Investigation of leukocyte *RHO/ROCK* gene expressions in patients with non-valvular atrial fibrillation

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Received December 13, 2018; Accepted May 8, 2019

DOI: 10.3892/etm.2019.7929

Abstract. Atrial fibrillation (AF) is an arrhythmia caused by disorganized electrical activity in the atria, and it is an important cause of mortality and morbidity. There is a limited data about Rho/Rho-kinase (ROCK) pathway contribute to AF development. The aim of the present study was to elucidate leukocyte *RHO/ROCK* gene expressions in patients with non-valvular AF (NVAF). A total of 37 NVAF patients and 47 age and sex-matched controls were included in this study. mRNA was extracted from leukocytes, and real-time polymerase chain reaction was used for gene expression analysis. A marked increase in *ROCK1* and *ROCK2* gene expressions in patients with NVAF was observed (P<0.0001). The present study detected significant elevations in *RHOBTB2, RND3 (RHOE), RHOC, RHOG, RHOH, RAC3, RHOB, RHOD, RHOV, RHOBTB1, RND2, RND1* and *RHOJ* gene expressions (P<0.01). However, there were marked decreases in *CDC42, RAC2*, and *RHOQ* gene expressions in patients with NVAF. No significant modifications were seen in the other Rho GTPase proteins *RHOA, RAC1, RHOF, RHOU* and *RHOBTB3*. To the best of our knowledge, the present study is the first to provide data that gene expression of leukocyte *RHO/ROCK* may contribute to the NVAF pathogenesis through activated leukocytes, which promotes the immune or inflammatory cascade.

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Key words: atrial fibrillation, gene expression, polymerase chain reaction, Rho proteins, Rho-kinase

Introduction

Atrial fibrillation (AF) is among the most common arrhythmia seen in clinical practice, with a prevalence approaching 2% of the general population. Its prevalence raises with advancing age, and is expected to enhance 3-fold in the next 3 decades (1). AF is associated with impaired functional status, hospitalization, decreased quality of life and augmented mortality. Furthermore, clinical characteristics of ischemic stroke from AF are severe and thromboembolism is considered the most significant cause (2). Risk factors for developing AF include both intrinsic cardiac disease such as left ventricular hypertrophy, valvular pathology, myocardial infarction and congestive heart failure and non-cardiac risk factors such as diabetes, smoking, hypertension, and obesity. It has been reported that AF is linked with a 2.3-fold risk of ischemic stroke, 2-fold risk of cardiovascular mortality, and 5-fold risk of incident congestive heart failure (3). AF development can be a multifactorial process, including susceptibility related to co-morbidities that promote early atrial enlargement, inflammation, ion channel abnormalities, conduction heterogeneity due to atrial fibrosis, and autonomic remodeling. However, the exact molecular mechanisms leading to AF are still unknown. In addition to conventional risk factors, a genetic predisposition has been shown to contribute to AF risk (4), and the genes responsible may provide important clues toward therapy. Additionally, treatment to restore sinus rhythm among patients with AF has limited long-term success rates. Therefore, identifying potential new therapeutic approaches and targets based on molecular mechanisms of AF is very important.

Rho-kinase (ROCK) is known as an effector of the small GTP-binding Rho proteins. Rho/ROCK pathway is involved in diverse cellular functions, including endothelial dysfunction, smooth muscle contraction, gene expression, actin cytoskeletal organization, apoptosis, proliferation, migration, inflammation, and cell polarity (5). Rho/ROCK system contributes to the pathogenesis of cardiovascular diseases including heart failure, hypertension, and angina pectoris (5). The Rho/ROCK

pathway has also been involved in regulating cardiac conduction system (6). It has been suggested that leukocyte ROCK activity may be a useful surrogate marker for cardiovascular outcomes and that ROCK inhibition may be a therapeutic target for inhibition of cardiovascular events (7). It has been shown that administration of selective ROCK inhibitor produces antiarrhythmic effects in experimental studies (8,9). Since there is no clinical study showing the contribution of leukocyte Rho/ROCK pathway on AF development, the goal of the present study was to determine the role of leukocyte *RHO*/*ROCK* gene expressions in patients with non-valvular atrial fibrillation (NVAF).

Materials and methods

A total of 37 NVAF patients followed up in Gaziantep 25 Aralik State Hospital were enrolled in this study. NVAF diagnosis was made by surface electrocardiogram and all of the patients had atrial fibrillation when the blood samples were taken. Patients who had heart failure, peripheral artery disease, valvular heart disease, coronary artery disease, diabetes mellitus, kidney failure, thyroid disorder, autoimmune disorder, pregnancy, hypertension, dyslipidemia, and cancer were excluded. Patients who had had prior cardiac surgery or an ablation procedure for AF management were also excluded. All the patients had persistent AF. Age and sex matched 47 healthy controls were included to the study. The control group was consisted of healthy individuals who had no history of cardiac arrhythmias or AF. Patients stopped taking medications for at least 24 h prior to venous blood sample collection, and blood samples were taken between 9:00 and 10:00 a.m. Medications used by the patients are presented in Table I. Written informed consent was obtained from subjects according to the Declaration of Helsinki, and the Ethics Committee of Gaziantep University approved the study with approval number 2015/193.

Gene expression studies. Blood samples (5 ml) were obtained from subjects, and RNA was purified from leukocytes using the High Pure RNA Isolation Kit (Roche Diagnostics) as described by the manufacturer. RNA quantities were spectrophotometrically measured by using a microplate spectrophotometer (Epoch; BioTek). Concentrations were kept constant according to measurements, and equal sample aliquots were stored at -80˚C until further use. RNA was converted to cDNA using the Qiagen miScript Reverse Transcription Kit (Qiagen) according to manufacturer's protocol.

PCR was performed using the high-throughput platform BioMark HD System (Fluidigm) that utilizes a fluorescent based PCR method. Expression of each gene was determined by performing primary probe design. Real-time PCR was done in BioMark 96.96 Dynamic Array (Fluidigm) using a set of TaqMan Gene Expression Assays (Life Technologies; Thermo Fisher Scientific, Inc.). mRNA expressions were determined by comparison with housekeeping gene β-actin (*ACTB*) and *GAPDH* from the same sample as internal control. We studied 2 ROCK and 21 Rho GTPases genes for expression study. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method, according to the formula: $\Delta Ct=C_{tRHO/ROCK}-C_{tACTB/GAPDH}$, where C_t is threshold cycle.

Statistical analysis. Data are expressed as the mean ± SD, SEM or percentage. Statistics were carried out using GraphPad Instat version 3.05 (GraphPad Software Inc.). Differences in the mean values of the two groups were determined using the unpaired Student's t-test. Categorical data were analyzed with Chi-square test. The gene expression analysis was performed by using online program, QIAGEN GeneGlobe (http://www.qiagen.com/geneglobe), and unpaired Student's t test was used to compare gene expression data. The level of statistical significance was set at $P<0.05$.

Results

General characteristics of study population. Table I shows clinical and demographic characteristics of the study groups. The average age, sex, percentages of smokers, body mass index, blood pressure, total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, and triglyceride levels in the NVAF group were similar when compared to the controls (Table I). The mean ejection fraction of the left ventricle was 61.28±2.47%, and the mean left atrium diameter was 33.96±1.74 mm in the patient group.

Comparision of gene expression between atrial fibrillation patients and healthy controls. Gene expression studies revealed that *ROCK1* and *ROCK2* mRNA contents in leukocytes were markedly elevated in NVAF patients when compared to the control groups ($P<0.0001$ for *ROCK1* and *ROCK2* (Fig. 1). There were also significant elevations in *RHOBTB2, RND3 (RHOE), RHOC, RHOG, RHOH, RAC3, RHOB, RHOD, RHOV, RHOBTB1, RND2, RND1,* and *RHOJ* gene expressions in NVAF patients (Fig. 2). However, there were marked depressions in *CDC42, RAC2,* and *RHOQ* gene expressions in NVAF patients (Fig. 3). No significant changes in gene expressions were detected in other Rho GTPase proteins (*RHOA, RAC1, RHOF, RHOU,* and *RHOBTB3,* $P>0.05$ (Figs. 2 and 3).

Discussion

This study evaluated 2 *ROCK* and 21 *RHO* gene expressions in patients with NVAF and compared to control subjects in this study. Suppressed (*CDC42, RAC2,* and *RHOQ,*) and elevated (*ROCK1, ROCK2, RND3, RHOBTB2, RHOC, RHOG, RHOH, RAC3, RHOD, RHOB, RHOV, RHOBTB1, RND2, RND1,* and *RHOJ*) gene expressions were observed in cases with NVAF. However, we have not observed marked changes in *RHOA, RAC1, RHOF, RHOU,* and *RHOBTB3* gene expressions. To the best of our knowledge, there is no published study to evaluate the leukocyte *RHO*/*ROCK* gene expressions in NVAF.

Several studies have suggested that the measurement of leukocyte ROCK activity is a valuable and alternative method to determine clinical ROCK activity in patients. ROCK activity in circulating leukocytes is considered to be a useful biomarker for the assessment of therapeutic responses and disease severity (7). Indeed, the elevated leukocyte ROCK activities in patients with hypertension (10), stable chronic congestive heart failure (11), metabolic syndrome (12), coronary artery disease (13), and angina pectoris (14) have previously been reported. Li *et al* (15) showed that ROCK1, but not ROCK2, activity in circulating leukocytes is increased in

Table I. Baseline demographic and clinical characteristics of patients with NVAF and controls.

Characteristics	Controls (n=47)	NVAF Patients (n=37)	P-value
Age (years)	57.76±6.05	55.82±8.19	0.2154
Sex			0.8669
Male, n (%)	25 (53.2)	19 (51.4)	
Female, n (%)	22 (46.8)	18 (48.6)	
Smoking status			
Current, n (%)	8 (17.0)	9 (24.3)	0.4415
Never, n (%)	33 (70.2)	21 (56.8)	
Past, n (%)	6 (12.8)	7 (18.9)	
BMI (kg/m^2)	25.52±4.28	24.47±6.21	0.3624
Systolic BP (mm Hg)	118.03±9.76	120.93±13.05	0.2473
Diastolic BP (mm Hg)	77.92±8.46	79.84±13.61	0.4303
Total cholesterol (mg/dl)	146.73±16.32	157.04±32.58	0.0622
Low density lipoprotein cholesterol (mg/dl)	99.72±11.62	109.02±30.93	0.0609
High density lipoprotein cholesterol (mg/dl)	43.18±7.06	41.32±8.79	0.2852
Triglyceride (mg/dl)	126.10±28.53	135.11±39.62	0.2293
Medications			
Antiplatelets, n (%)	-	25 (67.6)	
Anticoagulants, n (%)	-	12 (32.4)	

Values are presented as mean ± SD or as percentage. NVAF, non-valvular atrial fibrillation; BMI, body mass index; BP, blood pressure; ACEIs, angiotensin-converting enzymes inhibitors; ARBs, angiotensin II receptor blockers.

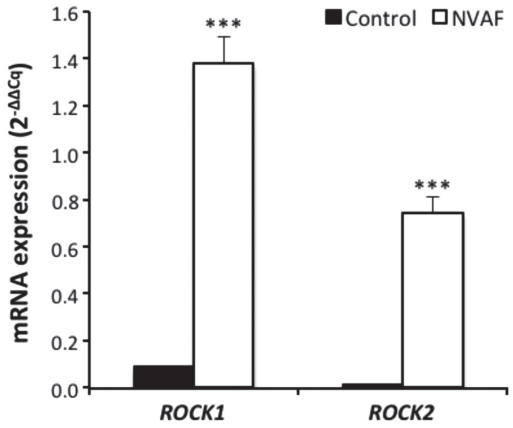

Figure 1. Comparison of the peripheral blood mRNA *ROCK1* and *ROCK2* expressions in healthy controls (n=47, solid bars) and in patients with non-valvular atrial fibrillation (NVAF, n=37, open bars). Values are given as mean ± SEM. P<0.0001 and P<0.0001 values were obtained for *ROCK1* and *ROCK2*, respectively. ***P<0.001.

ST-segment elevation myocardial infarction (STEMI) patients with diabetes mellitus. However, protein expression levels of ROCK1 and ROCK2 in circulating leukocytes were found to be both greater in STEMI patients with diabetes than those without diabetes (15). The protein expressions of ROCK1 and ROCK2 in the myolytic left atrial myocytes of mitral regurgitation AF patients were found to be markedly higher than that of the normal subjects (16). We have observed an increased *ROCK1* and *ROCK2* gene expressions in circulating leukocytes of NVAF patients.

Rnd3 (known as RhoE) functions as a repressor of ROCK1 (17,18). Since members of the Rnd subfamily are defective in GTPase activity, even in the presence of RhoGAPs, they bind but do not hydrolyze GTP (19). It has been demonstrated that Rnd3 (RhoE) is able to bind to and inhibit the function of ROCK1 but not that of ROCK2 (18). Additionally, Rnd3 (RhoE) binding to ROCK1 prevents RhoA binding to the Rho-binding domain (18). Rnd3 (RhoE) is the only endogenous ROCK1 antagonist discovered to date (18). Overexpression of Rnd3 (RhoE) diminished ROCK1-mediated biological effects including myosin light chain phosphatase phosphorylation, stress fiber formation, and apoptosis (18). Rnd3-null mice died due to fetal arrhythmias at the embryonic stage (20). We have observed augmented both *RND3 (RHOE)* and *ROCK1* gene expressions in our study. This data may suggest that Rnd3 (RhoE) inhibition of ROCK1 signaling does not occur at the gene expression level.

Gene expression of *RHOA* from left atrial tissue is significantly increased in patients with severe mitral valve disease and persistent AF when compared to sinus rhythm (21). However, we have not noted any significant change in *RHOA* gene expression in this study. This may be related to the fact that Rnd proteins function as RhoA antagonists (17). We have detected increased expressions of *RND1, RND2, RND3* as well as *RHOG* genes. Up-regulation RhoG has also been observed to counteract the effects of RhoA (19).

The atypical Rho GTPases, RhoU and RhoV, which are constitutively in an active GTP-bound state, may regulate cell-cell adhesions (22). We detected elevated *RHOV*, but not

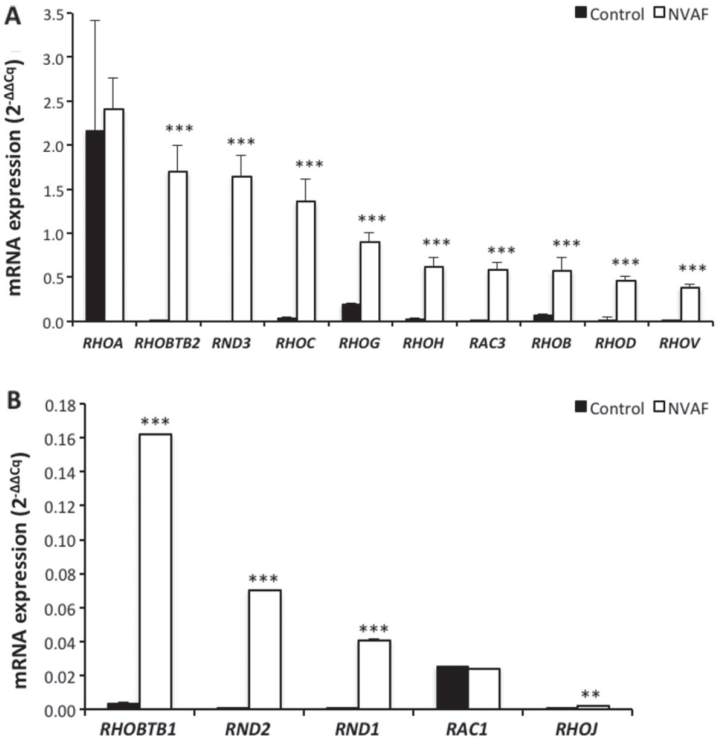

Figure 2. Comparison of the peripheral blood mRNA *RHOA, RHOBTB2, RND3 (RHOE), RHOC, RHOG, RHOH, RAC3, RHOB, RHOD, RHOV,* (A) and *RHOBTB1, RND2, RND1, RAC1, RHOJ* (B) expressions in healthy controls (n=47, solid bars) and in patients with non-valvular atrial fibrillation (NVAF, n=37, open bars). Values are given as mean ± SEM. P=0.8478, P<0.0001, P<0.0001, P<0.0001, P<0.0001, P<0.0001, P<0.0001, P=0.0003, P<0.0001, P<0.0001, P<0.0001, P<0.0001, P<0.0001, P=0.9273, and P=0.0084 values were obtained for *RHOA, RHOBTB2, RND3 (RHOE), RHOC, RHOG, RHOH, RAC3, RHOB, RHOD, RHOV, RHOBTB1, RND2, RND1, RAC1 and RHOJ,* respectively. **P<0.01 and ***P<0.001. NVAF, non-valvular atrial fibrillation.

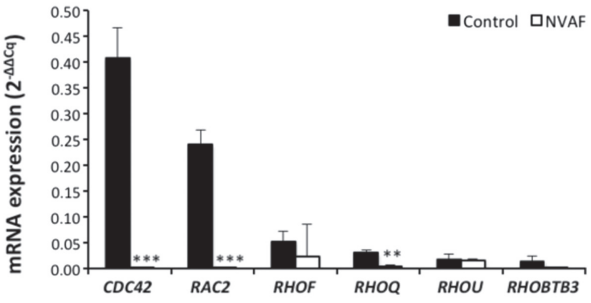

Figure 3. Comparison of the peripheral blood mRNA *CDC42, RAC2, RHOF, RHOQ, RHOU,* and *RHOBTB3* expressions in healthy controls (n=47, solid bars) and in patients with non-valvular atrial fibrillation (NVAF, n=37, open bars). Values are given as mean ± SEM, P<0.0001, P<0.0001, P=0.1511, P=0.0060, P=0.7739, and P=0.0511 values were obtained for *CDC42, RAC2, RHOF, RHOQ, RHOU,* and *RHOBTB3*, respectively. **P<0.01 and ***P<0.001. NVAF, non-valvular atrial fibrillation.

RHOU, gene expression in our study. Significance of this observation in NVAF remains to be identified.

The RhoBTB family consists of three members, namely RhoBTB1, RhoBTB2 and RhoBTB3. They are considered atypical Rho GTPases, because they are not regulated by the conventional GTPase cycle (23). Augmentation in *RHOBTB1* and *RHOBTB2,* but no change in *RHOBTB3,* gene expressions was observed in our study. However, their roles in the NVAF are currently unknown.

AF is frequently associated with cerebral and cardiac atherothromboembolism (2). It is known that Rac2 and RhoH are the only Rho GTPases with expression restricted to the hematopoietic cells (24). We detected depressed *RAC2* and elevated *RHOH* gene expressions in this study. Significance of these changes in AF-related thrombogenesis is not known, and requires further studies.

Rac1 is a membrane-bound signal transducing molecule involved in the regulation of adhesion and cell motility as well as mitosis, gene expression, cell cycle progression, and cell death (25). Rac1 GTPase is one of the main regulators of cell motility through actin reorganization and of reactive oxygen species (ROS) formation through regulation of NADPH oxidase activity (25). However, we have found no change in leukocyte *RAC1* gene expression in this study.

CDC42 activity is essential for endothelial barrier repair, adherens junction stability, and restoration of permeability (26). Thus, depressed *CDC42* gene expression seen in this study may contribute to the development of AF.

Accumulating evidence indicate that generation of ROS may play an important role in the induction and maintenance of AF (27). Levels of the serum oxidative stress biomarker are elevated in patients with AF (27). AF in human is linked with a significant reduction in the expression of antioxidant genes as well as a significant increase in the expression of 5 genes related to ROS, supporting a shift toward prooxidation state in AF (28). There is evidence that augmented oxidative stress triggers the activation of ROCK (29). ROCK also up-regulates NADPH oxidases, and increases Ang II-induced ROS production (30). Taken together, these findings may imply that ROCK is involved in the pathogenesis of AF.

There are several limitations of this study. Firstly, the gene expression profiles in this study were measured in isolated

leukocytes, but the ideal tissue for study NVAF is the left atrium. However, Lin *et al* (31) studied the association of whole blood gene expression with AF in a large community-based cohort, and identified 7 genes markedly upregulated with prevalent AF. Raman *et al* (32) also elucidated peripheral blood gene expression in patients with persistent AF that underwent electrical cardioversion. In another study, peripheral monocyte toll-like receptor (TLR) expression levels have been investigated and elevated TLR-2 and TLR-4 expressions were detected in patients with AF (33). Recently, we have also shown that all transient receptor potential channels gene expressions are upregulated in leukocytes of the NVAF patients (34). The increased leukocyte *RHO/ROCK* gene expressions observed in this study should be studied at the cardiac level. However, it should be emphasized that this group patient with NVAF has no indication for cardiac operation. Additionally, we were not able validate the Rho/ROCK pathway in AF development using another method such as western blot analysis. Finally, medications can modulate Rho/ROCK pathway and can induce gene expressions. However, in order to eliminate this, subjects stopped taking medications for at least 24 h prior to blood collection.

These data may imply that Rho/ROCK pathway can contribute to the pathogenesis of NVAF through activated leukocytes which stimulates the immune or inflammatory cascade. The findings of this study may provide novel pathophysiological insights for NVAF, and new potential intervention targets that can be tested in future studies. Therefore, these findings may also suggest that Rho/ROCK inhibitors can serve as a potential novel antiarrhythmic approach for the treatment of AF.

Acknowledgements

The authors wish to thank Ebru Temiz (Gaziantep University, Department of Medical Biology) for her contribution to gene expression analysis.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

IVD designed and performed experiments. IVD, FY, EV, ES, FP and HA collected and analysed the data. IVD, YC, HG, BC and MS interpreted results of the study. ATD and IVD performed the statistical analyses, prepared figures, and wrote the paper. All authors edited and revised manuscript and approved final submission of manuscript.

Ethics approval and consent to participate

The study protocol of the present experiment was reviewed and approved by the Ethics Committee at Ethics Committee of Gaziantep University (Approval number 2015/193). Written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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