Original Research

Amlodipine and atorvastatin improved hypertensive cardiac hypertrophy through regulation of receptor activator of nuclear factor kappa B ligand/receptor activator of nuclear factor kappa B/osteoprotegerin system in spontaneous hypertension rats

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Abstract

The present study aims to study the role of receptor activator of nuclear factor kappa B ligand/receptor activator of nuclear factor kappa B/osteoprotegerin (RANKL/RANK/OPG) system in cardiac hypertrophy in a spontaneous hypertension rat (SHR) model and the effects of amlodipine and atorvastatin intervention. Thirty-six-week-old male SHRs were randomly divided into four groups: 1) SHR control group; 2) amlodipine alone (10 mg/kg/d) group, 3) atorvastatin alone (10 mg/kg/d) group, 4) combination of amlodinpine and atorvastatin (10 mg/kg/d for each) group. Same gender, weight, and age of Wistar-Kyoto (WKY) rats with normal blood pressure were used as normal control. Drugs were administered by oral gavage over 12 weeks. The thicknesses of left ventricle walls, left ventricle weight, and cardiac function were measured by transthoracic echocardiography. Left ventricular pressure and function were assessed by hemodynamic examination. Cardiomyocyte hypertrophy and collagen accumulation in cardiac tissue were measured by hematoxylin and eosin (HE) and Masson staining, respectively. The hydroxyproline content of cardiac tissue was examined by biochemistry technique. RANKL, RANK and OPG mRNA, protein expression and tissue localization were studied by RT-PCR, Immunohistochemistry and Western blot. Treatment with amlodipine or atorvastatin alone significantly decreased left ventricular mass index, cardiomyocyte cross-sectional area and interstitial fibrosis in SHR (each $P < 0.05$). Moreover, combined amlodipine and atorvastatin treatment induced significant reversal of left ventricular hypertrophy and decreased cardiomyocyte cross-sectional area and interstitial fibrosis in SHR to a greater extent than each agent alone ($P < 0.05$). Compared with WKY rats, the myocardial expression of RANKL, RANK, and OPG was increased. Both amlodipine and atorvastatin reduced RANKL, RANK, and OPG expression, with the best effects seen with the combination. Based on our results, activation of the RANKL/RANK/OPG system may be an important factor leading to ventricular remodeling in SHR rats. Amlodipine and atorvastatin could improve ventricular remodeling in SHR rats through intervention with the RANKL/RANK/OPG system.

Keywords: Receptor activator of nuclear factor kappa B, receptor activator of nuclear factor kappa B ligand, osteoprotegerin, amlodipine, atorvastatin, cardiac hypertrophy, spontaneously hypertension

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Introduction

Receptor activator of nuclear factor kappa B ligand (RANKL) is a member of the tumor necrosis factor superfamily ligands discovered in 1997. Earlier studies have shown that RANKL, via binding to its receptor, receptor activator of nuclear factor kappa B (RANK), was involved in the regulation of genesis of osteoclasts and antigen presentation by dendritic cells. Osteoprotegerin (OPG), as a decoy receptor for RANKL, can weaken or neutralize the biological effects of the latter by binding to it, is a negative regulator of RANKL/RANK signaling pathway. Recent studies have found that, RANKL/RANK/OPG system was not only involved in bone formation, but also closely related to the embryonic heart development, ventricular remodeling after myocardial infarction (MI), heart failure, immune-inflammatory cardiomyopathy among others, and is a group of cytokines with multiple functions.[1–6]

As a member of the tumor necrosis factor superfamily, whether RANKL/RANK/OPG system is involved in the process of left ventricular hypertrophy is not clear. Clinical data showed that the plasma OPG level in hypertension patients is higher than the general population[7,8];

for heart failure patients undergoing aortic valve replacement surgery, preoperative serum OPG levels were significantly higher than normal, and serum OPG levels decreased significantly after valve replacement surgery[9]; in multiple sclerosis patients, hypertension and left ventricular hypertrophy are independent risk factors for elevated levels of plasma OPG[10]; in the general population, higher plasma OPG levels are associated with oversize of the left ventricle and increase of the wall thickness[11]; increased plasma sRANKL level is an independent risk factor for development of cardiovascular disease in the general population in the future.[3] In addition, *in vitro* studies demonstrated that cardiac pro-hypertrophy factor ET-1 may promote the expression of RANKL mRNA in osteoblast,[12] and prorenin can induce the expression of OPG mRNA in cardiomyocytes.[13] In recent years, studies have shown that activation of NF-κB signaling pathway plays an important role in the pathogenesis of hypertensive cardiac hypertrophy, whereas binding of RANKL to its membrane receptor RANK can activate intracellular NF-κB signaling pathway to induce biological effects.[14] Based on these clinical data and laboratory reports, we hypothesized that RANKL/RANK/OPG system may be involved in the process of hypertensive left ventricular remodeling, and it may be a new target for treating cardiac hypertrophy.

In this study, we studied 36-week-old Wistar-Kyoto (WKY) rats and SHR by comparing the RANKL/RANK/OPG system in normal myocardial tissue and hypertrophic myocardial tissue, with the purpose to explore whether the RANKL/RANK/OPG system is involved in the regulation of cardiac hypertrophy and understand whether Amlodipine and atorvastatin improved left ventricular remodeling through the RANKL/RANK/OPG system.

Materials and methods

Materials

Animals. The male WKY rats used in this study were purchased from Chinese Academy of Sciences Shanghai Experimental Animal Center at 16 weeks of age, body weight ($332 \pm s$ 18) g, and clean grade with certificate: SCXK (Shanghai) 2003-0003. The male SHR rats used in this study were purchased from Beijing Wei Tong Lihua Experimental Animal Center at 16 weeks of age, body weight (325 ± 20) g, and clean grade with certificate: SCXK (Beijing) 2007-2001. WKY rats and SHR rats were hosted till 36 weeks old for experiment in clean level environment, with light/dark cycle 12/12 h, a relative humidity of 50–60%, the ambient temperature 22–25°C, 4–5 rats per cage, free access to food and water. All procedures were approved by the Animal Care and Use Committee at Hebei Medical University.

Experimental reagents. Amlodipine and atorvastatin were from Pfizer Inc., USA. Trizol was from Invitrogen Inc., USA. Diethylpyrocarbonate (DEPC) and PCR primers were from Beijing Parkson Gene Technology Ltd, China. dNTP, Moloney murine leukemia virus (MMLV) reverse transcriptase, and random hexamer were from Promega Inc., USA. RNAsin, Taq DNA polymerase, and DNA ladder were from Sino-American Inc, Beijing, China. Goat anti-OPG polyclonal antibody, rabbit anti-RANKL polyclonal antibody, and rabbit anti-RANL polyclonal antibody were from Santa Cruz Inc., USA.

Methods

Group and treatment of the experimental animals. Thirty-six-week-old male SHR rats were randomly divided into four groups: 1) SHR control group: 18 rats; 2) amlodipine alone (10 mg/kg/d) group: 16 rats; 3) atorvastatin alone (10 mg/kg/d) group: 14 rats; 4) combination of amlodinpine and atorvastatin (10 mg/kg/d for each) group: 18 rats. Fourteen WKY rats with normal blood pressure were used as normal control. Drugs were crashed, dissolved in water, and administered by oral gavage over 12 weeks for experimental groups. Same volume of water was administered by oral gavage over 12 weeks for control groups.

Echocardiography. After 12 weeks, the rats were weighed and anesthetized with pentobarbital (40 mg/kg) by intraperitoneal injection. Acuson Sequoia 512 Ultrasound system (Siemens, Germany) was used with probe frequency of 14 MHz, depth of 3.0 cm, and speed of 100–200 mm/s. Left ventricular long axis was taken, left ventricular end-diastolic dimension (LVEDd), left ventricular end-systolic dimension, (LVEDs), left ventricular end-diastolic interventricular septum thickness (IVSd), end-systolic interventricular septum thickness (IVSs), left ventricular end-diastolic posterior wall thickness (LVPWd), and end-systolic posterior wall thickness (LVPWs) were routinely measured. The average of three consecutive cardiac cycles was taken. According to the formula[13]: LV weight (LVW) = $1.04 \times [(\mathrm{IVSd} + \mathrm{LVEDd} + \mathrm{LVPWd})^3 - (\mathrm{LVEDd})^3]$, weight of left ventricle was calculated. Meanwhile, left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were determined to assess the systolic function of the left ventricle. The sample volume was placed between the left ventricular outflow tract and inflow tract, and the time between the end of aorta blood flow to the beginning of mitral blood flow was determined as the isovolumic relaxation time (IVRT), and was used to evaluate the diastolic function of left ventricle.

Measurement of hemodynamic parameters. After echocardiography was completed, the rats were fixed on the operating table in supine position. Neck middle incision was taken and right common carotid artery was separated for about 2 cm. A 2 F pressure conduit (SPR320, Millar company, USA) was inserted into the ventricle through the right common carotid artery, and ventricular pressure waveform was recorded using BioPac MP150 Multi-channel polygraph (BioPac company, USA). Left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), the maximum rise and fall rate of left ventricular pressure change (dP/dt_{max} and dP/dt_{min}), and relaxation time constant (τ) were determined. As per previous literature,[14] because dP/dt_{min} value was affected by LVSP, LVSP was used to correct dP/dt_{min} to get dP/dt_{min}/LVSP.

dP/dt_{max} was used as an index for left ventricular systolic function, LVEDP, dP/dt_{min}/LVSP, and relaxation time constant (τ) were used as indexes for left ventricular diastolic function.

Measurement of left ventricular mass index. After all measurements, the chest of the rat was immediately open and the heart was quickly removed. After rinsing with ice saline, atrium and right ventricular free wall were cut along the atrioventricular ring. After dried with filter paper, the remaining septal and left ventricular free wall were weighed as left ventricular mass. Left ventricular mass index (LVMI) = left ventricular mass/body weight (mg/g). The left ventricle was cut perpendicular to the long axis, LV central section was harvested and fixed in 10% neutral formalin, and the remaining portion of the left ventricle was snap frozen in liquid nitrogen and then stored in −80°C refrigerator.

Pathological study of cardiac hypertrophy

HE staining. Five microliters of serial sections were cut and conventionally dewaxed to water (xylene I, xylene II, 100% alcohol I and 100% alcohol II, 10 min each. Then, 95% ethanol, 90% ethanol, 80% ethanol, 70% ethanol for 10 min each and distilled water); stained with hematoxylin for 1 min with tap water; differentiated in 1% hydrochloric acid, rinsed with water once; stained with eosin for 2 min; then dehydrated with conventional gradient alcohol (70% ethanol, 80% ethanol, 90% ethanol and 95% ethanol, 2 min each; 100% ethanol I, 100% alcohol II, 10 min each); cleared with xylene, and finally mounted with neutral gum. Image analysis software (Image-Pro plus, American Media cybernotis. Company) was used to quantitatively analyze the myocardial cell cross-sectional area. According to the formula: shape factor = $4 \times 3.14 \times (\text{area}/\text{perimeter}^2)$, the shape factor of cardiomyocytes were calculated. Approximately 100 cardiomyocytes with form factor >0.75 were selected and average area of cardiomyocytes was calculated.

Masson trichrome staining. The 5 μm serial sections were first dewaxed to water as described above. Then the sections were placed in composite Masson staining solution for 5 min; washed with 0.2% acetic acid solution of 1 min; stained with 5% phosphotungstic acid solution for 5 min; dipped in 0.2% acetic acid solution for 2 min; stained with brilliant green staining solution for 5 min, dipped in 0.2% acetic acid solution twice; separated in 95% alcohol, dehydrated in gradient alcohol, cleared with xylene, and mounted with neutral gum. With Masson trichrome staining, myocardial cells were red, collagen was green. Collagen content was observed under light microscope, and 10 perspectives were randomly analyzed using image analysis software (Image-Pro plus, American Media cybernotis. Company), myocardial collagen volume fraction (CVF, CVF = collagen area/total area) was measured and average value was taken.

Tissue protein extraction and Western blot

Tissue protein extract. Hundred milligrams of cardiac tissue were homogenized in 1 mL RIPA lysis buffer (150 mmol/L NaCl, 50 mmol/L pH 7.8 of Tris-HCl, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1% EDTA, 1 mmol/L PMSF, 1 mmol/L DTT) on ice bath. The homogenate was centrifuged at 4°C, 12,000 rotations per minute (rpm) for 10 min, the supernatant was harvested as myocardial tissue total protein extract. Sample was aliquoted and stored at −70°C for use. The protein content of the extract was measured using a modified Lowry method.

Western blot

Certain amount of protein samples were mixed with 5 × SDS sample buffer (0.1 mmol/L pH 6.8 of Tris-HCl, 20% glycerol, 0.1% bromophenol blue, 10% β-mercaptoethanol, 4% SDS) and boiled at 100°C for 5 min and then loaded onto 10% PAGE gel for electrophoresis. Protein sample was then transferred onto polyvinylidene difluoride (PVDF) membrane, which was then immersed with primary antibody after blocking with 5% skim milk. The primary antibodies used were anti-OPG antibody (1:400), anti-RANKL antibody (1:400), anti-RANK antibody (1:400), and anti-GAPDH antibody (1:400). After overnight incubation with primary antibody, the membrane was washed three times with Tween Tris-buffered saline (TTBS) and then incubated with horseradish peroxidase conjugated secondary antibody for 2 h. After washing with TTBS for three times, the membrane was immersed with 3,3′-diaminobenzidine (DAB) chromogenic assay reagents till clear bands appeared. Gel-pro gel image analysis software was used for quantitative analysis of the Western blot results, and GAPDH was used as reference.

Immunohistochemistry study

Six micrometers of serial sections of paraffin-embedded heart tissue were immunohistochemically stained as following. Paraffin-embedded sections were first dewaxed to water, incubated in 3% methanol and hydrogen peroxide for 10 min, washed with distilled water, and 0.01 mol/L phosphate-buffered saline (PBS) (pH 7.4) for 5 min, then incubated in 0.1 mol/L citrate buffer (pH 6.0) at 98°C for 20 min, washed with 0.01 mol/L PBS for 5 min, incubated with 10% normal rabbit serum at 37°C for 30 min, and then incubated with 1:50 dilution of primary antibody at 4°C overnight, washed with 0.01 mol/L PBS (pH 7.4) buffer three times for 5 min, incubated with biotinylated secondary antibody (goat anti-rabbit or horse anti-goat) at 37°C for 30 min, then washed with 0.01 mol/L PBS (pH 7.4) buffer three times for 2 min, then incubated with streptavidin labeled horseradish peroxidase working solution at 37°C for 30 min, washed with 0.01 mol/L PBS (pH 7.4) buffer three times for 5 min, and finally incubated with DAB chromogenic reagent for 5 min, water was used to stop color development. The sections were then dehydrated conventionally with gradient alcohol, cleared with xylene, and mounted with neutral gum. In negative control, PBS was used instead of primary antibody, the remaining steps were

the same as above. Positive reaction was brown or yellow staining. Average integrated optical density (IOD) was used to quantitatively analyze protein expression.

RNA extraction and RT-PCR

RNA extraction. Total RNA was extracted from cardiac tissue in the different treatment groups using Invitrogen's Trizol one-step protocol according to Manufacturer's manual.

Reverse transcription (RT) reaction. Two micrograms of total RNA were added into RT reaction system (AMV buffer, dNTPs, oligo dT primer, AMV, Rnase inhibitor, etc.) which was topped to 50 μL with DEPC-treated water, centrifuge briefly after the mix, mineral oil was added onto the top of the reaction solution and was incubated in PCR machine at 42°C for 60 min (cDNA synthesis).

RT-PCR. Three microliters of RT product were used as template for PCR reaction, dNTPs (final concentration 0.2 mmol/L), $MgCl_2$ 2.5 mmol/L, rat OPG, RANKL, RANK or GAPDH primers (final concentration of 50 pmol/L), 10 × PCR buffer, 2.5 μL, Taq DNA polymerase 2U, sterile deionized water were added to the mixture accordingly to a total volume of 25 μL. The PCR reaction conditions were to 94°C for 2 min for initial denaturation, then 94°C for 40 s, 58°C for 50 s and 72°C for 90 s, after 30 cycles, extension at 72°C for 10 min. PCR amplification products were then loaded onto 1% agarose gel for electrophoresis. BIO-PROFIF gel image analysis system and Bio-1D ++ software were used for analyzing the electrophoresis results. GAPDH was used as a reference. The primers were synthesized by Beijing Parkson Gene Technology Co. The primer sequences are as follows:

OPG upstream primer 5′-CACTGCACAGTCAGGAG GAA-3′
Downstream primer 5′-TGCTTTCGATGACGTCTCAC-3′
RANKL upstream primer 5′-AGCCGAGACTACGGCA AGTA-3′
Downstream primer 5′-GCGCTCGAAAGTACAGG AAC-3′
RANK upstream primer 5′-TTAAGCCAGTGCTTCACG GG-3′
Downstream primer 5′-ACGTAGACCACGATGATG TCG-3′
GAPDH forward primer 5′-CGCTAACATCAAATGGG GTG-3′
Downstream primer 5′-ACAACCTGGTCCTCAGTGTA-3′

Statistical analysis. SPSS 11.0 software was used for statistical analysis. The experimental data were expressed as mean ± standard deviation. Comparison between groups was performed using single factor ANOVA and least significant difference (LSD)-*t* test. $P < 0.05$ was considered statistically different.

Table 1 Effects of amlodipine, atorvastatin, and their combination on cardiac remodeling and cardiac function estimated by echocardiography of SHR ($n = 8$, $\bar{x} \pm s$)

Groups	LVEDs (cm)	LVEDd (cm)	IVSs (cm)	IVSd (cm)	LVPWs (cm)	LVPWd (cm)	LVW (g)	LVEF (%)	LVFS (%)	IVRT (ms)
WKY	0.45 ± 0.02	0.77 ± 0.03	0.25 ± 0.03	0.16 ± 0.01	0.23 ± 0.01*#	0.14 ± 0.01*#	0.78 ± 0.06*#	80.3 ± 5.1	42.3 ± 3.4	27.1 ± 6.2*
SHR	0.49 ± 0.05	0.80 ± 0.04	0.30 ± 0.05	0.18 ± 0.02	0.30 ± 0.03	0.20 ± 0.02	1.14 ± 0.12	77.2 ± 6.4	39.3 ± 5.2	39.8 ± 4.7
SHR + AM	0.43 ± 0.05	0.75 ± 0.04	0.29 ± 0.03	0.18 ± 0.03	0.28 ± 0.04	0.19 ± 0.03	0.99 ± 0.10*#	79.5 ± 5.2	42.4 ± 5.1	33.9 ± 6.1
SHR + AT	0.45 ± 0.06	0.79 ± 0.06	0.28 ± 0.05	0.18 ± 0.03	0.29 ± 0.02	0.18 ± 0.02	1.01 ± 0.11*#	81.4 ± 3.9	40.9 ± 3.4	36.8 ± 4.6#
SHR + AM + AT	0.43 ± 0.03	0.74 ± 0.03	0.27 ± 0.04	0.17 ± 0.04	0.27 ± 0.02	0.17 ± 0.02	0.88 ± 0.08*	80.1 ± 4.2	41.8 ± 3.7	29.2 ± 3.5*

SHR: SHR treated with vehicle; SHR + AM: SHR treated with amlodipine; SHR + AT: SHR treated with atorvastatin; SHR + AM + AT: SHR treated with combination of amlodipine and atorvastatin.
*$P < 0.05$ *vs.* SHR; #$P < 0.05$ *vs.* SHR + AM + AT.

Results

Changes in rat echocardiography

There was no significant difference in LVEDd, LVEDs, IVSs, IVSd, LVEF, and LVFS between WKY group and SHR control group, and within each drug treatment group ($P > 0.05$). The LVPWs and LVPWd of SHR control group were significantly higher than those of WKY rats ($P < 0.05$), but no significant difference among different SHR groups ($P > 0.05$). At 48 weeks, LVW of SHR control group was significantly higher than that of WKY rats ($P < 0.05$). LVWs of amlodipine group and atorvastatin group were significantly decreased than those of SHR control group (all $P < 0.05$), and LVW of the combination group was decreased even further ($P < 0.05$). There was no significant difference in LVEF and LVFS among all groups ($P > 0.05$), suggesting that cardiac systolic function was normal. And compared to that of W KY rats, IVRT of rats in SHR control group was significantly longer ($P < 0.05$), indicating that left ventricular diastolic dysfunction was impaired. After amlodipine or atorvastatin treatment, IVRT had a tendency to decrease, but there was no significant difference ($P > 0.05$), however combination of amlodipine and atorvastatin administration could significantly decrease IVRT ($P < 0.05$) (Table 1).

Changes in rats' hemodynamics

Compared with that of WKY rats with the same age, LVSP of SHR rats was significantly increased ($P < 0.05$). Atorvastatin treatment slightly decreased LVSP but the difference was not statistically significant ($P > 0.05$). Amlodipine alone and combination of amlodipine and atorvastatin could both significantly decrease LVSP ($P < 0.05$), but the difference between the two groups was not significantly different ($P > 0.05$). Compared with those of WKY rats, the LVEDP in SHR rats was increased, τ was extended, and dp/dt_{min}/LVSP was decreased ($P < 0.05$), but dp/dt_{max} was the same, suggesting that SHR rats have only left ventricular diastolic dysfunction, and the overall cardiac systolic function was normal. After amlodipine and/or atorvastatin treatment, LVEDP and τ were significantly decreased, dp/dt_{min}/LVSP was significantly increased, with the most significant effects shown when treated with the combination of amlodipine and atorvastatin treatment ($P < 0.05$). Consistent with echocardiographic results, these results further indicated that amlodipine and/or atorvastatin not only reversed the ventricular hypertrophy, but also improved left ventricular diastolic function (Table 2).

Changes in body weight, heart rate, and LVMI

There was no significant difference in body weight among all rats ($P > 0.05$). Heart rate in all SHR groups was significantly higher than that of WKY control group ($P < 0.05$), but there was no significant difference among different SHR groups ($P > 0.05$). LVMI in SHR control group was significantly higher than that of WKY rats ($P < 0.05$), after amlodipine or (and) atorvastatin treatment, LVMI was significantly decreased, with the most significant effects shown when treated with the combination of amlodipine and atorvastatin treatment ($P < 0.05$) (Table 3).

Comparison of pathological changes in cardiac tissues after treatment

As shown in HE staining, compared with that of WKY rats, the cross-sectional area of cardiomyocytes in SHR rats was significantly increased ($P < 0.05$). Both amlodipine and atorvastatin were able to reduce the cross-sectional area of cardiomyocytes ($P < 0.05$), and the combination treatment was able to reduce it further compared with monotherapy group ($P < 0.05$), indicating that drugs intervention was able to improve the degree of hypertrophy of cardiomyocytes, with the best results shown with combination of the two drugs (Figure 1).

As shown in Masson staining, compared with that of WKY rats, myocardial interstitial collagen fibers in SHR were significantly increased and disorganized, the wall of small vessels became thicker, and surrounding matrix was

Table 3 Effects of amlodipine, atorvastatin, and their combination on cardiac morphology of SHR ($n = 8$, $\bar{x} \pm s$)

Groups	Body weight (g)	HR (beats/min)	LVMI (mg/g)
WKY	385 ± 30	$322 \pm 31^{*\#}$	$2.05 \pm 0.18^{*\#}$
SHR	365 ± 25	371 ± 17	3.01 ± 0.21
SHR + AM	360 ± 18	370 ± 21	$2.51 \pm 0.16^{*\#}$
SHR + AT	358 ± 20	360 ± 41	$2.68 \pm 0.11^{*\#}$
SHR + AM + AT	359 ± 24	357 ± 22	$2.27 \pm 0.17^{*}$

*$P < 0.05$ *vs.* SHR; #$P < 0.05$ *vs.* SHR + AM + AT.

Table 2 Effects of amlodipine, atorvastatin, and their combination on hemodynamics of SHR rats ($n = 8$, $\bar{x} \pm s$)

Groups	LVSP (mm Hg)	LVEDP (mm Hg)	DP/dt_{max} (mm Hg/ms)	DP/dt_{min} (mm Hg/ms)	DP/dt_{min}/LVSP (s^{-1})	τ (ms)
WKY	$122 \pm 11^{*\#}$	$1.8 \pm 0.3^{*}$	$7.1 \pm 0.6^{*\#}$	$6.2 \pm 0.8^{*\#}$	$53.5 \pm 8.8^{*}$	$14.7 \pm 2.1^{*}$
SHR	228 ± 10	4.4 ± 0.6	11.5 ± 1.2	8.7 ± 0.9	36.5 ± 5.5	18.9 ± 2.7
SHR + AM	$185 \pm 10^{*}$	$2.8 \pm 0.5^{*\#}$	$9.7 \pm 1.1^{*}$	8.4 ± 2.0	$45.4 \pm 6.8^{*\#}$	15.1 ± 2.9
SHR + AT	$220 \pm 13^{*\#}$	$3.5 \pm 0.4^{*\#}$	11.3 ± 1.4	9.1 ± 1.4	$42.5 \pm 5.1^{*\#}$	15.4 ± 2.6
SHR + AM + AT	$178 \pm 14^{*}$	$2.1 \pm 0.4^{\#}$	10.3 ± 0.7	9.4 ± 1.2	$52.7 \pm 6.5^{*}$	$13.8 \pm 1.9^{*}$

*$P < 0.05$ *vs.* SHR; #$P < 0.05$ *vs.* SHR + AM + AT.

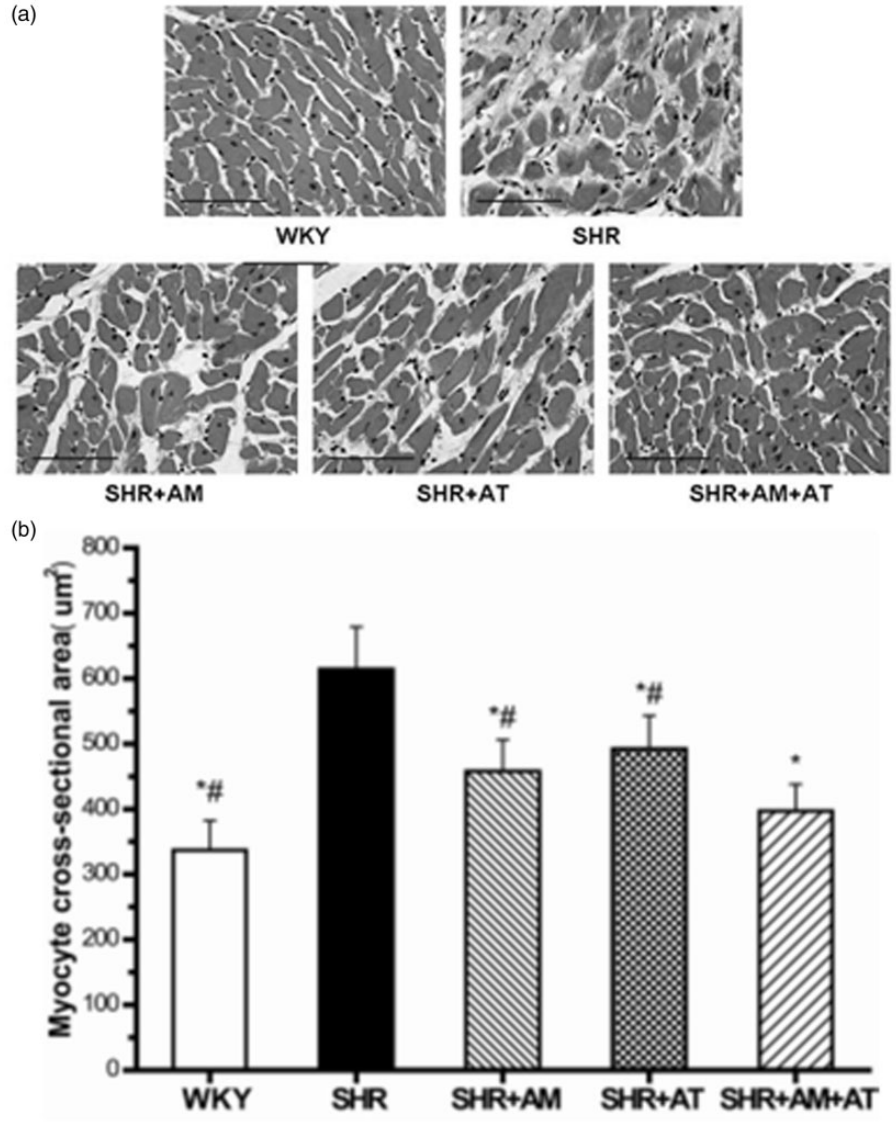

Figure 1 Effects of amlodipine, atorvastatin, and their combination on cardiomyocyte hypertrophy in SHR. (a) Representative micrographs in each group. (b) Cardiomyocyte cross-sectional area in each group. Data are expressed as mean ± SD ($n = 5$). *$P < 0.05$ compared with SHR; #$P < 0.05$ compared with SHR + AM + AT. Magnification, ×400. Bar, 50 μm

also increased ($P < 0.05$). Both amlodipine and atorvastatin intervention significantly reduced myocardial interstitial collagen accumulation, made its alignment more organized, and reduced the degree of small vessel wall thickening and the extent of perivascular fibrosis. The combination intervention showed more pronounced improvement in the myocardial perivascular fibrosis compared with that of monotherapy intervention ($P < 0.05$) (Figures 2 and 3).

Effects of amlodipine, atorvastatin, and combination treatment on RANKL mRNA and protein expression in SHR rats

As shown by RT-PCR and Western blot, compared with that of WKY rats, both cardiac mRNA and protein levels of RANKL were higher in SHR rats ($P < 0.05$). Both amlodipine and atorvastatin intervention significantly reduced RANKL mRNA and protein expression ($P < 0.05$), with the best results shown when treated with combination of the two drugs ($P < 0.05$) (Figures 4 and 5).

As shown by immunohistochemistry study, compared with that of WKY rat, cardiomyocytes of SHR had increased the numbers of positively stained cells for RANKL. Both amlodipine and atorvastatin statin intervention significantly decreased the numbers of RANKL positive cardiomyocytes, also made the stain lighter ($P < 0.05$), with the best results shown with combination of the two drugs ($P < 0.05$) (Figure 6).

Effects of amlodipine, atorvastatin, and combination treatment on RANK mRNA and protein expression in SHR rats

As shown by RT-PCR and Western blot, compared with that of WKY rats, both cardiac mRNA and protein levels of

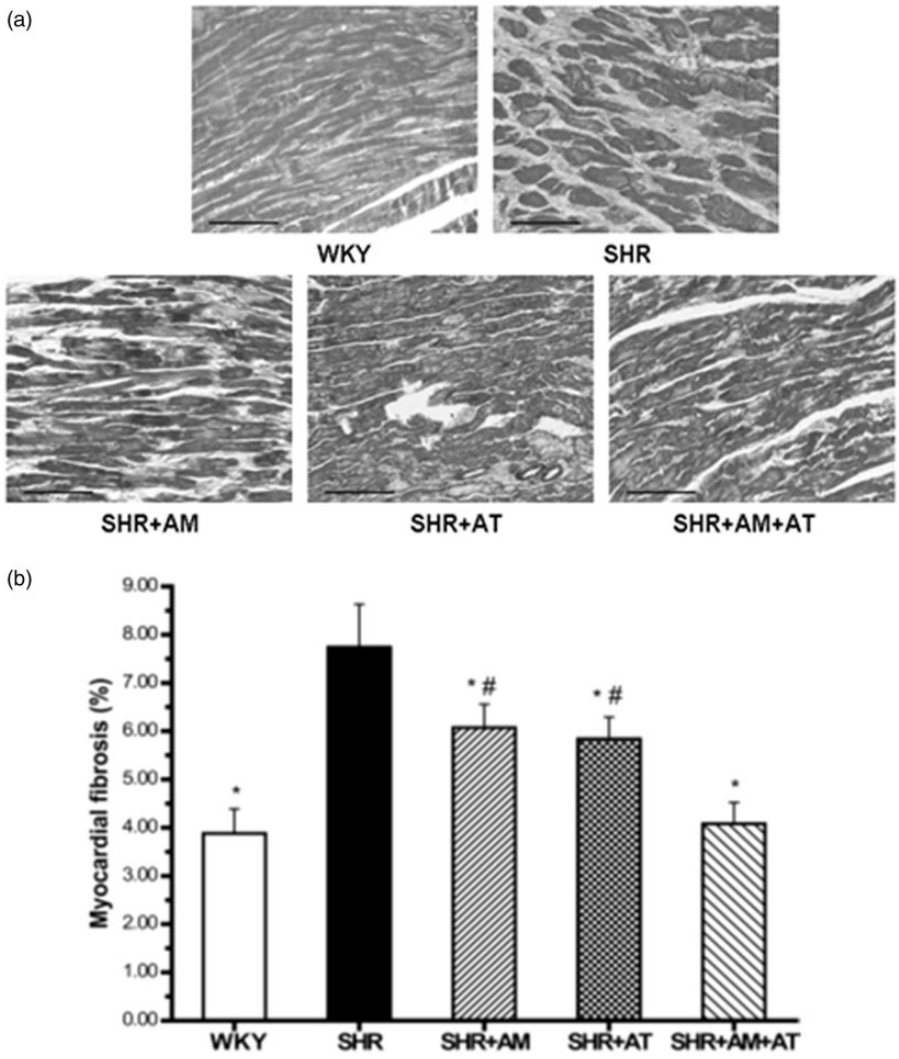

Figure 2 Effects of amlodipine, atorvastatin, and their combination on cardiac interstitial fibrosis in SHR. (a) Representative micrographs in each group. (b) Interstitial fibrosis in each group. Data are expressed as mean ± SD ($n = 5$). *$P < 0.05$ compared with SHR; #$P < 0.05$ compared with SHR + AM + AT. Magnification, ×200. Bar, 100 μm

RANK were higher in SHR rats ($P < 0.05$). Both amlodipine and atorvastatin intervention significantly reduced RANK mRNA and protein expression ($P < 0.05$), with the best results shown with combination of the two drugs ($P < 0.05$) (Figures 4 and 5).

As shown by immunohistochemistry study, compared with that of WKY rat, cardiomyocytes of SHR had increased numbers of positively stained cells for RANK. Both amlodipine and atorvastatin statin intervention significantly decreased the numbers of RANK positive cardiomyocytes, also made the stain lighter ($P < 0.05$), with the best results shown with combination of the two drugs ($P < 0.05$) (Figure 7).

Effects of amlodipine, atorvastatin, and combination treatment on OPG mRNA and protein expression in SHR rats

As shown by RT-PCR and Western blot, compared with that of WKY rats, both cardiac mRNA and protein levels of OPG were higher in SHR rats ($P < 0.05$). Both amlodipine and atorvastatin intervention significantly reduced OPG mRNA and protein expression ($P < 0.05$), and there was no significant difference in terms of effect among the three intervention groups ($P > 0.05$) (Figures 4 and 5).

As shown by immunohistochemistry study, compared with that of WKY rat, cardiomyocytes of SHR had increased numbers of positively stained cells for OPG. Both amlodipine and atorvastatin statin intervention significantly decreased the numbers of OPG positive cardiomyocytes, also made the stain lighter ($P < 0.05$), and there was no significant difference in terms of effect among the three intervention groups ($P > 0.05$) (Figure 8).

Discussion

Our study showed that amlodipine and atorvastatin significantly reduced LVMI and LVW, reversing left ventricular hypertrophy. Amlodipine, atorvastatin, and their

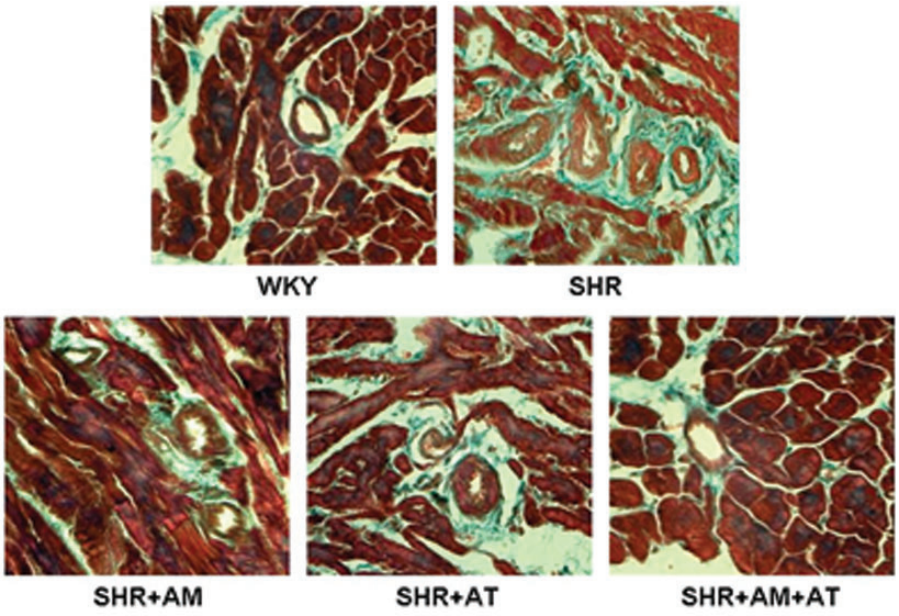

Figure 3 Effects of amlodipine, atorvastatin, and their combination on small artery remodeling and perivascular fibrosis in SHR. Representative micrographs in each group. Magnification, ×400. (A color version of this figure is available in the online journal.)

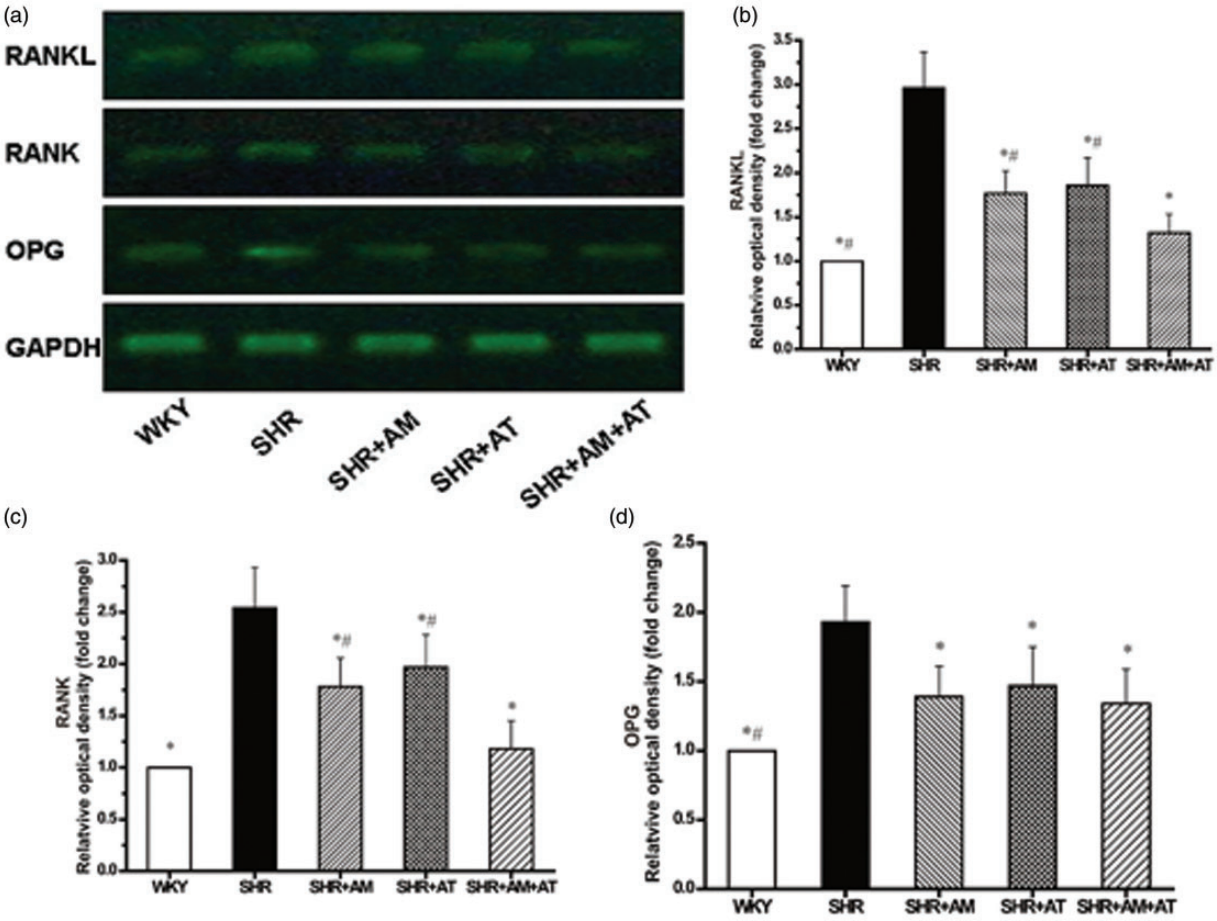

Figure 4 Effects of amlodipine, atorvastatin, and their combination on RANKL, RANK, OPG mRNA expression by RT-PCR. (a) Representative results of RT-PCR. (b) Densitometry analysis of RT-PCR for RANKL, RANK, OPG. Data are expressed as mean ± SD ($n = 5$). $^{*}P < 0.05$ compared with SHR; $^{\#}P < 0.05$ compared with SHR + AM + AT. (A color version of this figure is available in the online journal.)

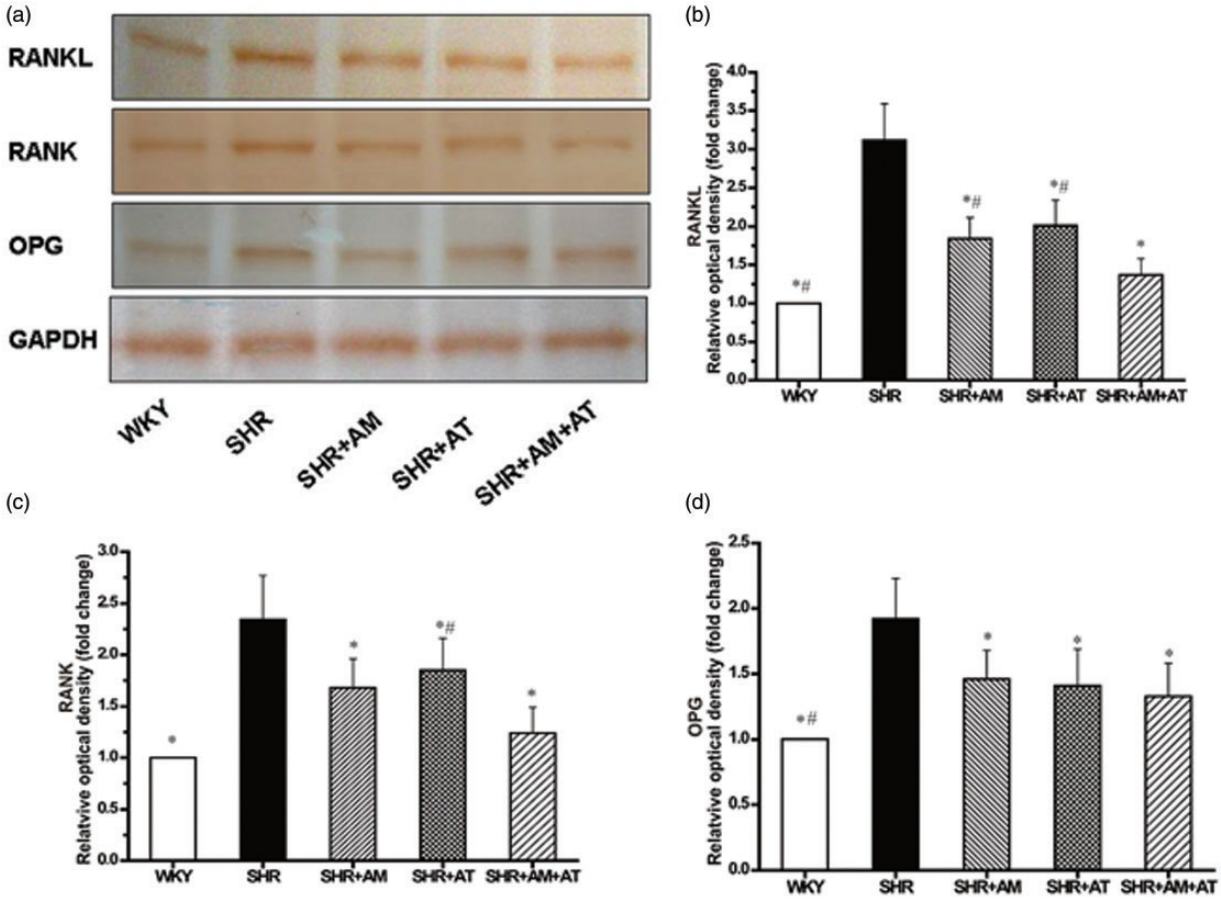

Figure 5 Effects of amlodipine, atorvastatin, and their combination on RANKL, RANK, OPG protein expression in SHR by Western blot. (a) Representative results of Western blot. (b) Densitometry analysis of Western blot for RANKL, RANK, OPG. Data are expressed as mean ± SD ($n = 5$). $^{*}P < 0.05$ compared with SHR; $^{\#}P < 0.05$ compared with SHR + AM + AT. (A color version of this figure is available in the online journal.)

combination also decreased IVRT, LVEDP, and relaxation time constant τ, increased dP/dt_{min}/LVSP, indicating that both monotherapy and combination therapy significantly improved cardiac diastolic function, with the best effects seen with combination therapy. We also showed that compared with WKY rats, in cardiomyocytes of SHR rats, the mRNA and protein of RANKL, RANK, and OPG were significantly increased, suggesting that the activity of RANKL/RANK/OPG system was increased in hypertrophic myocardial tissue, which may play an important role in the development of cardiac hypertrophy and heart failure. Amlodipine, atorvastatin, and their combination intervention significantly reduced the mRNA and protein expression of RANKL, RNAK, and OPG, with the best effects seen with the combination therapy.

Recent studies have found, RANKL/RANK/OPG system not only plays an important role in bone metabolism, but also has great significance in the pathogenesis of many cardiovascular diseases. It has been reported that vascular calcification and arterial aneurysm was developed in aorta of OPG gene deficient mice, suggesting that OPG plays a protective role in the vascular system by inhibiting vascular wall matrix remodeling and endothelial cell apoptosis.[15] It has also been shown that RANKL was able to promote vascular smooth muscle cell calcification and endothelial cell apoptosis, OPG could antagonize the adverse effects of RANKL.[16,17] In addition, clinical data showed that RANKL/RANK/OPG system might also play an important role in ventricular remodeling and development of heart failure.[18] Increased plasma OPG level is an important symbol of RANKL/RANK/OPG system activation.[19] Clinical studies have shown that plasma OPG levels were correlated with left ventricular hypertrophy,[9,11] animal studies also showed that activation of RANKL/RANK/OPG system was an important pathogenic factor for ventricular remodeling after either MI or inflammatory cardiomyopathy.[4,6] In this study, compared to normotensive rats, hypertensive rats' myocardial tissue showed increased RANKL, RANK, and OPG mRNA and protein expression, which provided further evidence that RANKL/RANK/OPG system plays an important role in development of cardiac hypertrophy and heart failure in rat.

The pathological features of left ventricular hypertrophy include myocardial hypertrophy, cardiac fibroblast proliferation and extracellular matrix deposition, small blood vessels remodeling within the myocardial wall. Although our results did not provide an exact mechanism how activation

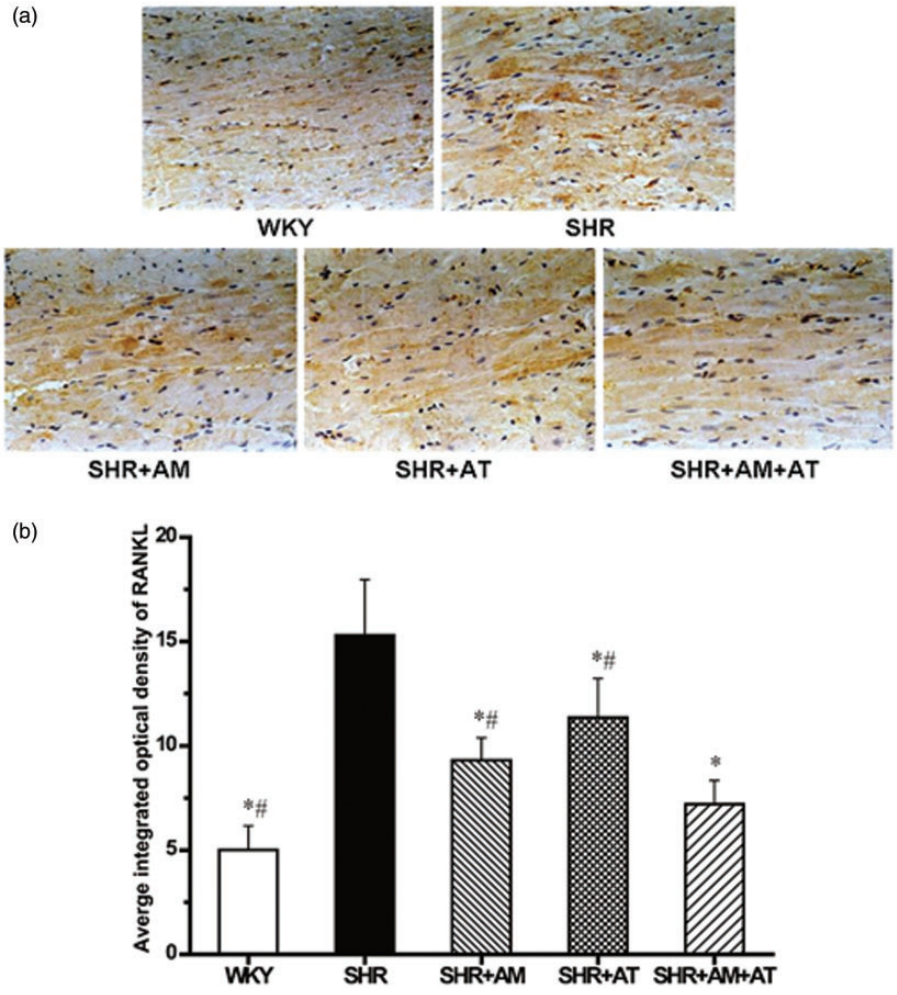

Figure 6 Effects of amlodipine, atorvastatin, and their combination on RANKL protein expression in SHR by immunohistochemical staining. Data are expressed as mean ± SD ($n = 5$). (a) Representative micrograph in each group. (b) Average IOD analyses of immunohistochemistry for RANKL. $^{*}P < 0.05$ compared with SHR; $^{\#}P < 0.05$ compared with SHR + AM + AT. Magnification, ×400. (A color version of this figure is available in the online journal.)

of RANKL/RANK/OPG system regulated hypertensive left ventricular remodeling, based on previous literature, we hypothesized that RANKL/RANK/OPG system may play a role through the following mechanisms:

1. Kaden *et al*.[20] reported that RANKL could induce heart valve myofibroblast proliferation, which suggested that RANKL had the potential to stimulate cardiac fibroblasts proliferation.
2. Ueland *et al*.[4] reported that, in animal models of heart failure after MI, mRNA and protein expression of RNAKL, RANK, and OPG were significantly increased in cardiomyocytes of ischemia region, and the ratio of RANKL/OPG was decreased. RANKL could promote myocardial matrix metalloproteinase 2 (MMP-2) and MMP-9 mRNA level, but had no effect on the expression of tissue inhibitor of metalloproteinase 1 (TIMP-1) and TIMP-2. In addition, RANKL could also promote fibroblast MMP-2 and MMP-9 mRNA level and enhanced their activity, and slightly decreased in TIMP 1-and TIMP-2 mRNA level. Ueland *et al*. thought that RANKL could induce myocardial remodeling and eventually led to heart failure through the net effect of matrix degradation. We previously showed enhanced MMP-2 and MMP-9 expression and activity were the leading causes of hypertensive myocardial fibrosis (data not published). In summary, we hypothesized that RANKL might promote myocardial fibrosis in SHR rats through enhancing MMP-2 and MMP-9 mRNA expression and activity in fibroblast and cardiomyocytes.
3. The results of our immunohistochemical study showed that compared with WKY rats, the expression of RANKL and RANK were significantly increased in vascular endothelial cells and vascular smooth muscle cells in SHR rats, OPG increased slightly, and the ratio of RANKL/OPG was imbalanced, indicating that the activated RANKL/RANK system possibly participated small blood vessel remodeling and perivascular

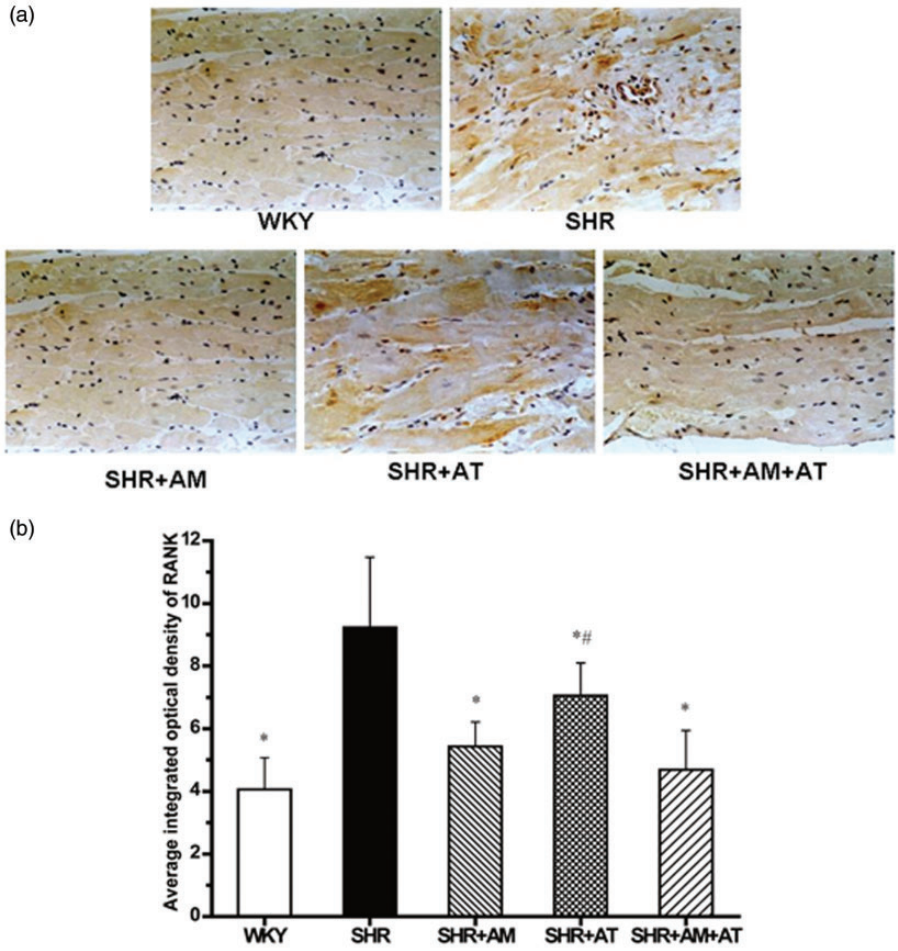

Figure 7 Effects of amlodipine, atorvastatin, and their combination on RANK protein expression in SHR by immunohistochemical staining. Data are expressed as mean ± SD ($n = 5$). (a) Representative micrograph in each group. (b) Average IOD analyses of immunohistochemistry for RANK. $^{*}P < 0.05$ compared with SHR; $^{\#}P < 0.05$ compared with SHR + AM + AT. Magnification, ×400. (A color version of this figure is available in the online journal.)

fibrosis within myocardial wall through regulating the autocrine and paracrine function of endothelial cells and vascular smooth muscle cells. In addition, decreased numbers of capillaries in myocardial tissue is an important reason that SHR rat had decreased coronary flow reserve in its hypertrophied myocardium tissue.[21] A study showed that RANKL could promote apoptosis in rat aortic endothelial cells and inhibit angiogenesis of nourishing blood vessels to aorta. In contrast, OPG activation could promote vascular endothelial cell proliferation and angiogenesis of nourishing blood vessels to aorta by inhibiting RANKL/RANK signaling pathway.[16] Thus, RANKL/RANK/OPG system activity may be associated with scarce capillaries in cardiac tissue in SHR rats.

What regulates the activation of the myocardial RANKL/RANK/OPG system in SHR rats is still not clear. Limited literature reports indicated that pro-renin could upregulate OPG mRNA level in cardiomyocytes, inflammatory cytokines IL-17 could promote OPG and RANKL mRNA expression in myocardial tissue.[6,13] Inflammatory cytokines tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and angiotensin II (Ang II) all could promote OPG mRNA expression in vascular endothelial cells and smooth muscle cells.[22–24] Recently, it was found that RANKL/RANK/OPG system is of particular importance in the pathogenesis of heart failure, and the pressure-overloaded myocardium could generate RANKL, which then induced TNF-α, IL-1α, and IL-1β production via a RANK-TRAF2/TRAF6-PLC-PKC-NF-κB-mediated autocrine mechanism.[25,26] Therefore, we hypothesized that activation of local RAS system and upregulation of pro-inflammatory cytokines may be the cause for RANKL/RANK/OPG system activation in myocardial tissue in SHR rats. In this study, we found that amlodipine, atorvastatin, and their combinations could significantly reduce the mRNA and protein expression of RANKL, RANK, and OPG in cardiomyocytes in SHR rats, suggesting that amlodipine and atorvastatin could improve cardiac interstitial fibrosis and small blood vessel remodeling possibly through intervention of the RANKL/RANK/OPG system.

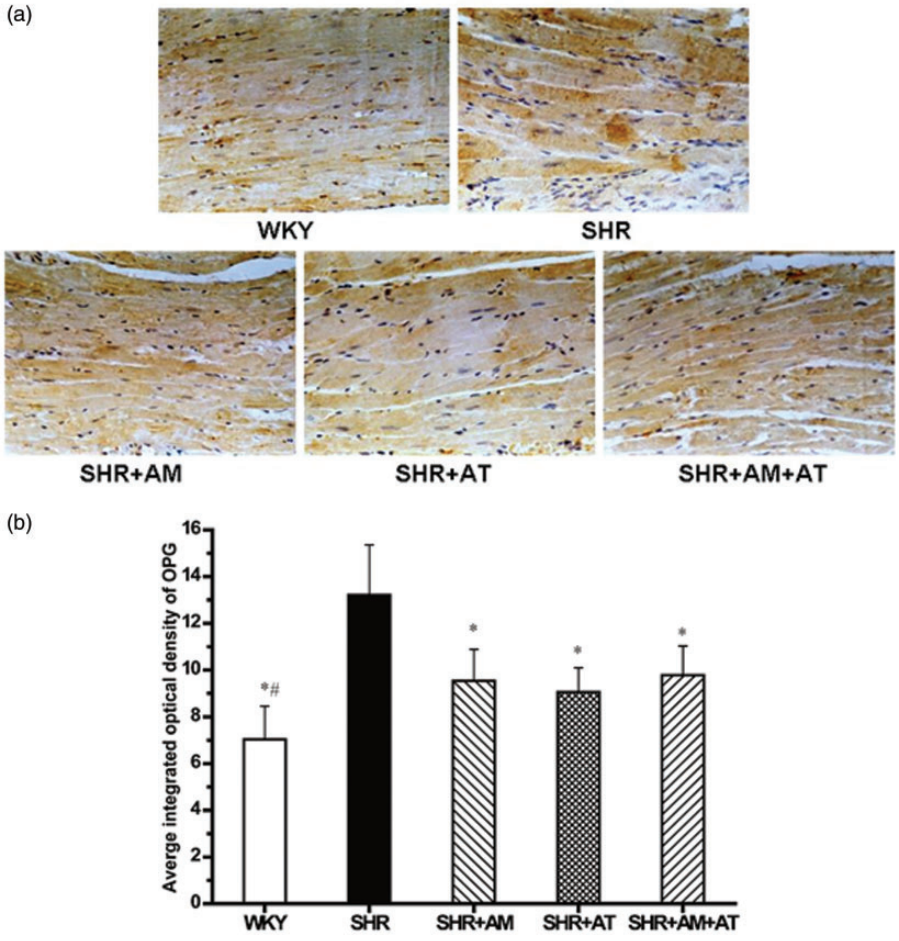

Figure 8 Effects of amlodipine, atorvastatin, and their combination on OPG protein expression in SHR by immunohistochemical staining. Data are expressed as mean ± SD ($n = 5$). (a) Representative micrograph in each group. (b) Average IOD analyses of immune histochemistry for OPG. $^{*}P < 0.05$ compared with SHR; $^{\#}P < 0.05$ compared with SHR + AM + AT. Magnification, ×400. (A color version of this figure is available in the online journal.)

Author contributions: LJC and CW contributed to the conception and design of the study; LDM and DH contributed to the acquisition of data; HJ and YXC performed the experiments; LDM and HJ contributed to the analysis of data; LJC wrote the manuscript; All authors reviewed and approved the final version of the manuscript.

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DECLARATION OF CONFLICTING INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Kartsogiannis V, Zhou H, Horwood NJ, Thomas RJ, Hards DK, Quinn JM, Niforas P, Ng KW, Martin TJ, Gillespie MT. Localization of RANKL (receptor activator of NF kappa B ligand) mRNA and protein in skeletal and extraskeletal tissues. *Bone* 1999;**25**:525–34
2. Omland T, Ueland T, Jansson AM, Persson A, Karlsson T, Smith C, Herlitz J, Aukrust P, Hartford M, Caidahl K. Circulating osteoprotegerin levels and long-term prognosis in patients with acute coronary syndromes. *J Am Coll Cardiol* 2008;**51**:627–33
3. Kiechl S, Schett G, Schwaiger J, Seppi K, Eder P, Egger G, Santer P, Mayr A, Xu Q, Willeit J. Soluble receptor activator of nuclear factor-kappa B ligand and risk for cardiovascular disease. *Circulation* 2007;**116**:385–91
4. Ueland T, Yndestad A, Oie E, Florholmen G, Halvorsen B, Froland SS, Simonsen S, Christensen G, Gullestad L, Aukrust P. Dysregulated osteoprotegerin/RANK ligand/RANK axis in clinical and experimental heart failure. *Circulation* 2005;**111**:2461–8
5. Lawrie A, Waterman E, Southwood M, Evans D, Suntharalingam J, Francis S, Crossman D, Croucher P, Morrell N, Newman C. Evidence of a role for osteoprotegerin in the pathogenesis of pulmonary arterial hypertension. *Am J Pathol* 2008;**172**:256–64
6. Liu W, Feng W, Wang F, Li W, Gao C, Zhou B, Ma M. Osteoprotegerin/RANK/RANKL axis in cardiac remodeling due to immuno-inflammatory myocardial disease. *Exp Mol Pathol* 2008;**84**:213–7
7. Kim SM, Lee J, Ryu OH, Lee KW, Kim HY, Seo JA, Kim SG, Kim NH, Baik SH, Choi DS, Choi KM. Serum osteoprotegerin levels are associated with inflammation and pulse wave velocity. *Clin Endocrinol* 2005;**63**:594–8
8. Rasmussen LM, Tarnow L, Hansen TK, Parving HH, Flyvbjerg A. Plasma osteoprotegerin levels are associated with glycaemic status, systolic blood pressure, kidney function and cardiovascular morbidity in type 1 diabetic patients. *Eur J Endocrinol* 2006;**154**:75–81
9. Helske S, Kovanen PT, Lindstedt KA, Salmela K, Lommi J, Turto H, Werkkala K, Kupari M. Increased circulating concentrations and augmented myocardial extraction of osteoprotegerin in heart failure due to left ventricular pressure overload. *Eur J Heart Fail* 2007;**9**:357–63

10. Dovio A, Data V, Carignola R, Calzolari G, Vitetta R, Ventura M, Saba L, Severino A, Angeli A. Circulating osteoprotegerin and soluble RANK ligand in systemic sclerosis. *J Rheumatol* 2008;**35**:2206–13
11. Omland T, Drazner MH, Ueland T, Abedin M, Murphy SA, Aukrust P, de Lemos JA. Plasma osteoprotegerin levels in the general population: relation to indices of left ventricular structure and function. *Hypertension* 2007;**49**:1392–8
12. Clines GA, Mohammad KS, Bao Y, Stephens OW, Suva LJ, Shaughnessy JD Jr, Fox JW, Chirgwin JM, Guise TA. Dickkopf homolog 1 mediates endothelin-1-stimulated new bone formation. *Mol Endocrinol* 2007;**21**:486–98
13. Saris JJ, t Hoen PA, Garrelds IM, Dekkers DH, den Dunnen JT, Lamers JM, Jan Danser AH. Prorenin induces intracellular signaling in cardiomyocytes independently of angiotensin II. *Hypertension* 2006;**48**:564–71
14. Baud'huin M, Lamoureux F, Duplomb L, Redini F, Heymann D. RANKL, RANK, osteoprotegerin: key partners of osteoimmunology and vascular diseases. *Cell Mol Life Sci* 2007;**64**:2334–50
15. Collin-Osdoby P. Regulation of vascular calcification by osteoclast regulatory factors RANKL and osteoprotegerin. *Circ Res* 2004;**95**:1046–57
16. McGonigle JS, Giachelli CM, Scatena M. Osteoprotegerin and RANKL differentially regulate angiogenesis and endothelial cell function. *Angiogenesis* 2009;**12**:35–46
17. Panizo S, Cardus A, Encinas M, Parisi E, Valcheva P, Lopez-Ongil S, Coll B, Fernandez E, Valdivielso JM. RANKL increases vascular smooth muscle cell calcification through a RANK-BMP4-dependent pathway. *Circ Res* 2009;**104**:1041–8
18. Ueland T, Aukrust P, Damas JK, Gullestad L, Yndestad A. The tumor necrosis factor superfamily in heart failure. *Future Cardiol* 2006;**2**:101–11
19. Kiechl S, Werner P, Knoflach M, Furtner M, Willeit J, Schett G. The osteoprotegerin/RANK/RANKL system: a bone key to vascular disease. *Expert Rev Cardiovasc Ther* 2006;**4**:801–11
20. Kaden JJ, Dempfle CE, Kilic R, Sarikoc A, Hagl S, Lang S, Brueckmann M, Borggrefe M. Influence of receptor activator of nuclear factor kappa B on human aortic valve myofibroblasts. *Exp Mol Pathol* 2005;**78**:36–40
21. Pu Q, Larouche I, Schiffrin EL. Effect of dual angiotensin converting enzyme/neutral endopeptidase inhibition, angiotensin converting enzyme inhibition, or AT1 antagonism on coronary microvasculature in spontaneously hypertensive rats. *Am J Hypertens* 2003;**16**:931–7
22. Ben-Tal Cohen E, Hohensinner PJ, Kaun C, Maurer G, Huber K, Wojta J. Statins decrease TNF-alpha-induced osteoprotegerin production by endothelial cells and smooth muscle cells in vitro. *Biochem Pharmacol* 2007;**73**:77–83
23. Zhang J, Fu M, Myles D, Zhu X, Du J, Cao X, Chen YE. PDGF induces osteoprotegerin expression in vascular smooth muscle cells by multiple signal pathways. *FEBS Lett* 2002;**521**:180–4
24. Moran CS, Cullen B, Campbell JH, Golledge J. Interaction between angiotensin II, osteoprotegerin, and peroxisome proliferator-activated receptor-gamma in abdominal aortic aneurysm. *J Vasc Res* 2009;**46**:209–17
25. Ueland T, Yndestad A, Dahl CP, Gullestad L, Aukrust P. TNF revisited: osteoprotegerin and TNF-related molecules in heart failure. *Curr Heart Fail Rep* 2012;**9**:92–100
26. Ock S, Ahn J, Lee SH, Park H, Son JW, Oh JG, Yang DK, Lee WS, Kim HS, Rho J, Oh GT, Abel ED, Park WJ, Min JK, Kim J. Receptor activator of nuclear factor-kappaB ligand is a novel inducer of myocardial inflammation. *Cardiovasc Res* 2012;**94**:105–14

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