Atorvastatin improves cardiac function of rats with chronic cardiac failure via inhibiting Rac1/P47^{phox}/P67^{phox}-mediated ROS release

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Abstract. – OBJECTIVE: To discuss the protective mechanisms of atorvastatin treatment for isoproterenol (ISO)-induced chronic heart failure.

MATERIALS AND METHODS: The rats were randomly divided into three groups: normal group (n = 15, age-matched normal adult rats), ISO group (n = 11, ISO induced heart failure) and atorvastatin group (n = 14, ISO induced lesion but received atorvastatin treatment). The cardiac function was evaluated by echocardiography and hemodynamics analysis. In addition, the Rac1 activity in the myocardium and the expression levels of Rac1, p47^{phox} and p67^{phox} were measured by RT-PCR and western blot.

RESULTS: Rats in ISO group developed into heart failure with decreased cardiac function. The Rac1, p47^{phox} and p67^{phox} mRNA expressions and ROS release were increased in ISO group. Atorvastatin treatment improved cardiac function of rats with isoproterenol-induced chronic heart failure and decreased the Rac1, p47^{phox} and p67^{phox} mRNA expressions. Also, membrane protein expression of Rac1 and ROS release decreased significantly.

CONCLUSIONS: Atorvastatin may improve cardiac function of rats with heart failure via inhibiting Rac1/P47^{phox}/P67^{phox}-mediated ROS release.

Key Words:
Heart failure, congestive, Atorvastatin, Rac1 GTP-binding protein, p47^{phox}, p67^{phox}.

Introduction

Chronic heart failure is a complex clinical syndrome defined by the inability of the heart to maintain a normal cardiac output without invoking maladaptive compensatory mechanisms, leading to signs of pulmonary and peripheral edema and symptoms of dyspnea and fatigue[1]. Heart failure has become a major and growing public health problem, affecting more than 5.7 million patients in the US[2]. Although its pathophysiology remains not fully understood, substantial evidence suggest that increased oxidative stress resulting from an increased cardiac generation of reactive oxygen species (ROS) is involved in the progression of heart failure[3]. Further studies indicate that nicotinamide adenosine dinucleotide phosphate (NADPH) oxidases are major sources of ROS in cardiomyocytes[4]. The translocation of the cytosolic regulatory subunits of NADPH (p40^{phox}, p47^{phox}, p67^{phox}, and rac1) to the plasma membrane is a prerequisite for oxidase activation and ROS production[5,6]. Thus, the approaches that can inhibit the NADPH oxidase activity and expression may be effective for alleviating cardiac dysfunction[7,8].

Atorvastatin is a selective competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase that is widely used for treatment of dyslipidemias and reducing the risk of cardiovascular diseases[9]. Recent studies have proved that in addition to lower lipid levels, atorvastatin also possess pleiotropic effects to inhibit oxidative stress via down-regulation of NADPH oxidase in patients with hypercholesterolemia[10], undergoing coronary artery bypass grafting[11], and rat models of cerebral infarction[12]. Although atorvastatin has been shown to suppress inflammation, extracellular matrix remodeling, improve both endothelial function and exercise capacity for heart failure patients[13], the mechanism for down-regulating NADPH oxidase is not reported.

The aim of this study was to investigate the effects of atorvastatin treatment on the expressions of NADPH oxidase subunit (p47^{phox}/p67^{phox}/Rac1) in rats with chronic heart failure, which may provide some theoretical foundations for use of atorvastatin to treat heart failures.

Materials and Methods

Animal Model and Grouping

The study protocol was approved by the Animal Use and Care Committee of Heilongjiang

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Provincial Hospital. All the rats were housed in accordance with the National Institutes of Health Care and Treatment of Laboratory Animals. Ninety-five adult male Wistar rats (220-300 g body weight, provided by the Experimental Animal Center of Harbin Medical University) were randomly divided into two groups: normal group (n = 15) and heart failure group (n = 80). The chronic heart failure model was induced by isoproterenol (ISO, Sigma, St Louis, MO, USA: 340 mg/kg) administration subcutaneously twice with a 24-hour interval. Only 34 rats survived in the heart failure model group after two months. Further, the survived rats were randomly divided into two subgroups: (1) Atorvastatin group (n = 17). The atorvastatin (Lipitor Pfizer Pharmaceuticals Ltd., Dalian, People Republic of China, lot number, 35837006) was dissolved in the normal saline and then given to the rats intragastrically once a day (50 mg/kg)[14]; (2) ISO group (n = 17). The rats were given normal saline intragastrically. After two weeks, only 14 rats survived in atorvastatin group, but 11 in ISO group.

Echocardiographic Measurements

After being anesthetized with 10% chloral hydrate (3 mL/kg), all rats underwent echocardiography with Philip SONOS 7500 sector scanner (Hewlett Packard Co, Andover, MA, USA) using a 12-MHZ imaging transducer. The cardiac function parameters included left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), and fractional shortening (FS).

Hemodynamic Measurements

The hemodynamic studies were performed after echocardiography. The right carotid artery was exposed and cannulated with a 2-Fr micromanometer-tipped catheter that was advanced into the left ventricle for measuring the left ventricular end-diastolic pressure (PED), end-systolic pressure (PES), the maximal rate of pressure rise and fall (dP/dt_{max} and dP/dt_{min}).

Biochemical Indicator Detection in Myocardial Tissues

The rats were sacrificed by injection with 3 mL 10% KCl via femoral vein and the heart was harvested. After the left and right atria, big blood vessels and the attached connective tissues were removed, the myocardial tissues were isolated from the left ventricle. Subsequently, the myocardial homogenate was prepared through grinding 0.2 g myocardial tissues that were mixed with 1.8 mL 0.86% cold normal saline. After 10 min of centrifugation at 3000 r/min at 4°C, the supernatants were collected and frozen until analysis. Some myocardial tissues (200 mg) were snap frozen in liquid nitrogen, and stored at −80°C.

The total protein content was measured by using Coomassie brilliant blue staining. The superoxide anion (O_2^-) level was determined by colorimetric method. Lipid peroxidation (LPO) was analyzed by measuring the thiobarbituric acid (TBA) reactive substances (Nanjing Jiancheng Science and Technology Co., Ltd, Nanjing, China).

RT-PCR for Rac1, $p47^{phox}$ and $p67^{phox}$ mRNA

Total RNA was isolated from myocardial tissues using a two-step method of guanidinium isothiocyanate-phenol-chloroform extraction and reverse transcribed at 42°C for 40 min. RT-PCR was performed in a 20 μL mixture according to manufacturer's protocol (Promega, Madison, WI, USA). The PCR procedure was initial denaturation at 94°C for 5 min, 25 cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s, with a 5 min final extension at 72°C. Each PCR was repeated for at least three times. The primers were synthesized by Shanghai Boya Biology Company: Rac1 with the forward primer: 5′-GTA AAA CCT GCC TGC TCA TC-3′and reverse primer: 5′-GCT TCA TCA AAC ACT GTC TTG-3′ (472 bp); $p47^{phox}$ with the forward primer: 5′-GGG TGA TGT GGT GGA TGT CG-3′ and reverse primer: 5′-TGT CCT TTG AGT CAG GGC TC-3′ (518 bp); $p67^{phox}$ with the forward primer: 5′-TCA GCC CAC GCA TCT CCC-3′ and reverse primer: 5′-ACA GCA AGC CTG CCC ACC-3′ (222 bp); β-actin with the forward primer: 5′-TCA TGC CAT CCT GCG TCT G-3′; and reverse primer: 5′-GCA TCG GAA CCG CTC ATT-3′ (276 bp). PCR product (5 μL) was run through a 1.5% agarose gel electrophoresis and visualized with Electrophoresis Gel Imaging (Kodak, New Haven, CT, USA). β-actin was used as an internal control to calculate the relative expression of Rac1, $p47^{phox}$ and $p67^{phox}$.

Western Blotting for Rac1 Protein in the Cytomembrane and Cytoplasm of Myocardial Tissues

The cytomembrane and cytoplasm of myocardial tissues (100 mg) was isolated by the sucrose density gradient[15]. The protein content was measured by Bradford assay. Next, 30 μg protein were

resolved by 15% SDS-PAGE gel (100 V) and transferred to nitrocellulose filter membranes (Amersham Biosciences, Piscataway, NJ, USA) at 80 V for 90 min. after blocking for 30 min, nitrocellulose membranes were incubated with rabbit anti-Rac1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:500) for 1 h at room temperature, followed by horseradish peroxidase-conjugated secondary antibody (1:5,000) for 30 min. Rac1 was visualized by enhanced chemiluminescence reagents and quantitated by densitometry.

Rac1 Activity Assay

A glutathione-S-transferase (GST)-PAK-CD (PAK-CRIB domain) fusion protein, containing the rac1 binding region from human PAK1B, was used to determine rac1 activity as described[4,16]. Briefly, myocardium was homogenized and resuspended in lysis buffer. After centrifugation, the supernatant protein were incubated with the GST-PAK-CD fusion protein bound to glutathione-coupled Sepharose beads at 4°C for 30 minutes. Beads were washed 3 times with lysis buffer, eluted in Laemmli buffer and analyzed for bound rac1 by Western blotting.

Statistical Analysis

The experiment data are expressed as mean ± SD (standard deviation), and analyzed on SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The measurement data were compared between groups using one-way analysis of variance (ANOVA) and q test. The correlation among some variables was analyzed by univariate linear correlation analysis. $p < 0.05$ was considered statistically significant.

Results

Echocardiographic Measurements

Compared with the normal control, the LVEDD and LVESD were significantly increased, but the FS was significantly decreased in the ISO group. However, after atorvastatin treatment, the above parameters were improved, showing reduced LVEDD and LVESD, but higher FS (Table I, Figure 1A, B, C).

Hemodynamic Measurements

In comparison with normal control, the PES and dp/dt_{max} were significantly reduced, but the dp/dt_{min} and PED were significantly increased in the ISO group. Atorvastatin treatment partially reversed the above changes, leading to increased PES and dp/dt_{max}, but decreased dp/dt_{min} and PED (Table II).

Effect of Atorvastatin on the Levels of O_2^- and LPO in Myocardial Tissues

As shown in Table III, the O_2^- and LPO in the myocardial tissues were significantly higher in the ISO group than those in normal control. Atorvastatin treatment could lower the free-radical production, resulting in decreased content of O_2^- and LPO in atorvastatin group.

Effect of Atorvastatin on the mRNA Expressions of Rac1, $p47^{phox}$ and $p67^{phox}$

Using the β-actin as an internal control, the relative expressions of Rac1, $p47^{phox}$ and $p67^{phox}$ mRNA were calculated and shown in Figure 2. As expected, atorvastatin treatment could significantly reduce the relative expressions of Rac1, $p47^{phox}$ and $p67^{phox}$ mRNA which were higher in the ISO group.

Effect of Atorvastatin on the Membranal and Cytoplasmic Rac1 Protein Expressions

Under normal condition, the Rac1 protein was mainly expressed in the cytoplasm, but it was expressed in the cell membrane in the patients with chronic heart failure. As expected, the Rac1 protein expression in the cell membrane was enhanced in ISO group, but was decreased after atorvastatin treatment (Figure 3A, B).

Table I. Echocardiographic results ($\bar{x} \pm s$).

Group	Sample	LVEDD (mm)	LVESD (mm)	FS
Normal control	15	4.2 ± 0.5	2.3 ± 0.4	0.46 ± 0.03
ISO	11	6.3 ± 0.5*	4.8 ± 0.3*	0.23 ± 0.04*
ISO + atorvastatin	14	5.8 ± 0.3*,#	3.8 ± 0.2*,#	0.34 ± 0.02*,#

ISO: isoproterenol; LVEDD; left ventricular end systolic diameter; LVESD: left ventricular end systolic diameter; FS: fractional shortening. *Compared with the normal control, $p < 0.01$; #Compared with the ISO group, $p < 0.01$.

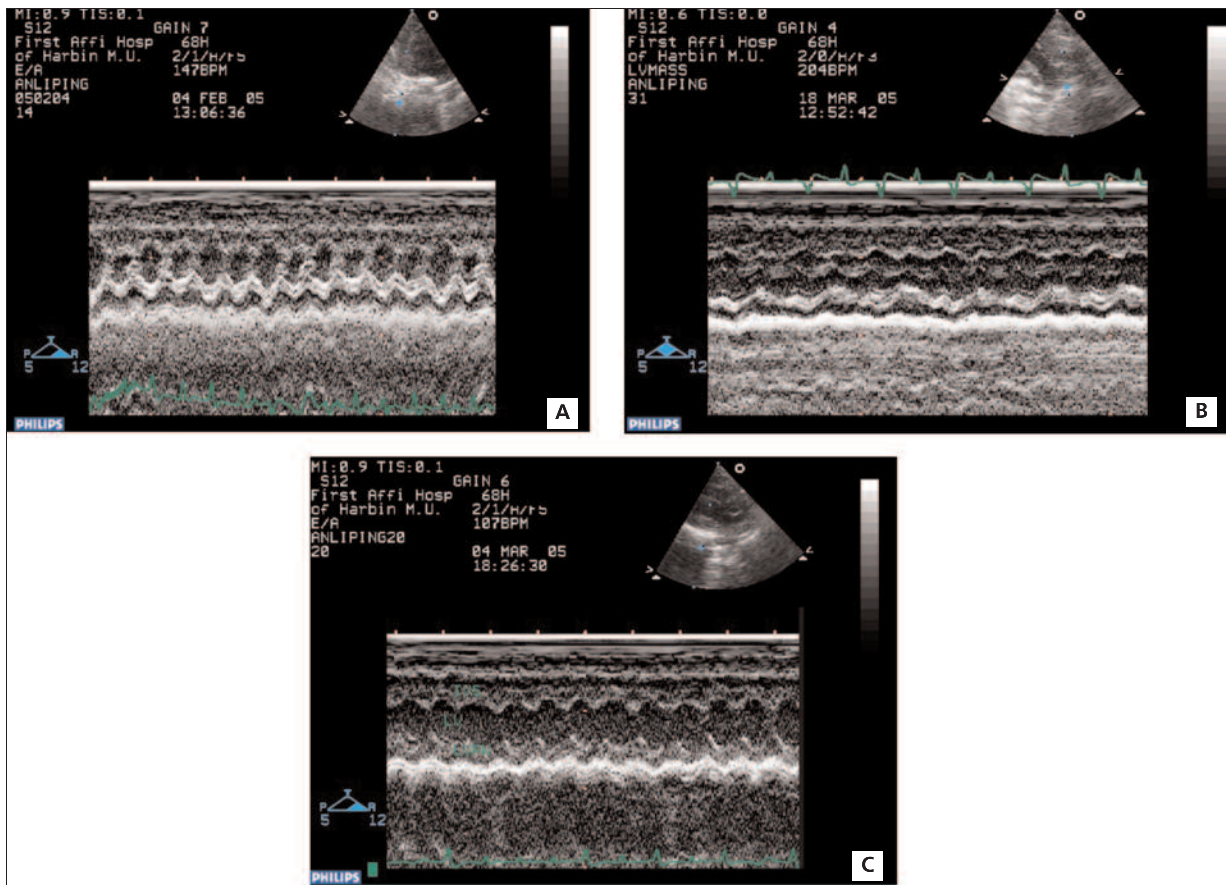

Figure 1. Echocardiographic measurements of the left ventricle in rats. ***A,*** Normal control group. ***B,*** Isoproterenol (ISO) group. ***C,*** ISO + atorvastatin group.

Table II. Hemodynamic results ($\bar{x} \pm s$).

Group	Sample	HR (beats/minute)	PES (mmHg)	PED (mmHg)	dp/dt_{max} (mmHg/s)	dp/dt_{min} (mmHg/s)
Normal control	15	249 ± 17	112.6 ± 3.4	2.6 ± 0.4	11294 ± 796	-7351 ± 684
ISO	11	260 ± 18*	96.8 ± 5.1*	7.2 ± 0.6*	6879 ± 512*	-5426 ± 526*
ISO + atorvastatin	14	252 ± 14*,#	106.3 ± 4.3*,#	4.3 ± 0.7*,#	8456 ± 873*,#	-5986 ± 398*,#

ISO: isoproterenol. *Compared with the normal control, $p < 0.01$; #Compared with the ISO group, $p < 0.05$.

Table III. The levels of O_2^- and LPO in myocardial tissues ($\bar{x} \pm s$).

Group	Sample	O_2^- (mmol/L)	LPO (nmol/L)
Normal control	15	2.35 ± 0.36	6.51 ± 0.60
ISO	11	7.63 ± 0.82*	10.51 ± 1.59*
ISO + atorvastatin	14	4.66 ± 0.66*,#	8.15 ± 0.95*,#

ISO: isoproterenol. *Compared with the normal control, $p < 0.01$; #Compared with the ISO group, $p < 0.05$.

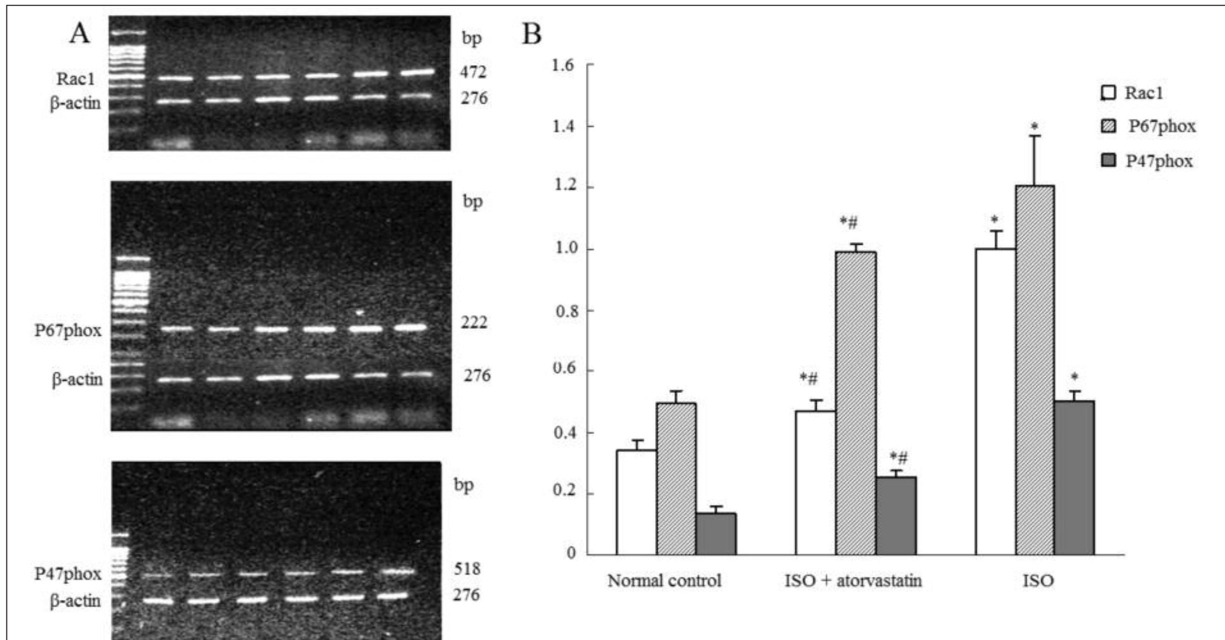

Figure 2. RT-PCR for Rac1, $p47^{phox}$ and $p67^{phox}$ expression. ***A,*** Electrophoretogram. Lane 1, 2: normal control group; lane 3, 4: isoproterenol (ISO) + atorvastatin group; lane 5, 6: ISO group. ***B,*** Statistical diagram. *Compared with the normal control, $p < 0.05$; #Compared with the ISO group, $p < 0.05$.

Effect of Atorvastatin on the Rac1 Activity

The Rac1-GTP binding activity is critical to activate the NADPH oxidase-dependent O_2^- generating. Therefore, we also investigated the Rac1 activity. As anticipated, the Rac1-GTP binding activity increased by 3.4 fold in the ISO group compared with the normal control, but decreased after atorvastatin treatment (Figure 3C).

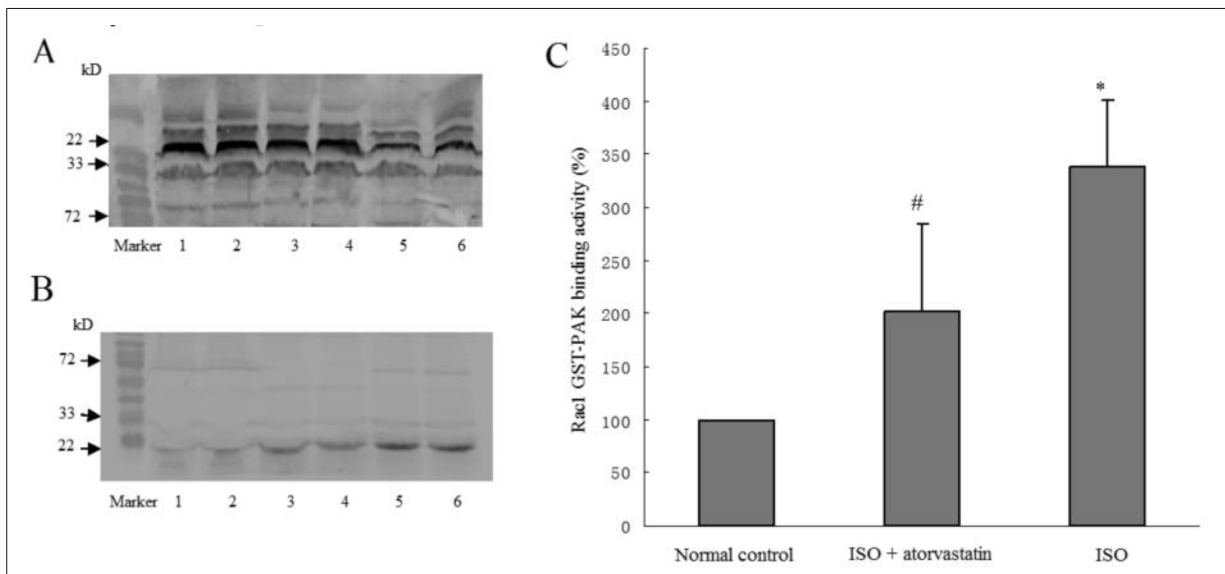

Figure 3. Rac1 protein expression and Rac1 activity. ***A,*** Rac1 protein expression in the cytoplasm of myocardial tissues. ***B,*** Rac1 protein expression in the cell membrane Rac1 protein expression in the cytoplasm. Lane 1, 2: normal control group; lane 3, 4: isoproterenol (ISO) + atorvastatin group; lane 5, 6: ISO group. ***C,*** Rac1 activity. *Compared with the normal control, $p < 0.01$; #Compared with the ISO group, $p < 0.05$.

Univariate Linear Correlation Analysis

Correlation analysis indicated that the Rac1-GTP binding activity was positively associated with the mRNA expressions of $p47^{phox}$ and $p67^{phox}$ ($r = 0.945$, $p < 0.01$; $r = 0.728$, $p < 0.05$). The mRNA expressions of $p47^{phox}$ and $p67^{phox}$ were also positively correlated with O_2^- and LPO content ($p47^{phox}$: $r = 0.926$, $p < 0.01$; $r = 0.972$, $p < 0.01$; $p67^{phox}$: $r = 0.775$, $p < 0.05$; $r = 0.808$, $p < 0.05$).

Discussion

Several studies have demonstrated that administration of high-dose ISO, a selective beta-adrenergic agonist (> 85 mg/kg), induces oxidative myocardial necrosis in rats which progressively develop into the dilated cardiomyopathy and heart failure[17,18]. This model could better reflect the pathphysiologic processes of heart failure and, thus, widely used. In this study, we also established the chronic heart failure by administration of 340 mg/kg ISO. The echocardiographic and hemodynamic measurements showed that compared with the normal control group, the FS, PES and dp/dt_{max} were significantly decreased in the ISO group, but dp/dt_{min} and PED were significantly increased in ISO, indicating the heart failure model was successfully induced.

Reactive oxygen species (ROS) are kinds of oxygenated products generated during the oxidative metabolism, including O_2^-, OH•, H_2O_2, and LPO. Recently, growing evidence suggests that ROS play a potentially deleterious role for heart failure[19,20]. Superoxide anion and H_2O_2 stimulate the downstream effectors, such as mitogen-activated protein kinases (MAPK), tyrosine kinases, Rho kinase, and inactivate protein tyrosine phosphatases, which lead to hypertrophy or apoptosis of cardiac myocytes[21,22]. In addition, ROS also increase intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) by activation of Ca/Calmodulin Kinase Iiδ. $CaMKII\delta^{-/-}$ knockout myocytes develop significantly less H_2O_2-induced arrhythmias and are more resistant to hypercontracture, ultimately preventing heart failure[23]. In this study, we also found that O_2^- and LPO were significantly increased in the ISO group.

Although the exact contribution to the generation of ROS is not clear, the activation of non-phagocytic NAD(P)H oxidases (particular $p47^{phox}$ and $p67^{phox}$ subunit) are suggested to play the predominant roles for ROS production in cardiac myocytes[21]. Thus, the $p47^{phox}$ and $p67^{phox}$ expressions were also analyzed. As expected, the mRNA expressions of $p47^{phox}$ and $p67^{phox}$ were significantly increased in ISO group. More importantly, their expression was positively related with the content of O_2^- and LPO in myocardial tissues, indirectly demonstrated the regulatory relationship between NADPH oxidases and ROS. Furthermore, it is reported that Rac1 subunit of NADPH is a small GTP binding protein of Rho family. The translocation of the Rac1 from the cytoplasm to the plasma membrane is a core prerequisite for NADPH oxidase activation and ROS production[5,6], which were also confirmed by our present study. The Rac1 activity was significantly increased in the plasma membrane of chronic heart failure rats and the Rac1 expression was positively associated with $p47^{phox}$ and $p67^{phox}$ expression. However, in a study described by Maack et al[16], $p67^{phox}$ was not significantly influenced by Rac1 activity. This may be attributed to different myocardial tissues types.

In addition to lower lipid levels, atorvastatin also inhibits inflammation, lessens the adhesion between leucocytes and endothelial cells, and regulates thrombogenesis, endothelial function, vascular smooth muscle cells proliferation[13]. These imply that atorvastatin may exert important protective roles for myocardial cells and heart failure. However, whether it also down-regulates NADPH oxidase and ROS has not been reported. Atorvastatin could improve cardiac function, reduce O_2^-, LPO, Rac1, $p47^{phox}$, and $p67^{phox}$.

Conclusions

These findings suggest that atorvastatin may improve cardiac function of rats with isoproterenol-induced chronic cardiac failure via inhibiting $P47^{phox}/P67^{phox}$-mediated NADPH oxidase regulated by the small GTPase Rac1.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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