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Effects of simvastatin on apolipoprotein M *in vivo* and *in vitro*

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Abstract

Objective: To investigate effects of lipid lowering drug, simvastatin, on apolipoprotein M expression in the hyperlipidemic mice and in hepatic cell line, HepG2 cells.

Methods: Swiss male mice were randomly divided into the high fat group and control group, and were intragastrically fed with 0.9% saline (control group) or lipid emulsion (high fat group) at the daily dosage of 15 ml/kg body weight, respectively. After 8 weeks feeding, the hyperlipidemic model was successfully induced and these hyperlipidemic mice were then randomly divided into three experimental groups: vehicle control group, high-dose simvastatin-treated group (100 mg/kg body weight), and low-dose simvastatin-treated group (10 mg/kg body weight). Mice were dosed daily for 6 weeks of simvastatin before mice were sacrificed for determining serum lipid profile and apoM protein levels that was determined by using dot blotting analysis. Effects of simvastatin on apoM mRNA expression in the HepG2 cells were determined by real-time RT-PCR.

Results: Comparing to high fat model mice without simvastatin treatment, 100 mg/kg simvastatin could significantly increase serum total cholesterol ($P < 0.05$). Serum apoM levels, in all mice, were significantly lower in the mice at the age of 26 weeks than the mice at 12 weeks old ($P < 0.05$), which indicated that serum apoM levels were significantly correlated to the mice age. It demonstrated also that treatment of simvastatin did not influence serum apoM levels in these mouse model, although serum apoM levels were increased by about 13% in the 10 mg/kg simvastatin group than in the vehicle control group without simvastatin. In HepG2 cell cultures, simvastatin could significantly decrease apoM mRNA levels with dose- and time-dependent manners. At 10 μ M simvastatin treatment, apoM mRNA decreased by 52% compared to the controls.

Conclusion: The present study suggested that simvastatin, *in vivo*, had no effect on apoM levels in the hyperlipidemic mouse model. ApoM serum levels in mice were significantly correlated to the animal's age, whereas in cell cultures simvastatin does inhibit apoM expression in the HepG2 cells. The mechanism behind it is not known yet.

Introduction

Apolipoprotein M (apoM) is one of the latest discovered lipoprotein-associated plasma protein that is mainly synthesized in the liver, and to a smaller amount, in the kidney [1]. In human plasma, most apoM are found in high-density lipoproteins (HDL) and small proportion present also in the apoB-containing lipoproteins, i.e. chylomicrons, very low-, and low-density lipoproteins (VLDL and LDL) [1,2]. Recent investigation has

demonstrated that apoM may participate in the HDL-related biological activities as an important component of HDL particle on the protection of endothelial cells [3]. Wolfrum, et al., [4] reported that apoM is required for pre β -HDL formation and cholesterol efflux to HDL as described for an initial and crucial stage of reverse cholesterol transport, and subsequently protects against atherosclerosis. In addition, the physiological and pathophysiological roles of apoM may also involve in the inflammatory activities and the potential immuno- and inflamm-reactive property of apoM may contribute to the anti-inflammatory function of HDL [5,6]. The statin class of drugs inhibits the enzyme 3-hydroxy-3-

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methylglutaryl coenzyme A (HMG-CoA) reductase, which is the first committed step of sterol synthesis, lead to a lowering of plasma cholesterol levels. In several large clinical trials, the use of statins reduces coronary events. Simvastatin has been shown to reduce total mortality rates in patients with coronary heart disease [7]. Previous studies have shown that the plasma apoM concentration is positively correlated with leptin levels and negatively correlated with total cholesterol in normal and obese subjects [8]. ApoM gene expression could be directly regulated by transcription factors including transforming growth factor (TGF)- β , hepatic nuclear factor (HNF)-1 α , liver receptor homolog (LRH)-1 and forkhead box A2 (Foxa2), all these could regulate hepatic lipid metabolism [9-12]. This may indicate that apoM is involved in lipid and glucose homeostasis. Evidence from different individual laboratories suggests that HMG-CoA reductase inhibitors can down-regulated apoA-IV apoB, apoC-III and apoE, while apoA-I was up-regulated in animal models and cultured hepatocytes [13]. The effect of simvastatin on apoM has not been studied. To examine whether cholesterol-lowering with statin therapy impact on plasma apoM concentration may provide greater insight into the role of apoM in human lipoprotein metabolism. In this study, we investigated if apoM expression is regulated by simvastatin *in vivo* and *in vitro*.

Materials and methods

Cells, animals, and reagents

The human hepatoblastoma cell line, HepG2, was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Male Swiss mice were purchased from Shanghai Slac Laboratory Animal Co. (Shanghai, China). Simvastatin was purchased from International Laboratory Co.(Utah, OREM, USA). Cholesterol, Propylthiouracil and Tween-80 were from Sigma Aldrich (St Louis, USA). Deoxycholic acid sodium salt was obtained from Merck (Germany). ABI PRISM 7700 sequence detection system, real-time RT-PCR reagents and control probe GAPDH were purchased from the Applied Biosystems Inc. (Foster City, CA, USA). Rabbit anti-mouse apoM was from Abnova Corporation, Taiwan. Alkaline phosphatase (AP) conjugated Affinipure goat anti-rabbit IgG was from Jackson ImmunoResearch Laboratories, Inc., USA. BCIP/NBT Color Development Substrate was from Sino-American Biotechnology Company (Luoyang, China). Quantity One Software V4.6.2 was from Bio-Rad. Cell culture flasks (25 cm²) and 6-well cell culture clusters were from Costar, USA.

Animal experiments

Fifty male Swiss mice weighing 30-32 g, at 12-weeks old, were subjected in the study. Mice were acclimatized one

week prior to the experiment and were housed in standard cages at 22°C and 40-60% relative humidity with a 12-hrs light/dark cycle, maintained on standard chow and water ad libitum. The mice were randomly assigned to and housed in ten different cages containing sawdust bedding. For inducing hyperlipidemia, mice were fed on a lipid emulsion that contains 20% (g/100 g) fat of lard, 10% cholesterol, 2% deoxycholic acid sodium salt, 1% propylthiouracil, 10% Tween-80 and double-distilled water. Mice were randomly divided into two groups, i.e., high fat group (fed lipid emulsion) and control group (fed 0.9% saline) at the daily dosage of 15 ml/kg body weight, respectively. After 8 weeks feeding, thirty-nine hyperlipidemic mice successfully induced, which were randomly divided into three experimental groups. And then animals were dosed daily via oral gavage with 10 or 100 mg/kg simvastatin in 0.5% methylcellulose or administered methylcellulose alone (vehicle control) starting at 20 weeks of age. After 6 weeks, mice were anesthetized and sacrificed. Blood samples were taken through tail vein and the serum was centrifuged and stored at -80°C. The housing care of the animals and all the procedures used in these studies were performed in accordance with the guidelines and regulations of the University of Suzhou Institutional Animal Care and Use Committee.

Cell cultures

HepG2 cells were grown in RPMI 1640 with 10% fetal calf serum (FCS) in the presence of benzylpenicillin (100 U/ml) and streptomycin (100 μ g/ml) under standard culture conditions (5% CO₂, 37°C). Cells were seeded in 25-cm² cell culture flasks or in 6-well cell culture clusters, and grown to 50-70% confluence.

Prior to experiments, cells were washed twice with phosphate buffered saline (PBS) and once with serum-free RPMI 1640 without antibiotics. Then the experimental medium, containing RPMI 1640 with 0.5% human serum albumin (HSA) and different concentrations of simvastatin (0 μ M, 3 μ M, 5 μ M, 10 μ M, and 30 μ M) were added. Cells were incubated at 37°C with different time intervals.

Isolation of total RNA and real-time RT-PCR

Total RNA of HepG2 cells was isolated by the guanidinium thiocyanate method [14]. Primer Express software (Applied Biosystems) was used to design human apoM primers and probes based on the TaqMan assay. In order to avoid amplifying the DNA template, the apoM specific primers that are spanned an 81-bp intron was designed to amplify a 66-bp product. The primers were 5'-tgccccggaatggatcta and 5'-cagggcgccctcagtt, and the probe was 5'-FAM-cacctgactgaagggagcacagatctca-TAMRA. Relative standard curves for apoM and

GAPDH were performed to compensate for the efficiency of PCR. A serial dilution of human apoM cDNA was used to generate a standard curve by plotting the cycle threshold versus the log of input cDNA. The apoM and GAPDH standards were linear with the input of cDNA. Quantification of apoM mRNA levels is relative to GAPDH mRNA levels, and was performed on an ABI PRISM 7700 Sequence Detector. The real-time RT-PCR was performed in two steps in a 25 µl reaction mixture containing 1 µl TaqMan Universal PCR Master Mix, 22.5 pmol of both forward and reverse primers, 5 pmol probes and 50 ng of the total RNA templates. Thermal cycling conditions included the following steps: 25°C 10 min, 48°C 30 min, and 95°C 5 min to do reverse transcription, and then the reaction mixture was preheated for 2 min at 50°C and for 10 min at 95°C to activate Taq polymerase. After that, a 40-cycle two-step PCR was performed consisting of 15s at 95°C and 1 min at 60°C. All experiments were performed at least three times in triplicate.

Determination of serum apoM

Serum apoM levels were semi-quantitatively examined by a dot-blot analysis with a specific rabbit anti-mouse apoM antibody. Two-microliter serum samples were applied to the Hybond-C membrane in triplicate. All samples were applied to the same membrane. The membrane was quenched in Tris-HCl buffer in presence of 4% Tween and 3% BSA for 3 hrs, and sequentially incubated with primary antibody (1:1000 dilution in Tris-HCl buffer) overnight at 4°C. After washing by Tris-HCl buffer three times membrane was then incubated with AP conjugated secondary antibody for 2 hrs at room temperature. The development of AP activity was performed with a commercial visualization system according to the manufacturer's instructions. The relative amount of apoM were analyzed with Quantity One Software, and presented as volume (intensity*mm²).

Statistical analyses

Statistical analysis was performed with Graphpad Prism 5.0 software (GraphPad Software Inc.). Results are expressed as means ± SEM. Multiple comparisons were performed with one-way ANOVA/Turkey, and

comparisons between after and before simvastatin treatment of each group were statistically evaluated by the paired *t*-test. Significance was established at a *P* value less than 0.05.

Results

Serum lipid profile in mice after a high fat diet and effects of simvastatin on serum levels of lipid and apoM

As shown in table 1, the serum profile of LDL-cholesterol, total cholesterol were significantly increased in the mice under oral gavaged for 8 weeks compared to the control group by 154% and 85%, respectively. Serum triglycerides were didn't change after 12 weeks high fat feeding, whereas after 20 weeks feeding, serum triglycerides were even slightly decreased in these mice compared to controls. In the hyperlipidemic mice after treated with simvastatin, either in the low dose (10 mg/kg body weight) or high dose (100 mg/kg body weight), unexpectedly, serum total cholesterol levels were significantly increased compared to the vehicle control mice and normal control mice (Table 2). The same phenomenon was seen in LDL-cholesterol levels too. As shown in Figure 1, there were no statistical significant differences on serum apoM levels in these mice at 26 weeks, although serum apoM levels were slightly increased (about 13%) in the 10 mg/kg simvastatin-treated mice compared to the vehicle control mice (Figure 1). As shown in Figure 2, serum apoM levels, in all mice, were dramatically lower in the mice at the age of 26 weeks than in the mice at 12 weeks old (*P* < 0.05). At the 26th week, serum apoM levels were decreased by 26% (*P* < 0.01) and 17% (*P* < 0.05), respectively, compared to 12 or 20 weeks of normal control mice. It is also demonstrated that neither low dose (10 mg/kg body weight) nor high dose (100 mg/kg body weight) influence serum apoM levels.

Effects of simvastatin on apoM mRNA levels in HepG2 cell cultures

As shown in Figure 3, simvastatin could significantly inhibited apoM expression in the HepG2 cells, with dose- and time-dependent manners. Simvastatin at 10 µM decreased apoM mRNA in HepG2 cells by about 52% compared to the controls (*P* < 0.01) (Figure 3).

Table 1 Serum lipid profile in mice fed a high fat diet

Lipids (mmol/L)	Control diet			High fat diet		
	12 weeks	20 weeks	<i>P</i>	12 weeks	20 weeks	<i>P</i>
TC	2.44 ± 0.22	2.64 ± 0.20	<i>P</i> > 0.05	2.97 ± 0.09	4.89 ± 0.18	<i>P</i> < 0.0001
LDL-C	0.40 ± 0.03	0.40 ± 0.02	<i>P</i> > 0.05	0.47 ± 0.01	0.99 ± 0.05	<i>P</i> < 0.0001
HDL-C	1.53 ± 0.10	1.61 ± 0.12	<i>P</i> > 0.05	1.73 ± 0.06	1.69 ± 0.06	<i>P</i> > 0.05
TG	1.53 ± 0.10	1.67 ± 0.15	<i>P</i> > 0.05	1.57 ± 0.07	0.98 ± 0.04	<i>P</i> < 0.0001

Data are shown as means ± SEM. TC (total cholesterol), HDL-C (high density lipoprotein cholesterol), LDL-C (low density lipoprotein cholesterol), TG (triglyceride).

Table 2 Effects of simvastatin on serum lipid levels (mmol/L)

Groups	N	TC	TG	HDL-C	LDL-C
Normal control	6	2.39 ± 0.17	2.30 ± 0.26	0.27 ± 0.03	0.26 ± 0.02
Vehicle control	9	3.59 ± 0.22 ^a	1.58 ± 0.11 ^b	1.17 ± 0.10 ^b	0.73 ± 0.06 ^b
Simvastatin (10 mg/kg)	10	3.84 ± 0.18	1.19 ± 0.08	1.34 ± 0.12	0.76 ± 0.04
Simvastatin (100 mg/kg)	14	4.49 ± 0.25*	1.33 ± 0.09	1.59 ± 0.17	0.93 ± 0.09

Data are shown as means ± SEM. TC (total cholesterol), HDL-C (high density lipoprotein cholesterol), LDL-C (low density lipoprotein cholesterol), TG (triglyceride). **P* < 0.05 vs. vehicle control, ^a*P* < 0.05 vs. normal control, ^b*P* < 0.01 vs. normal control.

Discussion

ApoM is one of the latest discovered human apolipoprotein predominantly present in HDL in human plasma, and its physiological and patho-physiological roles remain to be clarified [1]. Experiments in transgenic mice suggested apoM may have anti-atherogenic properties and the possible mechanisms include increased formation of pre-β HDL, enhanced cholesterol mobilization from foam cells, and increased antioxidant properties [2,4]. Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, have revolutionized the treatment of hypercholesterolemia. They are the most efficient agents for reducing plasma cholesterol, being also appreciated for their good tolerance. Angiographic studies have demonstrated that these compounds reduce the progression and may induce the regression of atherosclerotic lesion in cardiovascular vessels, which could lead to a significant decrease of cardiovascular morbidity and mortality [15]. Statins have been shown to inhibit hepatic production of apoA-IV apoB, apoCIII and apoE-containing lipoproteins both *in vitro* and *in vivo* [13,16].

In the present study, we demonstrated that simvastatin, *in vivo*, had no effect on apoM levels in the

hyperlipidemic mouse model or in normal mice. Interestingly apoM serum levels in mice were significantly correlated to the animal's age. In wild-type mouse plasma, cholesterol was predominantly found in HDL and apoM was also recovered in the same fractions as HDL [17]. This may partly explain the reason why normal control mice had a pronounced decrease in HDL cholesterol [18]. The detailed mechanism needs further investigation. However, in HepG2 cell cultures, simvastatin does inhibit apoM expression. We don't have the reasonable explanation on such difference between the animal models and cell cultures. In addition, in the present study, we demonstrated that serum triglyceride levels were significantly decreased in the high fat diet group mice compared to normal control mice at 20 weeks, which may be possible that the high fat diets contain some cholates. As a bile salt, it is a ligand for the nuclear hormone receptor FXR, whose activity regulates the expression of multiple genes involved in lipoprotein metabolism. Among these genes are apoCII and apoCIII, whose alterations of expression could account for the fact that the presence of cholate in the diet is associated with a lower plasma triglyceride than is the

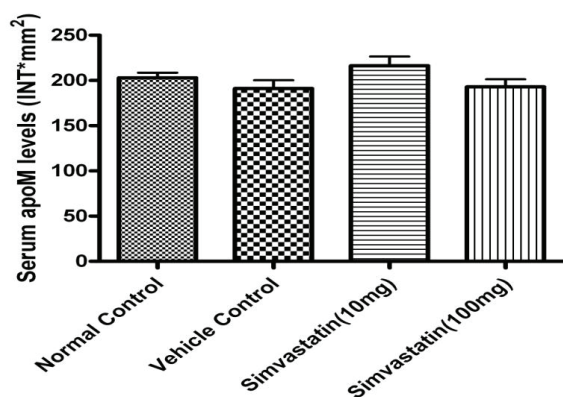


Figure 1 Serum apoM levels in mice treated with simvastatin for 6 weeks. ApoM concentrations were determined by dot-blotting analyses as described in the materials and methods. Data are expressed as the intensity*mm² that was analyzed by the software of Quantity One. Data are means ± SEM.

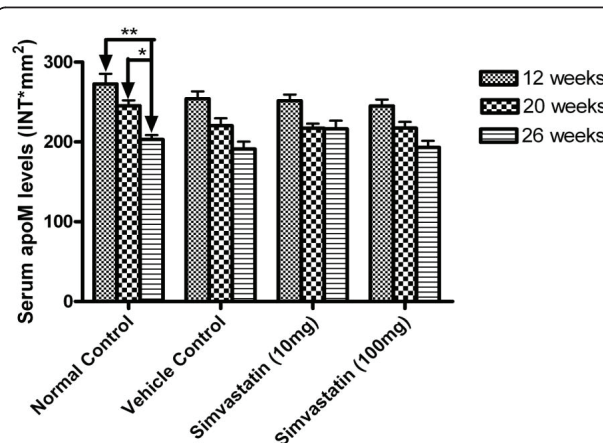
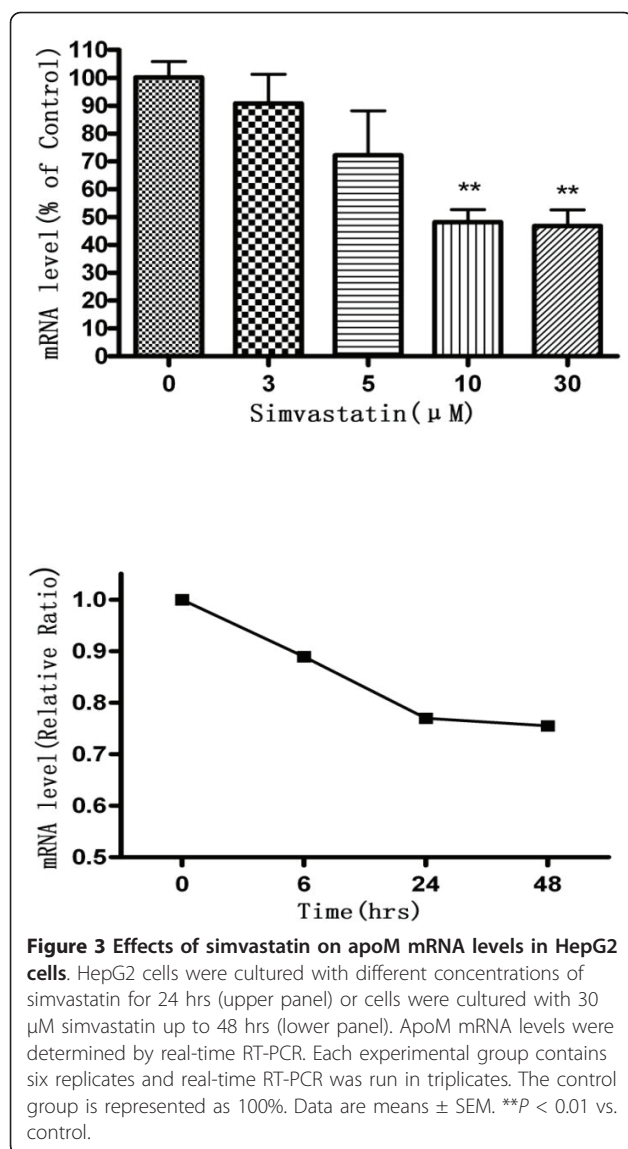


Figure 2 Serum apoM levels in mice at different time intervals. ApoM concentrations were determined by dot-blotting analyses as described in the materials and methods. Data are expressed as the intensity*mm² that was analyzed by the software of Quantity One. Data are means ± SEM. **P* < 0.05 vs. 20-week normal control, ***P* < 0.01 vs. 12-week normal control.



case for similar diets without cholate [19]. It is interesting to note that simvastatin could elevate serum cholesterol in Swiss mice when dosed at 100 mg/kg body weight/daily for 6 weeks in this study, which is consistent with previous observations by Wang et al [20], who reported that simvastatin could increase serum cholesterol in mice. The mechanism responsible for this unexpected increase of serum cholesterol is unknown yet. One possible explanation may be related to the discrepancies of various species. It may also be possible that higher continuous dose of simvastatin had stimulatory effects on the expression and activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the liver [21], which lead to an increased cholesterol levels in these mice. The detailed mechanism needs further investigation.

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Authors' contributions

XZ, SM, GL and JW carried out experiments, data collection, performed the statistical analysis and drafted the manuscript. MBS, PNE and NX participated in the design of the experiments and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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