Effect of simvastatin on the expression and regulation mechanism of apolipoprotein M

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Abstract. Apolipoprotein M (ApoM) is a recently discovered human apolipoprotein predominantly present in high-density lipoprotein (HDL) in the plasma. Statins have effects on many HDL-associated apolipoproteins. However, it is unknown whether statins have effects on ApoM. In the present study, we investigated the effects of simvastatin on ApoM expression and the underlying mechanism(s). Simvastatin up-regulated hepatic ApoM mRNA and protein expression in mice. In HepG2 cells, simvastatin significantly enhanced ApoM mRNA and protein expression in a dose-dependent manner. Simvastatin increased hepatic hepatocyte nuclear factor-1α (HNF-1α) mRNA and reduced liver X receptor-α (LXRα) mRNA expression in mice. The simvastatin-induced up-regulation of ApoM was blocked by an HNF-1α inhibitor (UCDA) or an LXRα agonist (TO901317) in HepG2 cells which indicates that this effect is mediated via the regulation of HNF-1α and LXRα. In conclusion, simvastatin significantly up-regulated ApoM expression *in vivo* and *in vitro*, which indicates that ApoM is another novel apolipoprotein regulated by simvastatin. The mechanism of this effect is related to the regulation of HNF-1α and LXRα.

Introduction

The level of high-density lipoprotein cholesterol (HDL-C) has a negative correlation with the incidence of coronary heart disease (1,2). The effect of HDL-C on anti-atherosclerosis is related to its key role in reverse cholesterol transport (RCT) (3). Apolipoprotein M (ApoM) is a newly discovered apolipoprotein related to HDL-C that plays an important role in HDL-C metabolism (4,5). ApoM is mainly expressed in hepatocytes and in the tubular epithelial cells of the kidney (6). Inhibiting apolipoprotein M production by the liver using siRNA results

in a decrease in HDL cholesterol levels, an increase in the size of HDL, and the absence of pre-β HDL (7). Moreover, ApoM-deficient HDL was not as effective as normal HDL in facilitating the transport of cholesterol out of macrophages to HDL (7). Furthermore, ApoM affects RCT mainly through the regulation of pre-β HDL generation. ApoM is irreplaceable in the formation of HDL-C and the process of RCT. Expression of the ApoM gene in the liver is regulated by transcription factors that control key steps in hepatic lipid and glucose metabolism. Hepatocyte nuclear factor-1α (HNF-1α) and liver X receptor-α (LXRα) are two key nuclear receptors regulating ApoM expression (8,9). Statins are widely used lipid-lowering drugs. Research has shown that statins can increase HDL-C levels and improve RCT, but the mechanism remains unclear. To discover whether statins can increase HDL-C and promote RCT through ApoM, we examined the effect of simvastatin on ApoM expression.

Materials and methods

Materials and reagents. Male 8-week-old C57BL/6N mice were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. The human hepatocellular carcinoma cell line, HepG2, was provided by the cell center of the Xiangya School of Medicine, Central South University. Other reagents included: a quantitative PCR kit (Promega); an ApoM antibody (Abcam); simvastatin original powder (Sigma); an HNF-1α inhibitor (UCDA, Sigma); and an LXRα agonist (TO901317, Wako Pure Chemicals, Inc.). ApoM, HNF-1α, and LXRα primers were provided by AuGCT Biotechnology (Beijing, China).

Animal experiments. This protocol was approved by the Second Xiangya Hospital, Central South University. Sixteen 8-week-old male C57BL/6N mice were fed in single-cages (rearing environment: light and dark cycles were changed every 12 h; temperature, 24-28˚C; relative humidity, 60-75%). Mice were acclimatized one week prior to the experiment. The mice were randomly divided into two groups and received different treatments (n=8/group): i) control group: normal diet; ii) statin group: 10 mg/kg/day simvastatin for 4 weeks of drug intervention; simvastatin was diluted by 0.9% saline and was administered by a gavage tube once daily. Four weeks later, the mice were sacrificed. The liver tissues were removed.

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Cell cultures. DMEM culture medium containing 10% fetal bovine serum was used for culturing HepG2 cells. The cells were cultured in a carbon dioxide incubator at 37˚C. The culture medium was supplemented with 1.0×10^5 U/l penicillin and 1.0×10^5 U/l streptomycin. The medium was changed every two days and the cells were passaged. The simvastatin groups were divided according to the concentration of simvastatin: group 0 (containing 0.1% DMSO, the control group) and 1, 5, 10, and 25 μmol/l simvastatin. Other groups included: simvastatin (25 μmol/l) + HNF-1α inhibitor (100 μmol/l UCDA) group and the simvastatin (25 μmol/l) + LXRα agonist (100 μmol/l TO901317) group; The expression of ApoM, HNF-1α and LXRa was determined in every group.

Real-time fluorescence relative quantitative RT-PCR. Total-RNA was isolated from mouse liver or HepG2 cells using TRI-Reagent. RNA solutions underwent formaldehyde denaturing and agarose gel electrophoresis. MOPS (1X) was used as the running buffer and a 5 V/cm constant current and constant voltage were set for electrophoresis. Ten minutes after staining, the gels were observed under ultraviolet light. Synthesis of cDNA was performed according to the instructions of a reverse transcriptase (AMV). The mRNA sequences of ApoM and GAPDH were designed and synthesized according to reports in the PubMed database and other related literature. The PAGE method was used for purification. ApoM primer sequences were the following: mApoM, F, 5'-CAGTGC CCTGAGCACAGTCAA-3' and R, 5'-GCTGCTCCCGCAA TAAAGTACC-3'; hApoM, F, 5'-CTGACAACTCTGGGC GTGGA-3' and R, 5'-CAGAGCCAGCAGCCATATTGAA-3'; HNF-1α primers: mHNF-1α, F, 5'-GCCTGCTGAGTACA GAAGCCAAG-3' and R, 5'-GGGTAGTGCATGACCTGTTC GTAG-3'; hHNF-1α, F, 5'-GAAAGCCGTGGTGGAGTCA-3' and R, 5'-GGATGTTGTGCTGCTGCAAG-3'; LXRα primer sequences: mLXRα, F, 5'-TCAGCATCTTCTCTGCAGAC CGG-3' and R, 5'-TCATTAGCAGCCGTGGGAACA-3'; hLXRα, F, 5'-AGAACAGATCCGCCTGAAGA-3' and R, 5'-AGCCTCTCCACCTGGAGCTGGT-3'. To ensure the uniformity of the amount of RNA in samples, GAPDH was used as an internal reference. GAPDH primers were as follows: mGAPDH, F, 5'-ACAGCAACAGGGTGGTGGAC-3' and R, 5'-TTTGAGGGTGCAGCGAACTT-3'; hGAPDH, F, 5'-CCA TGTTCGTCATGGGTGTGAACCA-3' and R, 5'-GCCAGTA GAGGCAGGGATGATGTTC-3'. The SYBR-Green real-time PCR reaction solution was prepared and the PCR conditions were set according to the manufacturer's instructions. The data were analyzed for target gene expression by the $2^{-\Delta\Delta Ct}$ method.

Western blotting for detection of ApoM expression. Proteins were isolated from mouse liver or HepG2 cells. The protein concentrations were determined by the BCA method. Protein (50 μg) was added to 2X SDS gel sample buffer and bathed in 100˚C water for 10 min followed by immediate placement on ice. Electrophoresis on a 6% SDS polyacrylamide gel was performed and the protein was transferred to a PVDF membrane. The membrane was blocked with 5% milk for 2 h. The ApoM antibody was added and incubated at 4˚C overnight. TBST was used for washing three times for 10 min. Horseradish peroxidase-labeled secondary antibody was added at a concentration of 1:2,000 and incubated for 1 h at
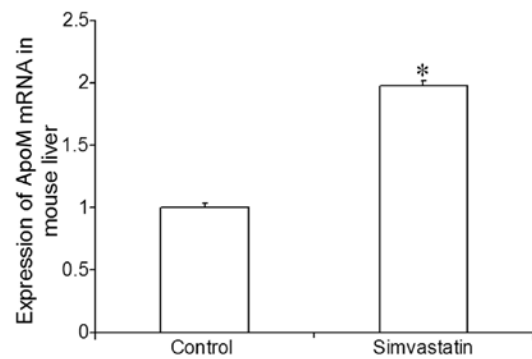

Figure 1. Effect of simvastatin on ApoM mRNA expression in mouse liver. ApoM mRNA levels were determined by real-time RT-PCR. Each experimental group contains 8 replicates. The control group is represented as 100%. Data are means ± SD.*P<0.05, as compared with control.
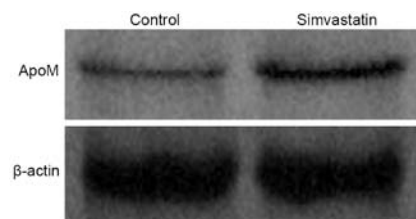

Figure 2. Effect of simvastatin on ApoM protein expression in mouse liver ApoM protein levels were measured by Western blotting. Each experimental group contains 8 replicates.

room temperature. The film was washed with TBST 3 times. A Western blotting fluorescence detection kit was used and the results were visualized on X-ray film. Absorbance was analyzed using image analysis software. The size of the gray value of the control group was set as 100% and compared with the experimental group. A semi-quantitative analysis was performed.

Statistical analysis. The experimental data are presented as the mean ± standard deviation. One-way ANOVA analysis was performed for comparisons among multiple groups. P<0.05 was set as statistical significance.

Results

Effects of simvastatin on the ApoM expression in mouse liver and HepG2 cells. Our experiments examined the effect of simvastatin on the ApoM expression in mouse liver and HepG2 cells. At the fourth week, ApoM gene (ApoM mRNA) expression in mouse liver was determined using real-time RT-PCR. The results showed that compared with the control group, ApoM mRNA levels were significantly increased by 2-fold in the statin group (Fig. 1). Furthermore, mouse liver ApoM protein expression detected by Western blotting (WB) showed consistent results with the ApoM mRNA expression (Fig. 2). To evaluate the effect of simvastatin on ApoM expression in HepG2 cells, we incubated HepG2 cells with increasing concenrations (1-25 μmol/l) of simvastatin for 24 h. As shown in Figs. 3 and 4, simvastatin dose-dependently up-regulated ApoM gene and protein expression in HepG2 cells.
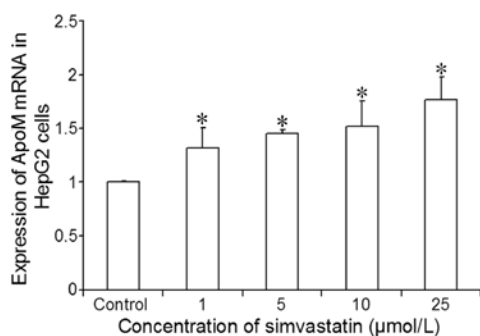
Figure 3. Effect of different concentrations of simvastatin on the expression of ApoM mRNA in HepG2 cells. HepG2 cells were grown in the culture medium with different concentrations of simvastatin for 24 h. ApoM mRNA levels were determined by real-time RT-PCR. Each experimental group contains 6 replicates and real-time RT-PCR was run in triplicates. The control group is represented as 100%. Data are means ± SD. *$P<0.05$, as compared with control.
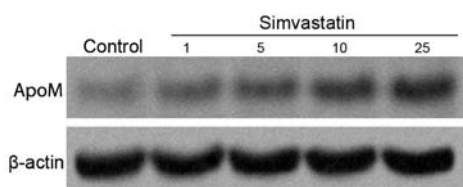

Figure 4. Effect of different concentrations of simvastatin on the expression of ApoM protein in HepG2 cells. HepG2 cells were grown in the culture medium with different concentrations of simvastatin (1-25 μmol/l) for 24 h. ApoM protein levels were measured by Western blotting. The experiment was run in triplicate.
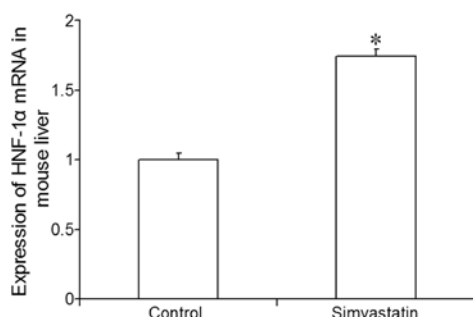

Figure 5. Effect of simvastatin on HNF-1α mRNA expression in mouse liver ApoM mRNA levels were determined by real-time RT-PCR. Each experimental group contains 8 replicates and real-time RT-PCR was run in triplicates. The control group is represented as 100%. Data are means ± SD. *$P<0.05$, as compared with control .

Effects of simvastatin on the HNF-1α and LXRα mRNA expression in mouse liver and HepG2 cells. HNF-1α and LXRα have been shown to be the key transcription factors regulating apolipoprotein M expression (8,9). We therefore also assayed the effect of simvastatin on HNF-1α and LXRα expression. HNF-1α and LXRα mRNA expression in mouse liver was determined by real time RT-PCR. The results showed that compared with the control group, HNF-1α mRNA expression in the statin group was significantly increased by 1.7-fold and LXRα mRNA in the statin group was decreased to 65% of control (Figs. 5 and 6). In HepG2 cells, simvastatin
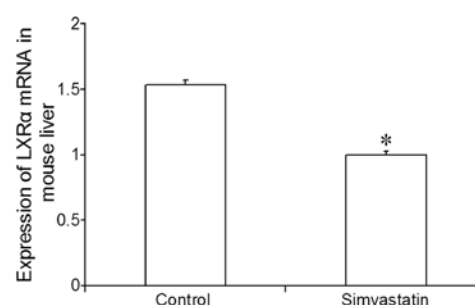

Figure 6. Effect of simvastatin on LXRα mRNA expression in mouse liver. ApoM mRNA levels were determined by real-time RT-PCR. Each experimental group contains 8 replicates and real-time RT-PCR was run in triplicates. The simvastatin group is represented as 100%. Data are means ± SD. *$P<0.05$, as compared with control.

Figure 7. Effect of different concentrations of simvastatin on the expression of HNF-1α mRNA in HepG2 cells. HepG2 cells were grown in the culture medium with different concentrations of simvastatin for 24 h. HNF-1α mRNA levels were determined by real-time RT-PCR. Each experimental group contains 6 replicates and real-time RT-PCR was run in triplicates. The control group is represented as 100%. Data are means ± SD. *$P<0.05$, as compared with control .

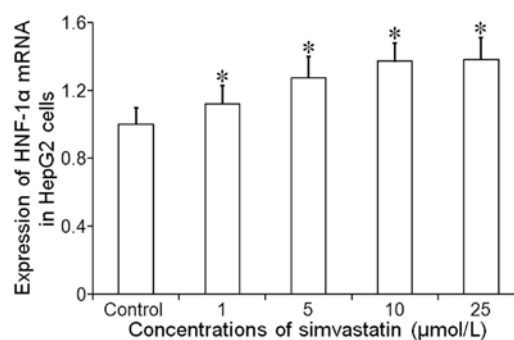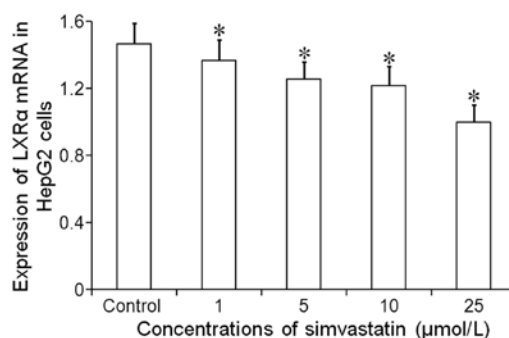

Figure 8. Effect of different concentrations of simvastatin on the expression of LXRα mRNA in HepG2 cells. HepG2 cells were grown in the culture medium with different concentrations of simvastatin for 24 h. LXRα mRNA levels were determined by real-time RT-PCR. Each experimental group contains 6 replicates and real-time RT-PCR was run in triplicate. The 25 μmol/l simvastatin group is represented as 100%. Data are means ± SD. *$P<0.05$, as compared with control.

dose-dependently up-regulated HNF-1α mRNA expression and down-regulated LXRα mRNA expression (Figs. 7 and 8).

Effect of an HNF-1α inhibitor and an LXRα agonist on simvastatin-induced ApoM expression in HepG2 cells. To further
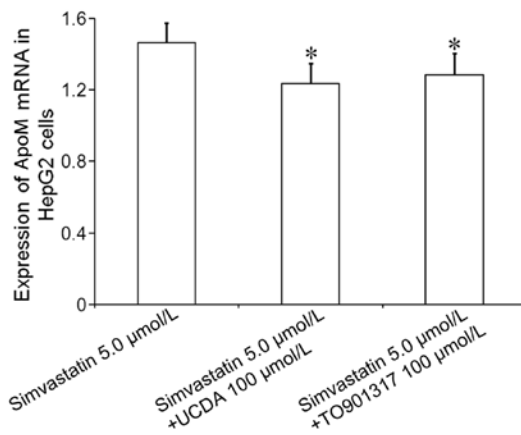
Figure 9. The HNF-1α inhibitor and LXRα agonist inhibit statin-induced up-regulation of ApoM mRNA expression in HepG2 cells. Cells were incubated with simvastatin (5 µmol/l) in the presence of the HNF-1α inhibitor (100 µmol/l UCDA) or LXRα agonist (100 µmol/l TO901317) for 24 h. ApoM mRNA levels were determined by real-time RT-PCR. Each experimental group contains 6 replicates and real-time RT-PCR was run in triplicate. Data are means ± SD. *P<0.05, as compared with control.

Figure 10. HNF-1α inhibitor and LXRα agonists inhibit statin-induced up-regulation of ApoM protein expression in HepG2 cells. Cells were incubated with simvastatin (5 µmol/l) in the presence of HNF-1α inhibitor (100 µmol/l UCDA) or LXRα agonist (100 µmol/l TO901317) for 24 h. ApoM protein levels were measured by Western blotting. The experiment was run in triplicate.

study the mechanism of simvastatin-induced ApoM expression, HepG2 cells were administered 5.0 µmol/l of simvastatin in the presence of a HNF-1α inhibitor (UCDA) or LXRα agonist (TO901317). As shown in Fig. 9, both UCDA and TO901317 could inhibit the up-regulation of ApoM mRNA caused by simvastatin. Consistently, UCDA and TO901317 could abolish the simvastatin-induced ApoM protein expression (Fig. 10).

Discussion

A large number of epidemiological studies have shown that HDL-C level is negatively correlated with the risk of coronary heart disease (1,2). Many clinical trials also indicate that the application of statins and fibrates in treatment of patients with low HDL-C level reduce cardiovascular events (10,11). The effect of HDL-C on anti-atherosclerosis is related to its key role in RCT. ApoM is a newly identified apolipoprotein that is an important component of high density lipoprotein (4). ApoM plays an important role in HDL formation and RCT (7).

Statins play an important role in the prevention and treatment of coronary artery disease and are used as a first-line therapy for the lowering of LDL-C. Statins can also slightly increase HDL-C and promote cholesterol efflux, but the mechanism is not clear. Research has shown that the effects of statins on HDL-C levels might be related to the decreased activity of cholesterol ester transfer protein and stimulation of apolipoprotein A I (apoA I, major protein of HDL) transcription and synthesis (12). One study showed that statin induced increases in apo A-I levels through the activation of its promoter, the peroxisome proliferator activator receptor-α (PPAR-α) (13). Statins also increase cholesterol efflux by up-regulating the HDL-C receptor, such as ATP binding transporter A1 (ABCA1) and Scavenger receptor class B type I (SRB1) (14,15). However, our study showed for the first time that simvastatin could up-regulate the ApoM gene and protein expression, suggesting that the regulation of HDL-C level and the promotion of cholesterol efflux by simvastatin are related to its stimulation of ApoM expression. These results provide
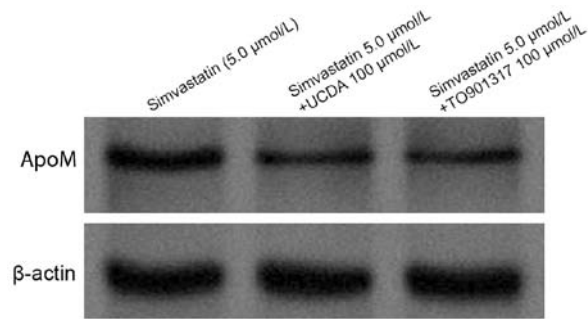
new clues for research on the mechanism of the effect of statins on increasing HDL-C and promoting cholesterol efflux and revealed that ApoM was one of the targets for regulating lipid by statins.

To elucidate the mechanisms regulating ApoM by statins, we further explored the effects of a statin on ApoM regulatory factors. HNF-1α is a major transcriptional regulator for a variety of liver genes. Studies have shown that HNF-1α is a highly efficient transcription activator for the coding of the ApoM gene and is a key factor for the regulation of ApoM (8). Our study showed that simvastatin increased the expression of HNF-1α *in vivo* and *in vitro*. *In vitro* experiments showed that HNF-1α inhibitors might weaken the role of increasing ApoM by simvastatin, suggesting that HNF-1α signaling pathway is an important way for regulating ApoM metabolism by simvastatin. Studies have confirmed that statins can up-regulate the expression of HNF-4 (16). HNF-4 is a member of the hepatic nuclear factors and can regulate other hepatocyte nuclear factors, including HNF-1α (17). Therefore, we speculated that simvastatin could affect the expression of HNF-1α indirectly through the regulation of HNF-4 and thereby affect the expression of ApoM.

Liver X receptor α (LXRα) belongs to the nuclear receptor superfamily and is a ligand-activated transcription factor involved in regulating lipid metabolism and inflammation (18,19). LXRα is first activated by a specific ligand and then forms a dimer with the retinol receptor. The dimer binds with the binding element of a promoter in the target gene and regulates target gene expression (20). LXRα is a key factor in the regulation of the metabolism of lipid and cholesterol (21-25). It has been shown that ApoM is a target gene of LXRα and LXRα may reduce ApoM expression just as it regulates other target genes (9). LXRα may inhibit ApoM expression through the LXR/RXR pathway. Our *in vivo* and *in vitro* experiments have shown that simvastatin inhibits the expression of LXRα and increases ApoM gene and protein expression. *In vitro* experiments showed that LXRα agonists reduced the role of statin in increasing ApoM, suggesting that the up-regulation of ApoM expression by simvastatin is related to the LXRα signaling pathway. Previous studies confirmed that statin inhibited LXRα expression and its mechanism was related to the inhibition of oxysterols (LXRα natural ligand) generation

by statins (26). The data support the results of our study in which statins up-regulated ApoM expression by inhibiting the expression of LXRα. However, contradictory results regarding the effect of LXRα on ApoM gene regulation have also been reported, showing that LXRα is recruited to the proximal ApoM promoter region -241/+42 and LXRα ligand (oxysterols) strongly induced human ApoM gene transcription and ApoM promoter activity in HepG2 cells (27). If based on the above theory, we are tempted to speculate that the down-regulation of LXR by simvastatin may inhibit the expression of ApoM, which is in conflict with the fact that simvastatin can increase ApoM and HDL-C. The down-regulation of LXRα by simvastatin will suppress and weaken the regulatory effect on ApoM. This phenomenon is called an off-target effect of the drug. Our *in vivo* and *in vitro* tests showed that simvastatin increased ApoM, suggesting that the off-target effects of simvastatin (i.e., the down-regulation of LXRα) is not stronger than the treatment effect (i.e., the up-regulation of HNF-1α). Off-target effects are also found with other drugs; it is one of the main mechanisms of side effects (28). This study showed that simvastatin may have an off-target effect, which provided new clues for the drug's clinical efficacy and safety evaluation. Previous studies have demonstrated that statins have effects on nuclear receptors PPARα (29) and HNF-4 (16) which are confirmed to be the regulators of ApoM. Whether simvastatin regulates ApoM through these nuclear receptors needs further studies.

In short, it was proven for the first time that ApoM is the target for the regulation of lipids by simvastatin. Regulation of ApoM may be a new way to increase HDL level and promote RCT by statin. Our study provides new clues for the anti-atherosclerosis mechanisms of statin.

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