Markedly increased Rho-kinase activity in circulating leukocytes in patients with chronic heart failure

María Paz Ocaranza, PhD,[a] Luigi Gabrielli, MD,[a] Italo Mora, Bq,[a] Lorena Garcia, PhD,[b,c,d] Paul McNab, MD,[a] Iván Godoy, MD,[a] Sandra Braun, MD,[a] Samuel Córdova, MD,[a] Pablo Castro, MD,[a] Ulises Novoa, PhD,[a] Mario Chiong, PhD,[b,c,d] Sergio Lavandero, PhD,[b,c,d,e] and Jorge E. Jalil, MD[a] *Santiago, Chile, and Dallas, TX*

Background The small guanosine triphosphatase Rho and its target Rho-kinase have significant roles in experimental remodeling and ventricular dysfunction, but no data are available on Rho-kinase activation in patients with heart failure (HF). We hypothesized that, in patients with chronic HF, Rho-kinase in circulating leukocytes is activated and related to left ventricular (LV) remodeling and dysfunction.

Methods Accordingly, Rho-kinase activity, assessed by the levels of phosphorylated to total myosin light chain phosphatase 1 (MYPT1-P/T) in circulating leukocytes, and echocardiographic LV function data were compared between patients with HF New York Heart Association functional class II or III due to systolic dysfunction (n = 17), healthy controls (n = 17), and hypertensive patients without HF (n = 17).

Results In the control subjects, mean MYPT1-P/T ratio was 1.2 ± 0.2 (it was similar in the hypertensive patients without HF), whereas in patients with HF, it was significantly increased by >100-fold ($P < .001$). Both MYPT1-P/T and log MYPT1-P/T ratios were inversely correlated with ejection fraction ($r = –0.54$, $P < .03$ and $r = –0.86$, $P < .001$, respectively). Furthermore, in patients with HF with LV end-diastolic diameter <60 mm, MYPT1-P/T ratio was 35.8 ± 18.1, whereas it was significantly higher in patients with LV diameter ≥60 mm ($P < .05$).

Conclusions Rho-Kinase activity is markedly increased in patients with stable chronic HF under optimal medical treatment, and it is associated with pathologic LV remodeling and systolic dysfunction. Mechanisms of Rho-kinase activation in patients with HF, its role in the progression of the disease, and the direct effect of Rho-kinase inhibition need further investigation. (Am Heart J 2011;161:931-7.)

The small guanosine triphosphatase Rho and its target, Rho-kinase, play important roles in both blood pressure regulation and vascular smooth muscle contraction. Rho is activated by agonists of receptors coupled to cell membrane G protein (such as angiotensin II, endothelin, or noradrenalin), by growth factors, or by cytokines.[1-3] Once Rho is activated, it translocates to the cell membrane where it activates Rho-kinase. Rho-kinase phosphorylates myosin light chain phosphatase, which is then inhibited. This sequence stimulates vascular smooth muscle contraction, stress fiber formation, and cell migration. In this way, Rho and Rho-kinase activation have important effects on several cardiovascular diseases.[1-3]

There is also experimental evidence on the significant role of Rho-kinase activation in the development of cardiac hypertrophy, remodeling, and ventricular dysfunction. In Dahl salt-sensitive, hypertensive rats, increased left ventricular (LV) weight in the hypertrophy stage was significantly ameliorated by the Rho-kinase inhibitor Y-27632.[4] Up-regulated RhoA protein, Rho-kinase gene expression, and myosin light chain phosphorylation in the hypertrophy stage were also suppressed by the Rho-kinase inhibitor.[4] In hypertensive rats, the Rho-kinase inhibitor fasudil attenuated myocardial fibrosis, possibly via suppression of monocyte/macrophage infiltration of the heart.[5] In mice with experimental myocardial infarction, chronic Rho-kinase inhibition with fasudil, prevented myocardial remodeling,[6] and also high levels of inflammatory cytokines in the infarcted area were suppressed by inhibiting Rho-kinase.[6]

However, there are no data on the role of Rho-kinase activation in patients with heart failure (HF). We

From the [a]Pontificia Universidad Católica de Chile, Escuela de Medicina, Departamento De Enfermedades Cardiovasculares, Santiago, Chile, [b]Universidad de Chile, Facultad de Ciencias Quimicas y Farmacéuticas, Santiago, Chile, [c]Centro FONDAP Estudios Moleculares de la Celula, Santiago, Chile, [d]Universidad de Chile, Facultad de Medicina Instituto de Ciencias Biomédicas, Santiago, Chile, [e]Cardiology Division, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX.

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Reprint requests: Jorge E. Jalil, MD, or Luigi Gabrielli, MD, Pontificia Universidad Católica de Chile, Escuela de Medicina, Departamento de Enfermedades Cardiovasculares, Marcoleta 367 Piso 8, Santiago, Chile.

E-mails: jjalil@med.puc.cl, lgabriel@med.puc.cl

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Table I. Demographics, blood pressure, and laboratory tests

	Controls (n = 17)	Hypertensive patients (n = 17)	Patients with HF (n = 17)	Intergroup differences
Age (y)	53.2 ± 1.2	56.5 ± 1.4	60.2 ± 2.7	NS
Men (%)	76	59	70	NS
Body mass index (kg/m^2)	25 ± 0.8	26 ± 0.7	26 ± 0.8	NS
Systolic BP (mm Hg)	115 ± 6	147 ± 5*	125 ± 6	$P < .01$
Mean BP (mm Hg)	88 ± 1	110 ± 9*	91 ± 5	$P < .01$
PWV (m/s)	8.3 ± 0.2	13.1 ± 1*	9.9 ± 0.4	$P < .01$
Heart rate (beat/min)	74 ± 1	77 ± 2	69 ± 2*	$P < .01$
Creatinine (S, mg/dL)	0.8 ± 0.01	0.8 ± 0.03	0.9 ± 0.01	NS
Potassium (S, mEq/L)	4.2 ± 0.1	4.2 ± 0.1	4.5 ± 0.2	NS
Hematocrit (%)	43 ± 0.9	43 ± 0.8	40 ± 1.1	NS
Total cholesterol (mg/dL)	200 ± 9	201 ± 3	183 ± 11	NS
LDL cholesterol (mg/dL)	116 ± 8	121 ± 6	103 ± 8	NS
AST (U/L)	27 ± 3	24 ± 2	29 ± 2	NS
MDA (PL, μmol/L)	0.51 ± 0.09	1.35 ± 0.43	0.71 ± 0.16	NS
8-isoprostane (PL, pg/mL)	23.5 ± 6.4	36.4 ± 10.1	50.5 ± 23.9	NS
hs-CRP (S, mg/L)	2.4 ± 0.8	5.5 ± 1.4	6.8 ± 1.4	NS
PICP (S, μg/L)	111.5 ± 17.5	90.2 ± 14.1	121.9 ± 19.7	NS

Values shown as mean ± SEM. *NS* indicates nonsignificant; *BP*, blood pressure; *LDL*, low-density cholesterol; *AST*, aspartate aminotransferase; *PL*, plasma, *S*, serum.
* $P < .05$ versus the other groups (after ANOVA).

hypothesized here that, in patients with HF due to systolic dysfunction, Rho-kinase is activated, and its activation is related to LV remodeling and dysfunction. Accordingly, Rho-kinase activity and LV function were simultaneously determinated in patients with HF and in healthy control individuals.

Methods

Study design

This was a cross-sectional study comparing patients with stable chronic HF due to systolic dysfunction with healthy controls matched by age and gender. The study was approved by the Research Committee of the Medical School, Pontifical Catholic University of Chile, and was funded by Fondecyt 1085208 (Chile). Participants were consecutive patients (n = 17) with stable chronic congestive HF functional class II or III (New York Heart Association) and LV systolic dysfunction (ejection fraction [EF] <40%) under optimal medical treatment; with serum creatinine <2 mg/dL; nondiabetic patients; and nonobese (body mass index <28 kg/m^2), all in sinus rhythm. Their characteristics are depicted in Table I. Controls (n = 17) were healthy normotensive subjects matched by age and gender, without any antihypertensive drug; nonobese; and nondiabetic patients. A group of hypertensive patients without HF and receiving antihypertensive treatment was also included as a second control group. Exclusion criteria were neoplastic disease in the last 4 years; active infection in the last 8 weeks; use of statins (because in addition to inhibiting cholesterol biosynthesis, statins also inhibit the formation of isoprenoid intermediates, which are required for the activation of the Rho/Rho-kinase pathway, and they inhibit circulating Rho-kinase activity in humans)[7,8]; and chronic lung, liver, or kidney disease. All the study participants signed an informed consent before participating in the study.

Echocardiographic measurements and noninvasive arterial stiffness evaluation

Echocardiograms were obtained with a 2.5-MHz transducer at the time of blood sampling with a Phillips IE-33 instrument (Andover, MA) to evaluate LV function, geometry, and mass. All measurements were performed blindly according to the recommendations of the American Society of Echocardiography.[9,10] The following variables in the parasternal short axis were measured: interventricular septal thickness and posterior wall thickness, end-diastolic dimension, and end-systolic dimension. With these variables, LV mass and LV mass indexes were calculated according to the formula developed by Devereux et al[11] and modified by the American Society of Echocardiography.[12] Ejection fraction was measured by the Simpson method.

Carotid-to-femoral pulse wave velocity (PWV), a noninvasive and indirect index of arterial stiffness, was determined in all subjects with a Complior 1 equipment (Createch Industrie, Massy, Cedex, France).[13]

Oxidative stress and other biomarkers

Two parameters for oxidative stress were determined in venous blood. In the 2 groups, we measured malondialdehyde (MDA) and 8-isoprostane plasma levels, for which we obtained 10 mL of blood via puncture of a peripheral vein. The sample was centrifuged at 3000 rpm for 10 minutes at a temperature of 4°C. The plasma was separated and stored at −20°C. Malondialdehyde levels were measured by determining the content of the reactive substances to thiobarbituric acid reactive substances,[14] and values were expressed as μmol/L. The 8-isoprostane plasma levels were evaluated with an enzyme immunoassay commercial kit (Cayman Chem Co, Ann Arbor, MI), and the values were expressed as pg/mL.

High-sensitivity C-reactive protein (hs-CRP) as well as the carboxy-terminal propeptide of procollagen type I (PICP) was also determined in serum by enzyme-linked immunosorbent assay as markers of inflammation and myocardial fibrosis, respectively.

Rho-kinase activity in circulating leukocytes

Rho-kinase activity was assessed by measuring the levels of phosphorylated to total myosin light chain phosphatase 1 (MYPT1-P/T), a direct downstream target of Rho-kinase, and by analysis of total Rho-kinase isoforms in circulating leukocytes from venous blood. Blood containing EDTA was poured over Histopaque (Histopaque-1077; Sigma Chemical Co, St Louis, MO) and centrifuged. The white cells were resuspended in phosphate-buffered saline (PBS). After determining cell yield and viability by using the trypan blue exclusion test ($4\text{-}80 \times 10^6$ viable cells, 95% viability), the cells were resuspended in lysis buffer. Protein content of supernatants was determined by Bradford assay. Soluble fractions were heated at 95°C with sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer for myosin light chain phosphatase 1 (MYPT1), Rho-associated kinase 1 (ROCK1), and Rho-associated kinase 2 (ROCK2) Western blot analysis.[15] The leukocyte protein extracts were matched for protein, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 6% polyacrylamide gels, and electrotransferred to nitrocellulose. Membranes were blocked with 7% nonfat milk in PBS containing 0.05% Tween-20 at room temperature. Antiphospho-Thr853-MYPT1 (Phospho-myosin-binding subunit/MYPT1-P-Thr853; Cyclex, Woburn, MA) or anti-MYPT1 (BD Transduction Laboratories, Becton, Dickinson and Company, Franklin Lakes, NJ), anti-ROCK1 monoclonal antibody, and anti-ROCK2 monoclonal antibody (BD Biosciences, San Jose, CA) primary antibodies were diluted in blocking solution (1:700, 1:1000, 1:500, and 1:2000, respectively). Nitrocellulose membranes were incubated with primary antibody overnight at 4°C. After washing in PBS containing 0.05% Tween-20, blots were incubated with horseradish peroxidase–linked secondary antibody, and specific binding was detected using enhanced chemiluminescence with exposure to Kodak film. Each blot was quantified by scanning densitometry with the Un-Scan-It software (Silk Scientific, Inc. Orem, UT).[15]

Statistical analysis

Results are presented as mean ± SEM or as a percentage. χ^2 Test (for categorical variables), analysis of variance (ANOVA), followed by Student-Newman-Keuls or Kruskal-Wallis test (for continuous variables), and linear regression were used. $P < .05$ was considered statistically significant.

The authors are solely responsible for the design and conduct of this study, all study analyses, the drafting and editing of the manuscript, and its final contents.

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Results

Clinical characteristics

Most of the general characteristics including age, gender, and body mass were similar among patients with HF, controls, and hypertensive patients (Table I). Heart rate was lower in patients with HF compared to both control groups. Blood pressure and carotid-to-femoral PWV were significantly higher in hypertensive patients.

The etiology of systolic HF was hypertensive (59%), idiopathic dilated cardiomyopathy (23%), and coronary heart disease (18%). All patients were in stable HF during the previous last 8 weeks as well as in sinus rhythm. Patients with HF were receiving as treatment angiotensin-converting enzyme (ACE) inhibitors (58%) or angiotensin receptor blockers (42%), β-blockers (100%), furosemide (47%), and spironolactone (53%). Hypertensive patients were receiving ACE inhibitors (18%), angiotensin receptor blockers (76%), calcium-channel blockers (12%), and β-blockers (6%). General laboratory tests and biochemistry, including MDA and 8-isoprostane plasma levels as well hs-CRP and PICP, were similar in the 3 groups (Table I).

Table II. Echocardiographic dimensions, LV mass, and systolic LV function

	Controls (n = 17)	Hypertensive patients (n = 17)	Patients with HF (n = 17)	Intergroup differences (ANOVA)
Left atrial area (cm^2)	19 ± 1	20 ± 1	33 ± 2*	<.01
End-systolic LV diameter (mm)	28 ± 1	27 ± 1	48 ± 2*	<.01
End-diastolic LV diameter (mm)	48 ± 1	47 ± 1	62 ± 2*	<.01
End-diastolic septal thickness (mm)	8 ± 0.3*	10 ± 0.4	10 ± 0.5	<.05
Posterior wall thickness (mm)	8 ± 0.3*	9 ± 0.3	10 ± 0.6	<.01
LVMI (g/m^2)	78 ± 3	86 ± 5	160 ± 12*	<.01
EF (%)	61 ± 1	59 ± 1	27 ± 2*	<.01

Values shown as mean ± SEM. *LVMI* indicates LV mass index.
* $P < .05$ versus the other groups (after ANOVA).

Echocardiographic dimensions, LV mass, and LV systolic function

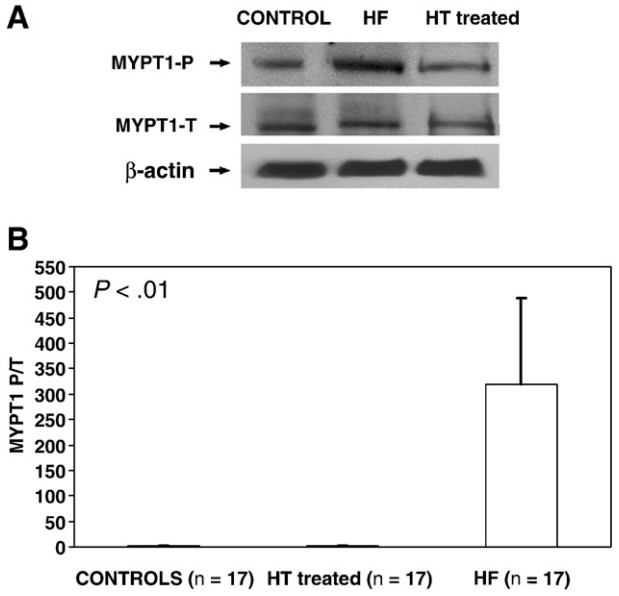
Compared with both control groups, left atrial area, LV systolic diameter, and LV diastolic diameter were significantly larger in patients with HF by 77%, 71%, and 29%, respectively, whereas the LV mass index was higher by 2-fold in patients with HF ($P < .05$) (Table II). Systolic LV function was markedly deteriorated in patients with HF (Table II).

Rho-kinase activation in patients with HF

In the control subjects, the mean ratio between MYPT1-P/T in circulating leukocytes, an evidence for Rho-kinase activation, was 1.2 ± 0.2. Similar levels were observed in the hypertensive patients. In patients with stable HF, the MYPT1-P/T ratio was significantly increased by >100-fold compared ($P < .01$) (Figure 1) with the control group and with the hypertensive patients. No differences were observed in the ROCK1 or ROCK 2 isoform measured in circulating leukocytes comparing the control group with the patients with stable HF (Figure 2).

In patients with HF, both the MYPT1-P/T ratio and log MYPT1-P/T ratio were inversely correlated with EF ($r = -0.54$, $P < .03$ and $r = -0.86$, $P < .001$, respectively)

Figure 1



Rho-kinase activity in patients with stable HF assessed by the ratio between MYPT1-P/T determined by Western blot in circulating leukocytes. **A**, Representative Western blots of MYPT1-P (130 kd) and MYPT1-P/T (130 kd) from 1 control, 1 patient with HF, and 1 hypertensive patient without HF (HT treated). **B**, Comparative MYPT1-P/T values (mean and SEM) in controls, hypertensive patients without HF (HT treated), and patients with HF.

(Figure 3). Besides, in patients with HF with LV end-diastolic diameter <60 mm (n = 8), MYPT1-P/T ratio was 35.8 ± 18.1, whereas it was significantly higher in patients with LV diameter ≥60 mm (n = 9) (Figure 4).

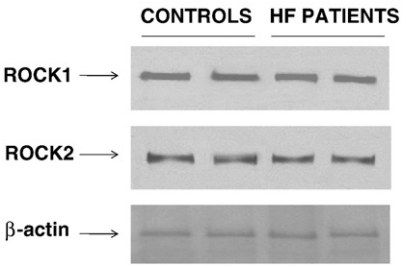
Discussion

The main findings were that, in patients with stable HF, Rho-kinase activity, determined by the MYPT1-P/T ratio in circulating leukocytes, was markedly elevated compared with healthy individuals and with treated hypertensive patients without systolic dysfunction. Increased Rho-kinase activity was correlated to the severity of LV systolic function deterioration and to ventricular remodeling.

This is the first observation about Rho-kinase activation in circulating leukocytes in patients with HF. Interestingly, increased Rho-kinase activation was observed despite optimal HF treatment and clinical stability.

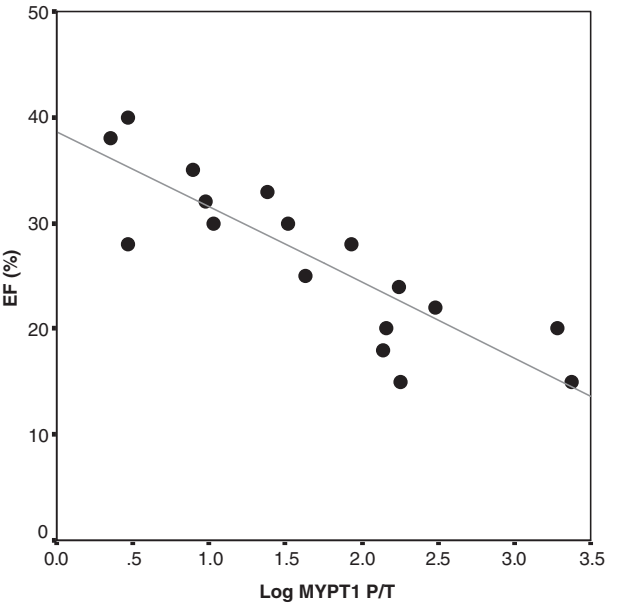
Few observations have been made on the role of the Rho-kinase signaling pathway in humans, and most of them are studies in patients with pulmonary hypertension.[16-18] In patients with pulmonary hypertension, Rho-kinase activity in circulating neutrophils is significantly increased compared with controls.[19] In these patients,

Figure 2



Representative Western blots of ROCK1 and ROCK 2 isoforms in circulating leukocytes in controls (n = 2) and patients with HF (n = 2).

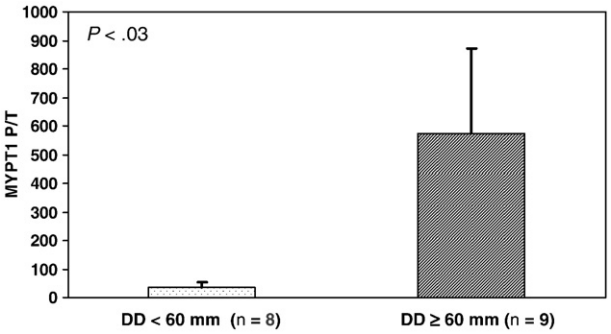
Figure 3



Inverse relationship between Rho-kinase activity assessed by MYPT1 ratio (as log MYPT1-P/T) determined by Western blot in circulating leukocytes and EF in the patients with HF ($R^2 = 0.74$, $P < .001$, n = 17).

Rho-kinase expression and activity in isolated lung tissue were also significantly increased compared with controls.[19] In patients with systemic hypertension, the Rho-kinase inhibitor fasudil induced a larger vasodilator response in the arm compared to control subjects, whereas the vasodilator response with nitroprusside (a direct vasodilator) was similar in both groups.[20] These data provided the first clinical evidence about the role of the RhoA/Rho-kinase pathway in the pathogenesis of increased systemic vascular resistance in hypertensive patients. In patients with coronary heart disease, fasudil enhanced vasodilation and reduced Rho-kinase activity in circulating leukocytes.[21] In patients with metabolic

Figure 4



Comparative MYPT1-P/T ratio (mean and SEM) in patients with HF with end-diastolic diameter <60 (n = 8) versus ≥60 mm (n = 9).

syndrome, Rho-kinase activity determined in circulating leukocytes was significantly increased compared to controls, and it was related to the number of components of the metabolic syndrome and also with inflammation markers such as C-reactive protein and adiponectin.[15] In patients with HF, with elevated arterial arm resistance, and reduced flow mediated vasodilatation, fasudil increased the blood flow as well as reactive hyperemia more than in controls, indicating the role of Rho-kinase in the increased vascular resistance and reduced forearm vasodilatation in these patients.[22]

Possible mechanisms explaining the current observations were not directly explored here, but some hypotheses may be considered. Circulating MDA and 8-isoprostane levels were similar in our stable patients with HF as in the controls, reflecting a similar and possibly normal level of oxidative stress in these stable patients. Levels of angiotensin II or aldosterone were not determined; however, all these patients were stable and receiving ACE inhibitors or angiotensin receptor blockers, and most of them were also receiving spironolactone. It is not possible, however, to rule out that higher and uncontrolled levels of angiotensin II or aldosterone due to the escape phenomenon[23-26] may activate Rho-kinase in these patients. In this regard, experimentally in healthy normotensive Brown Norway rats, with genetically high levels of ACE and angiotensin II,[27] Rho-kinase is significantly activated in the aortic wall, and it directly activates genes that promote both vascular remodeling and oxidative stress.[28]

In our patients with HF patients, cathecolamine levels were neither measured. However, these stable patients were all receiving β-blockers, and their heart rate was lower compared to both control groups. Compared to healthy individuals, we previously observed in patients with HF New York Heart Association functional class II or III (without β-blockers) that resting, circulating cathecolamine levels were similar but were significantly higher than in controls during submaximal exercise.[29]

The current observations suggest that, in these patients with HF, pathologic cardiac remodeling by itself could be a direct major determinant of Rho-kinase activation. This is consistent with several experimental observations on the role of Rho-kinase inhibition and its effects on cardiac remodeling and LV dysfunction. Long-term Rho-kinase inhibition with fasudil ameliorated diastolic HF in Dahl salt-sensitive, hypertensive rats,[30] and in rats with pressure overload hypertrophy, selective Rho-kinase inhibition with GSK-576371 (GlaxoSmith-Kline) improved LV geometry, collagen deposition, and diastolic function.[31] ROCK1 gene deletion prevented LV dilatation and systolic dysfunction in mice overexpressing Gαq.[32] Using the Langendorff preparation in the isolated, perfused rabbit heart, Rho-kinase inhibition improved cardiac function after 24-hour heart preservation.[33] Furthermore, in cultured cardiomyocytes, RhoA/Rho-kinase activation up-regulates Bax through p53 to induce mitochondrial death pathway and cardiomyocyte apoptosis.[34] Alternatively, it is not possible to rule out in our patients with HF a primary or initial role of Rho-kinase activation in cardiac dysfunction and ventricular remodeling. In this regard, in transgenic mice overexpressing myosin light chain phosphatase 2 (MYPT2), an isoform of MYPT1 associated with endogenous 1 phosphatase catalytic subunit δ isoform, increased formation of the cardiac myosin phosphatase holoenzyme is observed.[35] In these transgenic mice overexpressing MYPT2, a higher level of myosin phosphatase activity caused LV dysfunction and enlargement, possibly via decreased calcium sensitivity, and also some deterioration of myofibrillar structure, the first report demonstrating the function of cardiac myosin phosphatase and MYPT2, and the role of cardiac myosin light chain phosphorylation in vivo.[35]

One limitation of the study is related to the biologic significance of Rho-kinase activation in circulating leukocytes in relation with HF and ventricular dysfunction, whether it directly mediates myocardial remodeling or it implies similar activation in other cell types in the myocardium. No information is available in the literature on this specific aspect. However, circulating lymphocytes have been used to study β-adrenergic receptor signaling and to make extrapolations to the cardiac β-adrenergic receptor system, and they represent a valuable and reliable marker of the functional state of cardiac β-adrenergic receptor signaling.[36-39] Furthermore, the G protein–coupled receptor kinase 2 (GRK2 or β-ARK1) regulates β-adrenergic receptors in the heart, and its cardiac expression is elevated in human HF. A direct correlation between myocardial and circulating lymphocytes GRK2 activity has been found in patients with HF, implying that myocardial GRK2 expression and activity are mirrored by lymphocyte levels of this kinase in human HF,[39] which might be similar but needs to be proven, with Rho-kinase activation. Our data suggest that Rho-kinase activity in peripheral leukocytes may have a role as

an HF biomarker. Another limitation in this study is the small sample size. Based on our current data, it is not possible to rule out that observed differences in Rho-kinase activity between cases and controls may be attributable to the clinical antecedent of HF. Besides, although a specific phosphorylation site was measured as a function of Rho-kinase activity, this does not exclude other mechanisms leading to myosin light chain phosphatase phosphorylation.

It will be relevant in future studies to explore relevant clinical questions raised from the current findings, concerning the role of Rho-kinase activation in decompensated or in more severe patients with HF, in patients with diastolic HF as well as in conditions such as left ventricular hypertrophy or atrial fibrillation, or in patients where LV dysfunction or remodeling can be further reduced by a more effective treatment. Most relevant, however, will be to evaluate the role of specific Rho-kinase inhibitors in patients with HF for symptoms, ventricular function, and remodeling.

In conclusion, Rho-kinase activity in circulating leukocytes is markedly increased in patients with stable chronic HF despite optimal medical treatment, and it is associated with pathologic LV remodeling and systolic dysfunction. The mechanisms of Rho-kinase activation in patients with HF, its role in the progression of HF, and the direct effect of Rho-kinase inhibition need further investigation.

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