone tended to be greater in VSA patients than in non-VSA patients. According to the definition, coronary vasoconstricting responses to ACh were significantly larger in VSA patients than in non-VSA patients. Rho-kinase activity in circulating leukocytes at spasm provocation test was significantly higher in VSA patients than in non-VSA patients (non-VSA 0.98±0.24 vs. VSA 1.19±0.13, P<0.001). Individual angiographical findings and Rho-kinase activity at ACh provocation test are summarized in Table S1. There was no significant difference in Rho-kinase activity between VSA patients with 1-vessel spasm and those with multivessel spasm (1.15±0.13 vs. 1.22±0.14, P=0.13). Also, we found no difference in Rho-kinase activity in terms of the spasm configurations (diffuse spasm 1.16±0.14 vs. focal spasm 1.22±0.14 vs. mixed spasm 1.23±0.15, P=0.52). Correlations between Rho-kinase activity in circulating leukocytes and basal coronary tone and vasoconstricting responses to ACh are shown in Figure 2. In non-VSA patients, no significant correlation was noted be-

Table 4. Parameters of Heart Rate Variability

Variables	Non-VSA (n=12)	VSA (n=19)	P-value
Time domain			
Mean RR (ms)	832.3±129.8	825.6±103.5	0.87
SDNN (ms)	118.7±31.9	116.0±23.5	0.68
Frequency domain			
Ln (LF) (ms^2)			
All day	5.5±1.1	5.7±0.9	0.60
16:00–24:00	5.4±1.1	5.5±0.7	0.76
24:00–08:00	5.6±1.2	5.8±0.9	0.66
08:00–16:00	5.3±1.0	5.5±1.0	0.53
Ln (HF) (ms^2)			
All day	4.8±1.1	4.9±0.9	0.69
16:00–24:00	4.4±1.0	4.4±0.9	0.87
24:00–08:00	4.7±1.1	4.8±0.9	0.75
08:00–16:00	4.1±1.0	4.2±1.1	0.82
LF/HF			
All day	3.8±2.2	3.9±2.2	0.94
16:00–24:00	4.4±2.1	3.9±1.8	0.56
24:00–08:00	4.0±2.1	3.7±2.1	0.66
08:00–16:00	4.6±2.0	4.8±2.8	0.76

Results are expressed as mean ± SD. LF and HF powers are expressed in natural logarithmic units.
HF, high-frequency power (0.15–0.40 Hz); LF, low-frequency power (0.04–0.15 Hz); mean RR, mean duration of all normal-to-normal intervals; SDNN, standard deviation of all normal-to-normal intervals. Other abbreviation as in Table 1.

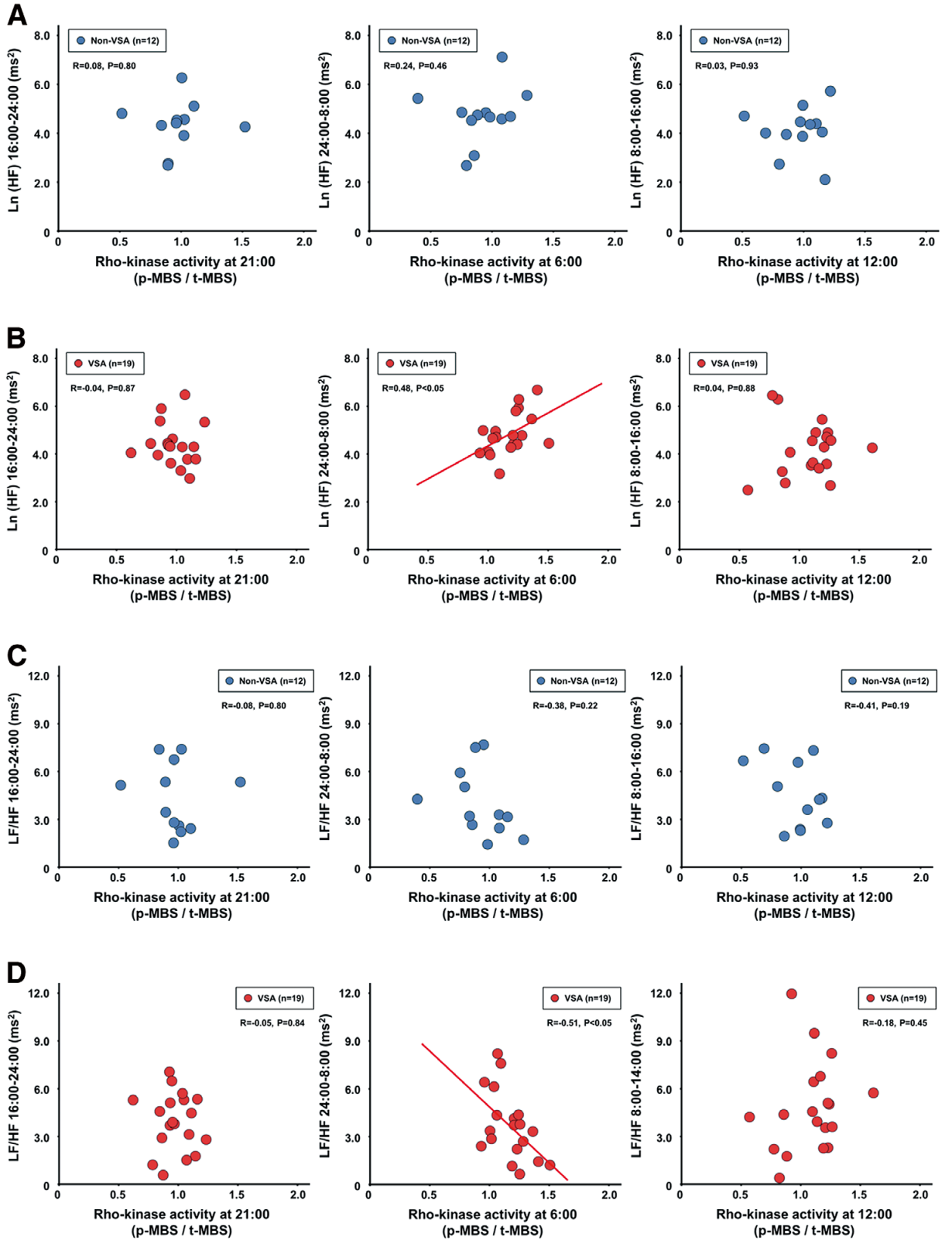

Figure 3. Relationship between Rho-kinase activity in circulating leukocytes and cardiac autonomic activity. (**A**,**B**) Correlations between Rho-kinase activity in circulating leukocytes and parasympathetic nervous activity of non-VSA (**A**) and VSA patients (**B**). (**C**,**D**) Correlations between Rho-kinase activity in circulating leukocytes and sympathetic nervous activity of non-VSA (**C**) and VSA patients (**D**). HF, high frequency; LF, low frequency; MBS, myosin-binding subunit; p-/t-, phosphorylated/total form; VSA, vasospastic angina.

tween Rho-kinase activity and basal coronary tone or vasoconstricting response to ACh (Figure 2A,C). In contrast, in VSA patients, we found a positive correlation between Rho-kinase activity and basal coronary tone (r=0.40, P<0.05; Figure 2B) and between Rho-kinase activity and vasoconstricting response to ACh (r=0.44, P<0.05; Figure 2D). Serum hsCRP levels again showed no significant correlation with basal coronary tone or vasoconstricting response in all patients (Figure S4).

Correlation Between Rho-Kinase Activity and Autonomic Nervous Activity

There was no difference in the baseline HRV parameters between the 2 groups (Table 4). In non-VSA patients, Rho-kinase activity at every sampling time was not correlated with parasympathetic (Figure 3A) or sympathetic nervous activity (Figure 3C). In contrast, in VSA patients, Rho-kinase activity at 6:00 was positively correlated with parasympathetic nervous activity from midnight to early morning (Figure 3B) and was negatively correlated with sympathetic activity (Figure 3D).

Discussion

The major findings of the present study were that (a) there was a significant circadian variation of Rho-kinase activity in circulating leukocytes with a peak at 6:00 of VSA patients, (b) Rho-kinase activity at ACh provocation test in the morning was associated with basal coronary tone and coronary vasoconstricting response to ACh of VSA patients, and (c) Rho-kinase activity at 6:00 of VSA patients was positively correlated with cardiac parasympathetic nervous activity from midnight to early morning. To the best of our knowledge, this is the first study to identify circadian variation of Rho-kinase activity of VSA patients, associated with alterations in coronary vasomotor responses and autonomic nervous activities.

Circadian Variation of Rho-Kinase Activity of VSA Patients

A circadian variation of symptoms from midnight to early morning is of value as a clue in the diagnosis of VSA.[5] As first demonstrated by Yasue et al, disease activity of VSA as determined by basal coronary tone and hypersensitivity to constrictive stimuli markedly varies throughout the day.[22] Although autonomic nervous system[6,7] and endothelial function[8] have previously been reported to be associated with a circadian variation of VSA attacks, it still remains unclear whether those factors could be directly involved in the elevated basal coronary tone or enhanced vasomotor responses in VSA patients. We have recently demonstrated that Rho-kinase activity in circulating leukocytes is a useful biomarker for diagnosis and disease activity assesment of VSA.[15,16] In the present study, we were able to demonstrate that Rho-kinase activity in circulating leukocytes of VSA patients exhibited a significant circadian variation with a peak noted in the early morning, when their spontaneous angina attacks occurred most frequently. In contrast, no such circadian variation of Rho-kinase activity was noted in non-VSA patients. Importantly, Rho-kinase activity in circulating leukocytes at spasm provocation test in the morning was positively correlated with basal coronary tone and vasoconstricting responses to ACh in VSA patients. These results indicate that Rho-kinase activity in circulating leukocytes is a useful biomarker of VSA and may be involved in the circadian variation of the disorder.

It was previously reported that serum levels of hsCRP were elevated in VSA patients than in non-VSA patients,[23] and that 6-month treatment with a statin significantly reduced the disease activity of VSA along with the decrease in hsCRP levels.[24] These results suggest that low-grade inflammation is involved in the pathogenesis of VSA and that hsCRP is useful for disease activity assessment of VSA. However, in the present study, we found no circadian variation of hsCRP levels in VSA patients and no correlation between serum levels of hsCRP and basal coronary tone or vasomotor responses to ACh. These findings are consistent with the previous report that CRP levels showed no circadian or seasonal variations.[25] Thus, Rho-kinase activity in circulating leukocytes may be a more sensitive and reliable biomarker for disease activity assessment of VSA than hsCRP.

The circadian variation in the onset of myocardial infarction, sudden death and stroke has also attracted attention.[2,26] The circadian patterns of these cardiovascular diseases are also characterized by a morning peak as in the case of VSA. Since Rho-kinase pathway plays an important role in the pathogenesis of not only VSA but also other cardiovascular diseases,[27–29] the morning increase in Rho-kinase activity may be involved in the circadian variation of the onset of those cardiovascular diseases.

Possible Mechanisms of Circadian Variation of Rho-Kinase Activity in VSA Patients

Autonomic dysfunction has been proposed as one of the most important mechanisms of circadian variation of VSA.[7,22] However, the relationship between autonomic nervous system and VSA, seems to be complex and even controversial. Previous studies reported that coronary spasm was induced by a sympathetic activity surge by way of α-adrenergic stimulation in the early morning.[6,22] However, in the present study, we found no apparent circadian variation in plasma noradrenaline levels and no correlation between the noradrenaline levels and Rho-kinase activity or coronary vasomotor responses in VSA patients. These results suggest that activation of sympathetic nervous system per se is less likely to be involved in the circadian variation of VSA. In contrast, parasympathetic nervous system is likely to be involved as coronary spasm often occurs at rest, particularly from midnight to early morning, when parasympathetic nervous activity increases.[22] Furthermore, intracoronary administration of ACh, which is a neurotransmitter of parasympathetic nerve endings, induces coronary spasm in VSA patients with a high specificity.[30,31] Analyses using MIBG scintigraphy also reported that enhanced cardiac parasympathetic nervous activity was an important factor for the induction of coronary spasm and could predict future cardiac events in VSA patients.[32] In the present study, although the HRV parameters were comparable between VSA and non-VSA patients, Rho-kinase activity of VSA patients in the early morning was significantly correlated with parasympathetic nervous activity in the midnight to early morning. Thus, it is conceivable that enhanced Rho-kinase activity of VSA patients in the early morning may be determined, at least in part, by nocturnal parasympathetic nervous activity with resultant increases in basal coronary tone and coronary vasoconstriction response.

Study Limitations

Several limitations should be mentioned for the present study. First, the present study was a single-center, exploratory study with a relatively small number of patients. Second, Holter ECG recording and blood sampling were not always performed on the same day. However, in order to adjust the surrounding environments with blood sampling, Holter ECG recording was performed during hospitalization. Third, we were unable to perform ACh provocation test for RCA in all patients with VSA. Thus, RCA spasm and multivessel spasm involving RCA may have been underestimated in the present study. Fourth, we did not follow the time-course of the diurnal pattern of Rho-kinase

activity in VSA patients after initiation of medical treatment with calcium channel blockers.[15]

Conclusions

The present study provides the first evidence that there is a distinct circadian variation of Rho-kinase activity in circulating leukocytes of VSA patients associated with alterations in coronary vasomotor responses and parasympathetic nervous activity, suggesting that Rho-kinase activity plays an important role in the circadian variation of coronary spasm.

Acknowledgments

The authors thank Akemi Saito, Teru Hiroi, Takeshi Kato, Hidetomo Ohnuma, and Dr Zhulanqiqige Do.e for technical assistance in this study. The present work was supported in part by grants-in-aid [18890018] from Scientific Research and the global COE project (F02), and grants-in-aid (H22-Shinkin-004) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan.

Disclosures

None.

References

1. Aschoff J. Circadian rhythms in man. *Science* 1965; **148:** 1427–1432.
2. Muller JE, Tofler GH, Stone PH. Circadian variation and triggers of onset of acute cardiovascular disease. *Circulation* 1989; **79:** 733–743.
3. Maseri A, L'Abbate A, Baroldi G, Chierchia S, Marzilli M, Ballestra AM, et al. Coronary vasospasm as a possible cause of myocardial infarction: A conclusion derived from the study of "preinfarction" angina. *New Engl J Med* 1978; **299:** 1271–1277.
4. Maseri A, Beltrame JF, Shimokawa H. Role of coronary vasoconstriction in ischemic heart disease and search for novel therapeutic targets. *Circ J* 2009; **73:** 394–403.
5. JCS Joint Working Group. Guidelines for diagnosis and treatment of patients with vasospastic angina (coronary spastic angina) (JCS 2008): Digest version. *Circ J* 2010; **74:** 1745–1762.
6. Yasue H, Touyama M, Shimamoto M, Kato H, Tanaka S. Role of autonomic nervous system in the pathogenesis of Prinzmetal's variant form of angina. *Circulation* 1974; **50:** 534–539.
7. Lanza GA, Pedrotti P, Pasceri V, Lucente M, Crea F, Maseri A. Autonomic changes associated with spontaneous coronary spasm in patients with variant angina. *J Am Coll Cardiol* 1996; **28:** 1249–1256.
8. Kawano H, Motoyama T, Yasue H, Hirai N, Waly HM, Kugiyama K, et al. Endothelial function fluctuates with diurnal variation in the frequency of ischemic episodes in patients with variant angina. *J Am Coll Cardiol* 2002; **40:** 266–270.
9. Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. *Nature* 1994; **372:** 231–236.
10. Shimokawa H, Takeshita A. Rho-kinase is an important therapeutic target in cardiovascular medicine. *Arterioscler Thromb Vasc Biol* 2005; **25:** 1767–1775.
11. Liao JK, Seto M, Noma K. Rho kinase (ROCK) inhibitors. *J Cardiovasc Pharmacol* 2007; **50:** 17–24.
12. Shimokawa H, Seto M, Katsumata N, Amano M, Kozai T, Yamawaki T, et al. Rho-kinase-mediated pathway induces enhanced myosin light chain phosphorylations in a swine model of coronary artery spasm. *Cardiovasc Res* 1999; **43:** 1029–1039.
13. Kandabashi T, Shimokawa H, Miyata K, Kunihiro I, Kawano Y, Fukata Y, et al. Inhibition of myosin phosphatase by upregulated Rho-kinase plays a key role for coronary artery spasm in a porcine model with interleukin-1β. *Circulation* 2000; **101:** 1319–1323.
14. Masumoto A, Mohri M, Shimokawa H, Urakami L, Usui M, Takeshita A. Suppression of coronary artery spasm by the Rho-kinase inhibitor fasudil in patients with vasospastic angina. *Circulation* 2002; **105:** 1545–1547.
15. Kikuchi Y, Yasuda S, Aizawa K, Tsuburaya R, Ito Y, Takeda M, et al. Enhanced Rho-kinase activity in circulating neutrophils of patients with vasospastic angina: A possible biomarker for diagnosis and disease activity assessment. *J Am Coll Cardiol* 2011; **58:** 1231–1237.
16. Nihei T, Takahashi J, Kikuchi Y, Takagi Y, Hao K, Tsuburaya R, et al. Enhanced Rho-kinase activity in patients with vasospastic angina after the Great East Japan Earthquake. *Circ J* 2012; **76:** 2892–2894.
17. Do.e Z, Fukumoto Y, Sugimura K, Miura Y, Tatebe S, Yamamoto S, et al. Rho-kinase activation in patients with heart failure. *Circ J* 2013; **77:** 2542–2550.
18. Liu PY, Liao JK. A method for measuring Rho kinase activity in tissues and cells. *Methods Enzymol* 2008; **439:** 181–189.
19. Shiroto T, Yasuda S, Tsuburaya R, Ito Y, Takahashi J, Ito K, et al. Role of Rho-kinase in the pathogenesis of coronary hyperconstricting responses induced by drug-eluting stents in pigs in vivo. *J Am Coll Cardiol* 2009; **54:** 2321–2329.
20. Egashira K, Katsuda Y, Mohri M, Kuga T, Tagawa T, Shimokawa H, et al. Basal release of endothelium-derived nitric oxide at site of spasm in patients with variant angina. *J Am Coll Cardiol* 1996; **27:** 1444–1449.
21. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. Heart rate variability: Standards of measurement, physiological interpretation and clinical use. *Circulation* 1996; **93:** 1043–1065.
22. Yasue H, Omote S, Takizawa A, Nagao M, Miwa K, Tanaka S. Circadian variation of exercise capacity in patients with Prinzmetal's variant angina: Role of exercise-induced coronary arterial spasm. *Circulation* 1979; **59:** 938–948.
23. Itoh T, Mizuno Y, Harada E, Yoshimura M, Ogawa H, Yasue H. Coronary spasm is associated with chronic low-grade inflammation. *Circ J* 2007; **71:** 1074–1078.
24. Yasue H, Mizuno Y, Harada E, Itoh T, Nakagawa H, Nakayama M, et al. Effects of a 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitor, fluvastatin, on coronary spasm after withdrawal of calcium-channel blockers. *J Am Coll Cardiol* 2008; **51:** 1742–1748.
25. Pepys MB, Hirschfield GM. C-reactive protein: A critical update. *J Clin Invest* 2003; **111:** 1805–1812.
26. Muller JE, Stone PH, Turi ZG, Rutherford JD, Czeisler CA, Parker C, et al. Circadian variation in the frequency of onset of acute myocardial infarction. *New Engl J Med* 1985; **313:** 1315–1322.
27. Dong M, Liao JK, Yan B, Li R, Zhang M, Yu CM. A combination of increased Rho kinase activity and N-terminal pro-B-type natriuretic peptide predicts worse cardiovascular outcome in patients with acute coronary syndrome. *Int J Cardiol* 2013; **167:** 2813–2819.
28. Oi K, Shimokawa H, Hiroki J, Uwatoku T, Abe K, Matsumoto Y, et al. Remnant lipoproteins from patients with sudden cardiac death enhance coronary vasospastic activity through upregulation of Rho-kinase. *Arterioscler Thromb Vasc Biol* 2004; **24:** 918–922.
29. Feske SK, Sorond FA, Henderson GV, Seto M, Hitomi A, Kawasaki K, et al. Increased leukocyte ROCK activity in patients after acute ischemic stroke. *Brain Res* 2009; **1257:** 89–93.
30. Okumura K, Yasue H, Matsuyama K, Goto K, Miyagi H, Ogawa H, et al. Sensitivity and specificity of intracoronary injection of acetylcholine for the induction of coronary artery spasm. *J Am Coll Cardiol* 1988; **12:** 883–888.
31. Takagi Y, Yasuda S, Takahashi J, Tsunoda R, Ogata Y, Seki A, et al. Clinical implications of provocation tests for coronary artery spasm: Safety, arrhythmic complications, and prognostic impact: Multicentre registry study of the Japanese Coronary Spasm Association. *Eur Heart J* 2013; **34:** 258–267.
32. Sakata K, Iida K, Kudo M, Yoshida H, Doi O. Prognostic value of I-123 metaiodobenzylguanidine imaging in vasospastic angina without significant coronary stenosis. *Circ J* 2005; **69:** 171–176.

Supplementary Files

Supplementary File 1

Figure S1. Representative images of quantitative coronary angiography (QCA).

Figure S2. Gender differences in circadian variation of Rho-kinase activity in circulating leukocytes.

Figure S3. Relationship between Rho-kinase activity and serum levels of hsCRP and noradrenaline in VSA patients.

Figure S4. Relationship between serum levels of hsCRP and basal coronary tone and coronary vasoconstricting responses.

Table S1. Individual angiographical findings and Rho-kinase activity of VSA patients

Please find supplementary file(s);
http://dx.doi.org/10.1253/circj.CJ-13-1458