Laboratory Research

Effects of simvastatin on hypertrophy and PTEN expression of rat cardiac myocytes

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Objective To study the effects of simvastatin on the hypertrophy of cultured rat cardiac myocytes induced by serum and the role of phosphatase and tensin homolog deleted on chromosome ten (PTEN) in the signal pathway. Methods Cultured neonatal Sprague-Dawley (SD) rat cardiac myocytes were treated with 15% fetal bovine serum, or without serum, or different concentrations of simvastatin. Image analysis system was used to measure the cardiac myocytes surface area. Protein synthesis of myocytes was measured via [3H]-leucine incorporation method. The expression level of atrial natriuretic peptide (ANP) mRNA in myocytes was determined with reverse transcription polymerase chain reaction (RT-PCR). The mRNA and protein expression levels of PTEN in cardiac myocytes were investigated with RT-PCR and Western blot respectively. Results At 24 hours, cardiac myocytes surface area was significantly higher in 15% serum group (1611.16±160.75 μm²) than in serum-free group (538.04±118.60 μm², P<0.01). Simvastatin decreased the cell surface area in a concentration dependent manner. The cell surface area in 10⁻⁵ and 10⁻⁴ mol/L simvastatin groups were 799.84±167.70 μm² and 1076.88±199.28 μm² respectively, which were both significantly lower than that in 15% fetal bovine serum group (P<0.01). Incorporation rate of [3H]-leucine was significantly higher in 15% fetal bovine serum group (2360±106cpm/well) than that in serum-free group (1305±92 cpm/well, P<0.01). Incorporation rate of [3H]-leucine in 10⁻⁵ and 10⁻⁴ mol/L simvastatin groups were 1707±101 cpm/well and 1962±125 cpm/well respectively, which were both lower than that in serum group (P<0.01). With the increase of simvastatin concentration, the expression level of ANP mRNA in cardiac myocytes was decreased gradually, which were 0.29±0.03 and 0.40±0.03 respectively in 10⁻⁴ and 10⁻³ mol/L simvastatin groups, and significantly lower than that in serum group(0.60±0.03, P<0.01). Simvastatin increased the expressions of PTEN mRNA and protein in cardiac myocytes in a concentration dependent manner. PTEN mRNA expression level in 10⁻⁵, 10⁻⁴ and 10⁻³ mol/L simvastatin groups were 0.38±0.03, 0.83±0.04 and 0.85±0.05, respectively, which were all higher than that in 15% fetal bovine serum group (0.29±0.04, P<0.05). Similarly, PTEN protein level in 10⁻⁵, 10⁻⁴ and 10⁻³ mol/L simvastatin groups were 39.25±3.41, 46.35±1.78 and 47.22±2.39 respectively) were also significantly higher than that in 15% fetal bovine serum group (32.21±4.06, P<0.05). Conclusion Simvastatin can inhibit the hypertrophy of cultured rat cardiac myocytes induced by serum, and the increase of expression level of PTEN might be involved in the mechanism (J Geriatr Cardiol 2010; 7:47-51).

Key words simvastatin; cardiac myocyte; hypertrophy; PTEN

Introduction

Cardiac myocytes hypertrophy is the leading cellular pathological basis for hypertensive left ventricular hypertrophy (LVH),¹ so the elucidation of the development and regulation mechanisms of cardiac myocytes hypertrophy is of great importance in preventing cardiovascular diseases. It has been reported that phosphatase and tensin homolog deleted on chromosome ten (PTEN), not only is an important antioncogene, but also is involved in modulation of many types of cells proliferation, hypertrophy and apoptosis.² Researchers transfected PTEN into cultured cardiac myocytes and found that it inhibited angiotensin II -induced cardiac myocytes hypertrophy, suggesting that PTEN was probably involved in negative modulation of cardiac myocytes hypertrophy.³ It has been reported that HMG-CoA reductase inhibitor, statins, inhibited the proliferation of vascular smooth muscle cells and cardiac fibroblasts,⁴ but it is still unknown whether statins has effects on cardiac myocytes hypertrophy, whether it is involved in the reversal of hypertensive LVH, and what kind of association exists between its action mechanisms and PTEN expression. In this study, we determined the effects of simvastatin on serum-induced cardiac myocytes hypertrophy and PTEN expression in the cells to investigate the role of simvastatin in improving cardiac myocytes hypertrophy and its signal pathway. The study may provide new theoretic basis and treatment insights at cellular and molecular levels for alleviating LVH clinically.
Methods

Culture and identification of cardiac myocytes

Either-sex of 1 to 3-day-old Sprague-Dawley (SD) rats were provided by Research Center of Experimental Animals in Fourth Military Medical University. The cardiac myocytes were cultured and identified according to previous report with little modification. Briefly, the ventricles of the rats were taken out aseptically, and scissored into 1mm pieces. The tissues were digested with 0.1% type I collagenase at 37°C for 5-10 min with assistance of magnetic stirring. The digestion procedures repeated for 5-7 times. After centrifugation, the cell pellet was collected and prepared into cell suspension upon pipetting. The cells were seeded and allowed to stand for 2 hours at 37°C in 5% CO₂ to attach the flask wall. Difference in adhesion ability made it possible to isolate cardiac myocytes. Cardiac myocytes were seeded into culture flask or plate in density of 4×10⁶/ml. At first 48 hours, 0.1mmol/L BrdU was added to inhibit the growth of non-cardiac myocytes. The medium was changed every 2 days. The cells were identified as cardiac myocytes by spontaneous beating 24 hours after attaching and positive staining for α-striated muscle actin. The cells purity was up to 95%. The cardiac myocytes were seeded at 4×10⁶/ml into 6-well plate with coverslip placed previously. The cells were cultured with medium supplemented with 15% fetal bovine serum, or serum free medium, or medium containing different concentrations of simvastatin (10⁻³, 10⁻⁵, 10⁻⁷, 10⁻⁹mol/L) and 15% fetal bovine serum.

Determination of cardiac myocytes surface area

After 24 hours of intervention, the cultivation was terminated, and the cells on the coverslip were fixed with 95% alcohol for 15 min for HE staining. Leica-Q500 image analysis system was used to determine the cardiac myocytes surface area. Five observation fields were selected randomly on each coverslip, and 5-8 cells within each observation field were chosen for determination of surface area. The mean surface area of the cells were calculated.

Determination of protein synthesis rate with [³H]-leucine incorporation

The cardiac myocytes were seeded in 24-well plate in a density of 4×10⁶/ml, with 1 ml in each well. Prewarmed [³H]-leucine (3.7×10⁶ Bq/ml) at 37°C was added to each well together with the intervention factors. At predetermined time points, the cells were washed with 4°C PBS twice. 4°C 10% trichloroacetic acid for 5min, then 0.5 ml of 0.3 mol/L NaOH-1% SDS was added. The mixture was allowed to stand for 30 min at room temperature. The cell lysates were harvested and transferred to glass-fiber filter paper, and subjected to baking. The radioactivity was demonstrated by liquid scintillation counting.

Extraction of total cellular RNA and RT-PCR

Total cellular RNA was extracted from cardiac myocytes following the instructions of Trizol kit. RT-PCR was performed according to the manufacturer’s instructions (Promega, USA). A final volume of 20 μl reaction mixture included: 10×cDNA buffer, MgCl₂, oligo (dT), 4×dNTP, RNase inhibitor, AMV trascriptase and deionized water. The mixture was subjected to waterbath at 42°C for 60 min. cDNA products 10 μl were taken for PCR of ANP, PTEN and GAPDH. The sequences of the primers used for the different RT-PCRs are shown in Table 1. PCR reaction mixture included 10×PCR buffer, 4×dNTP, sense primer, anti-sense primer, cDNA template, Taq DNA polymerase. The final volume was adjusted to 50 μl with deionized water. The condition of PCR were as follows, denaturation at 95°C for 40 sec, then 35 cycles of annealing at 53°C (ANP), or 60°C (PTEN), or 55°C (GAPDH) for 40 sec, extension at 72°C for 1 min. The PCR products ran on 2% agarose gel. The measured amounts of each mRNA were normalized to the amounts of GAPDH mRNA.

Western blot analysis

At designated time points, the cells were washed with cold PBS, scraped in culture medium, pelleted by centrifugation. The cell pellets were resuspended in 100 μl of 85°C pre-warmed 1×SDS lysis buffer. The lysates were collected in Eppendorf tube, heated for 10 min, centrifuged at 10000 rpm for 10min at room temperature. The supernatant was collected and the protein concentration was determined by Bio-Rad method. A 20 μg of protein from each sample was boiled at 100°C for 5min, then loaded onto 5%-8% SDS-PAGE followed by transfer to nitrocellulose membrane. The membranes were blocked overnight at 4°C with 3% BSA solution. The detection of different proteins was carried out by overnight incubation of the membrane at 4°C with the required dilution of specific antibodies. After washing, the membrane was further incubated with HRP-coupled secondary antibodies. The protein bands were visualized with DAB. LabWork 3.0 UVP software was used to analyze OD to represent relative protein concentration.

Statistical analysis

The data were expressed as means±SD. SPSS10.0 Soft-

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<th>Table1</th>
<th>Primers used for RT-PCR analysis and products length</th>
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<tr>
<td>Primer</td>
<td>Sequence</td>
</tr>
<tr>
<td>ANP</td>
<td>Sense: 5'-TCG AGC AGA TCG CAA AAG ATC-3'</td>
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<td>Anti-sense: 5'-CAC ACT AAA CCA CTC ATC TAC-3'</td>
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<tr>
<td>PTEN</td>
<td>Sense: 5'-TCT ACT CCT CCA ACT CAG GAC-3'</td>
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<td></td>
<td>Anti-sense: 5'-CAT TAT CCG CAC GCT CTA TAC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense: 5'-GTC CAT GCC TGC TTC ACC ACC TCC TTG-3'</td>
</tr>
<tr>
<td></td>
<td>Anti-sense: 5'-GCC GCC TGC TTC ACC ACC TCC TTG-3'</td>
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ware was used. Single factor variance analysis (ANOVA) and LSD test were used. A $P$ value of less than 0.05 was considered significant.

Results

Effects of simvastatin on cardiac myocytes surface area
After treatment with 15% fetal bovine serum, cardiac myocytes surface area was 1611.16±160.75 μm$^2$, significantly higher than that in serum-free control group (538.04±118.60 μm$^2$, $P<0.01$). After intervention with different concentrations of simvastatin together with 15% fetal bovine serum, cardiac myocytes surface area was significantly lower in $10^{-5}$ and $10^{-6}$ mol/L simvastatin groups (799.84±167.70 μm$^2$ and 1076.88±199.28 μm$^2$ respectively) than in 15% fetal bovine serum group ($P<0.01$), while that in $10^{-7}$ and $10^{-8}$ mol/L simvastatin groups (1529.32±212.83 μm$^2$, 1606.84±220.8 μm$^2$) was not significantly different with that in 15% fetal bovine serum group (Fig.1).

Effects of simvastatin on incorporation rate of [3H]-leucine of cardiac myocytes
Incorporation rate of [3H]-leucine in 15% fetal bovine serum group (2360±106 cpm/well) was much higher than that in serum-free group (1305±92 cpm/well, $P<0.01$). It was 1707±101 and 1962±125 cpm/well in $10^{-5}$ and $10^{-6}$ mol/L simvastatin groups respectively, which was lower than that in serum group ($P<0.01$). In $10^{-7}$ and $10^{-8}$ mol/L simvastatin groups, it was 2280±105 and 2311±80 cpm/well respectively, with no significant difference with serum group (Fig.2).

Effects of simvastatin on ANP mRNA expression in cardiac myocytes
The ANP mRNA expression level was significantly higher in serum group than in control group (0.60±0.03 vs 0.21±0.03, $P<0.01$). With the increase of simvastatin concentration, the ANP mRNA expression level was decreased gradually. It was 0.29±0.03 and 0.40±0.03 respectively in $10^{-5}$ and $10^{-6}$ mol/L simvastatin groups, lower than that in serum group ($P<0.01$). In $10^{-7}$ and $10^{-8}$ mol/L groups, it was 0.56±0.03 and 0.59±0.04 respectively, with no difference with serum group (Fig.3).

Effects of simvastatin on PTEN mRNA expression in cardiac myocytes
PTEN mRNA expression level in cardiac myocytes was 0.29±0.04 in 15% fetal bovine serum group, which was significantly higher than that in control group (1.11±0.08, $P<0.01$). It was 0.85±0.05, 0.83±0.04, 0.38±0.03, and 0.29±0.05 in $10^{-5}$, $10^{-6}$, $10^{-7}$ and $10^{-8}$ mol/L simvastatin groups respectively. It was significantly higher in $10^{-5}$, $10^{-6}$ and $10^{-7}$ mol/L simvastatin groups than in serum group ($P<0.05$, Fig.4)
Effects of simvastatin on PTEN protein expression in myocytes

PTEN protein expression level was significantly lower in serum group than in control group (32.21±4.06 vs 56.53±4.36, P<0.01). Simvastatin increased the expression of PTEN protein in myocytes in a concentration dependent manner. PTEN protein level in 10⁻¹, 10⁻⁴, and 10⁻⁷mol/L simvastatin groups was 7.22±2.39, 46.35±1.78, and 39.25±3.41 respectively, significantly higher than that in serum group (P<0.05, Fig. 5).

Fig. 5  PTEN protein expression in myocytes treated with different concentrations of simvastatin. A: control group; B: serum group; C: 10⁻⁴ mol/L simvastatin group; D: 10⁻³ mol/L simvastatin group; E: 10⁻⁷ mol/L simvastatin group; F: 10⁻⁴ mol/L simvastatin group.

Discussion

Researches have shown that statins not only have good lipid-regulation roles, but also inhibit proliferation of vascular smooth muscle cells and cardiac fibroblasts. It might have preventive efficacy on atherosclerosis, restenosis post PTC and cardiac fibrosis. However, it is still unclear about its effects on cultured cardiac myocytes and the specific molecular mechanisms underlying its action.

Fetal bovine serum, containing endothelin-1, angiotensin-II, and alkline fibroblast growth factors, could induce hypertrophy of cardiomocytes. In this study, we found that simvastatin inhibited serum-induced increase of cardiac myocytes surface area and incorporation rate of [³H]-leucine, and decreased the ANP mRNA expression level significantly. Leucine is the precursor of protein synthesis, and examination on incorporation of [³H]-leucine is widely used for detection of protein synthesis rate in cells. Elevated ANP transcription level has been widely accepted as a sensitive index for LVH, cardiac myocytes hypertrophy, and phenotype change. Normally, ANP is mainly secreted by fetal cardiomyocytes and adult atrial myocytes. Adult ventricular cells secreted few ANP. Under stimulation of local pro-hypertrophy factors, ANP gene expression was stimulated in ventricular myocytes, which is not only the compensatory mechanism responsive to injury, but also the molecular marker for myocardium injury. The hypertrophic cardiac myocytes have decreased contraction function and abnormal conductivity, and secrete multiple bioactive factors, which accelerates heart remodeling process. The results in this study indicated that simvastatin inhibited hypertrophy, protein synthesis and phenotype change of cardiac myocytes, which is significant in alleviating hypertensive LVH, improving heart function, and delaying the development of cardiac myocardium from hypertrophy to failure.

PTEN, currently as the only antioncogene with phosphatase activity, can arrest phosphatidylinositol 3-kinase/protein kinase B (PI3K/PKB) pathway by phosphorylating some key enzymes, thus to modulate the cell proliferation, hypertrophy, apoptosis, and survival process. The mice with PTEN gene knock-out showed dramatic increase in heart size and decrease in myocardial contractility, indicating that PTEN might be involved in negative regulation of cardiac myocytes hypertrophy. Till now, it is still unclear how PTEN acts in pathogenesis of hypertensive LVH, and whether it is involved in statins-modulated hypertrophy process of cardiac myocytes. We found that PTEN expression in cardiac myocytes was lower in serum group than in serum-free control group, and that elevated expression level of PTEN was associated with inhibition of serum-induced cardiac myocytes hypertrophy. The results indicate that down-regulation of PTEN expression level might be involved in myocardium hypertrophy, which can be interfered by simvastatin through elevating PTEN expression level. The external pro-hypertrophic factors down-regulated the PTEN expression level in cardiac myocytes, which activated the downstream kinase and transcript factor through PI3K/PKB pathway, thus to motivate myocardium hypertrophy. The cardiovascular protection effects of statins were associated with inhibition of formation of intermediate products isoprene during biosynthesis process of cholesterol and with prohibition of isoprenization and membrane translocation of Ras protein.

Taken together, simvastatin can reverse myocytes hypertrophy induced by serum. Up-regulation of PTEN expression level might be involved in the mechanism. This study provides new theoretical and experimental basis for improvement of LVH at cellular and molecular levels, also offers new thoughts for clinical prevention of hypertensive LVH.

References


