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Enhanced Rho-Kinase Activity in Circulating Neutrophils of Patients With Vasospastic Angina

A Possible Biomarker for Diagnosis and Disease Activity Assessment

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Objectives The aim of this study was to examine whether Rho-kinase activity is systemically enhanced in patients with vasospastic angina (VSA) and, if so, whether a noninvasive diagnostic method could be developed to improve practice.

Background The activated Rho-kinase pathway plays a central role in the molecular mechanism of coronary vasospasm in animal models and patients with VSA. Recently, it has been reported that Rho-kinase activity in circulating leukocytes is associated with various diseases.

Methods Fifty-three consecutive patients with chest pain who underwent acetylcholine provocation testing for coronary spasm were examined. Patients were divided into 2 groups depending on their response to the test: VSA ($n = 33$) and non-VSA ($n = 20$) groups. Venous blood samples were collected to measure Rho-kinase activity in circulating neutrophils, determined by the extent of phosphorylation of myosin-binding subunit (MBS), a substrate of Rho-kinase.

Results Rho-kinase activity was significantly higher in the VSA group than in the non-VSA group (phosphorylated MBS/total MBS ratio 1.33 ± 0.37 vs. 0.95 ± 0.22, $p < 0.001$). In the VSA group, no correlation was noted between Rho-kinase activity and high-sensitivity C-reactive protein, smoking, or accumulated number of coronary risk factors. After the 3-month medical treatment, Rho-kinase activity in the VSA group was significantly decreased to 1.08 ± 0.31 ($p < 0.001$). On receiver-operating characteristic curve analysis, a phosphorylated MBS ratio of 1.18 was identified as the best cutoff level to predict the diagnosis of VSA.

Conclusions These results indicate that Rho-kinase activity in circulating neutrophils is enhanced in patients with VSA and may be a useful biomarker for diagnosis and disease activity assessment of the vasospastic disorder. (J Am Coll Cardiol 2011;58:1231–7) © 2011 by the American College of Cardiology Foundation

Coronary artery spasm plays an important role in the pathogenesis of a wide variety of ischemic heart diseases, including myocardial infarction and sudden cardiac death (1–5). However, the diagnosis of vasospastic angina (VSA) is not always easy on the basis of symptoms alone and often requires invasive provocation testing. Importantly, medical treatment with calcium-channel blockers (CCBs) could reduce symptomatic angina but did not completely normalize coronary vasoconstricting responses to acetylcholine (ACh) (6). These findings indicate the limitation of symptom-oriented diagnosis and treatment in patients with VSA and warrant a reliable biomarker representing the pathogenesis and severity of coronary spasm in clinical practice.

See page 1238

We have previously demonstrated that the activated Rho-kinase pathway plays a central role in the molecular mechanism of coronary vasospasm (7–14). Rho-kinase, 1 of the effectors of small guanosine triphosphate–binding protein Rho (15,16), enhances myosin light chain phosphorylation through the inhibition of the myosin-binding subunit (MBS) of myosin phosphatase, leading to vascular hyperconstriction or vasospasm (17,18).

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Abbreviations and Acronyms

ACh = acetylcholine
CCB = calcium-channel blocker
hsCRP = high-sensitivity C-reactive protein
MBS = myosin-binding subunit
ROC = receiver-operating characteristic
VSA = vasospastic angina

Recently, we and others have demonstrated that systemic activation of Rho-kinase, measured by Rho-kinase activity in peripheral blood leukocytes, is associated with coronary artery disease (19), acute ischemic stroke (20), pulmonary artery hypertension (21), smoking (22), metabolic syndrome (23), and essential hypertension (24). We also have demonstrated that activities of small G proteins (including Rho) and Rho-kinase in circulating leukocytes could be used for the assessment of medical treatment (25). However, it remains to be examined whether Rho-kinase activity is systemically enhanced in patients with VSA and, if so, whether Rho-kinase activity measured in circulating leukocytes could reflect the disease activity.

In the present study, we addressed these important issues in patients with VSA using the method developed in our laboratory (21,25).

Methods

All procedures were performed according to protocols approved by the Institutional Review Board of Tohoku University (No. 2008-470). Written informed consent was obtained from all patients before study entry.

Study population. From January 2009 to June 2010, a total of 702 patients were consecutively referred for diagnostic catheterization for evaluation of chest pain and/or electrocardiographic abnormalities at Tohoku University Hospital. Of these, 60 patients did not have significant coronary lesions (luminal narrowing <50%) on control angiography of the major coronary arteries. Because 4 patients did not give informed consent for ACh provocation testing and 3 patients had insufficient isolation of leukocytes, the remaining 53 patients were ultimately enrolled and divided into the 2 groups, depending on their response to the provocation test: the VSA group (n = 33) and the non-VSA group (n = 20). Hypertension, diabetes mellitus, dyslipidemia, and obesity were diagnosed on the basis of the guidelines of the Japanese Society of Hypertension (26), the Japan Diabetes Society (27), the Japan Atherosclerosis Society (28), and the Japan Society for the Study of Obesity (29), respectively. Obesity was defined as a body mass index >25 kg/m^2 (29). Coronary risk factors included hypertension, diabetes mellitus, dyslipidemia, smoking habit, obesity, and family history. Serum high-sensitivity C-reactive protein (hsCRP) levels were measured using a highly sensitive latex aggregation immunoassay with a biochemical analyzer.

Provocation test and diagnosis of VSA. All vasoactive drugs, including long-acting nitrates, CCBs, and beta-receptor blockers, were discontinued at least 24 h before catheterization, except for sublingual nitroglycerin when needed, whereas angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, and statins were not discontinued in the present study. Our protocol has been described previously (30). Briefly, ACh was administrated into the coronary artery (12.5, 25, 50, and 100 μg) with careful monitoring of arterial pressure and 12-lead electrocardiograms and serial coronary angiograms at 1-min intervals. The diagnosis of VSA was made on the basis of the "Guidelines for Diagnosis and Treatment of Patients With Vasospastic Angina" of the Japanese Circulation Society when patients had total or subtotal coronary artery narrowing (>90% stenosis) during the provocation test, accompanied by chest pain and/or transient ST-segment elevation or depression of more than 0.1 mV or new appearance of a negative U-wave in at least 2 related leads (31).

Rho-kinase activity in circulating neutrophils. Venous blood samples were collected just before the provocation test. To measure Rho-kinase activity, Western blot analysis was performed by an independent physician who was blinded to the clinical information of the patients, as previously described (21,25,32). NIH 3T3 cell lysates were used as a positive control and to standardize the results of Western blot analyses from several membranes. Finally, Rho-kinase activity was determined by the extent of phosphorylation of MBS, a substrate of Rho-kinase (21,25,32). The interobserver and intraobserver correlation coefficients were 0.97 and 0.98, respectively. We have previously reported that the level of Rho-kinase activity in circulating neutrophils was 0.58 ± 0.22 in healthy subjects (8 men, 32 women; mean age 44 ± 7 years) (21). Also, in a pilot study of 11 patients with stable effort angina due to severe organic stenosis who required coronary stent implantation (6 men, 5 women; mean age 65 ± 12 years; mean number of coronary risk factors 3.0 ± 1.4), the level was 1.31 ± 0.44.

In the present study, MBS phosphorylation was also measured with and without 10 μmol/l fasudil, to which isolated leukocytes were exposed for 5 min. Because fasudil is a potent and selective inhibitor of Rho-kinase and has an inhibitory effect on Rho-kinase 10 and 100 times more than on protein kinase C and myosin light-chain kinase, respectively (8), Rho-kinase activity could be defined to assess the component of MBS inhibited by fasudil, which was determined as a difference in the phosphorylated MBS/total MBS ratio between the absence and the presence of fasudil (21,25,32).

Follow-up study. Three months after the medical treatment, 21 of the 33 patients with VSA underwent venous blood sampling and were then examined using a questionnaire of anginal stability, in a prospective manner as performed at admission (blood sampling and then the provocation test). The questionnaire included the number, duration, and frequency of chest pain episodes, the frequency of nitroglycerin use, and the circumstances of pain appearance.

Statistical analysis. Continuous variables are expressed as mean ± SD and categorical variables as percents. Unpaired

Table 1 Baseline Clinical Characteristics

Variable	Non-VSA (n = 20)	VSA (n = 33)	p Value
Age (yrs)	60 ± 14	58 ± 12	0.54
Men/women	11/9	21/12	0.53
BMI (kg/m^2)	23.9 ± 2.9	24.1 ± 3.6	0.86
Waist circumference (cm)	87.0 ± 10.1	87.5 ± 10.1	0.86
HT	11 (55%)	15 (45%)	0.50
DM	4 (20%)	4 (12%)	0.35
Dyslipidemia	12 (60%)	23 (70%)	0.47
Smoking habit	6 (30%)	15 (45%)	0.27
Current smoking	1 (5%)	12 (36%)	0.01
Number of coronary risk factors	1.9 ± 1.5	2.2 ± 1.1	0.49
LVEF (%)	76.5 ± 7.3	70.6 ± 12.8	0.08
Statin use	7 (35%)	10 (30%)	0.98
hsCRP (mg/dl)	0.072 (0.034/0.220)	0.074 (0.031/0.187)	0.98

Values are mean ± SD, n, n (%), or median (25th percentile/75th percentile).
BMI = body mass index; DM = diabetes mellitus; hsCRP = high-sensitivity C-reactive protein; HT = hypertension; LVEF = left ventricular ejection fraction; VSA = vasospastic angina.

Student *t* tests for normal distribution and Mann-Whitney *U* tests for asymmetric distribution were used to analyze differences in continuous variables. Fisher and chi-square tests were used to analyze differences between categorical variables. One-way analysis of variance followed by the Tukey test was used to test difference between multiple variables. Test for trend was used to assess the trend in multiple variables. The McNemar test for correlated proportions was performed to determine whether significant changes existed in medication from the baseline to follow-up. Paired Student *t* tests were used to compare Rho-kinase activity before and after intensive medical treatment. Receiver-operating characteristic (ROC) curve analysis was performed to determine the best cutoff value of Rho-kinase activity to differentiate patients with from those without VSA. Cutoff values were determined as the sum of sensitivity and specificity. Statistical analysis was performed using SPSS (SPSS Inc., Chicago, Illinois), and p values <0.05 were considered statistically significant.

Results

Clinical characteristics. Baseline clinical characteristics are shown in Table 1. There was no difference in the prevalence of coronary risk factors between the 2 groups, except for current smoking habit (non-VSA 5% vs. VSA 36%, p = 0.01). The mean number of coronary risk factors was relatively small and comparable between the 2 groups (non-VSA 1.9 ± 1.5 vs. VSA 2.2 ± 1.1). Left ventricular ejection fractions were within the normal range in both groups. In the VSA group, coronary spasm was induced at 41 ± 32 μg of ACh in the left anterior descending (n = 19), left circumflex (n = 9), or right (n = 14) coronary artery, including 9 patients with multivessel spasm. Also, the type of spasm was characterized as focal (n = 9), diffuse (n = 21), or mixed (n = 3). In the non-VSA group, 5 patients showed vasodilating responses, and the remaining 15 patients showed no changes or insignificant vasoconstricting responses.

Rho-kinase activity in the non-VSA and VSA groups. Rho-kinase activity in circulating neutrophils was normally distributed and significantly higher in the VSA group than that in the non-VSA group (non-VSA 0.95 ± 0.22 vs. VSA 1.33 ± 0.37, p < 0.001) (Fig. 1A). In both groups, there was no significant correlation between Rho-kinase activity and the number of risk factors, and no effect of smoking habit was noted on the Rho-kinase activity (data not shown). Also, there was no difference between current smoking group and noncurrent smoking group (including previous smokers and nonsmokers) (1.41 ± 0.45 vs. 1.29 ± 0.32) in the VSA group. Levels of hsCRP were comparable between the 2 groups (Table 1) and tended to correlate with the Rho-kinase activity in the VSA group (R = 0.34, p = 0.08). Importantly, Rho-kinase activity was significantly associated with anginal duration and frequency (Figs. 2A and 2B).

Diagnostic significance of Rho-kinase activity for VSA. We then examined whether Rho-kinase activity in circulating neutrophils could predict the coronary response to ACh provocation testing using ROC curve analysis. As shown in Figure 1B, Rho-kinase activity was a powerful predictor of VSA, with an area under curve of 0.85 (95% confidence interval: 0.74 to 0.96). In this ROC curve analysis, a ratio of 1.18 was identified as the best cutoff level for the diagnosis of VSA. The sensitivity and specificity of this ROC curve analysis for detecting VSA were 82% and 90%, respectively. Also, the positive and negative predictive values were 93% and 75%, respectively.

Validation of Rho-kinase activity using fasudil, a selective Rho-kinase inhibitor. Rho-kinase activity was also validated by using fasudil, a selective Rho-kinase inhibitor, in 48 of 53 patients, 18 in the non-VSA group and 30 in the VSA group. The ratio of phosphorylated MBS to total MBS was well correlated, with a difference in the phosphorylated MBS/total MBS ratio between the absence and the presence of fasudil (R = 0.82, p < 0.001). Rho-kinase activity measured in isolated leukocytes using fasudil was significantly higher in the VSA group than in the non-VSA group (non-VSA 0.56 ± 0.31 vs. VSA 1.01 ± 0.44, p < 0.001).

Rho-kinase activity before and after intensive medical treatment. In the VSA group (n = 21), we compared Rho-kinase activity before and 3 months after the intensive medical treatment. Medications are shown in Table 2. Although 8 patients (38%) had already been treated with CCBs, there was no difference in baseline Rho-kinase activity between patients with and those without CCBs (1.56 ± 0.34 vs. 1.35 ± 0.35, p = 0.20). After the diagnosis of VSA was made, all patients were treated with CCBs, 14 with dihydropyridine agents alone, 1 with diltiazem, and 6 with combinations of CCBs. The use of nicorandil and aspirin was increased, whereas that of statins was unaltered between baseline and 3-month follow-up (Table 2). There was no difference in baseline Rho-kinase activity between patients with and those without statins (1.29 ± 0.29 vs.

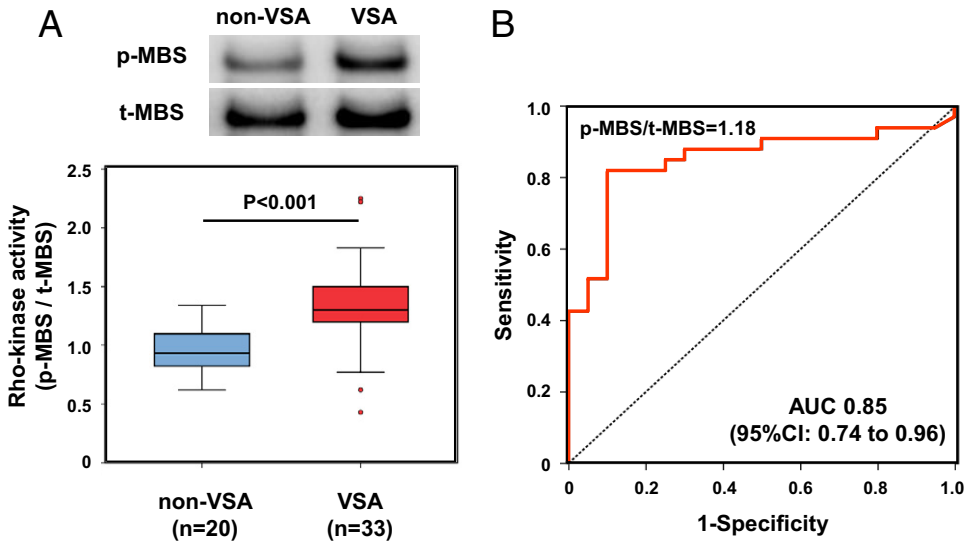

Figure 1 **Increased Rho-Kinase Activity in Circulating Leukocytes of Patients With VSA and ROC Curve Analysis for Detecting VSA**

(A) Rho-kinase activity was determined by the extent of phosphorylation of myosin-binding subunit (MBS), a substrate of Rho-kinase, and was expressed as the ratio of phosphorylated myosin-binding subunit (p-MBS) to total myosin-binding subunit (t-MBS). Results are expressed as box-and-whisker plots in Figures 1 to 3; the central box covers the interquartile range, with the median indicated by the line within the box. The whiskers extend to the most extreme values within 1.5 interquartile ranges. More extreme values are plotted individually. **(B)** In the present receiver-operating characteristic (ROC) curve analysis, a ratio of 1.18 was identified as the best cutoff level for Rho-kinase activity, with 82% sensitivity and 90% specificity. The calculated area under the curve (AUC) was 0.85 (95% confidence interval [CI]: 0.74 to 0.96). VSA = vasospastic angina.

1.54 ± 0.37, p = 0.11). Importantly, Rho-kinase activity was significantly decreased after 3-month medical treatment from 1.43 ± 0.36 to 1.08 ± 0.31 ($p < 0.001$) (Fig. 3A). Moreover, there was a trend toward an association between percent change in Rho-kinase activity and the reduction of symptoms at 3-month follow-up (Fig. 3B).

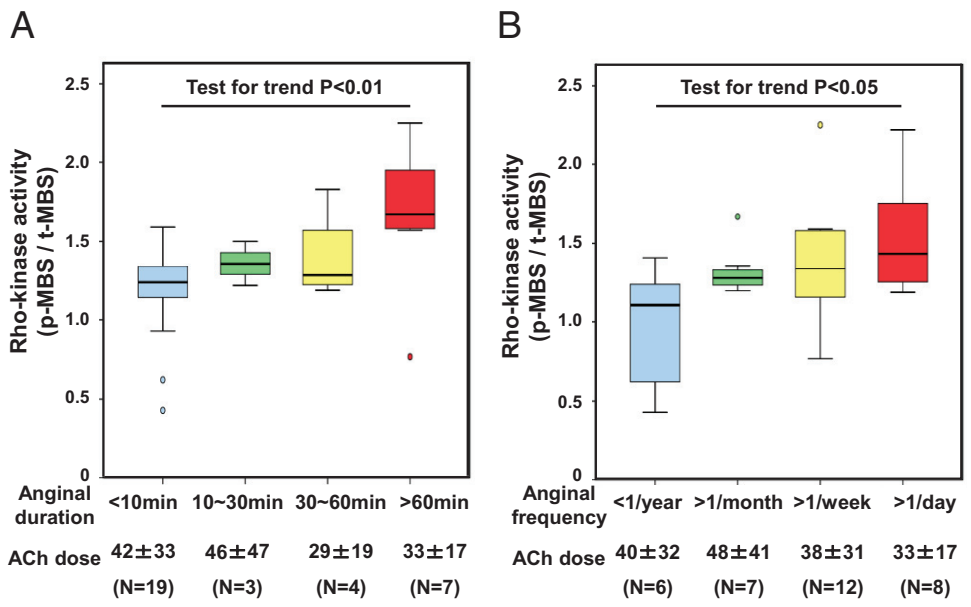

Figure 2 **Linear Trend of Anginal Duration and Frequency With Rho-Kinase Activity in Circulating Leukocytes**

(A) Anginal duration was classified into the following 4 grades: <10, 10 to 30, 30 to 60, and ≥60 min. **(B)** Anginal frequency was classified into the following 4 grades: >1/year, 1/year to 1/month, 1/month to 1/week, and 1/week to 1/day. The doses of acetylcholine (ACh) needed to induce coronary spasm are shown for each category. Abbreviations as in Figure 1.

Table 2 Medical Treatment at Baseline and 3-Month Follow-Up in Patients With VSA (n = 21)

Medication	Baseline	3 Months	p Value
CCBs	8 (38%)	21 (100%)	<0.01
Dihydropyridine	6 (29%)	20 (95%)	<0.01
Diltiazem	4 (19%)	7 (24%)	0.25
Nitrates	1 (5%)	4 (19%)	0.25
Nicorandil	1 (5%)	7 (24%)	0.03
Statins	8 (38%)	12 (57%)	0.25
ACE inhibitors/ARBs	7 (24%)	8 (38%)	1.00
Aspirin	2 (10%)	8 (38%)	0.07

Values are n (%).

ACE = angiotensin-converting enzyme; ARB = angiotensin II receptor blocker; CCB = calcium-channel blocker; VSA = vasospastic angina.

Discussion

The major findings of this study were that: 1) Rho-kinase activity in circulating leukocytes, determined by 2 different assessments, was significantly higher in the VSA group than in the non-VSA group; 2) the level of Rho-kinase activity was associated with vasospastic activity and decreased at 3 months after the medical treatment; and 3) a phosphorylated ratio of Rho-kinase of 1.18 was identified as the best cutoff level for the diagnosis of VSA. To the best of our knowledge, this is the first study to demonstrate the clinical significance of Rho-kinase activity in circulating leukocytes as a biomarker for diagnosis and assessment of disease activity in patients with VSA.

Measurement of Rho-kinase activity in circulating leukocytes. Accumulating evidence indicates that Rho-kinase activity in circulating leukocytes is associated with cardiovascular diseases and their risk factors, including coronary artery diseases (19), acute ischemic stroke (20), pulmonary artery hypertension (21), smoking (22), metabolic syndrome (23), and essential hypertension (24). Nohria et al. (19) reported that the functional improvement in flow-mediated vasodilation by fasudil, a selective Rho-kinase inhibitor, was related to the extent of Rho-kinase inhibition of circulating leukocytes in patients with coronary artery diseases, of which inflammation is a well-recognized component. In histological studies of coronary plaque in VSA, neointimal hyperplasia with infiltration of inflammatory cells was reported (33).

In patients with VSA, plasma levels of hsCRP, a sensitive marker of inflammation, are increased compared with those without spasm (34,35). A recent study demonstrated that 6-month treatment with a statin (fluvastatin) significantly reduced the occurrence of coronary spasm along with the decrease in hsCRP levels (6). Our previous findings with porcine models of spasm induced by long-term treatment with interleukin-1-beta (9,10,36) also indicate that chronic low-grade inflammation may be involved in the pathogenesis of coronary spasm. However, in the present study, hsCRP levels were comparable between the 2 groups, whereas Rho-kinase activity in circulating leukocytes was

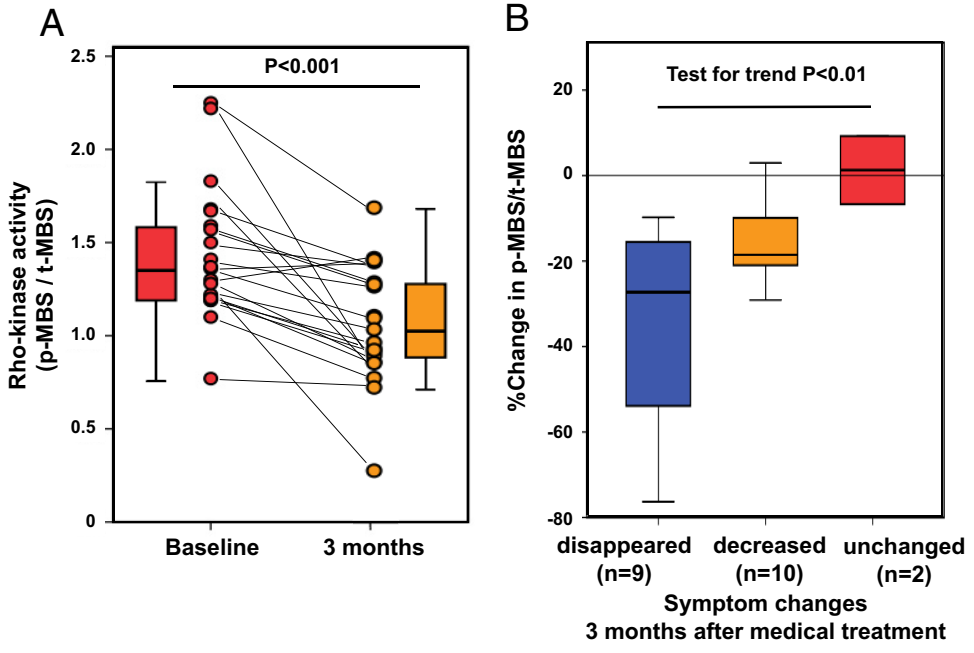

Figure 3 Rho-Kinase Activity in Circulating Leukocytes of Patients With VSA After 3-Month Medical Treatment

(A) After 3-month medical treatment, Rho-kinase activity in circulating leukocytes was significantly reduced in patients with vasospastic angina (VSA). **(B)** Patients' symptoms at 3 months were classified into the following 3 grades: disappeared, decreased, and unchanged. There was a linear trend between percent decrease in Rho-kinase activity in circulating leukocytes and symptom reduction during 3-month follow-up in patients with VSA. Abbreviations as in Figure 1.

significantly increased in the VSA group, suggesting that the Rho-kinase activity could be a more sensitive serological marker of coronary spasm than hsCRP.

Potential association of Rho-kinase activity with disease activity of coronary spasm and CCBs. Rho-kinase activity was significantly associated with anginal duration and frequency. After 3-month medical treatment, there was a trend between the extent in the decrease of Rho-kinase activity and the reduction of symptoms. These findings indicate that Rho-kinase activity in circulating leukocytes could be potentially associated with disease activity of coronary spasm as well. Also, the level of Rho-kinase activity in the patients with VSA seems to be comparable with that in patients with stable angina with severe organic stenosis, suggesting that the presence of active coronary spasm is equivalent to that of significant coronary stenosis in terms of Rho-kinase activation.

In the present study, Rho-kinase activity was significantly decreased after 3-month medical treatment with CCBs, the mainstay of current clinical practice in VSA. In a recent double-blind, randomized study of patients with hypertension (24), after 4 and 12 weeks of treatment, Rho-kinase activity in circulating neutrophils was significantly decreased in the CCB group but not in the renin-angiotensin system inhibitor group, whereas the antihypertensive effects were similar in the 2 groups. Although the precise mechanisms remain unclear and further studies are needed, long-term treatment with CCBs may have potential inhibitory effects on Rho-kinase.

Rho-kinase activity in circulating leukocytes as a possible diagnostic tool for VSA. The diagnosis of VSA is not necessarily easy (37). The attack is transient, often lasts only a few seconds to minutes, and is unpredictable. Even with 24-h ambulatory electrocardiographic monitoring, the attacks may not appear during the recording period, especially when the disease activity appears not to be high and the attacks are not frequent. Therefore, most patients with suspected VSA undergo provocation testing with ACh or ergonovine, which might cause complications such as ventricular fibrillation, myocardial infarction, or even death (38–40).

In the present study, we were able to demonstrate that Rho-kinase activity (as expressed by the ratio of phosphorylated to total Rho-kinase) of 1.18 is the best cutoff level to predict the diagnosis of VSA. Because the sensitivity and specificity of this ratio were reasonably high, Rho-kinase activity in circulating leukocytes may be a useful diagnostic biomarker for VSA. The advantages of the present method are that it is noninvasive and requires only a small volume of blood and thus can be easily performed in the clinical setting.

To date, only 1 study has reported that the best cutoff value for Rho-kinase activity in circulating neutrophils is 0.39 to predict the diagnosis of metabolic syndrome (23), a much lower value compared with patients with VSA. Further studies are needed to determine whether the analysis might be related to the specific diseases or the experimental conditions.

Study limitations. First, the present study was a single-center, exploratory study whose sample size was not calculated beforehand and consequently consisted of a relatively small number of patients. Second, medications after diagnosis were individualized at the discretion of each attending physician on the basis of symptoms and risk factors. Third, for ethical reasons, we were unable to follow the time course of Rho-kinase activity in patients with VSA without CCBs and to perform second ACh provocation tests after intensive medical therapy. Fourth, in the present study, there was no correlation between Rho-kinase activity and risk factors, which differs from previous studies (22–24). The difference may be related in part to the relatively small number of study patients and accumulating risk factors in the present study. Fifth, the present results suggest that the measurement of Rho-kinase activity does not distinguish between patients with different causes of angina.

Conclusions

The present study demonstrates that the noninvasive measurement of Rho-kinase activity in circulating leukocytes may be useful for diagnosis and disease activity assessment in patients with VSA.

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