



Rho-Kinase Activation in Patients With Heart Failure

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Background: Heart failure (HF) is a complex clinical syndrome, resulting from structural and/or functional cardiac disease. The aim of this study was to determine whether the activity of Rho-kinase, which has been identified as an important therapeutic target of cardiovascular disease, is enhanced in HF patients.

Methods and Results: Total and phosphorylated forms of myosin binding subunit (t-MBS and p-MBS), a substrate of Rho-kinase, were measured on western blotting in circulating leukocytes, and the p-MBS/t-MBS ratio was defined as an index of systemic Rho-kinase activity. First, during the time-course of acute HF (n=12), Rho-kinase activity was significantly elevated in the acute phase compared to the chronic phase (1.19 ± 0.06 vs. 0.97 ± 0.04 , $P < 0.05$). Next, Rho-kinase activity was examined in 30 controls and 130 chronic HF patients (cardiomyopathy, n=57; valvular heart disease, n=35; ischemic heart disease [IHD], n=33; and others, n=5). As compared with the controls, Rho-kinase activity was significantly elevated in the total HF group (1.14 ± 0.02 vs. 0.77 ± 0.05 , $P < 0.0001$) and in each underlying heart disease ($P < 0.05$ each). Importantly, in the high-risk non-IHD group, Rho-kinase activity was significantly associated with plasma brain natriuretic peptide level. Finally, p-MBS was expressed in myocardial biopsy samples (immunohistochemistry) in chronic HF patients (n=36), independent of Rho-kinase activity in leukocytes.

Conclusions: Rho-kinase is activated in HF patients, suggesting that it could be a new therapeutic target of the disorder. (*Circ J* 2013; **77**: 2542–2550)

Key Words: Biomarker; Circulating leukocytes; Heart failure; Myocardial biopsy; Rho-Kinase

Heat failure (HF) is a complex clinical syndrome resulting from any structural and/or functional cardiac disorder that impairs the ability of the ventricle to fill with and/or eject blood, where both HF with preserved ejection fraction (HFpEF) and HF with reduced EF (HFrEF) are substantially involved.^{1,2}

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Rho-kinase/ROK/ROCK was identified as an effector of the small GTP-binding protein Rho, and its activity is enhanced by binding of the GTP-binding active form of RhoA with subsequent enhancement of myosin light chain (MLC) phosphorylation through activation of MLC kinase and inhibition of myosin binding subunit (MBS) of myosin phosphatase.^{3–5} Rho-kinase plays an important role in various cellular functions, including smooth muscle contraction, stress fiber formation, focal adhe-

sion, migration, cytokinesis, and gene expressions.^{6–9} Recently, we and others have demonstrated that Rho-kinase activity in circulating leukocytes is elevated in various diseases, including coronary artery disease,¹⁰ acute ischemic stroke,¹¹ pulmonary artery hypertension,^{12,13} smoking,¹⁴ metabolic syndrome,¹⁵ essential hypertension,¹⁶ and coronary spasm.^{17–19}

We have previously demonstrated that Rho-kinase enhances myocardial stiffness, cardiac hypertrophy, ventricular fibrosis, and superoxide production in animal models of HFpEF in vivo, all of which abnormalities were ameliorated by long-term treatment with a selective Rho-kinase inhibitor, fasudil.²⁰ Importantly, there was a significant correlation between the extent of myocardial stiffness and that of myocardial Rho-kinase activity.²⁰ It remains to be determined, however, whether Rho-kinase is actually activated in patients with HF in both HFpEF and HFrEF. In the present study, we thus addressed this important issue in patients with HF.

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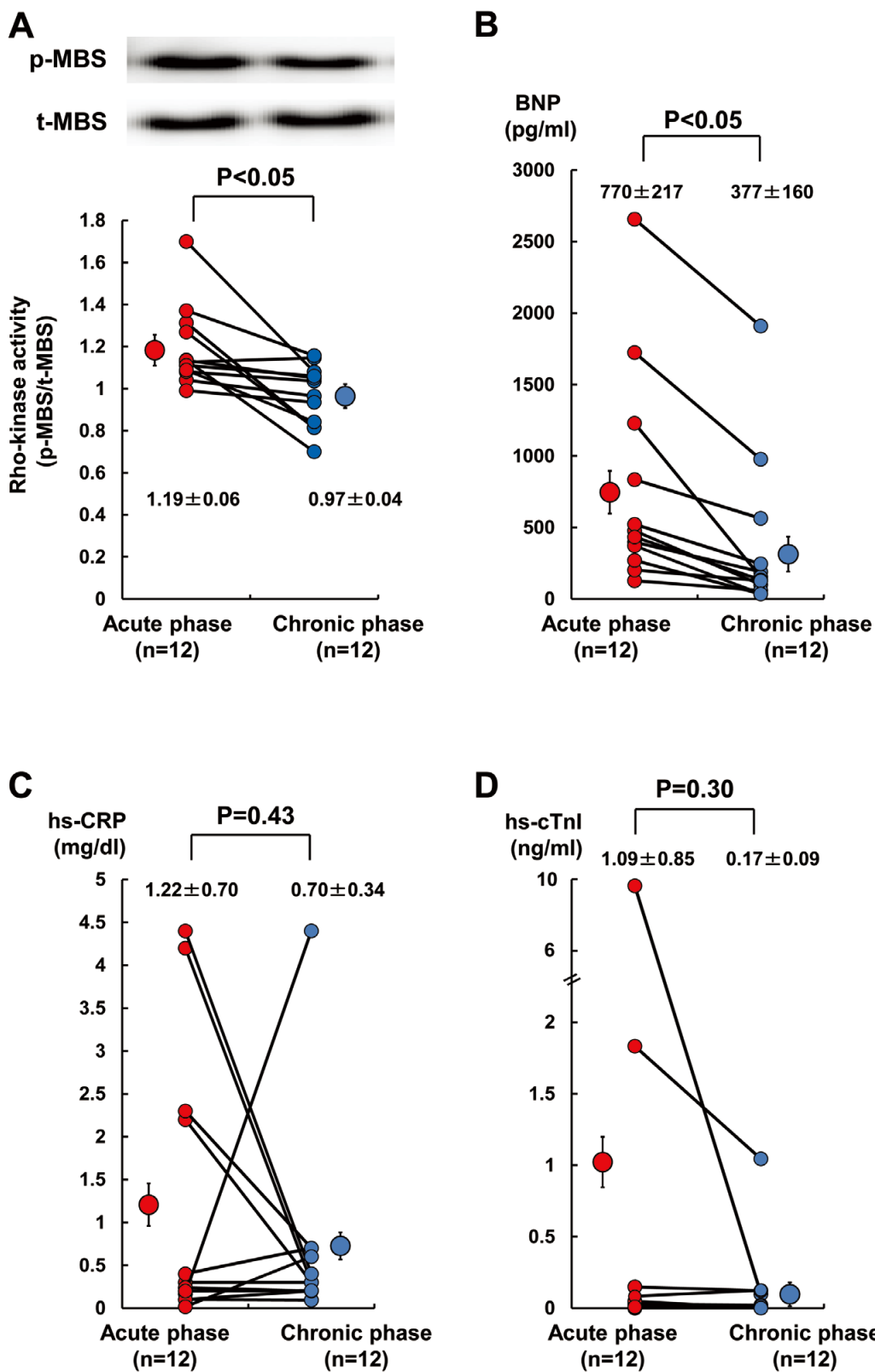


Figure 1. Rho-kinase activity in circulating leukocytes of patients with acute heart failure. **(A)** Representative western blotting for p-MBS and t-MBS of circulating leukocytes from patients in the acute and the chronic phases of HF. Rho-kinase activity was calculated as p-MBS/t-MBS. **(B)** Plasma BNP, **(C)** serum hs-CRP, and **(D)** plasma hs-cTnI levels in the acute and chronic phases of HF. Results given as mean ± SEM. BNP, brain natriuretic peptide; HF, heart failure; hs-CRP, high-sensitivity C-reactive protein; hs-cTnI, high-sensitivity cardiac troponin I; p-MBS, t-MBS, phosphorylated and total forms of myosin binding subunit, respectively.

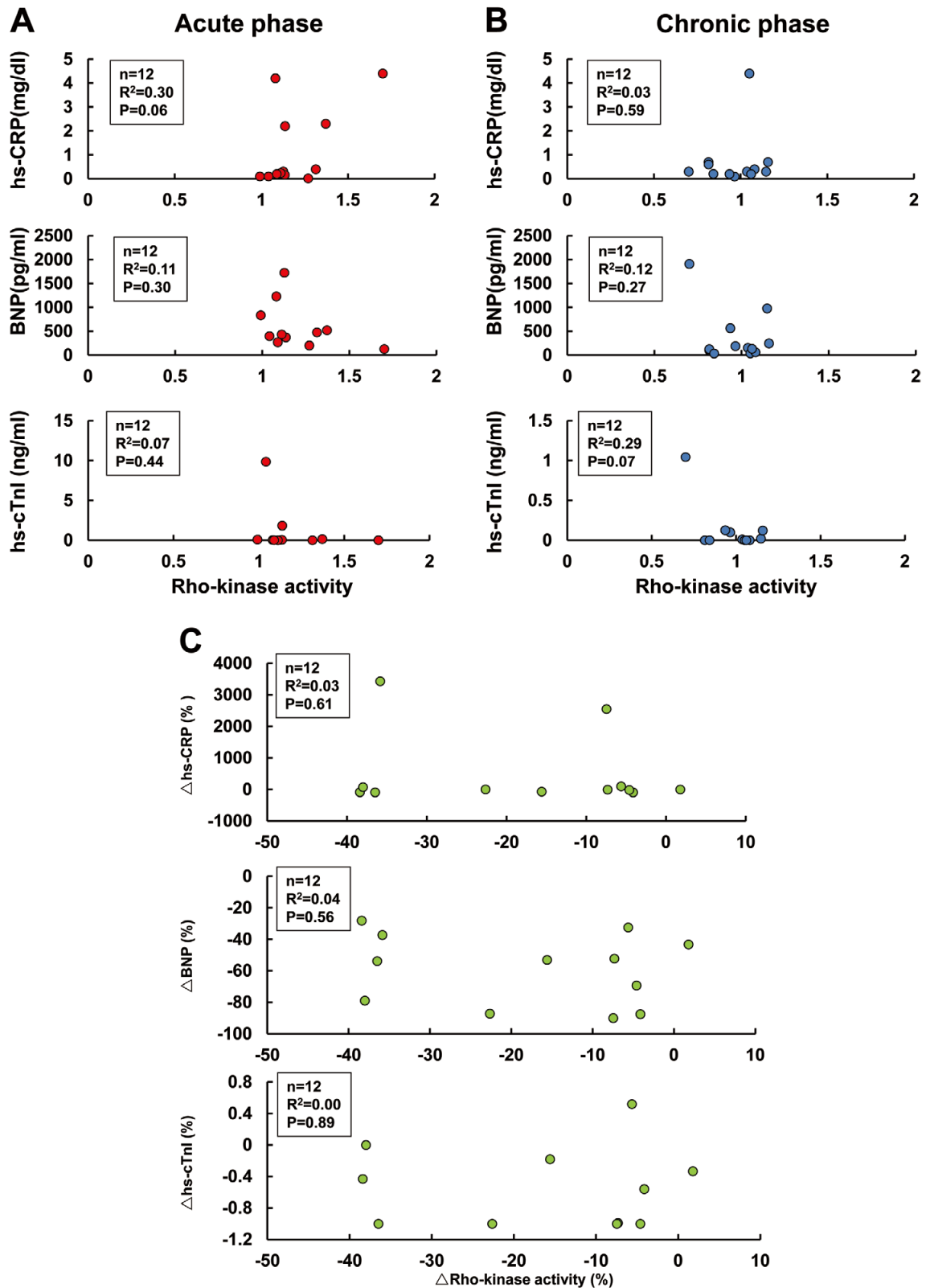


Figure 2. Correlation between Rho-kinase activity in circulating leukocytes and other markers of CHF. (A,B) Correlation between Rho-kinase activity of circulating leukocytes and hs-CRP, BNP and hs-cTnl levels in the (A) acute phase and (B) chronic phase of HF. (C) Correlations between the changes in Rho-kinase activity of circulating leukocytes and those in hs-CRP, BNP and hs-cTnl levels. Results given as mean \pm SEM. BNP, brain natriuretic peptide; HF, heart failure; hs-CRP, high-sensitivity C-reactive protein; hs-cTnl, high-sensitivity cardiac troponin I.

Methods

This study complies with the Declaration of Helsinki, and the Ethics Committees of Tohoku University Hospital approved the study protocol and all patients provided written informed consent. The authors had full access to the data and take full responsibility for its integrity.

Subjects

Twelve patients with acute HF (AHF; 3 men, 9 women; mean age, 63±6 years), 130 chronic HF patients (CHF; 82 men, 48 women; mean age, 58±1 years) and 30 control subjects (19 men, 11 women; mean age, 54±2 years) were enrolled in the present study. The etiology of AHF was hypertrophic cardiomyopathy in 6, dilated cardiomyopathy in 2, hypertensive heart disease in 1, mitral regurgitation in 1, mitral stenosis in 1, and congenital heart disease in 1. In the CHF patients, the etiology was ischemic heart disease (IHD) in 33 and non-IHD in 97, including cardiomyopathy (CM) in 57, valvular heart disease (VHD) in 35, and others in 5. All patients with HF were diagnosed according to the Framingham criteria.²¹ Physical activity was determined on the basis of the American College of Cardiology/American Heart Association guidelines for the diagnosis and management of CHF in the adult,¹ and all patients were in stage C. Control subjects were matched by age and gender to CHF patients without any cardiac risk factors, such as hypertension, diabetes mellitus, dyslipidemia and obesity. Plasma/serum levels of high-sensitivity C-reactive protein (hs-CRP), brain natriuretic peptide (BNP)²² and high-sensitivity cardiac troponin I (hs-cTnI),^{23,24} were also determined as markers of inflammation, HF severity and myocardial damage, respectively.

Data Collection

Baseline demographic data were collected based on the medical records, including age, sex, height, body weight, waist, medication (angiotensin-converting enzyme inhibitor [ACEI], angiotensin receptor blocker [ARB], calcium channel blocker [CCB], β -blocker, and statin), risk factors (hypertension, glucose intolerance/diabetes mellitus and dyslipidemia), blood pressure, pulse rate, heart rate, blood data (lipid profile and glucose), plasma BNP, and comorbidities (IHD, hypertensive heart disease, CM, VHD, and congenital heart disease). Left ventricular ejection fraction (LVEF) was measured on echocardiography. According to the European Society of Cardiology 2007 guidelines, we divided the patients into 2 groups: HFpEF (LVEF $\geq 50\%$) and HFrEF (LVEF $< 50\%$).²⁵

Leukocyte Isolation and Rho-Kinase Activity

Venous blood samples were collected from peripheral veins in the acute and chronic phases in AHF patients and during cardiac catheterization in CHF patients. Circulating leukocytes were isolated from venous blood samples, as previously reported.^{12,13,17} To quantify Rho-kinase activity in circulating leukocytes, we performed western blot analysis for phosphorylated forms of MBS (p-MBS) and total forms of MBS (t-MBS), a substrate of Rho-kinase, as previously described.^{12,13,17} NIH 3T3 cell lysates were used as a positive control. Briefly, cell extracts were loaded on 7.5% SDS-PAGE gel and membrane was incubated with rabbit anti-phospho-MBS polyclonal antibody (Upstate, Tokyo, Japan) or mouse anti-MBS antibody (BD Biosciences, Tokyo, Japan). Subsequently, membrane was incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Cell Signaling) or rabbit anti-mouse IgG (Sigma) as the second antibody. Signals were visualized with the use of the ECL detection kit (Amersham Biosciences), and were then

enhanced by the chemiluminescence system. Densitometric analysis was performed using Scion Image.^{12,13,17}

Immunohistology of Myocardial Biopsy Samples

Trans-venous endomyocardial biopsy samples were obtained from the interventricular septum using 6-Fr Biotom (Cordis, Bridgewater, NJ, USA) when CHF patients underwent cardiac catheterization (n=36). The tissues were immediately fixed in 4% paraformaldehyde and embedded in paraffin.²⁶ Immunohistochemical staining was performed using anti-rabbit phospho-MBS antibody (Upstate), and negative controls were performed for each slide. The positive rate of p-MBS was calculated as p-MBS-positive cell number/total cell number. As previously reported, immune-positive p-MBS was used as a marker of Rho-kinase activity.^{13,27,28}

Statistical Analysis

All results are expressed as mean \pm SEM. We assessed the differences in measured parameters in the acute and chronic phases during HF therapy using the paired and unpaired t-tests for changes caused by each intervention in the acute and chronic phases during HF therapy. Independent-sample t-test was used for comparison of mean Rho-kinase activity between CHF patients and control subjects. Receiver operating characteristic (ROC) curve analysis was performed to determine the best cut-off for Rho-kinase activity to differentiate between those with and those without CHF. The cut-off was determined as the sum of sensitivity and specificity.^{15,17} Statistical analysis was performed using SPSS (SPSS, Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

Results

Rho-Kinase Activity in AHF

In AHF patients, Rho-kinase activity was elevated in the acute phase and was then significantly decreased in the chronic phase (Figure 1A). Compared with the acute phase, plasma BNP level was significantly decreased in all patients in the chronic phase (Figure 1B). No significant differences, however, were noted in serum hs-CRP or plasma hs-cTnI (Figure 1C,D). In both the acute and chronic phases, no significant correlation was noted between Rho-kinase activity and plasma/serum hs-CRP, BNP or hs-cTnI (Figure 2A,B). Furthermore, no significant correlation was noted between the changes in Rho-kinase activity and those in hs-CRP, BNP or hs-cTnI (Figure 2C).

Rho-Kinase Activity in CHF

The CHF patients were treated with ACEI, ARB, β -blocker, CCB and statins (Table 1). In those patients, serum hs-CRP and plasma BNP were significantly elevated compared with the control subjects, whereas plasma hs-cTnI was similar between the 2 groups (Table 1). Regarding the effect of IHD as the underlying cause of CHF, plasma BNP was significantly higher in non-IHD compared with IHD, whereas serum hs-cTnI was significantly higher in IHD compared with non-IHD (Table S1). Serum hs-CRP was similar between the 2 groups (Table S1).

In the CHF patients, Rho-kinase activity was significantly elevated compared with the controls (Figure 3A). Rho-kinase activity was significantly and equally elevated in both the IHD and non-IHD groups including the CM and VHD groups (Figure 3B). Importantly, there was no significant difference in Rho-kinase activity between HFpHF (n=90) and HFrEF (n=40; Figure 3C). In the CHF patients, Rho-kinase activity in circulating leukocytes was independent of other markers of HF se-

Table 1. Clinical Patient Characteristics vs. Presence of CHF

	Controls (n=30)	CHF (n=130)	P-value
Age (years)	54±2	58±1	n.s.
Gender			
Male	19 (63)	82 (64)	n.s.
Female	11 (37)	48 (36)	n.s.
Type of HF			
CM	–	57 (44)	
VHD	–	35 (27)	
IHD	–	33 (25)	
Others	–	5 (4)	
Treatment			
ACEI	0	52 (40)	
ARB	0	35 (27)	
β-blocker	0	53 (41)	
CCB	0	49 (38)	
Statin	0	34 (26)	
Laboratory data			
BNP (pg/ml)	21±5	213±34	<0.05
hs-CRP (mg/dl)	0.05±0.01	0.58±0.19	<0.05
hs-cTnI (ng/ml)	0.05±0.03	0.09±0.06	n.s.

Results given as mean±SEM or n (%). ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BNP, brain natriuretic peptide; CCB, calcium channel blockers; CHF, chronic heart failure; CM, cardiomyopathy; HF, heart failure; hs-CRP, high-sensitivity C-reactive protein; hs-cTnI, high-sensitivity cardiac troponin I; IHD, ischemic heart disease; VHD, valvular heart disease.

verity, including hemodynamic variables, hs-CRP, BNP, and hs-cTnI (Table S2).

Rho-kinase activity was found to be a powerful predictor of CHF, with an area under the curve (AUC) of 0.87 (95% confidence interval: 0.80–0.93), and a ratio of 0.93 was identified as the best cut-off level for the diagnosis of CHF (Figure 3D). The sensitivity and specificity of the ROC curve analysis for detecting CHF were 84% and 80%, respectively (Figure 3D).

Rho-Kinase Activity and Cardiovascular Events in CHF

In the present 130 CHF patients, 5 died during the follow-up period of 408±32 days, due to HF in 1 and non-cardiovascular causes in 4 (eg, cancer). Furthermore, 31 (23.8%) repeatedly needed hospitalization (5 in IHD and 26 in non-IHD). The best cut-offs for BNP to predict re-hospitalization were 141.8 pg/ml for IHD (AUC, 0.86; sensitivity, 80%; specificity, 89%) and 266.4 pg/ml for non-IHD (AUC, 0.73; sensitivity, 62%; specificity, 83%), respectively (Figure S1).

In the IHD group, no significant correlation was noted between Rho-kinase activity and age, serum hs-CRP, plasma BNP or hs-cTnI in both the low-risk group (BNP ≤141.8 pg/ml) and the high-risk group (BNP >141.8 pg/ml; Table 2). In the non-IHD group, however, significant correlation was noted between Rho-kinase activity and plasma BNP in the high-risk group (BNP >266.4 pg/ml; Table 2).

Rho-Kinase Activity in Myocardial Biopsy Samples from CHF Patients

We examined Rho-kinase activity in myocardial biopsy samples from CHF patients (n=36) using immunohistochemistry for p-MBS expression, as a marker of Rho-kinase activity.^{13,27,28} Immuno-positive p-MBS staining was noted in myocardial

biopsy samples from CHF patients (Figure 4A,B), but no significant correlation was noted between Rho-kinase activity in circulating leukocytes and that in myocardial biopsy samples in the CHF patients (Figure 4C). Due to ethical reasons, no myocardial samples were available from the control subjects.

Discussion

The novel findings of the present study are as follows: (1) Rho-kinase activity was elevated in the acute phase and was then significantly decreased in the chronic phase; (2) even in the chronic phase, however, Rho-kinase activity in HF patients was significantly elevated compared with the controls; (3) increased Rho-kinase activity was significantly correlated with BNP level in the high-risk non-IHD patients; and (4) p-MBS immunoreactivity as a marker of Rho-kinase activity was noted in myocardial biopsy samples from CHF patients without correlation between local (in myocytes) and systemic (in blood) activity of Rho-kinase. These results indicate that Rho-kinase is systemically activated in patients with HF, suggesting that inhibition of Rho-kinase could be a new therapeutic target for CHF. To the best of our knowledge, this is the first study that provides direct evidence for Rho-kinase activation in patients with AHF and those with CHF.

Role of Rho-Kinase Pathway in the Pathogenesis of HF

We and others have previously demonstrated that the Rho-kinase pathway is involved in the pathogenesis of HF in animal models.^{29–31} We also have recently shown that Rho-kinase inhibition with fasudil, a selective Rho-kinase inhibitor, suppresses the development of cardiac hypertrophy and diastolic HF in Dahl salt-sensitive rats.²⁰ Furthermore, Rho-kinase is substantially involved in the angiotensin II (AngII)-induced signaling pathway.^{32–34} Because AngII plays a key role in the pathophysiological processes of cardiomyocytes, including cardiac hypertrophy,^{35–37} it is important to elucidate the alterations in the Rho-kinase pathway in CHF patients. We have previously demonstrated that intra-arterial infusion of fasudil caused a preferential increase in forearm blood flow in patients with CHF compared with control subjects, suggesting an involvement of the Rho-kinase pathway in the increased peripheral vascular resistance in CHF.³⁸ Direct evidence for Rho-kinase activation in patients with CHF, however, is still lacking. In the present study, we thus addressed this important issue.

Systemic Activation of Rho-Kinase in AHF

In the present study, Rho-kinase activity in circulating leukocytes was elevated in the acute phase and was then significantly decreased in the chronic phase. hs-CRP and hs-cTnI levels, however, were similar between the acute and chronic phases, whereas Rho-kinase activity was significantly decreased in the chronic phase, along with the decrease in plasma BNP level. Interestingly, no significant correlation was noted between Rho-kinase activity and hs-CRP, BNP or hs-cTnI levels, or between the changes in Rho-kinase activity and those in hs-CRP, BNP or hs-cTnI. These results suggest that Rho-kinase activity could be an independent biomarker of HF compared with hs-CRP, BNP and hs-cTnI.

Systemic Activation of Rho-Kinase in CHF

The present study shows that Rho-kinase is systemically activated in CHF patients and that Rho-kinase activity is markedly increased in all subgroups of CHF, with no significant difference between HFpHF and HFrfEF. Although it was recently reported that Rho-kinase activity in circulating leukocytes was

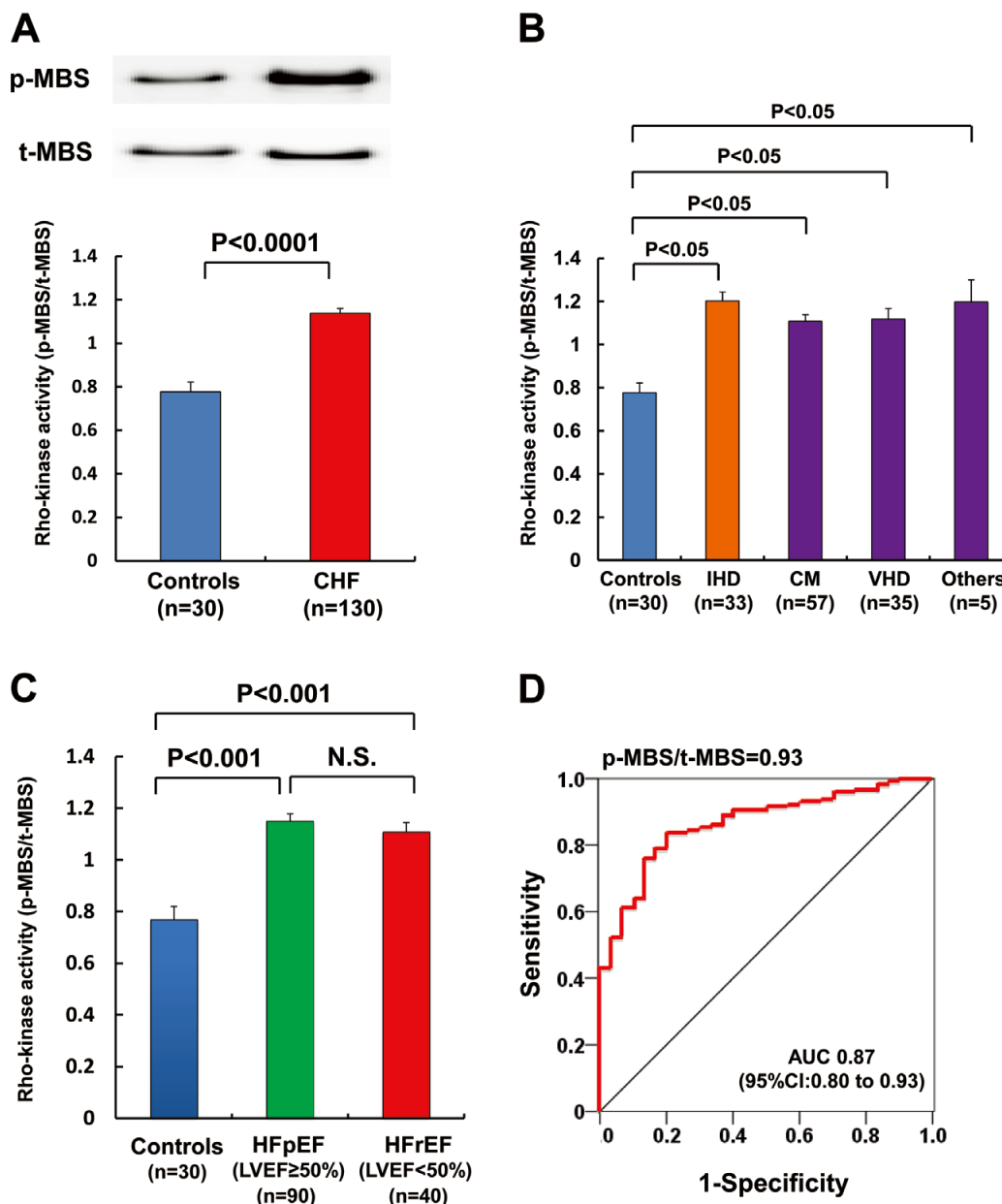


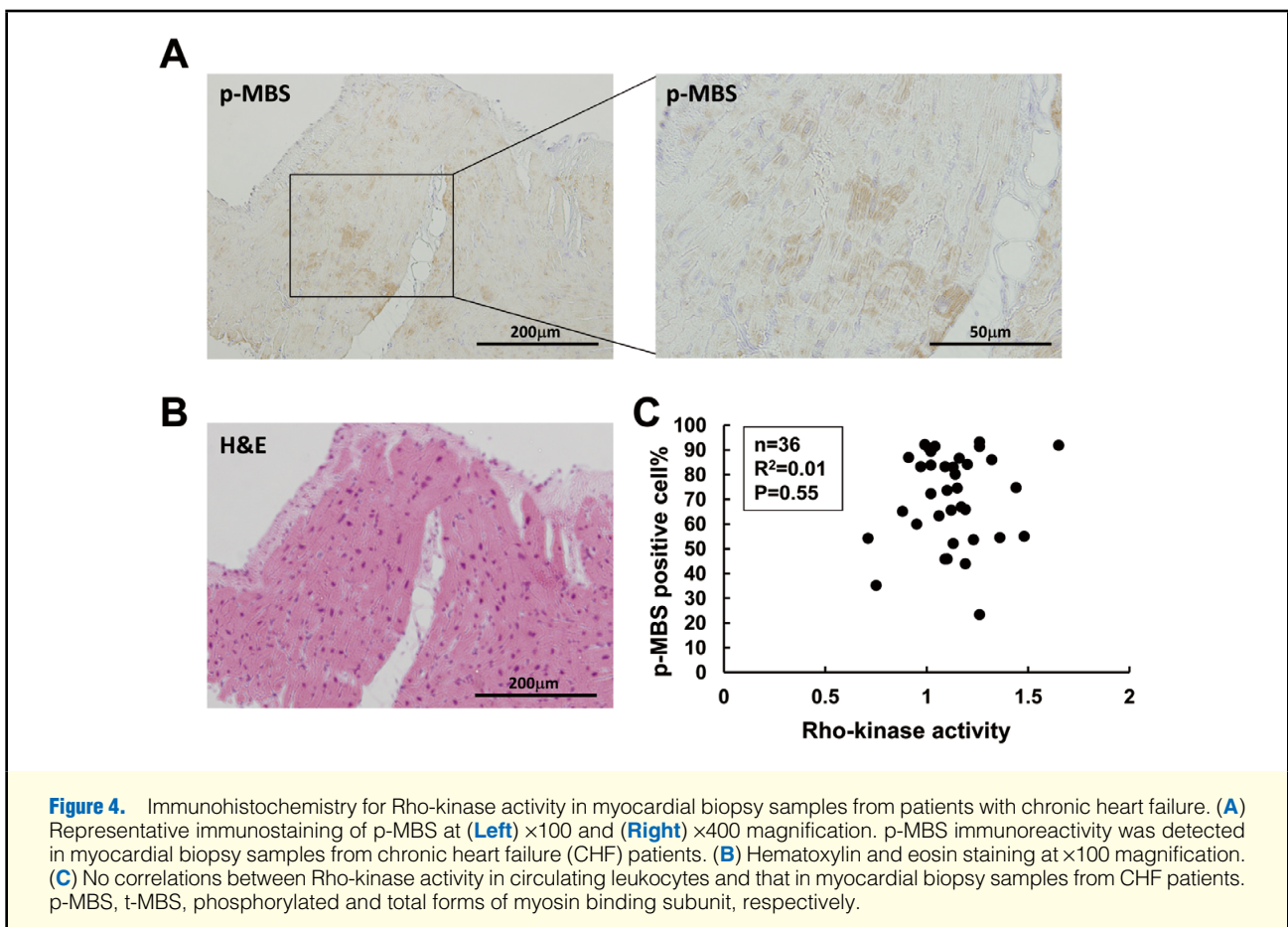
Figure 3. Rho-kinase activity in circulating leukocytes of patients with chronic heart failure and receiver operating characteristic (ROC) curve analysis. **(A)** Representative western blotting for p-MBS and t-MBS of circulating leukocytes from a control subject and a chronic HF (CHF) patient. Rho-kinase activity, as determined by p-MBS/t-MBS ratio, was significantly increased in patients with HF compared with control subjects. **(B)** Rho-kinase activity was significantly increased in all the 4 subgroups of CHF. **(C)** No significant difference in Rho-kinase activity was noted between the patients with HFpEF and those with HFrEF. **(D)** ROC curve analysis showed that a ratio of 0.93 was the best cut-off for Rho-kinase activity with 84% sensitivity and 80% specificity. The calculated area under the curve (AUC) was 0.87 (95% confidence interval [CI]: 0.80–0.93). Results given as mean \pm SEM. CHF, chronic heart failure; CM, cardiomyopathy; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; IHD, ischemic heart disease; p-MBS, t-MBS, phosphorylated and total forms of myosin binding subunit, respectively; VHD, valvular heart disease.

markedly increased in patients with HFrEF and that Rho-kinase activity was inversely correlated with LVEF,³⁹ the present study with a larger number of patients has shown that Rho-kinase activity in circulating leukocytes has no correlation with LVEF or other markers of HF severity, including hemodynamic variables, plasma/serum hs-CRP, BNP or hs-cTnI in CHF patients

(Table S2), suggesting that Rho-kinase activity is affected not only by LVEF but also by other comorbidities, such as hypertension. It was also recently reported that combined analysis of Rho-kinase activity and N-terminal pro-BNP increased the effectiveness of prediction of long-term mortality in CHF patients.⁴⁰ We were unable, however, to address this issue in the

Table 2. Rho-Kinase Activity and Age and Laboratory Markers in CHF			
CHF classification	Measurements	Correlation coefficient (R ²)	P-value
IHD, low-risk group (BNP≤141.8 pg/ml; n=27)	Age (years)	0.00	0.98
	hs-CRP (mg/dl)	0.02	0.54
	BNP (pg/ml)	0.07	0.19
	hs-cTnI (ng/ml)	0.02	0.58
IHD, high-risk group (BNP>141.8 pg/ml; n=6)	Age (years)	0.47	0.13
	hs-CRP (mg/dl)	0.06	0.63
	BNP (pg/ml)	0.09	0.56
	hs-cTnI (ng/ml)	0.22	0.54
Non-IHD, low-risk group (BNP≤266.4 pg/ml; n=70)	Age (years)	0.00	0.92
	hs-CRP (mg/dl)	0.04	0.12
	BNP (pg/ml)	0.00	0.62
	hs-cTnI (ng/ml)	0.02	0.22
Non-IHD, high-risk group (BNP>266.4 pg/ml; n=27)	Age (years)	0.01	0.71
	hs-CRP (mg/dl)	0.01	0.72
	BNP (pg/ml)	0.19	0.02
	hs-cTnI (ng/ml)	0.04	0.34

Data given as mean ± SEM. non-IHD, non-ischemic heart disease. Other abbreviations as in Table 1.



present study because only 1 of the present 130 CHF patients had a cardiovascular cause of death (HF death).

In the present study, the CHF patients were treated with ACEI, ARB, β -blocker, CCB and statins (Table 1). In a recent double-blind, randomized study of patients with hypertension, it was reported that long-term treatment with CCB may have a

potential inhibitory effect on Rho-kinase compared with ACEI, ARB and β -blocker, although the precise mechanisms remain unclear.¹⁵ The present results, however, suggest that Rho-kinase activation has no correlation with the use of these drugs. Given that HF is a clinical syndrome, various causes are involved in its pathogenesis in addition to the Rho-kinase pathway. Statins

might be a potential Rho-kinase inhibitor, but we have previously shown that clinical doses of statins lack the inhibitory effect on the Rho-kinase pathway but preferentially inhibit the Rac1 pathway.⁴¹

In the present study, we were able to show that Rho-kinase activity (calculated as the ratio p-MBS/t-MBS) of 0.93 is the best cut-off level for the diagnosis of CHF. Because the sensitivity and the specificity of the diagnosis were reasonably high (84% and 80%, respectively), Rho-kinase activity in circulating leukocytes could be a useful diagnostic biomarker of CHF.

Furthermore, p-MBS immunoreactivity was noted in myocardial biopsy samples from CHF patients. No significant correlation, however, was noted between Rho-kinase activity in circulating leukocytes and that in myocardial biopsy samples in CHF patients, suggesting that CHF is a complex disorder that is linked not only with cardiomyocytes but also with systemic organs (eg, blood vessels and the kidneys). In this sense, it is important to pay attention not only to the heart but also to the systemic organs when treating CHF patients.

Rho-Kinase Activity and Cardiovascular Events in HF

BNP is an established prognostic factor of CHF patients.^{42–44} In the present study, 23.8% of CHF patients needed re-hospitalization. Because a significant correlation was noted between Rho-kinase activity and plasma BNP in the high-risk and non-IHD group, the present results suggest that Rho-kinase activation is also involved in the pathogenesis of worsening of HF. Thus, the Rho-kinase pathway could be an important therapeutic target, especially for high-risk CHF patients.

Study Limitations

Several limitations should be mentioned for the present study. First, the prognostic impact of Rho-kinase activity in circulating leukocytes of CHF patients remains to be examined in future studies. This point is important especially because Rho-kinase activity had no significant correlation with the established prognostic markers, such as hemodynamic variables (eg, cardiac index) and biomarkers (eg, hs-CRP, BNP, and hs-cTnI). Second, the effects of medication on Rho-kinase activity in circulating leukocytes remain to be fully elucidated. Indeed, we were recently able to show that Rho-kinase activity could be altered by CCB in patients with vasospastic angina.¹⁷ Third, the discrepancy between the Rho-kinase activity in circulating leukocytes and that in myocardial biopsy samples remains to be examined, especially because we were unable to perform other Rho-kinase assay (eg, Rhotekin-Rho binding domain used for pull-down assay). As discussed here, it is conceivable that Rho-kinase activity in circulating leukocytes reflects the influence of systemic organs in addition to the heart. Fourth, it remains to be examined in future studies how Rho-kinase activity is correlated with elevated plasma BNP level only in the high-risk non-IHD group. Fifth, it also remains to be elucidated how activated Rho-kinase in circulating leukocytes affects target organs, including the signaling pathway to those organs, possible via cell surface proteins. We consider that the blood vessel and the kidney are also important for HF development, especially in relation to Rho-kinase activation.^{45,46}

Conclusions

Rho-kinase is systemically activated in patients with HF, suggesting that Rho-kinase could be a new therapeutic target of CHF, and that Rho-kinase activity in circulating leukocytes may be a novel biomarker of the disorder.

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Supplementary Files

Supplementary File 1

Table S1. CHF patient clinical characteristics vs. presence of IHD

Table S2. Rho-kinase activity and clinical variables in CHF

Figure S1. Classification of CHF patients (using best cut-off value of BNP to predict the presence or absence of re-hospitalization).

Please find supplementary file(s);
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