Original Paper

Atorvastatin Attenuates Myocardial Hypertrophy in Spontaneously Hypertensive Rats via the C/EBP β /PGC-1 α /UCP3 Pathway

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Key Words

Atorvastatin • Spontaneously hypertensive rats • Myocardial hypertrophy • Apoptosis • C/EBP β • UCP3

Abstract

Background/Aims: Many clinical and experimental studies have shown that treatment with statins could prevent myocardial hypertrophy and remodeling induced by hypertension and myocardial infarction. But the molecular mechanism was not clear. We aimed to investigate the beneficial effects of atorvastatin on hypertension-induced myocardial hypertrophy and remodeling in spontaneously hypertensive rats (SHR) with the hope of revealing other potential mechanisms or target pathways to interpret the pleiotropic effects of atorvastatin on myocardial hypertrophy. **Methods:** The male and age-matched animals were randomly divided into three groups: control group (8 WKY), SHR (8 rats) and intervention group (8 SHR). The SHR in intervention group were administered by oral gavage with atorvastatin (suspension in distilled water, 10 mg/Kg once a day) for 6 weeks, and the other two groups were administered by gavage with equal quantity distilled water. Blood pressure of rats was measured every weeks using a standard tail cuff sphygmomanometer. Left ventricular (LV) dimensions were measured from short-axis views of LV under M-mode tracings using Doppler echocardiograph. Cardiomyocyte apoptosis was assessed by the TUNEL assay. The protein expression of C/EBP β , PGC-1 α and UCP3 were detected by immunohistochemistry or Western blot analysis. **Results:** At the age of 16 weeks, the mean arterial pressure of rats in three groups were 103.6 $\pm$ 6.1, 151.8 $\pm$ 12.5 and 159.1 $\pm$ 6.2 mmHg respectively, and there wasn't statistically significant difference between the SHR and intervention groups. Staining with Masson's trichrome demonstrated that the increased interstitial fibrosis of LV and ventricular remodeling in the SHR group were attenuated by atorvastatin treatment. Echocardiography examination exhibited that SHR with atorvastatin treatment showed an LV wall thickness that was obviously lower than that of water-treated SHR. In hypertrophic myocardium, accompanied by increasing C/EBP β expression and the percentage of TUNEL-positive cells, the expression of Bcl-2/Bax ratio, PGC-1 α and UCP3 were reduced, all of which could be abrogated by treatment with atorvastatin for 6 weeks. **Conclusion:** This study further confirmed that atorvastatin

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could attenuate myocardial hypertrophy and remodeling in SHR by inhibiting apoptosis and reversing changes in mitochondrial metabolism. The C/EBP β /PGC-1 α /UCP3 signaling pathway might also be important for elucidating the beneficial pleiotropic effects of atorvastatin on myocardial hypertrophy.

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Introduction

Atorvastatin is a hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor and is one of the most frequently prescribed medications for lowering serum cholesterol levels and the primary prevention of cardiovascular disease in clinical use. Atorvastatin reduces serum cholesterol levels by inhibiting the synthesis of mevalonate, which is the rate-limiting step in the cholesterol biosynthetic pathway [1]. It is known that atorvastatin brings many cardiovascular benefits, which directly result from its lipid-lowering properties [2, 3]. It is also widely accepted that atorvastatin has beneficial pleiotropic effects that are independent of its lipid-lowering activity [4]. These effects bring additional benefits for the cardiovascular system and include anti-inflammatory activity, decreasing oxidative stress, improving endothelial function, promoting angiogenesis, and reducing myocardial hypertrophy and remodeling [5, 6].

Many clinical and experimental studies have shown that treatment with statins could prevent myocardial hypertrophy and remodeling induced by hypertension and myocardial infarction [7-10] and even reduce mortality due to heart failure [11]. However, the further elucidation of the molecular mechanisms of these functions needs more study. As the engine of the generation of energy and reactive oxygen species (ROS) [12], the mitochondrion plays an important role in the myocardium. The heart, which is an organ with a high energy consumption, also exhibits changes or impairment in mitochondrial function during the progression of hypertrophy or heart failure [13]. Some studies reported that treatment with atorvastatin could regulate the metabolism of the myocardium [14]. However, there were some controversial issues. Some researchers considered that atorvastatin could improve the function of mitochondria [12], but others found that atorvastatin impaired mitochondrial function [15]. Our previous study concluded that atorvastatin could inhibit myocardial hypertrophy and reverse the mitochondrial membrane potential (MMP) *in vitro* [16]. In the present study, we aimed to investigate the beneficial effects of atorvastatin on hypertension-induced myocardial hypertrophy and remodeling in spontaneously hypertensive rats (SHR) with the hope of revealing other potential mechanisms or target pathways to interpret the pleiotropic effects of atorvastatin on myocardial hypertrophy.

Materials and Methods

Animals and ethics statement

The male SHR rats and age-matched male Wistar-Kyoto (WKY) rats used in this study were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. at an age of 11 weeks with body weight 254.5 ± 5.6 g and 248.8 ± 5.4 g respectively, and SPF grade with certificate: SCXK (Beijing) 2016-0011. They were housed under a barrier system with SPF level feeding standard in Animal Laboratory Center of China Medical University. All procedures and experimental protocols involving animals in present study were performed in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978), which were also approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (Shenyang, China) (IACUC issue No.: 2017010). Every effort was made to minimize the number and suffering of animals in this study.

Experimental protocols

Animals were randomly divided into three groups: control group (8 WKY), SHR (8 rats) and intervention group (8 SHR). One rat from intervention group was dead in second week, possibly it's because the gavage

operation. Thus, eventually there were 7 SHR in intervention group. The SHR in intervention group were administered by oral gavage with atorvastatin (suspension in distilled water, 10 mg/Kg once a day) for 6 weeks, and the other two groups were also administered by gavage with equal quantity distilled water. Atorvastatin was provided by Pfizer Inc. USA. In China. Blood pressure of rats was measured every weeks using a standard tail cuff sphygmomanometer (BP-2010A System, Softron), and mean arterial pressure (MAP) was calculated by (systolic pressure + 2* diastolic pressure)/3. The body weights of each rat were recorded once a week. After 6 weeks, the rats were anesthetized using sodium pentobarbital (40 mg/kg, i.p.) to remove the heart. After rinsing with cold saline, atrium and right ventricular free wall were cut along the atrioventricular ring. Then the remaining septal and left ventricular free wall were dried with filter paper which were weighed as left ventricular mass.

Ecocardiographic analysis

After intervention with 6 weeks, ecocardiographic analysis was taken to detect the hypertrophic degree of heart with rats in left lateral decubitus position. Rats were anesthetized with Isoflurane (2.5% isoflurane for induction and 0.5% for maintenance). Echocardiographic measurements were performed using a commercially available Doppler echocardiograph (Vivid E9, GE Healthcare, USA) with a 4-12 MHz MicroScan transducer (S12-4). Heart rate and left ventricular (LV) dimensions, including interventricular septal thickness (IVSd), posterior wall thickness (PWTd), LV end-diastolic (LVdD) and end-systolic chamber dimensions (LVdS) were measured from short-axis views of LV under M-mode tracings at or just below the tip of the mitral valve leaflets. All M-mode tracings were obtained at 100 mm/s. The Teichholz equations [17] was used to determine the LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV): $LVEDV = LVdD^3 \times 7.0 / (2.4 + LVdD)$, $LVESV = LVdS^3 \times 7.0 / (2.4 + LVdS)$. The LV ejection fraction (EF) was calculated as $[(LVEDV - LVESV) / LVEDV] \times 100 \%$ and LV fractional shortening (FS) was calculated as $[(LVdD - LVdS) / LVdD] \times 100 \%$.

Histopathology staining and Immunohistochemistry

After anaesthesia, animal hearts were dissected, thoroughly washed with cold saline, and fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. After extensive washes, heart samples were dehydrated to be embedded into paraffin wax. Paraffin-embedded slides (5 µm thickness) were stained with Masson's trichrome (Solarbio, China) for interstitial fibrosis determination. The interstitial fibrosis fraction was calculated as area of Masson's trichrome-stained connective tissue divided by total area of the image using Image J 1.47 software (NIH, USA).

The commercial immunohistochemistry kit (MXB biotechnologies, China) was used in this study. Tissue sections were deparaffinized and exposed to 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. For heat-induced epitope retrieval, the sections were placed in a 0.01 mol/L citrate buffer and heated at 120 °C for 15 min. The nonspecific binding was blocked by preincubation with 5% normal goat serum in phosphate-buffered saline (blocking buffer) for 60 min at room temperature. Individual slides were then incubated overnight at 4 °C with an anti-C/EBPβ (CCAAT/enhancer-binding protein β) antibody (diluted 1:100; Abcam, USA), UCP3 (Uncoupling protein) (diluted 1:100; Proteintech, China) at a final concentration of 2 µg/mL in the blocking buffer. The slides were washed with phosphate-buffered saline and then incubated with a peroxidase-labeled polymer conjugated to goat anti-rabbit immunoglobulin G (MXB biotechnologies, China) for 45min at room temperature. After extensive washing with phosphate-buffered saline, the color reaction was developed using 2% 3, 3'-diaminobenzidine in 50mmol/L Tris buffer (pH 7.6) containing 0.3% hydrogen peroxide for 5-10 min. The sections were counterstained with Meyer's hematoxylin, dehydrated and mounted. Images were visualized and captured using a microscope (Leica, Germany). The mean integrated optical density (IOD) of each image was analyzed by Image Pro Plus software (Media Cybernetics, USA).

Tunnel analysis of apoptosis

Cardiomyocyte apoptosis was assessed by the TUNEL assay (KeyGEN BioTECH, China) as described in previous study [18]. In brief, nuclear DNA strand breaks were end-labeled with digoxigenin-conjugated dideoxy-UTP by terminal transferase and visualized immunohistochemically with digoxigenin antibody conjugated to alkaline phosphatase. The assay was standardized using the control sections treated with DNase I to induce DNA fragmentation as a positive control of apoptosis. Cardiomyocytes with a dark brown-

stained nucleus were identified as positive. Images were captured using a microscope (Leica, Germany). The percentage of TUNEL-positive cardiomyocytes was calculated in a transverse left-ventricle tissue section by IPP software (Media Cybernetics, USA). There were at least 10 Figures from each rat.

Western blot analysis

Myocardial tissue of rats were lysed in a cold radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, China) according to standard protocols, and the protein concentrations of lysates were determined using the Pierce BCA Protein Assay Kit (Beyotime Biotechnology). Equal quantities of proteins were separated by 8-15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The proteins in the gels were transferred to polyvinylidene difluoride (PVDF) membranes, blocked, and then incubated with the following primary antibodies overnight at 4°C: C/EBP β (Abcam, USA), PGC-1 α , BNP and ANP (Santa Cruz, USA), UCP3, Bax, Bcl-2 and α -tubulin (Proteintech, China). The positive signals were detected after incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies (Proteintech) for 2 hours at room temperature. Protein bands were detected using the enhanced chemiluminescence (ECL) western blotting substrate (Thermo Scientific, USA), and the intensity was quantified using Image J 1.47 software (NIH, USA).

Statistical Analysis

The experimental results were expressed as mean $\pm$ SD and analyzed by Student's t-test or one-way ANOVA with post hoc comparisons using the LSD test. All statistical analyses were performed using SPSS 22.0 statistical software (SPSS, Inc, Chicago, USA). $P < 0.05$ was considered to be statistically significant.

Results

Atorvastatin attenuated ventricular hypertrophy and remodeling in SHR

At an age of 16 weeks, the mean arterial pressure (MAP) of the rats in the three groups was 103.6 ± 6.1 , 151.8 ± 12.5 , and 159.1 ± 6.2 mmHg, respectively (Fig. 1F). There was no statistically significant difference between the SHR and intervention groups. There was also no significant difference in body weight between the three groups (Fig. 1D). After the removal of the atrium and right ventricle, the weight of the left ventricle (LV) was recorded. We found that the ratio of the LV weight to the body weight was significantly higher in the SHR group than in the control group, and the difference between the respective values could be reduced by atorvastatin (Fig. 1E). Furthermore, staining with Masson's trichrome demonstrated that the increased interstitial fibrosis of the LV and ventricular remodeling in the SHR group were attenuated by atorvastatin treatment (Fig. 1A). The blue fibrotic filaments were more prominent and covered a greater area in the SHR group.

In addition, an echocardiographic examination of each rat was performed at 16 weeks. In comparison with rats in the control group, water-treated SHR exhibited an increase in LV wall thickness, including the IVSd and PWTd (Fig. 2). SHR after atorvastatin treatment for 6 weeks exhibited an LV wall thickness that was obviously lower than that of water-treated SHR. However, indicators of cardiac function, including the FS and EF, did not display significant differences between the three groups, which implied that hearts with myocardial hypertrophy and remodeling were still in the compensatory stage.

Atorvastatin reduced the expression of C/EBP β and proteins related to myocardial hypertrophy

As indicators of pathological cardiac hypertrophy, the protein expressions of atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) were detected in this study (Fig. 3C). It was shown that the protein expressions of ANP and BNP increased significantly in water-treated SHR but could also be reduced by atorvastatin treatment. From the immunohistochemical analysis (Fig. 3A), the expression of C/EBP β increased in the myocardium of SHR, in which the gray color of not only the cytoplasm but also the nucleus was darker in comparison with WKY rats. This showed that C/EBP β was mainly expressed and functioning

Fig. 1. Atorvastatin attenuated ventricular remodeling in SHR. (A) Masson's trichrome was used for interstitial fibrosis determination. (B) The interstitial fibrosis fraction was calculated as area of Masson's trichrome-stained connective tissue divided by total area of the image using Image J 1.47 software. (C) The appearance of the whole hearts from WKY rats, water-treated SHRs and atorvastatin-treated SHRs. (D) The mean body weights in three groups at 16 weeks of age. (E) After removing the atrium and right ventricle, the weight of

left ventricle was recorded. The Fig. showed the ratio of LVM to the body weight. (F) The systolic blood pressure and diastolic blood pressure were measured by tail cuff sphygmomanometer during the 6 weeks. The mean artery pressure was calculated by $(SBP+2*DBP)/3$. (G) Heart rate was recorded while blood pressures were measured. The heart rate at 16 weeks of age was showed in the Fig. . Data are shown as means $\pm$ SD. * $P < 0.05$ vs. control, # $P < 0.05$ vs. SHR group.

in the nucleus. From the optical density analysis, the numbers of dark brown nuclei and the mean integrated optical density (IOD) of the expression of C/EBP β decreased in the myocardium of SHR treated with atorvastatin. Similarly, the western blot analysis also showed that the protein expression of C/EBP β was higher in water-treated SHR than in the other two groups.

Atorvastatin improved mitochondrial function and prevented apoptosis in hypertrophic myocardium

An increase in apoptosis in pathological cardiac hypertrophy was found in SHR in the present study. By the TUNEL assay (Fig. 4), in comparison with WKY rats the percentage of TUNEL-positive cells was higher in SHR, which was accompanied by a decrease in the ratio of the expression of the apoptosis-related protein Bcl-2 to that of Bax. Treatment with atorvastatin for 6 weeks reversed the decrease in the Bcl-2/Bax ratio in SHR and reduced the percentage of TUNEL-positive cells. Furthermore, we detected the expression of some mitochondrion-related proteins in rat myocardial tissue, including peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) and uncoupling protein-3 (UCP3). The immunohistochemical analysis showed that the expression of UCP3 in water-treated SHR was significantly reduced, with a decrease in mean IOD. Equally, the protein expression levels of PGC-1 α and UCP3 were also shown by the western blot analysis to be lower in water-treated SHR than in the control group. However, the decreases in the expression of PGC-1 α and UCP3 could be inhibited by treatment with atorvastatin in SHR.

Fig. 2. Atorvastatin attenuated left ventricular hypertrophy in SHR. (A) Examples of M-mode echocardiographic recordings for WKY, SHR and atorvastatin-treated SHRs at 16 weeks age. (B, C) Interventricular septal thickness (IVSd) and posterior wall thickness (PWTd) were measured from short-axis views of LV under M-mode tracings. (D, E) LV end-diastolic (LVDD) and end-systolic chamber dimensions (LVDS) were also measured under M-mode tracings at or just below the tip of the mitral valve leaflets. Functional parameters such as percentage of fractional shortening (FS, %) and ejection fraction (EF, %) were calculated by LVDD and LVDS. Data are shown as means $\pm$ SD. *P<0.05 vs. control, #P<0.05 vs. SHR group.

Fig. 3. Atorvastatin reduced the C/EBP β increasing in SHR. (A, B) The expression of C/EBP β in rats was detected by Immunohistochemistry method, and the mean integrated optical density of C/EBP β was analyzed by Image Pro Plus software (C) The hypertrophic marker BNP and ANP, and C/EBP β protein expression were detected by western blot. (D, E) The quantitative analysis for protein level expression of BNP, ANP and C/EBP β . Data are shown as means $\pm$ SD. *P<0.05 vs. control, #P<0.05 vs. SHR group.

Discussion

At the age of 16 weeks, in comparison with age-matched WKY rats SHR exhibited significant myocardial hypertrophy and remodeling with normal heart function. Although it

Fig. 4. Atorvastatin rescued the function of mitochondrion. (A) The TUNEL assay was used to assess the apoptosis of myocardium of rats. The positive control of apoptosis was induced by DNase I. And nucleus with dark brown-stained were identified as positive. (B) The quantitative analysis of TUNEL positive cells. (C, D) Western blot analysis of Bcl-2 and Bax expression. The Bcl-2/Bax ratio was calculated and compared. (E, F) The expression of UCP3 in rats was detected by Immunohistochemistry method, and the mean integrated optical density of UCP3 was analyzed by Image Pro Plus software. (G, H) Western blot analysis of PGC-1α and UCP3 expression and the corresponding quantitative analysis. Data are shown as means $\pm$ SD. * $P < 0.05$ vs. control, # $P < 0.05$ vs. SHR group.

was difficult to compare the differences between, as well as the volumes of, isolated hearts from the three groups on the basis of their gross appearance (Fig. 1C). Given the results of the echocardiographic examination and staining with Masson's trichrome, treatment with atorvastatin could attenuate myocardial hypertrophy and remodeling in SHR. Accompanied by decreases in the expressions of indicators of pathological cardiac hypertrophy (ANP and BNP), the expression of C/EBP β , which increased significantly in the myocardium of water-treated SHR, was also reduced after treatment with atorvastatin for 6 weeks. C/EBP β is a member of a family of basic leucine zipper transcription factors (CCAAT/enhancer-binding proteins) [19]. Previous studies demonstrated that C/EBP β is a central indicator in both physiological and pathological hypertrophy [20, 21]. In addition, it was reported that a pivotal role of C/EBP β in inflammation and metabolism had been identified [22]. Our previous study also found that the overexpression of C/EBP β influenced mitochondrial function by reducing the MMP and prevented the beneficial effects of atorvastatin [16]. Thus, we considered that atorvastatin could inhibit cardiac hypertrophy and improve mitochondrial function via the downregulation of C/EBP β .

In fact, we found that the expression of C/EBP β was markedly increased in the myocardium of SHR, in particular in cell nuclei. Moreover, the results of the TUNEL assay indicated that the numbers of apoptotic cells in SHR were higher than in the control group. The ratio of mitochondrial apoptosis-related proteins (Bcl-2/Bax) was also reduced in hypertrophic myocardium. However, treatment with atorvastatin for 6 weeks significantly inhibited apoptosis in hypertrophic myocardium. Myocardial apoptosis is one of the characteristics of pathological myocardial hypertrophy and contributes to the transition from compensatory hypertrophy to heart failure [23, 24]. Thus, the protective effect of atorvastatin on hypertrophic myocardium might be at least partly attributed to the inhibition of apoptosis [25]. Although there was no sign of heart failure at the age of 16 weeks in SHR in the present study, the inhibition of apoptosis by atorvastatin also had an important protective effect against the further progress of pathological myocardial hypertrophy.

As mentioned previously, we speculated that the impairment of mitochondrial function is strongly associated with myocardial apoptosis. Therefore, we detected some additional proteins related to mitochondrial metabolism. In accordance with a previous *in vitro* study [16], the levels of an important regulator of mitochondrial biogenesis and energy metabolism [26], namely, PGC-1 α , also exhibited reductions in hypertrophic myocardium in SHR, which were reversed by atorvastatin. In addition, treatment with atorvastatin prevented reductions in levels of UCP3 in hypertrophic myocardium in SHR. As well as UCP2, UCP3 is a major isoform of UCP in the heart and uncouples respiration from the synthesis of ATP by providing an alternative route for protons to enter the mitochondrial matrix [27]. Besides thermogenesis and the regulation of ATP synthesis, there are two other primary hypotheses regarding the physiological roles of UCP3: the regulation of fatty acid oxidation and the reduction of the mitochondrial generation of ROS [28]. And previous study showed that myocardial hypertrophy was accompanied by increasing of ROS production [29, 30]. The downregulation of UCP3 has previously been reported in hypertrophic myocardium and heart failure [31, 32]. It was shown that a decrease in the expression of UCP3 contributed to the production of ROS and cell death [32]. PGC-1 α has been suggested to regulate the expression of UCP3 [33]. Our previous study also found that the overexpression of C/EBP β reduced the expression of PGC-1 α in H9c2 cardiomyocytes [16]. Thus, atorvastatin possibly attenuated myocardial hypertrophy and inhibited myocardial apoptosis via the C/EBP β /PGC-1 α /UCP3 signaling pathway, which could regulate fatty acid oxidation and the generation of ROS.

Conclusion

This study further confirmed that atorvastatin could attenuate myocardial hypertrophy and remodeling in SHR by inhibiting apoptosis and reversing changes in mitochondrial metabolism. The C/EBP β /PGC-1 α /UCP3 signaling pathway might also be important for elucidating the beneficial pleiotropic effects of atorvastatin on myocardial hypertrophy. Each factor in this pathway promises to be a novel target for the prevention and treatment of pathological myocardial hypertrophy.

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Disclosure Statement

The authors declare no conflict of interest.

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