Cardiac Pharmacology

Atorvastatin, Valsartan, and N-Acetylcysteine Prevent Cardiac Hypertrophy and Overexpression of Myocardin in Pressure-Overloaded Rat Heart

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Background: The use of statin has emerged as a beneficial treatment for cardiac hypertrophy. Myocardin, a cardiac restricted gene, is up-regulated in the hypertrophic myocardium. However, the effect of statin on myocardin expression due to pressure overloaded cardiac hypertrophy is not well understood.

Methods: To evaluate the effect of statin on myocardin expression and cardiac hypertrophy, infrarenal aortic banding was performed for 4 weeks in adult Sprague-Dawley rats to induce cardiac hypertrophy. Atorvastatin (30 mg/kg), valsartan (30 mg/kg), N-acetylcysteine (NAC; 250 mg/kg), and the above three agents combined were given daily after surgery.

Results: After aortic banding for 4 weeks, the heart weight, the ratio of heart/body weight, and mean arterial pressure (MAP) increased significantly. However, echocardiography showed concentric hypertrophy after aortic banding. Elevated MAP and increased wall thickness and heart weight were reversed after treatment with atorvastatin, valsartan, NAC, and a combination of the three agents. Myocardin, myosin heavy chain (MHC), brain natriuretic peptide (BNP) proteins, myocardin mRNA, angiotensin II (AngII), and superoxide dismutase (SOD) expressions were up-regulated in the banding group, which were inhibited by atorvastatin, valsartan, NAC, and the three agents combined. Increased immunohistochemical labelings of myocardin and MHC in the ventricular myocardium were observed in the banding group, and again atorvastatin, valsartan, or NAC reversed the labelings.

Conclusion: Myocardin, MHC, BNP proteins, myocardin mRNA, AngII, and SOD expressions were up-regulated in the rat model of pressure-overloaded cardiac hypertrophy. Treatment with atorvastatin, valsartan, NAC, or three agents put together is associated with a reversal of abnormal regulation of myocardin in the hypertrophic myocardium.

Key Words: Aortic banding • Cardiac hypertrophy • Myocardin • Pressure overload • Statin

INTRODUCTION

Adult cardiomyocytes retain the ability to adapt to mechanical, hemodynamic, hormonal, and pathologic stimuli by hypertrophic growth.1,3 However, cardiac hypertrophy after sustained stress stimuli may finally lead to heart failure, which contributes to a major cause of morbidity and mortality in modern society.1,2 Cardiac hypertrophy as a response to different stress signals was thought to be conducted by way of certain fetal cardiac...
genes. Myocardin, a recently discovered cardiac restricted gene, has potent effects on the cardiovascular system, and has been proven in prior studies to be up-regulated to result in cardiomyocyte hypertrophy after stress signals. Different pharmacological agents are thought to be effective in preventing cardiac hypertrophy, including statin, angiotensin II (AngII) type 1 (AT1) receptor blocker (ARB), and beta-blocker. Statin, a drug with both pleiotropic and lipid-lowering effects, has been documented to be effective to prevent cardiac hypertrophy through several different mechanisms. However, the effect of statin on myocardin in pressure-overloaded rat myocardium was not well-known until now. The primary focus of our investigation is to study the effects of atorvastatin on myocardin expression and cardiac hypertrophy in the pressure-overloaded rat heart.

MATERIALS AND METHODS

Rat model of abdominal aortic constriction

On the day of surgery, adult Sprague-Dawley rats (250-300 g) were anesthetized with pentobarbital sodium (80 mg/kg), and then the aorta was exposed via abdominal midline incision. Rats were randomly divided into four groups: (1) sham operated; (2) sham-operated and treated with atorvastatin; (3) pressure-overloaded (aortic banding); and (4) pressured-overloaded and treated with atorvastatin. A polyethylene catheter (PE10) was placed on the surface of the abdominal aorta distal to the renal arteries. Then, the catheter and aorta were tightly constricted with a 6-0 silk suture. Sham-operated control animals were prepared in the same manner, except that the aorta was not constricted. After the procedure, the catheter was fully withdrawn, the abdominal wound was sutured, and the rats were given an opportunity to recover. Aortic banding was performed in the pressure overloaded group for 3 days, 7 days, 2 weeks, and 4 weeks, respectively. In the treatment group, the rats were administered atorvastatin after surgery in doses of 10, 30, or 50 mg/kg body weight per day in drinking water. The perioperative mortality rate in the aortic banding group was around 10%. In addition to atorvastatin, valsartan (an ARB) was administered at 30 mg/kg body weight per day, N-acetyl-cysteine (NAC; an antioxidant) was administered at 250 mg/kg body weight per day, and the above three agents combined was also given to different banding rats.

All animal procedures were performed in accordance with institutional guidelines, and in conformity with the Guide for the Care and Use of Laboratory Animals as published by the United States National Institutes of Health.

Hemodynamic monitor

Four weeks after the surgery, the rats were anesthetized with pentobarbital sodium (80 mg/kg), and their carotid arteries were cannulated with polyethylene catheters to measure mean arterial pressure (MAP). Heart rate was measured through a Grass model tachograph preamplifier. After hemodynamic data were measured, the rats were sacrificed for heart removal, to perform the following experiments. Each hemodynamic data was presented as a mean from six measurements.

Assessment of cardiac hypertrophy and function

Cardiac function of rats with aortic banding was evaluated noninvasively by echocardiography performed with an Acuson Sequoia 512 machine using a 15-MHz probe on the day of aortic banding, and 4 weeks after surgery. Left ventricular percent fractional shortening, left ventricular end-diastolic dimension, left ventricular end-systolic dimension, interventricular septum thickness, and left ventricular posterior wall thickness were calculated. The sonographer was blinded to the randomization of rats. The following experiments were performed by a technician blinded to the design of the study.

Western blot analysis

Tissue samples from left ventricle were homogenized in modified RIPA buffer (50 mmol/l tris [pH 7.4], 1% IGEPAL CA-630 (Sigma), 0.25% sodium deoxycholate, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, and 1 lg/ml aprotinin, leupetin, and pepstatin). Nuclear and cytosolic protein samples were mixed with sample buffer, boiled for 10 min, separated by SDS-PAGE under denaturing conditions, and electroblotted to nitrocellulose membranes. The nitrocellulose membranes were blocked by incuba-
tion in blocking buffer, incubated with anti-myocardin, anti-BNP (B type natriuretic protein), or anti-MHC (myosin heavy chain) antibody, washed, and incubated with horseradish peroxidase-conjugated secondary antibody. Signals were visualized by chemiluminescent detection.

**RNA isolation and reverse transcription**

Total RNA was isolated from frozen left ventricle using the single-step acid guanidinium thiocyanate/phenol/chloroform extraction method. Total RNA (1 µg) was incubated with 200 U of Moloney Murine Leukemia Virus reverse transcriptase in a buffer containing a final concentration of 50 mmol/l Tris-Cl (pH 8.3), 75 mmol/l KCl, 3 mmol/l MgCl2, 20 U of RNase inhibitor, 1 lmol/l polydT oligomer, and 0.5 mmol/l of each dNTP in a final volume of 20 µl. The reaction mixture was incubated at 42 °C for 1 h and then at 94 °C for 5 min to inactivate the enzyme. A total of 80 µl of diethyl pyrocarbonate treated water was added to the reaction mixture before storage at -70 °C.

**Real-time PCR**

A Lightcycler (Roche Diagnostics, Mannheim, Germany) was used for real-time PCR. cDNA was diluted 1 in 10 with nuclease-free water. 2 µl of the solution was used for the Lightcycler SYBR-Green mastermix (Roche Diagnostics): 0.5 lmol/l primer, 5 mmol/l magnesium chloride, and 2 µl Master SYBR-Green in nuclease-free water in a final volume of 20 µl. The primers used for myocardin, MHC, and BNP were: forward: 5'-GGACTGCTCTGGCAACCCAGTGC-3'; reverse: 5'-CATCTGCTGAC-TCCGGGTCATTTGC-3', forward: 5'-GGACTGCTCTGGCA ACCCAGTGC-3'; reverse: 5'-CATCTGCTGACTCCGGGTCATTTGC-3', and forward: 5'-CTCAAAGGACCAAGGC-3'; reverse: 5'-GTCGGTAAGGTAGAGGC-3'. GAPDH gene expression was used as internal controls (forward: 5'GAGAGGCTCTCTGTGACTAC-3'; reverse: 5'TAGTGTAGGTTGGGCCTCAA-3'). The initial denaturation phase for myocardin was 5 min at 95 °C followed by an amplification phase as detailed below: denaturation at 95 °C for 10 s; annealing at 57 °C for 10 s; elongation at 72 °C for 15 s; detection at 80 °C and for 36 cycles. The amplification phase for rat BNP was denaturation at 95 °C for 5 s; annealing at 60 °C for 3 s; elongation at 72 °C for 5 s; detection at 72 °C and for 40 cycles. Amplification, fluorescence detection, and post-processing calculation were performed using the Lightcycler apparatus.

Individual polymerase chain reaction (PCR) product was analyzed for DNA sequence to confirm the purity of the product. The lengths of the PCR products for myocardin, MHC, BNP, and GAPDH were 421, 436, 294, and 405 bp, respectively.

**Immunohistochemistry**

Slides were dried overnight at room temperature. Snap-frozen sections were postfixed in 4% paraformaldehyde for 20 min, treated in 3% hydrogen peroxide/PBS for 25 min, blocked in 5% normal rabbit serum for 15 min, and incubated with the following: primary antibody (anti-myocardin, and anti-MHC) for 2 h at room temperature, biotinylated rabbit-anti mouse IgG at 1:400 for 30 min, and Vector Elite ABC biotin-avidin-peroxidase complex for 30 min; sections were then developed with diaminobenzidine and diaminobenzidine enhancer (Vector), counterstained with hematoxylin, and mounted.

**Enzyme-linked immunosorbent assay (ELISA) for AngII and superoxide dismutase (SOD)**

AngII (nM) and SOD (unit/ml) activities were measured by cell lysates and the culture medium by a quantitative, competitive ELISA, using a specific anti-AngII (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) and anti-SOD antibodies (Cayman Chemical Company, Ann Arbor, MI, USA), as previously described.3

**Statistical analysis**

All results were expressed as mean ± standard deviation. Statistical significance was evaluated using analysis of variance followed by Tukey-Kramer multiple comparisons test (GraphPad Software Inc., San Diego, CA, USA). A value of p < 0.05 was considered to denote statistical significance.
RESULTS

Myocardin, MHC, and BNP proteins expression increases after aortic banding

Western blot for myocardin, MHC, and BNP were performed after induction of aortic banding for 3 days to 4 weeks. Myocardin, MHC, and BNP protein expression increased and was noted to have the most evident effect after aortic banding for 4 weeks (Figure 1). Consequently, aortic banding for 4 weeks was selected to conduct the following experiments.

Myocardin mRNA expression increases after aortic banding

Real-time PCR for myocardin mRNA was performed after induction of aortic banding for 3 days to 4 weeks. Myocardin mRNA expression increased and was noted to have the most evident effect after aortic banding for 4 weeks (Figure 2).

Atorvastatin treatment inhibits myocardin expression after aortic banding

The effect of atorvastatin on myocardin expression after aortic banding was evaluated with different concentrations of atorvastatin (10, 30, and 50 mg/kg) administered daily for 4 weeks. Treatment with atorvastatin at 10 mg/kg/day did not block the increased expression of myocardin protein induced by aortic banding. Treatment with atorvastatin at 30 and 50 mg/kg/day in the banding group significantly blocked the increased expression of myocardin protein (from 3.9 ± 0.2-fold to 2.0 ± 0.2-fold; p < 0.05, n = 6) induced by aortic banding. Atorvastatin at either 30 or 50 mg/kg has an evident and similar inhibitory effect on myocardin expression and the dosage of 30 mg/kg body weight was selected in the following experiments according to the dose response effect (Figure 3).

Hemodynamic and echocardiographic changes after banding and treatment with atorvastatin, valsartan, NAC, or the 3 agents combined

The mean of baseline body weight was 289 gm. The mean of baseline thickness of interventricular septum and left ventricular posterior wall was 1.2 mm and 1.2 mm, respectively. The baseline body weight and echocardiographic parameters were similar in each group (n = 42 for all groups). As shown in Tables 1 and 2, heart rate increased significantly after aortic banding for 4 weeks from 325 ± 24 bpm in the sham group to 356 ± 18 bpm (p < 0.05) in the banding group. MAP increased

![Figure 1. Effect of aortic banding on myocardin protein expression. (A) Representative Western blot for myocardin, myosin heavy chain (MHC), and brain natriuretic peptide (BNP) after induction of aortic banding for 3 days to 4 weeks. (B) Quantitative analysis of protein expression. Myocardin, MHC, and BNP proteins were noted to have the most evident effects after aortic banding for 4 weeks (n = 6). * p < 0.05 vs. Sham group. # p < 0.01 vs. Sham group.](image1)

![Figure 2. Effect of aortic banding on myocardin messenger RNA expression. Quantitative analysis of myocardin mRNA expression after induction of aortic banding for 3 days to 4 weeks. Myocardin mRNA was noted to have the most evident effect after aortic banding for 4 weeks (n = 6). * p < 0.05 vs. Sham group. # p < 0.01 vs. Sham group.](image2)
from 81 ± 9 mmHg in the sham group to 94 ± 6 mmHg (p < 0.01) in the banding group. Echocardiography showed that the thickness of interventricular septum and left ventricular posterior wall also increased significantly after aortic banding (p < 0.01; Table 1). Heart weight and the ratio of heart weight to body weight increased significantly after aortic banding (p < 0.01), which was reversed after treatment with atorvastatin, valsartan, NAC, or the 3 agents put together (p < 0.05; Tables 1 and 2). MAP also decreased after treatment with atorvastatin, valsartan, NAC, or the 3 agents combined (p < 0.05; Tables 1 and 2). The increased wall thickness and chamber size of left ventricle after banding were reversed by atorvastatin, valsartan, NAC, and the 3 agents put together (p < 0.05; Tables 1 and 2).

**Western blot analysis after aortic banding and treatment with atorvastatin, valsartan, NAC, and 3 agents put together**

To evaluate the effect of aortic banding and statin on myocardin protein expression, western blotting was performed (Figures 4 and 5). The myocardin protein increased 3.96-fold at 4 weeks of induction of aortic banding, as compared to the sham group (p < 0.001). MHC and BNP proteins also increased 4.0-fold at 4 weeks of induction of aortic banding as compared to the sham group (Figures 4 and 5). Treatment with atorvastatin in the sham group did not affect the protein expression of myocardin, MHC, and BNP (Figure 4). Treatment with atorvastatin, valsartan, NAC, and the above 3 agents put together significantly blocked the increase of myocardin, MHC, and BNP proteins expression induced by aortic banding.

**Myocardin mRNA expression increases after aortic banding and is reversed after treatment with atorvastatin**

We used real-time PCR to evaluate the effect of aortic banding and atorvastatin on myocardin mRNA expression. The myocardin mRNA increased 4.55-fold at 4 weeks of induction of aortic banding, as compared to the sham group (p < 0.001). Treatment with atorvastatin in the sham group did not affect the mRNA expression of myocardin (Figure 4). Treatment with atorvastatin, valsartan, NAC, and the above 3 agents put together significantly blocked the increase of myocardin mRNA expression induced by aortic banding.

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**Figure 3. Effect of atorvastatin on myocardin protein expression induced by aortic banding.** Representative Western blot for myocardin after induction of aortic banding for 4 weeks with treatment with 10, 30, and 50 mg/kg atorvastatin (n = 6). * p < 0.01 vs. Sham group. # p < 0.05 vs. Banding group without atorvastatin.

**Table 1. Hemodynamic and echocardiographic parameters after 3 agents**

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<td>897 ± 53*</td>
<td>757 ± 47*</td>
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<td>LVEDD, mm</td>
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Data are means ± SD. FS, fraction shortening; IVST, interventricular septum thickness; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; LVPWT, left ventricular posterior wall thickness; MAP, mean arterial pressure. * p < 0.05 vs. Sham group. † p < 0.05 vs. Banding group.
Table 2. Hemodynamic and echocardiographic parameters after 3 agents (atorvastatin, valsartan, and NAC) put together

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<td>897 ± 53*</td>
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<tr>
<td>Heart rate, min</td>
<td>325 ± 24</td>
<td>356 ± 18*</td>
<td>338 ± 34</td>
<td>324 ± 27†</td>
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<tr>
<td>MAP, mmHg</td>
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<td>84 ± 6†</td>
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<td>LVPWT, mm</td>
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<td>LVESD, mm</td>
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<td>FS, %</td>
<td>48 ± 6</td>
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</table>

Data are means ± SD. FS, fraction shortening; IVST, interventricular septum thickness; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; LVPWT, left ventricular posterior wall thickness; MAP, mean arterial pressure. * p < 0.05 vs. Sham group. † p < 0.05 vs. Banding group.

Figure 4. Effect of atorvastatin, valsartan, or NAC on myocardin protein expression induced by aortic banding. (A) Representative Western blot for myocardin, MHC, and BNP after induction of aortic banding for 4 weeks with or without treatment with atorvastatin, valsartan, or NAC. (B) Quantitative analysis of protein expression. Myocardin, MHC, and BNP proteins increased after aortic banding for 4 weeks, which was inhibited by atorvastatin, valsartan, or NAC (n = 6). Atorvastatin did not have any effect on Sham group. * p < 0.01 vs. Sham group. # p < 0.05 vs. Banding group.

Figure 5. Effect of three agents put together (atorvastatin, valsartan, and NAC) on myocardin and MHC protein expression induced by aortic banding. (A) Representative Western blot for myocardin and MHC after induction of aortic banding for 4 weeks with or without treatment with three agents put together. (B) Quantitative analysis of protein expression. Myocardin and MHC proteins increased after aortic banding for 4 weeks, which was inhibited by three agents put together (n = 6). Atorvastatin did not have any effect on the Sham group. * p < 0.01 vs. Sham group. # p < 0.05 vs. Banding group.
level. As shown in Figure 6, myocardin mRNA increased 3.98-fold at 4 weeks of induction of aortic banding as compared to the sham group (p < 0.001). Treatment with atorvastatin, valsartan, and NAC in the banding group significantly inhibited the increased expressions of myocardin mRNA induced by aortic banding (p < 0.05). Treatment with atorvastatin in the sham group did not affect the mRNA expression of myocardin.

Increased immunohistochemical labelings of myocardin after aortic banding

Immunohistochemical stain confirmed the previous findings from Western blots and real-time PCR. Increased labelings of myocardin and MHC in the ventricular myocardium were observed after induction of aortic banding for 4 weeks (Figure 7). Treatment with atorvastatin, valsartan, and NAC in the banding group decreased the immunohistochemical labelings of myocardin and MHC.

**Increased AngII expression after aortic banding was inhibited by atorvastatin, valsartan, NAC, and the 3 agents combined**

To evaluate the effect of aortic banding on AngII expression, ELISA was performed (Figure 8). The AngII concentration increased significantly (from 77.1 ± 24.8 to 209.5 ± 29.5 ng/ml) at 4 weeks of induction of aortic banding as compared to the sham group (p < 0.001). Treatment with atorvastatin in the sham group did not affect the expression of AngII (Figure 8). Treatment with atorvastatin, valsartan, NAC, and the above 3 agents put together in the banding group significantly blocked the increased expression of AngII induced by aortic banding (p < 0.05; Figure 8). Treatment with the 3

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**Figure 6.** Effect of atorvastatin, valsartan, or NAC on myocardin mRNA expression induced by aortic banding. Quantitative analysis of myocardin mRNA after induction of aortic banding for 4 weeks with or without treatment with atorvastatin, valsartan, or NAC was performed as follows. The quantification of myocardin expression in the fixed time was performed by real-time PCR using an ABI PRISM 7300 sequence detection system. The relative level of myocardin expression was obtained by calculating the ratio of threshold cycle numbers of the initial exponential amplification phase as determined by the sequence detection system for the myocardin. The values from experiment groups have been normalized to match GAPDH measurement and then expressed as a ratio of normalized values to mRNA in sham group. Myocardin mRNA level increased after aortic banding for 4 weeks, which was inhibited by atorvastatin, valsartan, or NAC (n = 6). Atorvastatin did not have any effect on the Sham group. * p < 0.01 vs. Sham group. # p < 0.05 vs. Banding group.

**Figure 7.** Immunohistochemical staining of left ventricular myocardin after induction of aortic banding for 4 weeks with or without treatment with atorvastatin, Valsartan, or NAC. Myocardin presentation in the nuclei of cardiomyocytes increased after aortic banding (yellow arrow), which was inhibited by atorvastatin, valsartan, or NAC (white arrows; n = 3). DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to A-T rich regions in DNA.
agents put together (full dose; half dose) further inhibited AngII expression compared with using NAC alone (p = 0.018; p = 0.028).

Increased SOD expression after aortic banding was inhibited by atorvastatin, valsartan, NAC, and the 3 agents put together

To evaluate the effect of aortic banding on SOD expression, ELISA was performed (Figure 9). The SOD activity increased significantly (from 3.0 ± 0.4 to 10.5 ± 1.0 unit/ml) at 4 weeks of induction of aortic banding as compared to the sham group (p < 0.001). Treatment with atorvastatin in the sham group did not affect the expression of SOD (Figure 9). Treatment with atorvastatin, valsartan, NAC, and the above 3 agents put together in the banding group significantly blocked the activity of SOD-induced by aortic banding (p < 0.05; Figure 9).

DISCUSSION

In this study, we demonstrated that both myocardin protein and mRNA expressions were up-regulated in a rat model of pressure overload-induced cardiac hypertrophy. The model of cardiac hypertrophy was confirmed by morphological and gene profile study. Blood pressure and heart weight increased after aortic banding. Echocardiography showed concentric hypertrophy of the left ventricle after aortic banding for 4 weeks. The hemodynamic and echocardiographic data that are presented certainly support a pressure overload state. Cardiac MHC and BNP protein expressions were measured to confirm the presence of cardiac hypertrophy. Expressions of MHC and BNP genes are two of the most reliable markers for activation of the hypertrophic program in clinical states and experimental models associated with hypertrophy.2,22 Although expressions of certain genes such as MHC and BNP have been extensively examined in cardiac hypertrophy, much less is known about myocardin.16,23,24 Aortic banding is known to increase concentrations of several humoral factors including AngII, epinephrine, endothelin-1 as well as blood pressure overload.25-28 The presence of circulating humoral factors secondary to pressure overload can trigger the cardiac hypertrophic response and alter gene expression.25-28 Reactive oxygen species also play a key role in pressure overload-induced cardiac hypertrophy, and statin, ARB, and NAC may exert their antioxidant effect to attenuate myocardial hypertrophy as shown in our experiments.27-29 This and other recent studies suggest that myocardin mRNA and protein can be induced under
hypoxia and pressure overload to cause cardiac hypertrophy in rat myocardium.\textsuperscript{3,12} Although increased wall stress and myocardial stretching might also be responsible for this change, humoral factors secondary to mechanical load could also contribute to the upregulation of myocardin mRNA and protein expressions in the model of pressure overload-induced cardiac hypertrophy.\textsuperscript{25-27} In the model of pressure overload, the blood pressure and left ventricular mass increased and cytokines were released due to neurohormonal activation. Accordingly, myocardin was up-regulated in the pressure overload cardiac hypertrophy. Statin-reversed cardiac hypertrophy as in our study may occur through its different pleiotropic effects, in addition to its classic cholesterol-lowering character.\textsuperscript{3,13,15,16,29} Statins have been shown to inhibit cardiomyocyte hypertrophy through the following pathways: 1) inhibition of Rac1 pathway to prevent ROS-related cardiomyocyte hypertrophy through NADPH (Nicotinamide adenine dinucleotide phosphate hydroxylase) oxidase inhibition (anti-oxidant); 2) inhibition of AngII expression by suppressing AT1 receptor, such that the effect may inhibit AngII-related cardiac hypertrophy through different pathways and inhibit Rac1 pathway through its upstream (AT1 receptor-mediated AngII expression); and 3) minor inhibition of the Rho/ROCK pathway to prevent cardiomyocyte hypertrophy.\textsuperscript{13,16,29} In addition to previous reports, we found that atorvastatin can inhibit myocardin expression in the pressure-overloaded model of cardiac hypertrophy. We also found that atorvastatin inhibited AngII and SOD activities after aortic banding through its anti-hypertrophic and antioxidant effects (Figures 8 and 9). Previous reports have proven that overexpression of myocardin may result in cardiac hypertrophy.\textsuperscript{3,11,12} Therefore, we can conclude that statin may prevent cardiac hypertrophy through the inhibition of myocardin expression.

AngII is another major contributor to cardiac hypertrophy when facing different stress stimuli.\textsuperscript{3,13-16} Systemic and cardiac renin-angiotensin systems are known to be activated in a pressure overload setting to result in AngII expression, ROS generation, and cardiomyocyte hypertrophy.\textsuperscript{13-16} This study and previous reports focusing on vascular smooth muscle cell (VSMC) and cardiomyocyte revealed that external stress stimuli may result in VSMC/cardiomyocyte hypertrophy and myocardin expression with earlier expression of Ang II through AT1 receptor.\textsuperscript{3,4,30} According to previous reports, we can hypothesize that ARB can suppress cardiomyocyte hypertrophy and myocardin expression through the inhibition of AT1 receptor and Ang II expression. In our study, we had similar findings as previous studies that valsartan inhibited cardiac hypertrophy and myocardin protein expression in rat pressure-overloaded myocardium.\textsuperscript{3,29,30} We also found that valsartan could effectively inhibit AngII and SOD after aortic banding through its anti-hypertrophic and antioxidant effects. We also found that both ARB and statin have inhibitory effects on myocardin and cardiac hypertrophy.

In our experiments, we also used ROS scavenger (NAC) to detect its effect on cardiomyocyte hypertrophy and myocardin expression. We found that NAC could inhibit both myocardin expression and cardiomyocyte hypertrophy in pressure-overloaded rat myocardium. So ROS generation after stress stimuli to cardiomyocytes may be a major contributor to myocardin expression and cardiomyocyte hypertrophy. Although NAC did not have an anti-hypertensive effect in clinical experiences, treatment with these agents did decrease blood pressure, heart rate, and heart weight/size in our experiments. This finding may have resulted from the antioxidant and anti-hypertrophic effects on ROS, myocardin, and cardiomyocytes.

In our study, we found that statin, ARB, and NAC had evident inhibitory effects on myocardin expression and cardiomyocyte hypertrophy. The effects may be either from the distinct character of each agent or from the crosstalk and synergetic value of actions among these three agents. For example, statin, ARB, and NAC have evident antioxidant effects on ROS. The anti-hypertrophic effect of statin is thought to have partially resulted from its inhibition to AT1 receptor and AngII. Both statin and ARB have inhibitory effects on myocardin and AngII expressions according to current and previous reports.\textsuperscript{3} The use of antioxidant may also inhibit ROS itself and AngII-related ROS generation and cardiac hypertrophy. The above hypothesis may be reflected in the results of our experiments with the combination of the three agents. We found that treatment with 3 agents put together (full dose; half dose) further inhibited AngII expression when compared with using...
NAC alone ($p = 0.018; p = 0.028$). But the inhibitory effect of 3 agents combined on AngII did not differ significantly from that of atorvastatin or valsartan used alone. This means that statin and/or ARB may have their further anti-hypertrophic effects in addition to an NAC-related antioxidant effect. We also found that the inhibitory effect of 3 agents put together on SOD did not differ significantly from that of atorvastatin, valsartan, or NAC used alone. This means that statin, ARB, and NAC may exert their antioxidant effect through similar pathways (ex: Rac1 pathway).

So statin, ARB, NAC, or a combination of these three agents should be considered for use in clinical settings to prevent oxidative stress, hypertension, cardiac hypertrophy, heart failure, or cardiac morbidity and mortality. The doses of atorvastatin, ARB, and NAC used in this paper were according to our experiments and previously published papers.20,21 If we applied these doses to human subjects, the doses will likely be quite high. Our explanation is that rodents and humans are different species of animals. The doses which are effective in mice may be unsuitable or toxic in human beings. So the effective doses of statin, ARB, and NAC to achieve their anti-hypertrophic or anti-oxidant effect should be studied and adjusted in human subjects.

Cardiac hypertrophy can be induced in many hypertensive animal models. The classic animal models include stroke-prone spontaneously hypertensive rats fed with a high-salt diet, uninephrectomized rats receiving deoxycorticosterone acetate and a high-salt diet, renovascular hypertensive rats, and rats infused with AngII or norepinephrine.15,19,21 The mechanisms of cardiac hypertrophy induced by these models may not be similar to that of our aortic banding model. It needs further study to investigate whether the effect of statin applies to these hypertensive animal models.

CONCLUSION

In conclusion, myocardin, MHC, and BNP proteins and myocardin mRNA expressions were up-regulated in the rat model of pressure-overloaded cardiac hypertrophy. Treatment with atorvastatin, valsartan, or NAC is associated with a reversal of abnormal regulation of myocardin in the hypertrophic myocardium.

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