Rosuvastatin-attenuated heart failure in aged spontaneously hypertensive rats via PKCα/β2 signal pathway

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Abstract

There are controversies concerning the capacity of Rosuvastatin to attenuate heart failure in end-stage hypertension. The aim of the study was to show whether the Rosuvastatin might be effective or not for the heart failure treatment. Twenty-one spontaneously hypertensive rats (SHRs) aged 52 weeks with heart failure were randomly divided into three groups: two receiving Rosuvastatin at 20 and 40 mg/kg/day, respectively, and the third, placebo for comparison with seven Wistar-Kyoto rats (WKYs) as controls. After an 8-week treatment, the systolic blood pressure (SBP) and echocardiographic features were evaluated; mRNA level of B-type natriuretic peptide (BNP) and plasma NT-proBNP concentration were measured; the heart tissues were observed under electron microscope (EM); myocardial sarcoplasmic reticulum Ca2+ pump (SERCA-2) activity and mitochondria cytochrome C oxidase (CCO) activity were measured; the expressions of SERCA-2a, phospholamban (PLB), ryanodine receptor2 (RyR2), sodium–calcium exchanger 1 (NCX1), Ca2+/calmodulin-dependent protein kinase II (CaMKII) and protein phosphatase inhibitor-1 (PPI-1) were detected by Western blot and RT-qPCR; and the total and phosphorylation of protein kinase Cα/β2 (PKCα/β2) were measured.

Aged SHRs with heart failure was characterized by significantly decreased left ventricular ejection fraction and left ventricular fraction shortening, enhanced left ventricular end-diastolic diameter and LV Volume, accompanied by increased plasma NT-proBNP and elevated BNP gene expression. Damaged myofibrils, vacuolated mitochondria and swollen sarcoplasmic reticulum were observed by EM. Myocardium CCO and SERCA-2 activity decreased. The expressions of PLB and NCX1 increased significantly with up-regulation of PPI-1 and down-regulation of CaMKII, whereas that of RyR2 decreased. Rosuvastatin was found to ameliorate the heart failure in aged SHRs and to improve changes in SERCA-2a, PLB, RyR2, NCX1, CaMKII and PPI-1; PKCα/β2 signal pathway to be suppressed; the protective effect of Rosuvastatin to be dose dependent. In conclusion, the heart failure of aged SHRs that was developed during the end stage of hypertension could be ameliorated by Rosuvastatin.

Keywords: spontaneously hypertensive rat • heart failure • Rosuvastatin • protein kinase Cα/β2 • sarcoplasmic reticulum calcium pump • phospholamban • ryanodine receptor2 • sodium–calcium exchanger

Introduction

Hypertension has become the most familiar ‘epidemic disease’ in the world. Because of its harmful complications in the cardiovascular system, it has been identified as the mayor risk factor for high cardiac mortality. Although plenty of new anti-hypertensive medications have been developed [1, 2], ventricular hypertrophy and the subsequent congestive heart failure are still the common occurrences in most aged hypertensives [3]. Therefore, an effective, safe and widely used agent is urgently needed to attenuate the progress of heart failure.

The HMG-CoA-reductase inhibitors (statins) exert a protective effect on the cardiovascular system [4, 5], in addition to their lipid-lowering action [6–8], as indicated by the heart function restoration in the hypertensive patients with or without coronary diseases [9–11]. Furthermore, the JUPITER trial suggested that Rosuvastatin could effectively decrease C-reactive protein (CRP) level, thereby reducing
cardiovascular event rates [12, 13]. Braunwald [14] doubted that this
effect might be based on reducing inflammation per se, without an
effect on LDL-C. However, the exact role and underlying mechanism
of these protective effects are not completely clear.

The purpose of this study was to observe the impairment of car-
diac systolic function in aged spontaneously hypertensive rats
(SHRs). We reported here that Rosuvastatin could ameliorate the
impairment that occurs at the end stage of hypertension in the aged
SHRs; and provide evidence indicating signalling pathways are
involved.

Material and methods

Animals

Twenty-one male SHRs, aged 52 weeks and weighing approximately
400 g, and seven age-matched normotensive male Wistar-Kyoto rats
(WKY) as controls (Animal Administration Center of Fudan University,
Shanghai, China) were bred ad libitum. SHRs were randomly divided
into three groups, the first two receiving Rosuvastatin at a dose of
20 mg/kg/day (SHR-LD, n = 7) and 40 mg/kg/day (SHR-HD, n = 7)
respectively; the third, placebo (SHR, n = 7) for comparison with
seven WKYs as controls. Rosuvastatin and vehicle were daily admin-
istered through an intra-gastric tube for 8 weeks. All animal experi-
mental procedures were approved by the Animal Care and Use
Committee of Fudan University and performed in accordance with the
guide for the care and use of laboratory animals (NIH publication
no. 85-23, National Academy Press, Washington, DC, USA, revised
1996).

Measurement of systolic blood pressure

All the rats were trained to adapt themselves to the restraining cages
and tail-cuff apparatus for the standard non-invasive tail-cuff method
before SBP measurement [15], which was performed at the beginning
without exception, and then every 4 weeks under their conscious condi-
tions. Each measurement was repeated thrice.

Determination of plasma NT-proBNP concentration

The animals were killed so that their blood samples were collected from
the abdominal aorta, the blood serum separated and NT-proBNP concen-
tration determined by ELISA according to the manufacturer’s
instructions (R & D Systems, Minneapolis, MN, USA).

Measurement of cardiac function

Trans-thoracic echocardiographic analysis was performed with an
animal-specific instrument (VisualSonics® Vevo770®; VisualSonicsInc,
Toronto, Canada), as previously described [16]. The animals were
anaesthetized with isoflurane before M-mode images of the left ven-
tricle were recorded when the heart rate was near 400 bpm. To eval-
uate the function of their hearts, the following parameters were
measured: the left ventricular end-diastolic diameter (LVDD), left ventri-
cular volume (LV Volume), left ventricular ejection fraction (LVEF),
left ventricular short-axis fractional shortening (LVFS), left ventricular
stroke volume (SV).

Transmission electron microscopy

From each group the cardiac tissues were minced into small pieces
(< 1 mm³) and fixed in 2.5% glutaraldehyde in 0.1 mol/l sodium caco-
dylate buffer (pH 7.3) for 2 hrs. The specimens were rinsed in buffer,
postfixed in cacodylate-buffered 2% OsO₄, stained en bloc in uranyl
acetate, dehydrated gradient in ethanol and embedded in epoxy resin.
Finally, 50–70 nm super thin slices were prepared, stained with uranyl
acetate and lead citrate and examined under an electron microscope
(Philips TECNA10; Philips, Amsterdam, The Netherlands).

Measurement of mitochondria cytochrome c oxidase activity

Mitochondria were isolated from left ventricle as described previously
[17]. The final crude mitochondrial pellet was resuspended in sucrose-
histidine-EDTA buffer, and the protein concentrations were determined
via bicinchoninic acid method. The cytochrome c oxidase (CCO) activity
was measured as described by Subbuswamy [18].

Quantitative RT-PCR

RNA was isolated and its concentrations were determined; quantitative
real-time PCR analyses were performed as previously described [15]. The
primers for BNP, SERCA2a, PLB, RyR2 type 1, RyR2 type 2, PKC β,
and glycerolaldehydes 3-phosphate dehydrogenase (GAPDH) were
designed from Takara: For BNP gene, sense, 5′-CAGTGAAGTGCTCGGTGGCTGTGTT-3′ and
antisense, 5′-GCAAGTCAAGGCCGGAGCTGAGT-3′; For SERCA2a gene, sense,
5′-TGAGGCCACCTCACAGGCAAC-3′ and antisense, 5′-CATAGCCGTTGGCTGATTGATG-3′;
For PLB gene, sense, 5′-AATCAAACATGCTTCATGACACC-3′ and antisense, 5′-
GGCGGGAGTGGATGTGGGA-3′. For RyR2 type 1 gene, sense, 5′-GGCCATCTTGGTGCACAGTACC-3′
and antisense, 5′-CTGCTGCCTATGTTAAAGGGCCATC-3′. For RyR2 type 2 gene, sense, 5′-
GAGCCCCGAAAGCTCTGAA-3′ and antisense, 5′-GGCAACTCATGGGCACACAC-3′. For PKCγ gene,
sense, 5′-TCGGATCTTGCACTGTAAGCTGAA-3′ and antisense, 5′-ATCGGCCATGGTTTTTGTGAG-3′.
The relative expression levels of the genes were normalized to
those of GAPDH using 2⁻¹⁴ΔΔCT method.
Western blot

The ventricle tissues were removed rapidly from the rats to be stored at −80°C. The expressions of phosphorylated PKC and calcium-handling proteins were measured via Western blot and normalized to the protein level of β-actin or GAPDH. From the frozen ventricle tissues were extracted the total proteins, whose concentration was determined with a BCA protein assay, SERCA2a, PLB, PPI-1, NCKX1 (1:1000; ABCAM, Cambridge, UK), CaMKII, RyR, PKCα, PKCβ1, PKCδ2, phospho-PKCα, phospho-PKCβ1 and phospho-PKCδ2 (1:300; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were examined by Western blot as previously described [15], and the optic densities were analysed using ImagePro 5.0 (Media Cybernetics, Inc., Silver Spring, MD, USA).

Statistical analysis

The results were presented as mean ± S.E.M. and analysed using one-way ANOVA followed by Fisher’s LSD test for multiple comparisons using the SPSS software package, version 16.0 (SPSS Inc., Chicago, IL, USA), P < 0.05 considered as statistically significant.

Results

Lowering effect of Rosuvastatin on blood pressure

The average SBP was found to be higher in SHR controls than in WKYs by 59.4% (P < 0.01). However, no significant difference was observed among SHR+LD, SHR+HD and SHR controls after 4 and 8 weeks, respectively (P > 0.05; Table 1).

Table 1 Changes in systolic blood pressure (SBP)

| Group      | Baseline  | In the fourth week | In the eighth week |
|------------|-----------|--------------------|--------------------|
| WKY        | 129.14 ± 7.76 | 124.22 ± 4.49 | 128.65 ± 4.44 |
| SHR        | 205.90 ± 8.56* | 206.07 ± 6.96* | 201.80 ± 2.95* |
| SHR+LD     | 200.35 ± 7.04* | 203.17 ± 7.51* | 206.07 ± 4.57* |
| SHR+HD     | 200.31 ± 7.89* | 198.07 ± 6.69* | 202.81 ± 6.39* |

Values are mean ± S.E.M., n = 5, *P < 0.01 versus WKYs.

Effect of Rosuvastatin on cardiac structure and function of SHRs

Echocardiographic measurements were conducted in vitro to prove whether myocardium of SHR underwent heart malfunction (Fig. 1A). It was found that LVEF and LVFS were lower in SHR controls than in WKYs by 37.5% and 45.5% respectively (P < 0.05). In SHR+HD, LVEF and LVFS increased by 52.9% and 66.3%, respectively, when compared with SHR controls (P < 0.05). However, SV showed no significant difference among these four groups (P > 0.05) (Fig. 1B and C).

The results also indicated an increase in LVIDd and LV volume in SHR controls by 34.8% and 80%, respectively, when compared with WKYs (P < 0.05). In SHR+HD, significant reductions developed in LVIDd and LV volume by 20.1% and 33.9%, respectively (P < 0.05; Fig. 1D and E).

According to the general changes, the results of the transmission electron microscopy showed the losses and damages of myofilaments in SHR controls when compared with WKYs (Fig. 2A). Swollen, fragmented and vacuolated mitochondria were observed to be evident in SHRs, and the cristae in the mitochondria appeared distorted and in some cases were completely lysed. Furthermore, there appeared dilated and swollen sarcoplasmic reticulum in SHRs (Fig. 2B). Treated with Rosuvastatin, SHRs showed improved cardiac structure as indicated by the reversed losses and damages of myofilaments, and increased volume of myofilaments. Moreover, the swollen, fragmented and vacuolated mitochondria were alleviated, whereas the sarcoplasmic reticulum was not as dilated and swollen as that of SHR controls. The improvement of the cardiac structure was more apparent in SHR+HD than in SHR+LD (Fig. 2C and D).

Furthermore, compared with WKY, the CCO activity of SHR controls was decreased by 31.2% (P < 0.05), but increased in SHR+LD and SHR+HD by 12.8% (P < 0.05) and 36.0% (P < 0.05) respectively (Table 2). In addition to the morphologic alterations, plasma NT-proBNP levels were examined to be significantly increased in SHR controls in comparison with WKYs, and to be decreased in SHR+LD and SHR+HD by 12.1% and 19.5% respectively (P < 0.01; Table 3). Meanwhile, the mRNA level of BNP examined by real-time RT-qPCR showed a 5.35-fold increase in SHR controls, and a decrease in SHR+LD and SHR+HD by 47.4% and 72.3% respectively (P < 0.01; Fig. 3).

Regulation of the mRNA and protein expressions of Ca2+-cycling protein in SHRs

To investigate the mechanism of Rosuvastatin in the attenuated cardiac function of SHRs, the expressions of myocardium Ca2+-handling proteins were evaluated.

Fig. 1 Echocardiographic data of %EF, %FS, SV, LVIDd and LV volume. (A) M-type echocardiographic images of WKYs, SHR controls, SHR+LD and SHR+HD. (B) Cardiac function decreased significantly in SHR controls compared with WKYs as indicated by both %EF and %FS (P < 0.05); %EF and %FS were significantly enhanced in SHR+HD (P < 0.05). (C) Stroke volume with no variety in all groups. (D) and (E) Left ventricle dilated significantly in SHR controls compared with WKYs as indicated by LVIDd and LV volume (P < 0.05), but reversed in SHR+HD (P < 0.05). Values are mean ± S.E.D., n = 4, *P < 0.05 versus WKYs; †P < 0.05 versus SHR controls. LVIDd: left ventricular end-diastolic diameter, LV volume: left ventricular volume, SV: left ventricular stroke volume, %EF: left ventricular ejection fraction, %FS: left ventricular short-axis fractional shortening.
PPI-1, dephosphorylation of the PLB and thus incapable of activating SERCA2a, was significantly up-regulated by 85.9% in SHR controls ($P < 0.01$), and significantly down-regulated in SHR+LD and SHR+HD by 56.4% and 77.3% respectively ($P < 0.01$). Meanwhile, the expression of CaMKII up-regulated significantly by 40.9% in SHR+HD ($P < 0.01$) but not SHR+LD ($P > 0.05$), when compared with SHR controls (Fig. 4A).

The results indicated that SERCA2a, RyR2 type 1 and RyR2 type 2 mRNA were significantly decreased by 78.4%, 41.3% and 91.1%, respectively ($P < 0.05$), whereas PLB expressions increased 4.8-fold in SHR controls when compared with WKYs. Furthermore, the expressions of SERCA2a, RyR2 type 1 mRNA were significantly up-regulated in SHR+HD ($P < 0.05$), whereas those of PLB were down-regulated ($P < 0.05$). However, RyR2 type 2 mRNA showed no significant changes among the three groups (Fig. 4C).

The expression of NCX1 was significantly up-regulated in SHR controls, but reversed in SHR+HD. Meanwhile, the protein expressions of SERCA2a, PLB and RyR2 were highly consistent with those of RT-qPCR, as indicated by Western blot (Fig. 4B and D).

The SERCA activity was significantly decreased by 20.6% in SHR when compared with WKY ($P < 0.05$), but increased by 11.7% and 19.7% in SHR+LD and SHR+HD ($P < 0.05$) respectively (Table 4).

### Table 2 Effect of Rosuvastatin on cytochrome c oxidase (CCO) activity (k/min./mg of protein)

| Group       | WKY       | SHR       | SHR+LD    | SHR+HD    |
|-------------|-----------|-----------|-----------|-----------|
| CCO activity| 5.69 ± 1.53| 3.92 ± 1.20 * | 4.42 ± 1.06 | 5.33 ± 1.32 # |

Values are mean ± S.E.M., $n = 7$, *$P < 0.01$ versus WKYs; # $P < 0.01$ versus SHR controls.

PKC signalling pathway has been identified as a given pathway in the Ca$^{2+}$-regulating process. It was found that Rosuvastatin down-regulated significantly the mRNA expression of PKC$\alpha$ and PKC$\beta$ by 64.5% and 67.7% in SHR+LD, respectively, and by 7.29% and 66.4% in SHR+HD respectively ($P < 0.01$). Furthermore, the ratios of phosphorylation to the total PKC$\alpha$ and PKC$\beta$ were significantly inhibited, but it was not true of PKC$\beta$1 in SHR+LD and SHR+HD when compared with SHR controls (Fig. 5).

### Discussion

Most of the hypertensives tend to develop cardiac hypertrophy and even heart failure, although they receive the administration of antihypertensive medications. Rosuvastatin has been reported to be controversial in attenuating cardiac function [9], improving survival rate in those with diastolic heart failure [10] and decreasing all-cause mortality following a diagnosis of heart failure [11]. However, the mechanisms of these protective effects remain unknown.

In the present study, we used 52-week-old SHR as the model of the end-stage hypertensive heart disease, finding that the aged SHRs developed systolic heart failure characterized by the abnormal parameters of echocardiography and the increased NT-proBNP. We
controls; 2012 The Authors 3057ª of SR can induce the impairments of Ca²⁺-handling proteins, thus NCX1 and PPI-1, suggesting a possible mechanism that the failure CA²a, RyR2 and CaMKII were accompanied by an increase in PLB, down-regulated. In addition, the decreased expressions of SER- swollen in SHRs. The mitochondria CCO and SERCA2 activity were vacuolated and the sarcoplasmic reticulum (SR) were dilated and also found that the mitochondria were swollen, fragmented and also found down-regulation of PKCα mRNA and protein expression of PKCα in the myocardium, and aetiological changes in heart failure, confusing the consistence aetiology were not differentially enrolled. Thus, the different inclu- sion and exclusion criteria could cause different pathophysiological and aetiological changes in heart failure, confusing the consistence of the results in the cohort.

Previous studies have shown that statins can ameliorate BNP up-regulation [25] and restore heart function in hypertensives [9] in addition to their lipid-lowering effects. This is also the case for the results from this study that a high concentration of Rosuvastatin could reverse the expressions of SERCA-2a, RyR2, PLB, NCX1, PPI-1 and CaMKII, thus ameliorating the cardiac malfunction at the end stage of hypertension.

However, there have been controversies around the therapeutic effect of statins on cardiac malfunction. In John Kjekshus’ trial, even Rosuvastatin could reduce the total number of hospitalizations for heart failure, but produce no effect on heart failure mortality [26]. The UNIVERSE trials also failed to demonstrate a therapeutic effect of statin on LV remodelling in the patients with chronic heart failure [27]. It could be that those enrolled had been well treated for heart failure with a standard therapy before Rosu- vastatin, which reduced significantly the population with potentially fatal heart failure enrolled in the trial. This could also be because the patients with the heart failure of ischaemic or non-ischaemic aetiology were not differentially enrolled. Thus, the different inclusion and exclusion criteria could cause different pathophysiological and aetiological changes in heart failure, confusing the consistence of the results in the cohort.

The previous literature has shown that PKCα/β function as funda- mental regulators of cardiac contractility and Ca²⁺-handling pro- teins in cardiomyocyte [28–31], and PKCβ2 inhibition attenuates myocardial infarction and hypertension-induced heart failure [32, 33]. Moreover, the enhancement in cardiac contractility associated with PKCα gene deletion protected the myocardium against pressure overload-induced heart failure and dilated cardiomyopathy [34].

Our previous study [15] indicated that Rosuvastatin could decrease the expression of angiotensin II type 1 receptor (AT1R), one type of G-protein coupling receptor, in the ventricle, and resultant inhibition of mitogen-activated protein kinase (MAPK) signal pathway, thus ameliorate cardiac hypertrophy in hypertensive heart disease. To investigate whether PKC is involved in the inhibitory effect of AT1R by Rosuvastatin, we examined the mRNA and protein expression of PKCα/β in the myocardium, and found down-regulation of PKCα/β as well as decreased ratios of phosphorylation to total PKCα and PKCβ2 after Rosuvastatin

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**Table 3 Effect of Rosuvastatin on plasma NT-proBNP (ng/ml)**

| Group     | WKY          | SHR          | SHR+LD       | SHR+HD       |
|-----------|--------------|--------------|--------------|--------------|
| NT-proBNP concentration | 158.27 ± 13.65 | 209.47 ± 20.91* | 184.18 ± 17.55ª | 168.62 ± 13.76ª |

Values are mean ± S.E.M., n = 7, BNP: B-type natriuretic peptide, *P < 0.01 versus WKYs; ªP < 0.01 versus SHR controls.

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**Fig. 3** BNP (Npb) gene expression by RT-qPCR. Real-time RT-qPCR analysis of BNP gene expression showing a significantly higher BNP expression in SHRs than WKYs (P < 0.01), but dose dependently down-regulated in SHR+LD and SHR+HD (P < 0.01). Values: mean ± S.E.M., n = 3, *P < 0.05 versus WKYs; ªP < 0.01 versus SHR controls; **P < 0.01 versus SHR+LD. BNP: B-type natriuretic peptide.
treatment. On the basis of the data presented, we concluded that Rosuvastatin could decrease the expression of AT1R, and consequently suppress the activation of Gα subunit [35–37], restrain G-protein coupling signal pathway, thus inhibit the phosphorylation of PKCa/b2 and ameliorate the impairment of calcium-handling proteins.
Table 4 Effect of Rosuvastatin on SERCA-2 activity [μmol/(mg/prot/hr)]

| Group        | WKY    | SHR    | SHR+LD  | SHR+HD  |
|--------------|--------|--------|---------|---------|
| SERCA-2 activity | 5.38 ± 0.46 | 4.27 ± 0.35* | 4.77 ± 0.58 | 5.11 ± 0.37# |

Values are mean ± S.E.M., n = 7, SERCA2a: sarcoplasmic reticulum calcium pump, *P < 0.01 versus WKYs; #P < 0.01 versus SHR controls.

Fig. 5: mRNA and protein expression of PKCα and PKCβ by real-time RT-qPCR and Western blot. (A) Expressions of total PKCα and PKCβ2 are significantly down-regulated in SHR+LD (P < 0.05), but not the same with the expression of total PKCβ1 (P > 0.05). (B) Expressions of phospho-PKCα and phospho-PKCβ2 are significantly down-regulated in SHR+LD and SHR+HD (P < 0.05), but no significant changes are found in the expression of phospho-PKCβ1 (P > 0.05). (C) Ratio of phosphor PKCα to total PKCα and the ratio of phosphor PKCβ2 to total PKCβ2 are down-regulated significantly in SHR+LD and SHR+HD (P < 0.01). (D) mRNA level of the whole PKCα (P < 0.05) and PKCβ (P > 0.05) gene expressions is down-regulated in SHR controls and lowered considerably (P < 0.05) in SHR+LD and SHR+HD. Values are mean ± S.E.M., n = 3, *P < 0.05 versus WKYs; #P < 0.05 versus SHR controls; ↨P < 0.01 versus SHR+LD. p-PKCα: phosphorylation of protein kinase Ca; p-PKCβ1: phosphorylation of protein kinase Cβ1; p-PKCβ2: phosphorylation of protein kinase Cβ2.
It was reported that the decreased expression of PKC resulted in inactivation of PPI-1, activating the phosphorylation of protein phosphatase-1, which in turn regulated PLB phosphorylation [38, 39], thereby promoting the function of SERCA-2a responsible for Ca^{2+} inactivation of PPI-1, activating the phosphorylation of protein phosphatase-1 and increased expression of CaMKII, which could result in the decreased expression of PLB and increased SERCA2a, RyR2 in the aged SHRs.

Beyond lipid lowering, statins have many pleiotropic effects, such as stimulating stem cells [42, 43], activating immune system [44], and having synergistic effects with telmisartan on endothelial progenitor cells [46]. Those we discovered above can be one of the statins’ pleiotropic effects, which can make statins potentially beneficial to patients with heart failure, for whom hypertension is the main risk factor.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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