

## Original Article

# Simvastatin inhibited cardiac hypertrophy and fibrosis in apolipoprotein E-deficient mice fed a "Western-style diet" by increasing PPAR $\alpha$ and $\gamma$ expression and reducing TC, MMP-9, and Cat S levels

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**Aim:** The examine the cardiac hypertrophy and fibrosis in apolipoprotein E-deficient mice (ApoE<sup>-/-</sup> mice) fed a "Western-style diet" and the effect of simvastatin intervention.

**Methods:** Male ApoE<sup>-/-</sup> mice ( $n=36$ ) were fed a "Western-style diet" from the age of 8 weeks. After 16 weeks, they were randomly given either simvastatin (25 mg·kg<sup>-1</sup>·d<sup>-1</sup>) or normal saline (control group) by gavage for 8, 16, or 24 weeks. The left ventricular (LV) wall thickness and diameter of the myocardial cells were determined with Hematoxylin-Eosin stain, and the level of fibrosis of the myocardial matrix was assessed with Masson stain. Real-time quantitative polymerase chain reaction and Western blotting analysis were used to determine the mRNA and protein expression of matrix metalloproteinase-9 (MMP-9), Cathepsin S (Cat S), and the peroxisome proliferator-activated receptors (PPARs) in the myocardium of ApoE<sup>-/-</sup> mice.

**Results:** ApoE<sup>-/-</sup> mice fed a "Western-style diet" showed an significant age-dependent increase in total cholesterol (TC), LV wall thickness, myocardial cell diameter and LV collagen content ( $P<0.05$ ). The simvastatin treatment group showed significantly reduced LV wall thickness, myocardial cell diameters and LV collagen content at 40 weeks when compared with the control group ( $P<0.05$ ). Furthermore, treatment with simvastatin also significantly inhibited the mRNA and protein expressions of MMP-9 and Cat S as well as increased the mRNA and protein expressions of PPAR alpha and PPAR gamma at 32 and 40 weeks compared with the control group ( $P<0.05$ ).

**Conclusion:** ApoE<sup>-/-</sup> mice fed a "Western-style diet" had cardiac hypertrophy and fibrosis, which worsened with age. Simvastatin treatment inhibits the development of cardiac hypertrophy and fibrosis, and this effect may be mediated through increased levels of PPAR alpha and PPAR gamma and reduced levels of TC, MMP-9, and Cat S.

**Keywords:** ApoE<sup>-/-</sup> mice; cardiac hypertrophy; cathepsin S; simvastatin; peroxisome proliferator-activated receptors

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## Introduction

Cardiac hypertrophy and fibrosis, which are the common responses of the heart to all forms of injury, are the major determinants of morbidity and mortality from cardiovascular disease in both developing and developed countries. Cardiac hypertrophy is one of the main responses of cardiomyocytes to mechanical and neurohormonal stimuli. Although cardiac hypertrophy may initially represent an adaptive response of the myocardium, ultimately, it often progresses to ventricular dilatation and eventually to heart failure, which is one of the

leading causes of mortality in the world. Cardiac hypertrophy is an independent risk factor for cardiovascular disease. Epidemiological studies<sup>[1, 2]</sup> showed that hypercholesterolemia is associated with higher left ventricular mass and that dyslipidemia is an independent determinant of increased left ventricular mass.

Wu *et al*<sup>[3]</sup> showed that the hypercholesterolemic ApoE<sup>-/-</sup> mice were more susceptible to cardiac hypertrophy. In their studies, the hearts of 2-month-old ApoE<sup>-/-</sup> mice had a significantly larger LV end diastolic dimension and a smaller ejection fraction than those seen in wild-type C57BL/6 mice. The heart weight/body weight ratios of ApoE<sup>-/-</sup> mice at 4 months of age were 15% larger than those of the wild-type mice with a similar genetic background. Other authors demonstrated<sup>[4]</sup>

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an age-dependent aortic stiffening, cardiac hypertrophy and increased cardiac output in ApoE<sup>-/-</sup> mice and that the long-term increase in cardiac afterload due to increased aortic stiffening may contribute to cardiac hypertrophy in ApoE<sup>-/-</sup> mice.

Statins, also known as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, have been reported to reverse established cardiac hypertrophy and interstitial fibrosis. This effect may be mediated by reducing the generation of reactive oxygen species and through the peroxisome proliferator activated receptor (PPAR) pathway<sup>[5-8]</sup>. PPAR alpha, the predominant PPAR isoform in the heart, has been implicated in hypertrophic signaling. The expression of PPAR alpha is significantly diminished during pressure overload-induced hypertrophy. In addition, cardiomyocyte-specific PPAR gamma deficiency has been reported to promote cardiac hypertrophy<sup>[9]</sup>. Several biochemical changes take place in the heart during interstitial fibrosis, including changes in the levels of matrix metalloproteinases (MMPs)<sup>[10]</sup> and the lysosomal cysteine protease, cathepsin S (Cat S)<sup>[11]</sup>.

Whether statins prevent cardiac hypertrophy and fibrosis in ApoE<sup>-/-</sup> mice, however, is not known. Thus, we examined the effects and potential mechanisms of action of simvastatin on cardiac hypertrophy and fibrosis in ApoE<sup>-/-</sup> mice fed a "Western-style diet".

## Materials and methods

### Reagents

Animals: Male ApoE<sup>-/-</sup> mice and C57BL/6J mice weighing 20.0±0.2 g were provided by Beijing Vital River Laboratory Animal Technology Co Ltd. (Certificate No: SCXK 2006-0008).

Simvastatin was kindly provided by MSD Pharmaceutical Co Ltd (Hangzhou, China). The assay kits for total cholesterol (TC), superoxide dismutase (SOD), malondialdehyde (MDA) and nitric oxide (NO) were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The BCA Protein Assay kit and Pierce NE-PER kit were purchased from Pierce Biotechnology (Rockford, USA). The primary antibodies for MMP-9 and the PPARs were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The mouse anti-Cat S monoclonal antibody was purchased from Abcam. Primers were synthesized by Beijing SBS Genetech Co, LTD (China). TRIzol reagent, random hexamer primers, and Superscript II reverse transcriptase were obtained from Invitrogen (Carlsbad, CA). Nitrocellulose membranes and chemiluminescence (ECL Western Blotting Detection Reagents) were purchased from Amersham Pharmacia Biotech. All other reagents were from Sigma Aldrich (St Louis, MO).

### Animals and experimental protocol

Male ApoE<sup>-/-</sup> mice ( $n=36$ ) at 8 weeks of age were housed in a normobaric hypoxic chamber (O<sub>2</sub>: 10.0%±0.5%) or under normoxic conditions and were fed a "Western-style diet" (0.15% cholesterol and 21% fat) for the next 8 weeks. All apoE<sup>-/-</sup> mice of 16 weeks were randomized for treatment with either simvastatin at a dose of 25 mg·kg<sup>-1</sup>·d<sup>-1</sup> or normal saline as the control group by gavage for 8, 16 or 24 weeks ( $n=6$ ). All mice

continued to be fed the "Western-style diet". The mice were cared for in accordance with the standards for laboratory animals established by the People's Republic of China (GB14925-2001).

After the experimental period, the mice were anesthetized by intraperitoneal injection of sodium pentobarbitone (50 mg/kg, ip). Blood samples were collected for the measurement of plasma TC.

All hearts were rapidly excised, immersed in ice-cold saline solution and blotted dry. Any excess connective tissue was removed and the hearts ( $n=3$ ) were fixed in 10% neutral buffered formalin and paraffin embedded. In the remaining hearts ( $n=3$ ), the atria were cut away and the left ventricle (including the intraventricular septum) was carefully dissected from the right ventricle. The left ventricles were stored in liquid nitrogen at -80 °C.

### Preparation of tissue samples

Tissue samples were homogenized in a buffer containing 2% sodium dodecyl sulfate (SDS), 10 mmol/L Tris-HCl (pH 7.4) freshly supplemented with sodium fluoride (10 mmol/L), sodium pyrophosphate (10 mmol/L), sodium orthovanadate (1 mmol/L), sodium molybdate (1 mmol/L), phenylarsine oxide (1 mol/L), and aprotinin (10 µg/mL). Protein concentration was determined in a homogenate aliquot with a BCA Protein Assay kit.

### Biochemical analysis

The plasma and myocardial levels of TC were determined by enzymatic colorimetric methods. The levels of MDA in myocardial homogenates were determined by the TBA method. The activity of SOD was determined by the xanthine oxidase method. NO production was determined with chemiluminescence using a commercial kit.

### Histopathological studies of cardiac hypertrophy

Transverse sections of LV were fixed in 10% buffered formalin and embedded in paraffin. Five-micron-thick sections were stained with hematoxylin and eosin and Masson trichrome. The left ventricular wall thickness and cardiomyocyte diameter were measured as previously described<sup>[12]</sup> using an image analysis program (Image-Pro Plus 3.0). The diameters of at least 100 cardiomyocytes were determined in randomly selected visual fields at 400-fold magnification, and the mean cardiomyocyte diameter was expressed in micrometers.

### Evaluation of collagen deposition

The collagen volume fraction was determined by Masson trichrome stain. Sections were stained by Masson's trichrome according to the manufacturer's guidelines, with red staining indicating muscle fibers, blue indicating collagen, and black indicating nuclei. The sections were analyzed using an Olympus microscope (DP 70, Olympus, Tokyo, Japan), and the images were analyzed using an image analysis program (Image-Pro Plus 3.0). The collagen volume fraction (%) was calculated by determining the area stained for collagen as a

percentage of the total area of sampled tissue and was also quantified using an analysis system. Ten areas were taken at random from each of 4 separate heart samples per group (40 images per group). Morphometric analysis<sup>[8]</sup> of 10 randomly selected fields per section from 10 sections per mouse was performed by an investigator who was blinded to the treatment protocol.

### RNA preparation

Myocardial homogenates were prepared from frozen myocardial specimens from the control and simvastatin treatment groups. Total RNA was extracted using the TRIzol reagent according to the manufacturer's guidelines. RNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm using a spectrophotometer. Only RNA with a 260:280 nm absorbance ratio between 1.7 and 2.1 was accepted as viable RNA. The integrity of the RNA was determined by gel electrophoresis on a standard 2% agarose gel stained with ethidium bromide and visualized by exposure to ultraviolet light.

### Real-time quantitative polymerase chain reaction

The amount of each mRNA was normalized using the corresponding amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Total RNA was extracted from each left ventricle, and 1 µg was used to synthesize cDNA. The expression of *MMP-9*, *Cat S*, *PPAR alpha* and *PPAR gamma* mRNAs was analyzed by real-time quantitative reverse transcription-polymerase chain reaction (real-time PCR). The reverse transcription reaction and PCR for each sample were performed in triplicate using the SYBR ExScript RT-PCR Kit (TaKaRa Bio, Shiga, Japan) and the ABI PRISM 7000 sequence detection PCR system (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. Sequence-specific PCR primers were designed using Beacon designer v4.0 (Premier Biosoft, Palo Alto, CA, USA) (see Table 1 for details). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. All reactions were performed utilizing the following conditions: 2 min at 50 °C; 10 min at 95 °C; 40 cycles of 15 s of melting at 95 °C followed by DNA synthesis for 30 s at 60 °C. Each sample was tested in triplicate. Results were expressed as fold differences for each gene after normalization against GAPDH using the  $2^{-\Delta\Delta CT}$  method. The results were validated by generating a single

PCR product of the expected size on gel electrophoresis and by dissociation curve analysis. Serial cDNA dilution curves were produced to calculate the amplification efficiency for all genes. A graph of the threshold cycle (Ct) versus the log<sub>10</sub> relative copy number of the sample from the dilution series was produced. The slope of the resulting curve was used to determine the amplification efficiency: efficiency=10 (-1/slope).

### Western analysis

Protein was extracted from myocardial tissue using 200 µL of ice-cold lysis buffer (pH 7.4) (50 mmol/L HEPES, 5 mmol/L EDTA, 100 mmol/L NaCl, 1% Triton X-100, protease inhibitor cocktail; Roche, Mannheim, Germany) in the presence of phosphatase inhibitors (50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate, 1 nmol/L microcystin). Nuclear proteins were isolated using the Pierce NE-PER kit according to the manufacturer's instructions. Protein concentrations were determined with the BCA protein assay kit. An equal amount of protein (30 µg) from each sample was separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were then incubated in a blocking solution of phosphate buffered saline (PBS), pH 7.4, containing 5% non-fat milk powder for 1 h at room temperature followed by an overnight incubation with specific antibodies against MMP-9 (1:200), Cat S (1:1000), PPAR alpha (1:500), PPAR gamma (1:1000) and β-tubulin (1:10000). After being washed in PBS containing 0.05% Tween 20, the membranes were incubated for 1 h with peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:1000). The antigen-antibody complexes were revealed by chemiluminescence and visualized by exposure to X-ray films. After being scanned, the optical densities of the bands were quantified with the use of Gel Doc 2000 (Bio-Rad). β-tubulin was used as a loading control to normalize the data. The results were quantified with a bioscanner and expressed as fold increase compared with control.

### Statistical analysis

Continuous variables were expressed as means±SEM. Differences among the groups were compared by ANOVA for phenotypes with equal variance and by the Kruskal-Wallis test for those with unequal variance. Differences at baseline and follow-up in each group were compared by paired Student's *t* tests. *P*<0.05 was considered significant.

**Table 1.** Primer sets for PCR amplification.

| Target gene | Forward primer               | Reverse primer               |
|-------------|------------------------------|------------------------------|
| MMP-9       | 5'-CCAGCTGGCAGAGGCATAC-3'    | 5'-GCTTCTCTCCCATCATCTGGG-3'  |
| Cathepsin S | 5'-AAGCGGTGTCTATGACGACCC-3'  | 5'-GAGTCCCATAGCCAACCCACAA-3' |
| GAPDH       | 5'-GGTTGAGTGAGCAGTTAC-3'     | 5'-GATAACCAGACCACACCTTAGC-3' |
| PPAR-α      | 5'-ACTATGGAGTCCACGCATGTGA-3' | 5'-TTGTCGTACGCCAGCTTTAGC-3'  |
| PPAR-γ      | 5'-AGGACATCCAAGACAACCTG-3'   | 5'-CTCTGTGACAATCTGCCTGA-3'   |

## Results

### Effect of simvastatin on cardiac hypertrophy and fibrosis in ApoE<sup>-/-</sup> mice fed a "Western-style diet"

The control group showed an age-dependent increase in the ratio of heart weight to body weight. Treatment with simvastatin did not affect body weight but did significantly inhibit the ratio of heart weight to body weight at 32 and 40 weeks ( $P<0.05$ ) (Table 2). LV wall thickness and myocardial cell diameter both increased with age (24 vs 40 weeks) in the control ApoE<sup>-/-</sup> mice ( $P<0.05$ ). Compared to those same clinical features, treatment with simvastatin reduced LV wall thickness ( $1.06\pm0.36$  mm vs  $1.30\pm0.21$  mm at 40 weeks,  $P<0.05$ ) and the diameter of myocardial cells ( $4.22\pm0.36$   $\mu$ m vs  $5.23\pm0.88$   $\mu$ m at 24 weeks,  $7.06\pm0.68$   $\mu$ m vs  $8.36\pm0.79$   $\mu$ m at 32 weeks,  $8.29\pm1.24$   $\mu$ m vs  $10.96\pm1.32$   $\mu$ m at 40 weeks;  $P<0.05$ ) (Figure 1).

The LV collagen content also showed an age-dependent

**Table 2.** Effects of simvastatin on the ratio of heart weight to body weight in ApoE<sup>-/-</sup> mice fed with "Western-style diet".  $n=6$ . Quantitative data represent are mean $\pm$ SD. <sup>b</sup> $P<0.05$  significant difference between the control with simvastatin groups of the same age. <sup>e</sup> $P<0.05$  significant difference between the control groups of the different ages.

| ApoE <sup>-/-</sup> Mice | Body weight (g)  | Heart/body weight ratio (mg/g) |
|--------------------------|------------------|--------------------------------|
| 24w                      | 29.89 $\pm$ 0.45 | 4.39 $\pm$ 0.21                |
| 24w+simvastatin          | 30.26 $\pm$ 0.65 | 4.32 $\pm$ 0.34                |
| 32w                      | 31.23 $\pm$ 0.74 | 4.91 $\pm$ 0.28 <sup>e</sup>   |
| 32w+simvastatin          | 32.22 $\pm$ 0.38 | 4.37 $\pm$ 0.41 <sup>b</sup>   |
| 40w                      | 32.87 $\pm$ 0.48 | 5.40 $\pm$ 0.36 <sup>e</sup>   |
| 40w+simvastatin          | 33.43 $\pm$ 0.56 | 4.68 $\pm$ 0.26 <sup>b</sup>   |

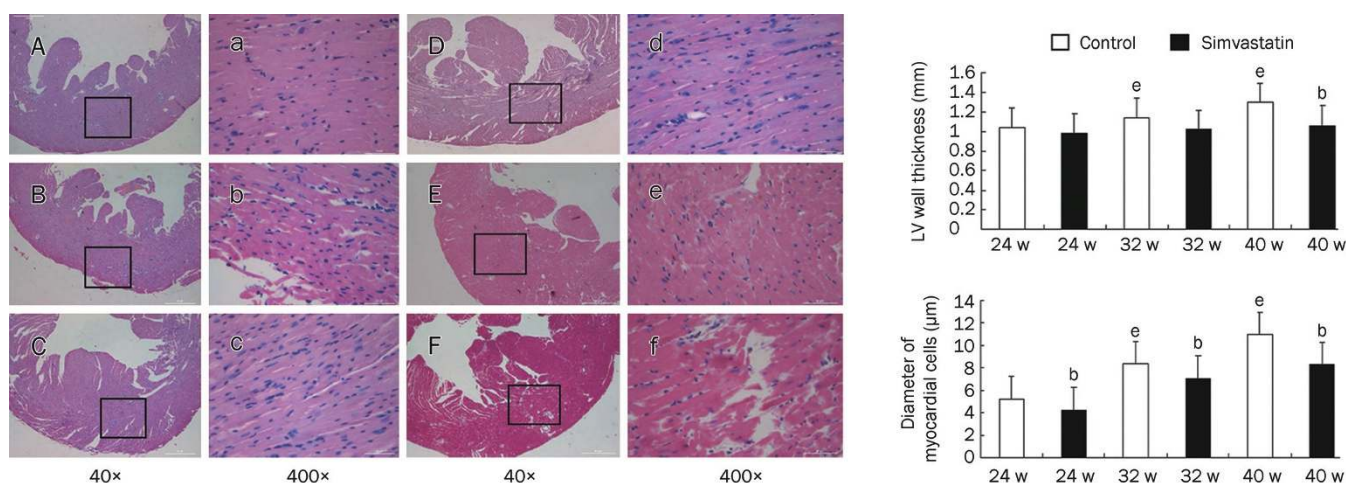
increase in the control mice ( $P<0.05$ ). The collagen volume fraction was reduced in the simvastatin group ( $0.20\pm0.02$  vs  $0.29\pm0.01$  at 32 weeks,  $0.31\pm0.02$  vs  $0.40\pm0.01$  at 40 weeks;  $P<0.05$ ) compared with that of the control group. Representative micrographs of Masson trichrome staining of the LV to determine the extent of fibrosis are shown in Figure 2.

### Effect of simvastatin on the serum and myocardial levels of TC in ApoE<sup>-/-</sup> mice fed a "Western-style diet"

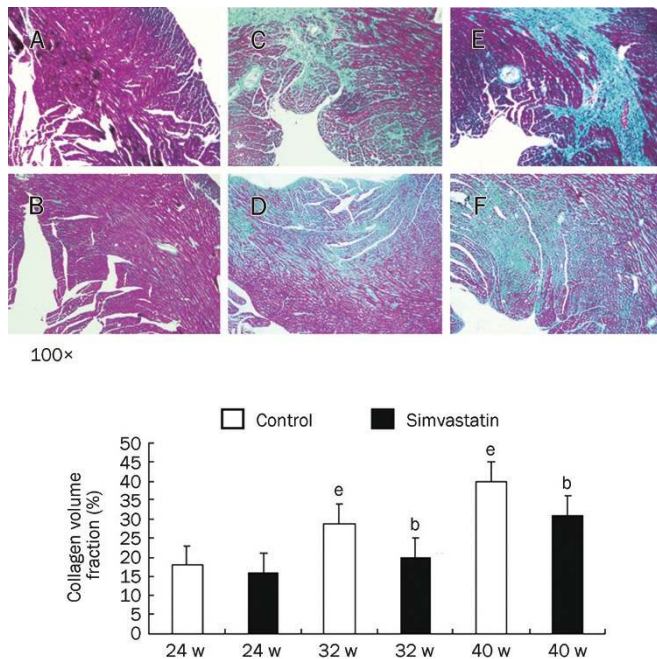
TC levels in the serum and myocardium were measured to investigate a possible relationship between cardiac hypertrophy and lipid levels. There was a significant age-related increase in the levels of serum and myocardium TC in the control ApoE<sup>-/-</sup> mice ( $P<0.05$ ). Serum TC levels were lower in the simvastatin group than in the control group ( $12.80\pm0.33$  mmol/L vs  $16.89\pm0.24$  mmol/L at 24 weeks,  $14.96\pm0.25$  mmol/L vs  $19.56\pm0.36$  mmol/L at 32 weeks,  $18.54\pm0.18$  mmol/L vs  $23.09\pm0.36$  mmol/L at 40 weeks;  $P<0.05$ ). The myocardial TC levels were also lower in the simvastatin group than in the control group ( $3.65\pm0.33$  nmol/mg vs  $4.36\pm0.16$  nmol/mg at 24 weeks,  $5.21\pm0.15$  nmol/mg vs  $6.83\pm0.38$  nmol/mg at 32 weeks,  $7.36\pm0.23$  nmol/mg vs  $10.37\pm0.28$  nmol/mg at the 40 weeks;  $P<0.05$ ) (Figure 3).

### Effect of simvastatin on the myocardial levels of NO, SOD, and MDA in ApoE<sup>-/-</sup> mice fed a "Western-style diet"

The myocardial levels of NO and SOD were decreased, whereas those of MDA were increased with age in the control mice ( $P<0.05$ ). Treatment with simvastatin significantly increased the myocardial levels of both NO ( $26.65\pm4.21$  nmol/mg vs  $21.69\pm1.24$  nmol/mg at 24 weeks,  $32.44\pm2.33$  nmol/mg vs  $18.33\pm2.32$  nmol/mg at 32 weeks,  $38.54\pm0.18$



**Figure 1.** Comparison of the LV wall and myocardial cell diameter in the LV myocardium between the control mice (A: 24 weeks, C: 32 weeks, E: 40 weeks) and the simvastatin-treated mice (B: 24 weeks, D: 32 weeks, F: 40 weeks). The sections were stained with hematoxylin and eosin and visualized at 40 $\times$  and 400 $\times$  magnifications. The control group showed an age-dependent increase in LV wall thickness and myocardial cell diameter. Simvastatin significantly inhibited the thickening of the LV wall at 40 weeks and the increase in the diameter of myocardial cells at 32 and 40 weeks. Representative light micrographs (left) and the combined quantitative data (right) are shown. The quantitative data are given as means $\pm$ SD. <sup>b</sup> $P<0.05$ , significant difference between the control and simvastatin groups of the same age. <sup>e</sup> $P<0.05$ , significant difference between control groups of different ages.

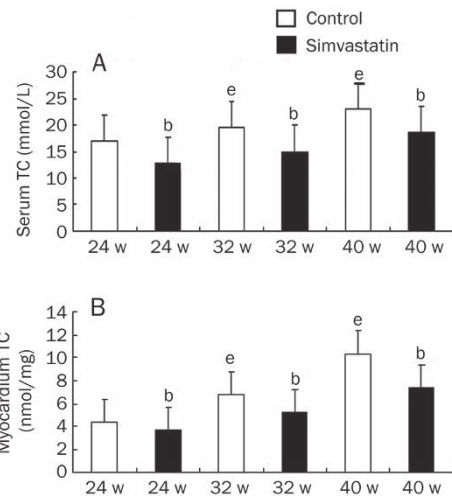


**Figure 2.** Comparison of the levels of fibrosis in the LV myocardium between the control (A: 24 weeks, C: 32 weeks, E: 40 weeks) and simvastatin-treated (B: 24 weeks, D: 32 weeks, F: 40 weeks) ApoE<sup>-/-</sup> mice fed a "Western-style diet". Sections were stained using Masson's trichrome and visualized at 100× magnification. Red indicates muscle fibers, blue indicates collagen, and black indicates nuclei. The control group showed an age-dependent increase in the LV collagen volume fraction, which was significantly inhibited by simvastatin at 32 and 40 weeks. Representative micrographs and the LV collagen volume fraction quantitative data are shown. The quantitative data are given as means±SD. <sup>b</sup>*P*<0.05, significant difference between the control and simvastatin groups of the same age. <sup>e</sup>*P*<0.05, significant difference between control groups of different ages.

nmol/mg vs 10.27±1.88 nmol/mg at 40 weeks; *P*<0.05) and SOD (69.32±1.89 U/mg vs 62.31±1.36 U/mg at 24 weeks, 68.66±2.03 U/mg vs 50.24±2.44 U/mg at 32 weeks, 60.35±2.88 U/mg vs 38.66±2.96 U/mg at 40 weeks; *P*<0.05) but decreased the myocardial levels of MDA (3.56±0.85 nmol/mg vs 5.36±0.39 nmol/mg at 24 weeks, 6.35±0.96 nmol/mg vs 8.66±1.54 nmol/mg at 32 weeks, 9.45±1.08 nmol/mg vs 17.68±3.21 nmol/mg at 40 weeks, *P*<0.05) (Figure 4).

#### Effect of simvastatin on the expression of MMP-9 and Cat S in ApoE<sup>-/-</sup> mice fed a "Western-style diet"

Real-time PCR and Western blot analyses were performed to determine the role of MMP-9 and Cat S in cardiac hypertrophy and fibrosis in ApoE<sup>-/-</sup> mice fed with a "Western-style diet". The expression of MMP-9 and Cat S at both the mRNA and protein levels significantly increased with age in the control mice (*P*<0.05). Simvastatin decreased the mRNA levels of MMP-9 and Cat S at 32 weeks and 40 weeks (Figure 5A and 5B). Similarly, the protein expression of MMP-9 and Cat S in the simvastatin-treated group decreased when compared with the control group (Figure 6).



**Figure 3.** Serum and myocardium TC levels after control and simvastatin treatment in ApoE<sup>-/-</sup> mice fed a "Western-style diet". (A) Serum TC levels. (B) Myocardium TC levels. The control group showed an age-dependent increase in serum and myocardium TC levels, which was significantly inhibited by simvastatin at 24, 32, and 40 weeks. The results are given as means±SD. <sup>b</sup>*P*<0.05, significant difference between the control and simvastatin groups of the same age. <sup>e</sup>*P*<0.05, significant difference between the control groups of different ages.

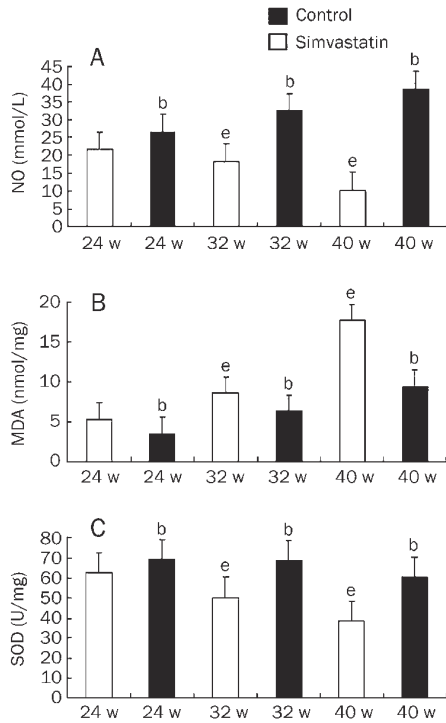
#### Effect of simvastatin on the expression of PPARs in ApoE<sup>-/-</sup> mice fed a "Western-style diet"

Because the activation of PPAR alpha and PPAR gamma has been shown to have a cardio-protective effect in cardiac hypertrophy, we examined the effects of simvastatin on PPAR alpha and PPAR gamma. There was a significant age-dependent decrease in the mRNA and the protein expression of both PPAR alpha and PPAR gamma in the control ApoE<sup>-/-</sup> mice (*P*<0.05). Simvastatin increased the levels of PPAR alpha and PPAR gamma mRNA (Figure 7) and protein (Figure 8) levels at 32 weeks and 40 weeks.

#### Discussion

Hypercholesterolemia is an independent determinant of increased left ventricular mass<sup>[1, 2]</sup>. ApoE<sup>-/-</sup> mice may have age-dependent cardiac hypertrophy<sup>[4]</sup> and fibrosis<sup>[13]</sup>. Several studies have suggested that age-dependent aortic stiffening<sup>[4]</sup> and the direct effects of ApoE that modulate cardiac hypertrophy<sup>[3]</sup> are the primary mechanism, but the exact mechanism is still not clear. The objectives of this study were to determine the possible mechanism responsible for the cardiac hypertrophy and fibrosis in ApoE<sup>-/-</sup> mice fed a "Western-style diet" and the effect of simvastatin intervention.

We performed a randomized study to investigate the cardiac hypertrophy and fibrosis in ApoE<sup>-/-</sup> mice fed a "Western-style diet" and treated with simvastatin for 8, 16, or 24 weeks. Our data show these ApoE<sup>-/-</sup> mice have significantly increased LV wall thickness, myocardial cell diameter and collagen volume fraction with age. Treatment with simvastatin significantly prevents the development of cardiac hypertrophy at the ages of 32 and 40 weeks. Simvastatin treatment was associated

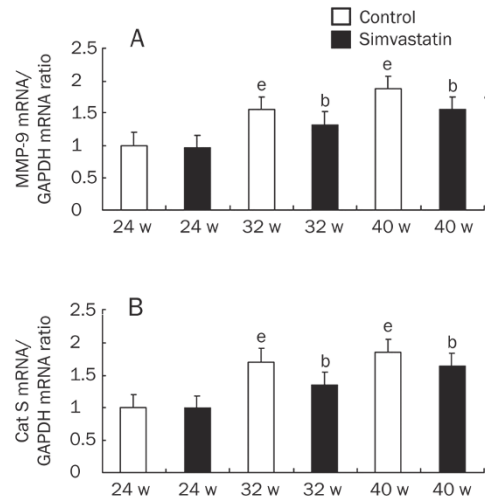


**Figure 4.** Levels of markers of oxidative stress in the myocardium of control and simvastatin-treated ApoE<sup>-/-</sup> mice fed a "Western-style diet". (A) Myocardial NO levels. (B) Myocardial MDA levels. (C) Myocardial SOD levels. The control group showed a significant age-dependent increase in the levels of MDA and a decrease in the levels of NO and SOD. Simvastatin treatment significantly decreased the levels of MDA and increased the levels of NO and SOD at 24, 32 and 40 weeks. The results are given as means±SD. <sup>b</sup>P<0.05, significant difference between the control and simvastatin groups of the same age. <sup>e</sup>P<0.05, significant difference between the control groups of different ages.

with reduced levels of TC, MDA, MMP-9, and Cat S as well as increased levels of NO, SOD, PPAR alpha and PPAR gamma. Taken together, these data indicate that simvastatin induced the regression of cardiac hypertrophy and fibrosis in ApoE<sup>-/-</sup> mice fed a "Western-style diet". Simvastatin had strong beneficial effects on the molecular (reduction of MMP-9 and Cat S levels and augmentation of PPAR alpha and PPAR gamma expression), histological (reduction of fibrosis), and structural (increased LV wall thickness and diameter of myocardial cells) phenotypes. Thus, simvastatin may have the expanded utility of preventing development of cardiac hypertrophy.

Histological analyses confirmed the cardiomyocyte hypertrophy and fibrosis in the ApoE<sup>-/-</sup> mice, as demonstrated by the increase in cardiomyocyte size and extensive collagen deposition. These data are consistent with the studies by Wang *et al*<sup>[4]</sup>, who showed cardiac hypertrophy in 13-month-old ApoE<sup>-/-</sup> mice, and studies by Nakashima *et al*<sup>[13]</sup>, who found myocardial fibrosis in the heart of 40-week-old ApoE<sup>-/-</sup> mice fed a "Western-style diet".

The mechanism by which simvastatin induces the reduction in the hypertrophy and fibrosis is likely to involve upregulat-

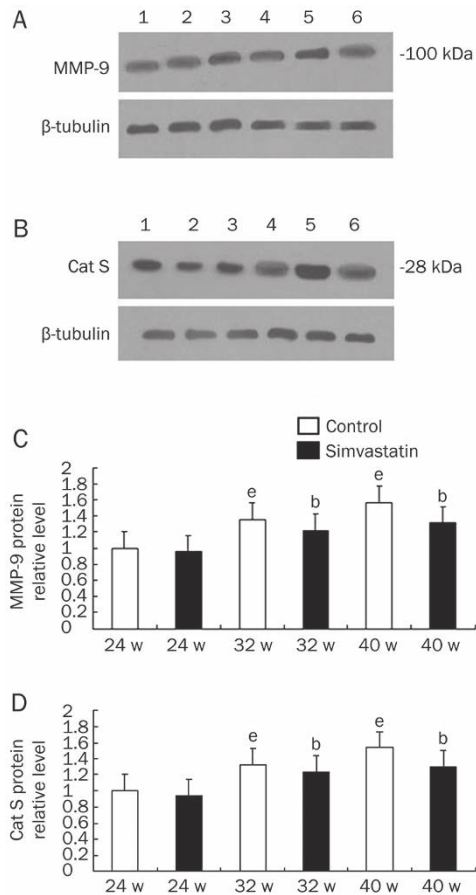


**Figure 5.** Relative quantitative RT-PCR analysis of the mRNAs for MMP-9 (A) and Cat S (B) in the LV myocardium of the simvastatin-treated group and their corresponding age-matched controls (n=6 for each group). The control group showed an age-dependent increase in the expression of MMP-9 and Cat S mRNAs. Simvastatin significantly reduced the expression of MMP-9 and Cat S mRNAs at 32 and 40 weeks (P<0.05). The data are normalized with respect to GAPDH mRNA levels and are given as means±SD. <sup>b</sup>P<0.05, significant difference between the control and simvastatin groups of the same age. <sup>e</sup>P<0.05, significant difference between the control groups of different ages.

ing the levels of activated PPAR alpha and gamma, which are members of the predominant signaling pathway involved in modulating cardiac hypertrophy. The PPARs belong to a superfamily of nuclear hormone receptors of the heart. In the present study, we demonstrated that there was a prominent age-dependent decrease in both the mRNA and the protein expression of PPAR alpha and PPAR gamma in control ApoE<sup>-/-</sup> mice fed with a "Western-style diet". Treatment with simvastatin prevented the cardiac hypertrophy and fibrosis with a significant increase in the mRNA and protein levels of PPAR alpha and PPAR gamma at 32 weeks and 40 weeks. The results are in accordance with the effects of statins for the prevention of cardiac hypertrophy by activating the PPAR pathway and reducing the generation of reactive oxygen species<sup>[8,9]</sup>.

PPAR alpha, the predominant PPAR isoform in the heart, has been implicated in hypertrophic signaling. Smeets *et al*<sup>[14]</sup> showed that the absence of PPAR alpha results in a more pronounced hypertrophic growth response and cardiac dysfunction, which are associated with the increased expression of inflammatory markers and extracellular matrix remodeling. PPAR gamma has been shown<sup>[15]</sup> to attenuate angiotensin-II-induced hypertrophic gene expression and increase cardiomyocyte size *in vitro*. Cardiomyocyte PPAR gamma suppresses cardiac growth and inhibits nuclear factor kappaB activity *in vivo*<sup>[16]</sup>.

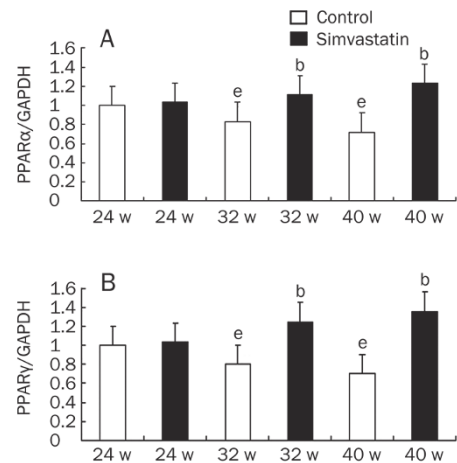
MMPs are a family of structurally and functionally related zinc-dependent endoproteinases that play a pivotal role in



**Figure 6.** Western blot analysis of MMP-9 (A) and Cat S (B) proteins in the LV myocardium of the simvastatin-treated group and their corresponding age-matched controls ( $n=6$  for each group). Tubulin was included as a loading control. From left to right, lane 1: 24-week-old control mouse, lane 2: 24-week-old simvastatin-treated mouse, lane 3: 32-week-old control mouse, lane 4: 32-week-old simvastatin-treated mouse, lane 5: 40-week-old control mouse, lane 6: 40-week-old simvastatin-treated mouse. The control group showed an age-dependent increase in the expression of MMP-9 and Cat S proteins. Simvastatin reduced the expression of MMP-9 and Cat S proteins at 32 and 40 weeks. Representative blots (A and B) and combined quantitative data (C and D) are shown. The levels of MMP-9 and Cat S were normalized against tubulin (bar diagram). The quantitative data represent the band intensity and are given as means $\pm$ SD. <sup>b</sup> $P<0.05$ , significant difference between the control and simvastatin-treated groups of the same age. <sup>e</sup> $P<0.05$ , significant difference between the control groups of different ages.

extracellular matrix (ECM) remodeling through their proteolytic effects<sup>[17]</sup>. The MMPs represent an important biological system within the myocardium and are involved in the maintenance of the complex and dynamic microenvironment of the extracellular matrix<sup>[18]</sup>. MMPs play an important role in the collagen degradation associated with cardiovascular remodeling. Aortic stenosis has been reported<sup>[19]</sup> to induce an increase in MMPs, which could be involved in the collagen degradation that is mitigated by PPAR gamma treatment.

Cat S, a lysosomal cysteine protease<sup>[20]</sup> that belongs to the

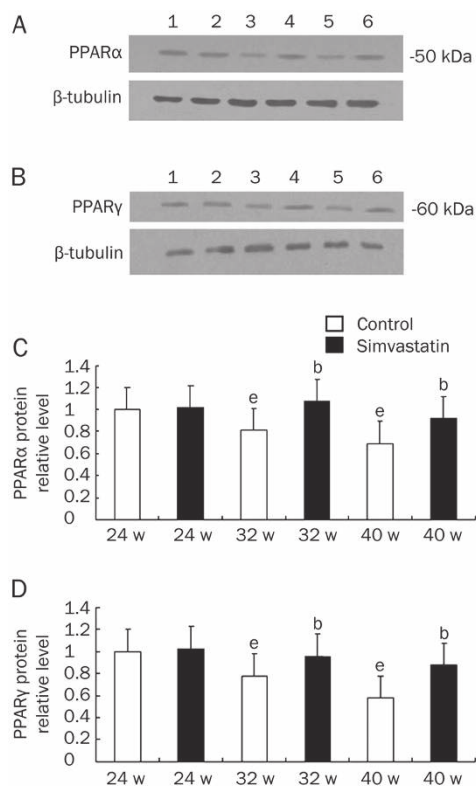


**Figure 7.** Relative quantification of PPAR $\alpha$  (A) and PPAR $\gamma$  (B) mRNAs by RT-PCR analysis in the myocardium of the simvastatin-treated group and their corresponding age-matched controls ( $n=6$  for each group). The control group showed an age-dependent decrease in the expression of PPAR $\alpha$  and PPAR $\gamma$  mRNAs. Simvastatin treatment increased the expression of PPAR $\alpha$  and PPAR $\gamma$  mRNAs at 32 and 40 weeks. The data were normalized against GAPDH and are given as means $\pm$ SD. <sup>b</sup> $P<0.05$ , significant difference between the control and simvastatin-treated groups of the same age. <sup>e</sup> $P<0.05$ , significant difference between the control groups of different ages.

family of papain-like peptidases, plays important roles in pathological LV remodeling by mediating ECM degradation in cooperation with MMPs. Cat S exhibits pronounced elastolytic and collagenolytic activities *in vitro* and *in vivo*<sup>[21]</sup>. Cheng *et al*<sup>[11]</sup> showed that Cat S is expressed at only a low level in the LV myocardium of control rats or normal humans. In contrast, the mRNA and protein levels of Cat S are markedly increased in rats or humans with hypertension-induced heart failure, suggesting that Cat S participates in pathological LV remodeling by mediating ECM degradation in cooperation with the MMPs. Tang *et al*<sup>[22]</sup> found that overexpression of cathepsin L inhibits cardiac hypertrophy and fibrosis by blocking Akt/GSK3 $\beta$  signaling. Blocking the angiotensin II type 1 receptor was reported<sup>[20]</sup> to attenuate cardiac remodeling and dysfunction by inhibiting the expression and activity of cathepsin. Taken together, these observations suggest the importance of cathepsins in cardiac remodeling.

Our study revealed that the abundance of MMP-9 and Cat S mRNAs and protein was significantly increased with age in control ApoE $^{-/-}$  mice fed a “Western-style diet” and that simvastatin decreased the levels of MMP-9 and Cat S mRNA and protein at 32 weeks and 40 weeks. These effects of statins may contribute to the reduction in cardiac hypertrophy and fibrosis noted in this study.

Statins likely attenuate cardiac hypertrophy through the inhibition of oxidative stress<sup>[23]</sup>. NO is an important reactive molecule that controls cardiovascular homeostasis. Mechanisms involving NO have been proposed to contribute significantly to the pathogenesis of cardiac hypertrophy<sup>[24]</sup>. NO can combine with the superoxide anion to form cytotoxic



**Figure 8.** Western blot of PPAR $\alpha$  (A) and PPAR  $\gamma$  (B) in the myocardium of the simvastatin group and their corresponding age-matched controls in ApoE $^{-/-}$  mice fed a "Western-style diet" ( $n=6$  for each group). Tubulin was used as a loading control. From left to right, lane 1: 24 weeks in control group, lane 2: 24 weeks in simvastatin group, lane 3: 32 weeks, control, lane 4: 32 weeks, simvastatin, lane 5: 40 weeks, control, lane 6: 40 weeks, simvastatin. The control group showed an age-dependent decrease in the levels of PPAR $\alpha$  and PPAR $\gamma$  protein expression. Simvastatin increased PPAR $\alpha$  and PPAR $\gamma$  protein expression at 32 and 40 weeks compared with the control group. Representative blots (A and B) and the combined quantitative data (C and D) are shown. The quantification of the PPAR $\alpha$  and PPAR $\gamma$  protein levels normalized using tubulin as a loading control is shown in the bar diagram. The quantitative data represent band intensity and are given as means $\pm$ SD. <sup>b</sup> $P<0.05$ , significant difference between the control and simvastatin groups of the same age. <sup>e</sup> $P<0.05$ , significant difference between the control groups of different ages.

peroxynitrite, a reactive species that is capable of triggering an array of cytotoxic processes, including lipid peroxidation and the activation of MMPs that contribute to cardiac remodeling<sup>[25]</sup>. In our study, the myocardial levels of NO and SOD decreased with age in the control ApoE $^{-/-}$  mice fed a "Western-style diet", and simvastatin significantly increased the myocardial levels of NO and SOD. Takemoto *et al*<sup>[5]</sup> suggested that statins prevent the development of cardiac hypertrophy through an antioxidant mechanism. Recent studies<sup>[25]</sup> have indicated the involvement of NO in cardiac hypertrophy, hypertension, hypercholesterolemia, diabetes, and atherosclerosis. These effects of simvastatin may contribute to the reduction in cardiac hypertrophy and fibrosis noted in ApoE $^{-/-}$

mice.

Although the precise mechanistic basis of cardiac hypertrophy and fibrosis is not known, it is thought to be related in part to a pro-oxidant state<sup>[20]</sup> and the release of angiotensin II and transforming growth factor  $\beta$ 1. In addition, cardiac hypertrophy is accompanied by changes in the cardiomyocyte phenotype characterized by the expression of fetal-type genes, such as the atrial natriuretic peptide and brain natriuretic peptide gene<sup>[26]</sup>. This area also requires further study.

Notably, simvastatin reduced the levels of serum TC in the ApoE $^{-/-}$  mice fed a "Western-style diet". Similarly, rosuvastatin was reported<sup>[26]</sup> to reduce the increased serum levels of TC in low-density lipoprotein receptor knockout mice fed a high cholesterol diet. These results, however, were in disagreement with the results of some prior studies<sup>[23]</sup>. Hypercholesterolemia commonly accompanies atherosclerosis and may be associated with cardiac hypertrophy<sup>[26]</sup>. Further studies on this association need to be performed.

The observed beneficial effects of simvastatin in the ApoE $^{-/-}$  mice fed a "Western-style diet" are in accordance with the effects of statins in the prevention of norepinephrine-induced myocyte hypertrophy<sup>[27]</sup>, a transgenic rabbit model of human hypertrophic cardiomyopathy<sup>[8]</sup> and pressure overload-induced hypertrophy in rats<sup>[7]</sup>. One study provided evidence that rosuvastatin ameliorated cardiac hypertrophy and fibrosis during hypercholesterolemia in low-density lipoprotein receptor knockout mice fed a high cholesterol diet<sup>[26]</sup>. Because the dose of simvastatin used in this study is higher than the conventional dose of simvastatin used in humans (up to 80 mg/d), whether the observed results could be extended to human patients with cardiac hypertrophy needs to be explored.

In summary, this study shows that ApoE $^{-/-}$  mice fed a "Western-style diet" develop age-dependent cardiac hypertrophy and fibrosis. Importantly, simvastatin, a pleiotropic HMG-CoA reductase inhibitor, induces the reduction of cardiac hypertrophy and fibrosis in these ApoE $^{-/-}$  mice, presumably as a result of the upregulation of PPAR alpha, PPAR gamma and NO, as well as the downregulation of MMP-9 and Cat S.

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### Author contribution

Ping YE designed research; Yan-wen QIN performed research; Jie DU contributed new analytical tools and reagents; Ji-qiang HE, Li SHENG, Lu-ya WANG analyzed data; Yan-wen QIN wrote the paper.

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