Increased leukocyte Rho-kinase activity in a population with acute coronary syndrome

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Abstract. Accumulating evidence suggests that Rho-associated kinase (ROCK) may be important in the pathogenesis of atherosclerosis and coronary vasospasm. In the present study, we investigated whether ROCK activity is increased in acute coronary syndrome (ACS) patients. Twenty-one patients with ACS (12 males, mean age 58.0±8.0 years) and 20 control subjects (10 males, mean age 55.0±6.0 years) were enrolled. Blood samples were obtained and demographics were recorded. Peripheral leukocyte ROCK activity was determined by the ratio of phospho-myosin-binding subunit (P-MBS) on myosin light-chain phosphatase to total MBS. Compared with the control subjects, ROCK activity was significantly increased in ACS patients (0.69±0.07 vs. 0.45±0.04, P<0.001). There was no apparent correlation between the lipid levels (total cholesterol and low-density lipoprotein) and ROCK activity (r=0.17, P>0.05; r=0.08, P>0.05; respectively). However, ROCK activity correlated with mean arterial pressure (r=0.58; P<0.01). ROCK activity is increased in ACS patients indicating that this may be a novel serological marker of ACS.

Introduction

According to the Heart Disease and Stroke Statistics 2010 Update, coronary heart disease caused ~1 of every 6 mortalities in the United States in 2006 (1). Coronary heart disease mortality in 2006 was 425,425. In 2010, 785,000 Americans were estimated to have a new coronary attack, and ~470,000 experience a recurrent attack (1). In China, mortalities from coronary heart disease were 57.1/100,000 among urban residents and 33.74/100,000 among rural residents. A total of 500,000 or more individuals are affected by myocardial infarction (MI) each year and the prevalence rate of MI patients is estimated to be >2,000,000 in China (2). Coronary heart disease has become a growing worldwide problem. Acute coronary syndrome (ACS) is a cluster of coronary atherosclerotic heart diseases, including unstable angina (UA), non-ST-segment elevation MI (NSTEMI) and ST-segment elevation MI (STEMI) (3). The most common pathophysiological basis of ACS is disrupted atherosclerotic plaques (4), caused by arterial inflammation, endothelial injury, microembolization of platelet aggregates and coronary spasm (5-7).

Ras homolog gene family, member A (RhoA) is one of the best-known members of the Rho protein family that, in addition to its effect on actin organization or through this effect, regulates a wide range of fundamental cell functions, including contraction, motility, proliferation and apoptosis (8). Stimulation of tyrosine kinase and G protein-coupled receptors recruits and activates Rho guanine nucleotide exchange factors (GEFs), leading to activation of RhoA, the direct upstream activator of Rho-associated kinases (ROCKs) (9).

Increasing evidence suggests that ROCK, a target of small glutamyltranspeptidase (GTPase) Rho, mediates various cellular physiological functions, including cell proliferation, migration, adhesion, apoptosis and contraction (10-12). Rho-kinase activity is increased in patients with atherosclerosis (13), hypertension (14), diabetes (15), metabolic syndrome (MetS) (16), stroke (17) and hyperlipemia (18), and in cigarette smokers (19). Atherosclerosis is the underlying disorder in the majority of patients with cardiovascular disease. Atherosclerosis is a complex process involving inflammatory cells, endothelial dysfunction, smooth muscle cell proliferation, extracellular matrix alteration and thrombosis. ROCKs have been shown to be upregulated in inflammatory arteriosclerotic lesions and have the ability to cause coronary vasospastic responses through the inhibition of myosin light-chain phosphatase (MLCP) in a porcine model of coronary artery spasm (20) and arteriosclerotic human arteries (21). Previously, the ROCK pathway has been shown to be involved in atherosclerotic lesion formation (22) and coronary artery disease (23). Furthermore, ROCK inhibitors, including fasudil and Y-27632, inhibited atherosclerosis and attenuated vasospastic angina (22,24), which suggests that ROCK may be important in the pathogenesis of coronary artery disease.

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Thus, we hypothesized that ROCK is increased in ACS. In the present study, we measured peripheral leukocyte ROCK activity in a Chinese population with ACS and determined whether ROCK activity is an independent marker of ACS and whether it correlates with other risk factors of ACS.

Materials and methods

Patients. Twenty-one patients with ACS (ACS group: 12 males, 9 females, mean age 58±8 years) and 20 age-matched control subjects (control group: 10 males, 10 females, mean age 55±6 years) were enrolled in the present study. All patients were from the Department of Cardiology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, Hubei, China). Patients with ACS were diagnosed under the American College of Cardiology/ American Heart Association (ACC/AHA) 2007 guidelines (3). This included patients who had typical acute chest pain syndrome ≥20 min and electrocardiogram (ECG) showing persistent ST-segment depression ≥1 mm, or patients who had typical acute chest pain syndrome ≥20 min and ECG showing persistent ST-segment elevation ≥1 mm in 2 contiguous leads (≥2 mm in V1-V3 leads), or patients with acute chest pain without ST-segment elevation; however, with elevated troponin levels.

Patients with stable angina or history of prior MI were excluded from the study. Other exclusion criteria included patients who had severe heart failure or any significant arrhythmias within 3 months of the study (25), patients with severe anemia or dysfunction of the kidney, liver or brain, those with a history of diabetes mellitus and patients taking statins prior to enrollment. Control subjects were those without any risk factors for coronary heart disease, symptoms and signs of heart disease or coronary angiography suggestive of atherosclerosis. The study was approved by the Human Research Committee at Tongji Hospital and written informed consent was obtained from all subjects.

Analytical methods. Blood samples (20 ml) were collected from the cubital vein of all subjects in sterile tubes containing ethylenediaminetetraacetic acid (EDTA) and stored at 4˚C for <1 h. Fasting serum lipids [total cholesterol (TC), low-density lipoprotein (LDL-C), high-density lipoprotein (HDL) and triglycerides] and glucose were measured in the clinical laboratory of Tongji Hospital. Mean arterial pressure (MAP) was approximated by dividing the pulse pressure by three and adding the value to the diastolic pressure. Blood pressure measurements were made with the patient sitting or recumbent, and were conducted using Korotkoff's method.

Leukocyte isolation. To isolate human leukocytes, whole blood samples were centrifuged at 2190 x g for 10 min at room temperature, and the supernatant was aspirated and discarded. The leukocyte pellet and 5-fold volume of the red cell lysis buffer (Red Blood Cell Lysing Buffer-R7757; Sigma, St. Louis, MO, USA) were added into a 15 ml centrifuge tube. The tube was then centrifuged at 716 x g for 10 min, and the supernatant was discarded. The leukocyte pellet was resuspended in 10 ml Hanks' balanced salt solution (HBSS) by pipetting the solution up and down, and the suspension was centrifuged at 716 x g for 10 min. The supernatant was discarded and the pellet was resuspended in 4 ml of M199 (Sigma). The trypan blue (Sigma) exclusion test was used to determine cell yield and viability, and the suspension was diluted with HBSS to achieve a concentration of $5x10^6$ cells/ml. After mixing the diluted cells with a transfer pipette to ensure a uniform suspension, 400 μl leukocyte suspension was transferred to sterile 1.5 ml tubes along with 400 μl fixative solution [50% trichloroacetic acid (Sigma), 50 mmol/l dichlorodiphenyltrichloroethane (Sigma) and protease inhibitors]. The suspension was vortexed and then centrifuged at 4˚C for 5 min at 14,000 x g. The supernatant was removed carefully and completely with a micropipette. The precipitate was stored at -80˚C for western blot analysis.

Measurement of ROCK activity by immunoblotting. Leukocyte pellets were diluted with 10 μl of 1 mol/l Tris base and 100 μl of extraction buffer (8 mol/l urea, 2% sodium dodecyl sulfate, 5% sucrose and 5% 2-mercaptoethanol). Equal amounts of protein extracts were used for separation by 8% SDS-PAGE and transferred onto a PVDF membrane. NIH3T3 cell lysates were used as a positive control. The experiments were repeated >3 times in order to standardize the results of western blot analysis. The proteins were detected with antibodies against MBS (Covance, Emeryville, CA, USA) and phospho-Thr696-MBS polyclonal antibody (Millipore, Temecula, CA, USA). Immunoblotting was performed according to the procedure described previously (26). Rho kinase activity was presented as the ratio between the phospho-myosin-binding subunit (P-MBS) and MBS normalized to the control.

Statistical analysis. SPSS 13.0 software was used to perform statistical analysis on the data. All quantitative data from the two groups are expressed as the means ± standard deviation (SD). The frequencies between ACS and controls were compared using Chi-square analysis. Due to heterogeneity of variance, age, heart rate, fasting glucose and triglycerides were analyzed using nonparametric methods. The student's unpaired t-test or Wilcoxon Rank Sum test were used to determine the significant differences between the two groups. Correlation of ROCK activity to the lipid levels and MAP was assessed by analysis of Pearson's correlation coefficient. All reported P-values were two-sided. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Baseline characteristics. The subjects in the control and ACS groups were age and gender matched. The risk factors for coronary artery disease are shown for the control and ACS groups (Table I). The two groups were comparable in heart rate, history of smoking, fasting glucose and triglyceride levels ($P>0.05$). Compared with the control group, the average body mass index, MAP, TC and LDL-C were significantly higher, and the average HDL was significantly lower, in the ACS group ($P<0.05$; Table I). These results were consistent with a previous study (16).

ROCK activity. Compared with the control subjects, ROCK activity, as measured by P-MBS/MBS, was significantly increased in ACS patients. The mean leukocyte ROCK

Table I. Clinical characteristics of controls and ACS patients.

	Control (n=20)	ACS (n=21)	P-value
Age (years)	55.0±6.0..	58.0±8.0..	0.146
Male (no.)	10 (50.0%).	12 (57.1%)..	0.647
BMI (kg/m^2)	23.0±2.9..	25.2±2.3..	0.012[a]
HR (bpm)	78.0±6.0..	80.0±7.0..	0.089
MAP (mmHg)	91.6±9.0..	100.0±14.0..	0.028[a]
Smokers (no.)	7 (35.0%)	9 (42.9%)	0.606
Glucose (mmol/l)	5.7±1.6	6.1±3.4	0.240
Total cholesterol (mmol/l)	4.6±0.5	5.3±0.5	0.001[a]
LDL-C (mmol/l)	2.6±0.5	3.2±0.5	0.002[a]
HDL (mmol/l)	1.4±0.3	1.2±0.3	0.043[a]
Triglycerides (mmol/l)	1.6±0.4	2.0±0.8	0.130

[a]P<0.05. Data are presented as the means ± SEM and no. (%). ACS, acute coronary syndrome. BMI, body mass index; HR, heart rate; MAP, mean arterial pressure; LDL-C, low-density lipoprotein; HDL, high-density lipoprotein; BPM, beats per minute.

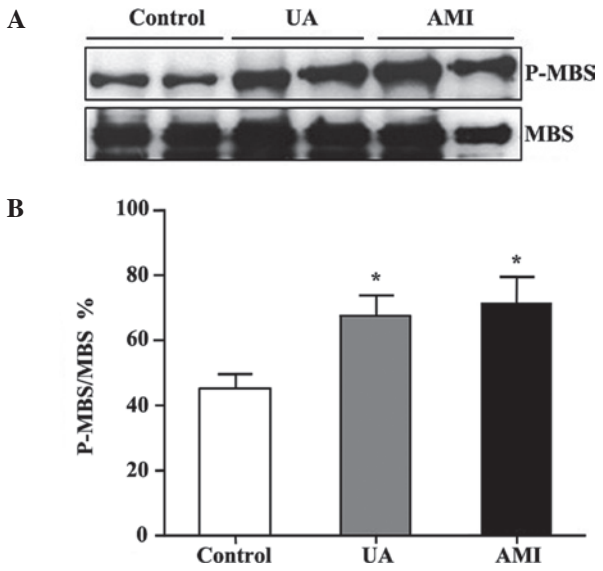

Figure 1. ROCK activity in ACS patients and control subjects. ROCK activity expressed as P-MBS/MBS. (A) Immunoblotting of ROCK activity in different groups. Each lane is one representative sample from one patient. (B) Histogram showing statistical analysis of relative density of immunoblotting. *P<0.01, compared with control. ACS, acute coronary syndrome; UA, unstable angina; AMI, acute myocardial infarction; P-MBS, phosphomyosin-binding subunit; MBS, myosin-binding subunit; ROCK, Rho-kinase; ACS, acute coronary syndrome.

activity levels were 0.45±0.04 in control subjects. In patients with ACS, the mean activity levels were 0.69±0.07 (P<0.001, Fig. 1). However, the ROCK activity levels did not significantly differ between the acute MI (AMI) patients and the UA patients (P=0.2).

Correlation between ROCK activity and parameters. To determine whether ROCK activity is a novel risk marker of ACS and whether it correlates with other risk factors of ACS,

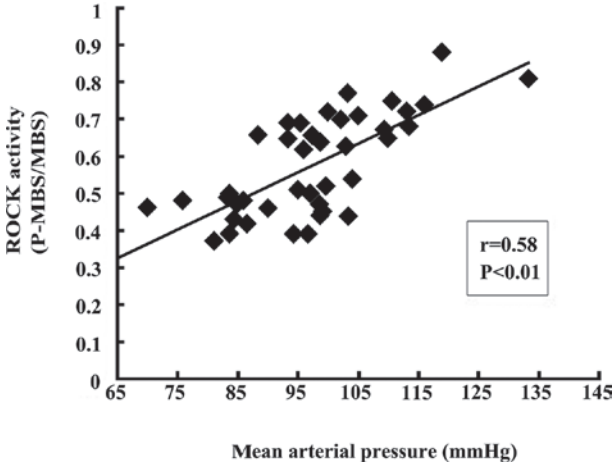

Figure 2. Correlation between ROCK activity and MAP. ROCK activity was positively correlated with the MAP of ACS patients (r=0.58, P<0.01). ROCK, Rho-kinase; MAP, mean arterial pressure; ACS, acute coronary syndrome.

correlation analysis was performed. Consequently, there was no correlation between the lipid levels (TC and LDL-C) and ROCK activity (r=0.17, P>0.05; r=0.08, P>0.05; respectively). However, MAP was significantly correlated with ROCK activity (r=0.58, P<0.01; Fig. 2). Multivariate analysis was performed to explain variability (blood pressure and smoking status being associated with ROCK activity). Thus, ACS status was an independent predictor of ROCK activity.

Discussion

The results of the present study demonstrate that peripheral leukocyte ROCK activity increased in patients with ACS. However, there were no significant differences between UA and AMI patients. In general, leukocyte ROCK levels represent the ROCK activity from systemic circulation and the ROCK levels in the blood vessels and myocardium represent tissue ROCK activity. The relevance of each level is currently unclear (27,28). Furthermore, the results revealed that higher ROCK activity was not correlated with lipid levels, whereas it was significantly positively correlated with MAP. These findings suggest that increased ROCK activity may be a novel marker of ACS, the activation of ROCK may contribute to the pathogenesis of ACS and therapies that inhibit ROCK may be clinically useful in the treatment of ACS.

It has been suggested that ROCK activity contributes to the development of early atherosclerosis, possibly through its modulation of NF-κB and activation of T lymphocyte proliferation (22). Furthermore, accumulating evidence indicates that coronary dysfunction and coronary arteriosclerosis are attenuated by inhibition of ROCKs (23,24,29,30). In the present study, ROCK activity was increased in subjects with ACS and there were no significant differences between AMI and UA patients, despite the possible higher ROCK activity in AMI patients.

Although the precise mechanism of increased ROCK activity in ACS patients is unclear, several possible mechanisms may explain the observed findings. Firstly, inflammation has a critical role in the occurrence and development of ACS. Recruitment of mononuclear leukocytes to the intima is one

of the earliest events in the formation of an inflammatory infiltrate and the active inflammation within plaques leads to plaque disruption (4,31). ROCK-mediated leukocyte recruitment in the vessel wall and enhanced inflammatory activity of the vessel wall contribute to the development of ACS (32). Furthermore, endothelial injury and dysfunction play a critical role in patients with atherosclerosis and acute coronary events. Notably, coronary artery spasm is one of the most significant features of ACS. Overactivity of ROCK in humans with atherosclerosis leads to reduced nitric oxide (NO) bioavailability and upregulated myosin light-chain (MLC) phosphorylation, which in turn leads to cellular contraction by inhibition of MLC phosphatase through phosphorylation of its regulatory MBS (20,23). Hence, Rho-kinase may contribute to the development of ACS. In addition, higher Rho-kinase activity in AMI patients may be due to increased damage to myocardial cells.

Blood pressure, fasting glucose, LDL-C, TG, BMI, hs-CRP and waist circumference were greater among the MetS subjects and lower HDL levels were observed in MetS patients. In the present study fasting glucose, TG, BMI, hs-CRP and waist circumference were positively associated with increased ROCK activity and HDL levels were negatively associated with ROCK activity (16). However, LDL-C and TC were not correlated with ROCK activity in ACS subjects. An earlier study demonstrated that the Rho-kinase inhibitor fasudil increased flow-mediated vasodilatation without altering lipid levels in patients with elevated baseline TC and LDL-C (23). This suggests that increased ROCK activity may be independent of lipid levels in ACS subjects.

Notably, in our study, ROCK activity significantly correlated with MAP in ACS subjects. Our results are supported by several studies demonstrating that ROCKs are involved in the pathogenesis of increased peripheral vascular resistance in hypertension (14,33). In cigarette smokers with normal blood pressure, a significant correlation was noted between the activity of ROCK and systolic blood pressure (19). Therefore, the current results indicate that activation of ROCK leads to VSMC contraction and contributes to coronary spasm in ACS patients.

Fasudil, a potent and selective inhibitor of Rho-kinase, is clinically used for the treatment of cerebral vasospasms following subarachnoid hemorrhage (34). It has been demonstrated that hydroxyfasudil, a major active metabolite of fasudil following oral administration, has a more selective inhibitory effect on ROCK compared with its parent drug (35). Fasudil inhibits Rho-kinase by competing with ATP for binding to the catalytic site of the kinase and therefore is equipotent in terms of inhibiting ROCK1 and ROCK2. Therefore, fasudil is currently being developed for the treatment of acute stroke and cardiovascular disorders. Inhibition of ROCK in atherosclerosis patients has been investigated in several previous studies using fasudil. A multicenter study demonstrated that fasudil significantly improved stable angina (36). In humans, leukocyte ROCK activity was increased in patients with acute ischemic stroke and reached maximal activity ~48 h after stroke onset. Fasudil additionally offers a safe option for the treatment of cerebral infarction in patients with acute thrombosis as well as cerebral vasospasm (17,37). These studies indicate that atherosclerosis and vascular injury may contribute to ROCK activity. The present findings suggest that fasudil inhibition of ROCK activity may have therapeutic benefits in patients with ACS.

This study has several limitations. Firstly, we measured the activity of ROCK by only measuring the ratio between P-MBS and MBS. Thus, further studies are required with antibodies from distal targets of ROCK in order to obtain precise results and to develop methods to distinguish between ROCK1 and ROCK2 activities. Secondly, all subjects in this study were Chinese. Therefore, the results may not be applicable to other ethnicities. As we were unable to determine the time of occurrence of unstable angina, we could not use the inhibitor of ROCK in these patients. Accordingly, future investigations should aim to use fasudil in patients with ACS and study the outcome.

In conclusion, we were able to demonstrate that peripheral leukocyte ROCK activity was increased in patients with ACS. This result suggests that inhibition of Rho-kinase may be regarded as a novel therapeutic method for the treatment of acute coronary events in humans. However, the precise molecular mechanism of increased ROCK in coronary atherosclerosis and its effects on subsequent acute coronary events remain to be elucidated.

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