Atorvastatin upregulates apolipoprotein M expression via attenuating LXRα expression in hyperlipidemic apoE-deficient mice

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Abstract. Apolipoprotein M (apoM) is a recently identified human apolipoprotein that is associated with the formation of high-density lipoprotein (HDL). Studies have demonstrated that statins may affect the expression of apoM; however, the regulatory effects of statins on apoM are controversial. Furthermore, the underlying mechanisms by which statins regulate apoM remain unclear. In the present study, *in vivo* and *in vitro* models were used to investigate whether the anti-atherosclerotic effects of statins are associated with its apoM-regulating effects and the underlying mechanism. Hyperlipidemia was induced by in apolipoprotein E-deficient mice by providing a high-fat diet. Atorvastatin was administered to hyperlipidemic mice and HepG2 cells to investigate its effect on apoM expression. The liver X receptor α (LXRα) agonist T0901317 was also administered together with atorvastatin to hyperlipidemic mice and HepG2 cells. The results revealed that atorvastatin increased apoM expression, which was accompanied with decreased expression of LXRα in the liver of hyperlipidemic apolipoprotein E-deficient mice and HepG2 cells. Additionally, apoM upregulation was inhibited following treatment with T0901317. In summary, atorvastatin exhibited anti-atherosclerotic effects by upregulating apoM expression in hyperlipidemic mice, which may be mediated by the inhibition of LXRα.

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Introduction

Coronary artery disease (CAD) is a leading cause of mortality and morbidity worldwide (1). Previous studies have reported that CAD is a complex, multi-factorial polygenic disorder that is mediated by genetic and environmental factors (2,3). Lipid metabolism disorders are a major factor contributing to coronary artery disease (4).

Apolipoprotein M (apoM), which is mainly expressed in the liver and kidney, has recently been identified (5). ApoM is considered to serve an important role in the generation of lipid-deficient pre-β high-density lipoprotein (HDL), an early receptor of cellular cholesterol in reverse cholesterol transport (RCT) (6). Ye *et al* (7) reported that dihydrotestosterone could downregulate apoM mRNA expression via the classical androgen receptor, independent of protein kinase C. In addition, Su *et al* (8) suggested that serum apoM protein levels are positively correlated with total cholesterol (TC) and serum HDL. ApoM overexpression in $Ldlr^{-/-}$ mice fed with a cholesterol-enriched diet was demonstrated to protect against atherosclerosis, indicating that apoM may exert anti-atherosclerotic effects *in vivo* (9). Christoffersen *et al* (10) reported that apoM, as a subpopulation of HDL, was able to protect against the oxidation of low-density lipoprotein (LDL) and stimulate cholesterol efflux more efficiently than apoM-deficient HDL. Together, these studies suggest that apoM is associated with HDL-mediated RCT and serves a crucial role in the development of CAD. However, the detailed mechanism of apoM in RCT and the pathogenesis of CAD remain unclear.

At present, statins are used as the first-line treatment for lowering plasma cholesterol levels (11). In addition to their inhibitory effect on cholesterol synthesis, statins have also been reported to have anti-oxidative (12), anti-inflammatory (13) and anti-thrombotic effects (14), as well as the ability to restore endothelial function and coronary microcirculation (15). Yang *et al* (16) administered healthy mice and HepG2 cells with simvastatin and observed that apoM mRNA and protein expression was upregulated *in vivo* and *in vitro*. Conversely, a study by Zhang *et al* (17) had contradictory results, suggesting that

simvastatin inhibits apoM expression in HepG2 cells, but had no effect *in vivo*. Therefore, the role of apoM in statin-regulated lipid metabolism requires further investigation.

Liver X receptor (LXR) was initially identified as an orphan member of the nuclear receptor superfamily that exhibits a stable regulatory effect on cholesterol and lipid metabolism (18,19). LXR is activated by synthetic agonists, including T0901317, and by endogenous oxysterols (20). Wong *et al* (21) postulated that statins inhibit the synthesis of an oxysterol ligand for LXR in human macrophages and decrease cholesterol efflux. They also demonstrated that supplementing human macrophages with cholesterol reverses the statin-mediated downregulation of ABC transporter expression, indicating that cellular lipid levels may influence the expression of LXR-target genes. Zhang *et al* (22) demonstrated that the administration of T0901317 resulted in hepatic apoM downregulation in healthy C57BL/6J mice and HepG2 cells. However, the association between apoM and LXRα in the hyperlipidemic microenvironment remains unclear. Considering the contradictory nature of previous studies, the present study was performed to investigate whether atorvastatin regulates apoM expression and to elucidate the potential underlying mechanisms.

Materials and methods

Cells, animals and reagents. The human hepatoblastoma cell line (HepG2) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A total of 16 male 8-week-old ApoE$^{-/-}$ (weight, 19.12±0.44 g) and 8 male 8-week-old C57BL/6 (weight, 20.08±0.31 g) mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). Atorvastatin original powder was purchased from Abcam (Cambridge, UK), LXR agonist T0901317 was from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and a quantitative polymerase chain reaction (qPCR) kit (SYBR® Premix Ex Taq™ II) was obtained from Takara Bio, Inc. (Otsu, Japan). Antibodies against apoM (cat. no. ab122896) and LXRα (cat. no. ab41902) were purchased from Abcam, while a pre-β HDL ELISA kit (cat. no. ml001270) was obtained from Mlbio (Shanghai, China). Reverse transcription (RT)-qPCR primers were obtained from GENEWIZ (South Plainfield, NJ, USA).

Animal experiments. Mice received humane care according to the Guidelines for the Care and Use of Research Animals established by Soochow University (Suzhou, China) and the experimental protocols were approved by the Ethics Committee of Soochow University. A total of 12 8-week-old apoE$^{-/-}$ mice and 4 8-week-old C57BL/6 mice were acclimated to housing in standard polycarbonate cages in the Animal Facility of Soochow University under a 12 h light/dark cycle for 1 week prior to experimentation. Mice were housed with free access to food and water, and under the temperature of 23˚C and air humidity of 55%. The *in vivo* study included four groups (16 mice total; 4 mice for each group): C57BL/6 mice and apoE$^{-/-}$ mice fed with regular diet (control and apoE$^{-/-}$ groups, respectively), apoE$^{-/-}$ mice fed with high-fat diet treated with normal saline (vehicle control group) and apoE$^{-/-}$ mice fed with high-fat diet and treated with atorvastatin (statin group). In the control and apoE$^{-/-}$ groups, mice were fed with regular chow for 8 weeks and administered with 0.2 ml (0.9%) normal saline by lavage every day. For the vehicle control and atorvastatin groups, mice were provided with high-fat feed (Suzhou Shuangshi Animal Feed Technology Co., Ltd., Suzhou, China), comprising 4% cholesterol, 0.5% sodium cholate, 10% lard, 0.2% propylthiouracil and 85.3% normal chow diet, for 8 weeks to induce hyperlipidemia. Following the successful establishment of hyperlipidemia, mice were randomly divided into the vehicle control and statin groups. In the statin group, mice were administered with 10 mg/kg/day atorvastatin dissolved in 0.2 ml (0.9%) normal saline by oral gavage for 4 weeks. Mice in the vehicle control group were administered with 0.2 ml (0.9%) normal saline only.

In addition to the groups described above, a total of 4 male 8-week-old apoE-deficient mice (Model Animal Research Center of Nanjing University) were housed with free access to food and water in standard polycarbonate cages under a 12 h light/dark cycle, and under the temperature of 23˚C and air humidity of 55%. The mice were used as the fifth experimental (agonist) group. Hyperlipidemia was induced in apoE-deficient mice as previously described. Thereafter, mice were administrated with 50 mg/kg/day of T0901317 + 10 mg/kg/day atorvastatin dissolved in 0.2 ml (0.9%) normal saline by lavage for 4 weeks. Following treatment, mice in all groups were anesthetized and sacrificed. Blood samples were collected through the tail vein and centrifuged at 4˚C at 5,000 x g for 20 min to collect the serum, which was subsequently stored at -80˚C. Liver samples were harvested and flash frozen for subsequent RT-qPCR and western blotting.

Cell culture. HepG2 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), 1.0x10^5 U/l penicillin and 1.0x10^5 U/l streptomycin at 37˚C. The medium was replaced every 2 days and cells were passaged. Cells were seeded in a 6-well plate at a density of ~5x10^5 cells/well and grown to 50-70% confluence. Prior to experimentation, cells were washed twice with PBS and the medium was replaced. *In vitro* experiments involved four groups: Control, dimethylsulfoxide (DMSO), atorvastatin and agonist. Cells in the control and DMSO groups were treated with 200 μl PBS or 200 μl DMSO, respectively. In the statin group, cells were treated with 20 nmol atorvastatin dissolved in 200 μl DMSO. In the agonist group, cells were treated with 200 nmol T0901317 + 20 nmol atorvastatin dissolved in 200 μl DMSO. Cells were harvested following 24 h of treatment at 37˚C for western blotting and RT-qPCR.

Serum lipid analysis. Mice were fasted for 12 h and sacrificed, following which blood samples were collected via the tail vein. Serum was separated by centrifugation at 600 x g under 4˚C for 20 min, following which HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), total cholesterol (TC; all cat. no. ab65390), and triglyceride (TG; cat. no. ab65336) levels were measured (23) with assay kits (Abcam).

Western blotting. ApoM and LXRα protein expression in liver samples and HepG2 cells were detected using western

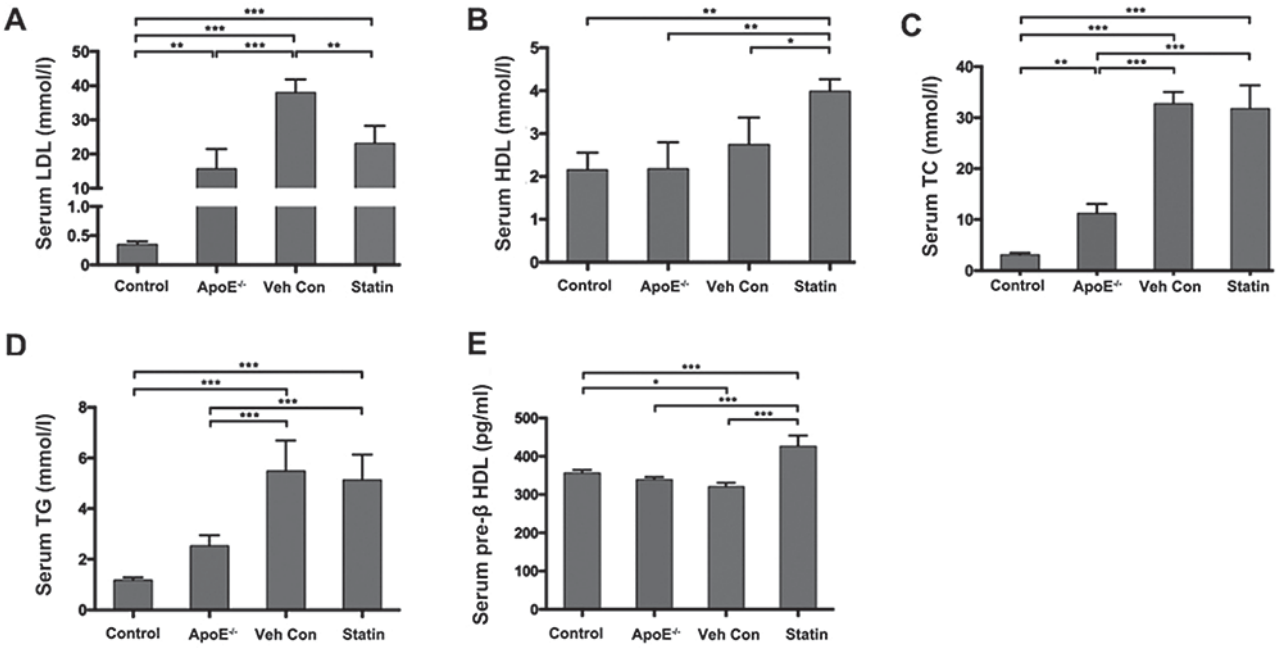

Figure 1. Serum lipid profiles of mice treated with atorvastatin for 4 weeks. Serum concentrations of (A) LDL, (B) HDL, (C) TC, (D) TG and (E) pre-β HDL. Data are presented as the mean ± standard deviation. n=4. *P<0.05, **P<0.01 and ***P<0.001. LDL, low-density lipoprotein; HDL, high-density lipoprotein; TC, total cholesterol; TG, triglyceride; Veh Con, vehicle control.

blotting as previously described (24,25). Briefly, 40 mg tissue or approximately $2x10^6$ cells in each group were homogenized to obtain lysates from which protein was extracted using ProteoPrep® Total Extraction Sample Kit (cat. no. PROTTOT-1KT; Sigma-Aldrich; Merck KGaA). The protein concentration was measured using a BCA protein assay kit according to the manufacturer's instructions. A total of 25 μg of each sample was separated by 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Blocking was then performed by overnight incubation at 4˚C in Tris-buffered saline/Tween 20 (TBST) containing 5% non-fat dried milk. Membranes were washed with TBST and incubated with anti-apoM (1:1,000), anti-LXRα (1:1,000), and anti-GAPDH (cat. no. AF0006; 1:1,000; Beyotime Institute of Biotechnology, Shanghai, China) primary antibodies for 1 h at room temperature. Subsequent to washing with TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. A0181; 1:500; Beyotime Institute of Biotechnology) for 1 h at 37˚C. A chemiluminescent substrate (cat. no. 34580; Thermo Fisher Scientific, Inc.) was used to detect the peroxidase-conjugated antibodies and membranes were exposed to X-ray film using BIO-RAD Gel Doc XR+ (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The intensity of bands was evaluated with ImageJ software (version 1.51; National Institutes of Health, Bethesda, MD, USA) and quantified relative to GAPDH bands from the same sample.

RT-qPCR. RT-qPCR was performed to determine the expression of LXRα and apoM mRNA in liver samples and HepG2 cells. Total RNA was extracted from liver tissues and HepG2 cells using TRIzol (Thermo Fisher Scientific, Inc.) and purified using a Qiagen RNeasy Mini kit (cat. no. 74104; Qiagen AG, Sollentuna, Sweden) as previously described (26). cDNA was synthesized from total RNA using the PrimeScript RT reagent kit (Takara Bio, Inc.). The temperature and duration for RT was 25˚C for 10 min, 37˚C for 60 min, 70˚C for 10 min and 4˚C on ∞ hold. A total of 2 μl cDNA was used for qPCR, which was performed using Power Syber Green and the StepOne-Plus real-time PCR system (Thermo Fisher Scientific, Inc.). The thermocycler protocol for qPCR was 95˚C for 3 min, then 40 cycles of 94˚C for 40 sec, 55˚C for 30 sec, 72˚C for 40 sec and 72˚C for 5 min, then 4˚C on ∞ hold. The endogenous housekeeping gene GAPDH was used to normalize expression levels. The sequences of primers used in qPCR are presented in Table I. The $2^{-\Delta\Delta Cq}$ method was used to evaluate changes in target gene expression relative to GAPDH (27).

ELISA for pre-β HDL quantification. Serum pre-β HLD levels were measured using a sandwich ELISA kit according to the manufacturer's protocol. Optical density (OD) values were measured at 450 nm (background reading at 620 nm) with an absorbance reader (BioTek Instruments, Inc., Winooski, VT, USA). The serum concentration of pre-β HLD (pg/ml) in each group was calculated using a standard curve.

Statistical analysis. Data are presented as the mean ± standard deviation. All data were analyzed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) using one-way analysis of variance with Bonferroni's correction for multiple group comparisons and Student's t-test for the comparisons between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Serum lipid profile in high-fat diet treated mice and the effects of atorvastatin. Hyperlipidemia was successfully

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Liver X receptor α	CTGTGCCTGACATTCCTCCT	CATCCTGGCTTCCTCTCTGA
Apolipoprotein M	GCGCCCAGACATGAAAACAG	AGGCCTCTTGATTCCTGGGA
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA

Table II. Mouse serum lipid following 8 weeks of regular or high-fat diet.

Group	n	TC (mmol/l)	LDL-C (mmol/l)	HDL-C (mmol/l)	TG (mmol/l)
C57BL/6 mice with regular chow	4	3.07±4.13	0.33±0.07	2.12±0.39	1.25±0.20
ApoE$^{-/-}$ mice with regular chow	4	11.18±1.65[a]	17.39±4.39[a]	2.19±0.67	2.54±0.54
ApoE$^{-/-}$ mice with high-fat diet	8	38.00±3.63[a,b]	37.97±3.98[a,b]	2.73±0.59	5.49±1.12[a,b]

Data are presented as the mean ± standard error of the mean. [a]P<0.001 vs. C57BL/6 mice with regular chow, [b]P<0.001 vs. ApoE$^{-/-}$ mice with regular chow. Apo, apolipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TC, total cholesterol; TG, triglyceride.

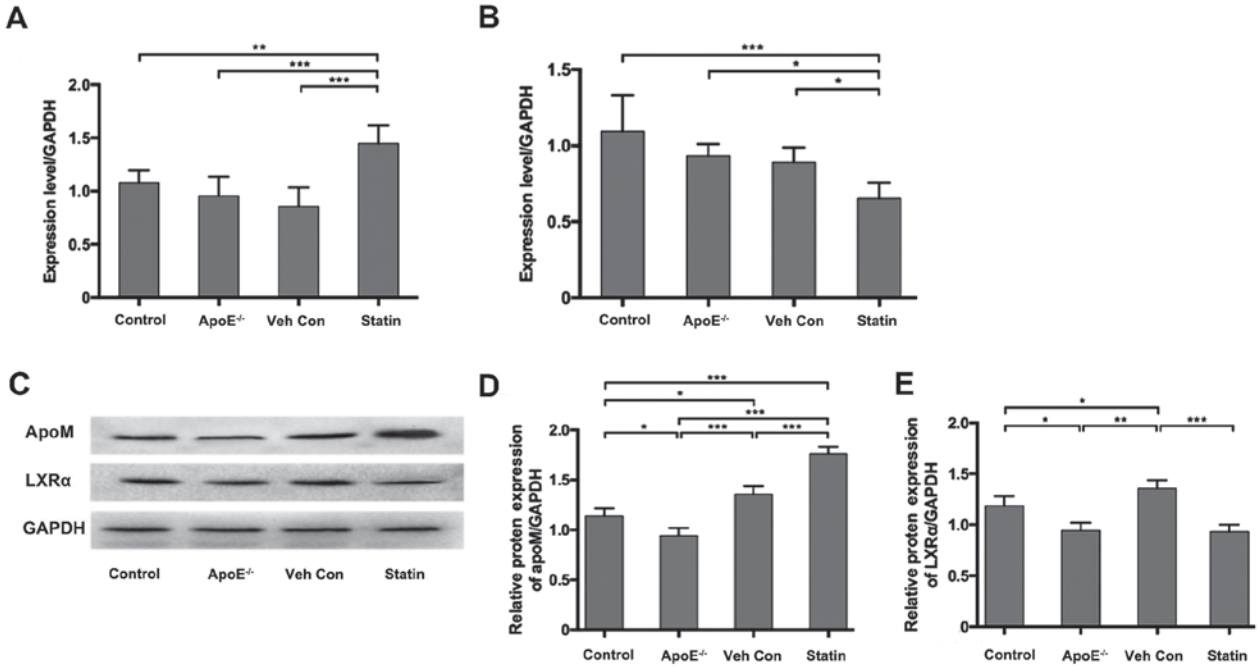

Figure 2. *In vivo* effects of atorvastatin. Relative expression of (A) apoM and (B) LXRα mRNA. (C) ApoM and LXRα expression in the liver was assessed using western blotting. Quantified expression of (D) apoM and (E) LXRα protein. Data are presented as the mean ± standard deviation. n=4. *P<0.05, **P<0.01 and ***P<0.001. ApoM, apolipoprotein; LXRα, liver X receptor α; Veh Con, vehicle control.

induced in apoE$^{-/-}$ mice following 8 weeks of high-fat diet administration. As presented in Table II, TC, LDL-C and TG were significantly increased in apoE$^{-/-}$ mice fed with a high-fat diet compared with C57BL/6 mice or apoE$^{-/-}$ mice fed with regular chow for 8 weeks. Following 4 weeks of statin or vehicle treatment, the serum concentration of LDL-C was significantly decreased in the statin group compared with the vehicle control group, while HDL-C levels were significantly increased (Fig. 1A and B). Serum TC and TG levels were decreased by 2.99 and 6.38%, respectively, in the statin group compared with the vehicle control group (Fig. 1C and D). However, no statistically significant differences were observed between the statin group and the vehicle control group. Serum pre-β HLD levels were reduced in the apoE$^{-/-}$ mice compared with the vehicle control group (Fig. 1E). However, reduced pre-β HLD levels were reversed in the statin group, resulting in significant pre-β HLD upregulation compared with the control group (Fig. 1E).

Effects of atorvastatin on apoM and LXRα expression levels in mouse liver tissues. ApoM and LXRα expression was assessed in mouse liver samples using RT-qPCR and western

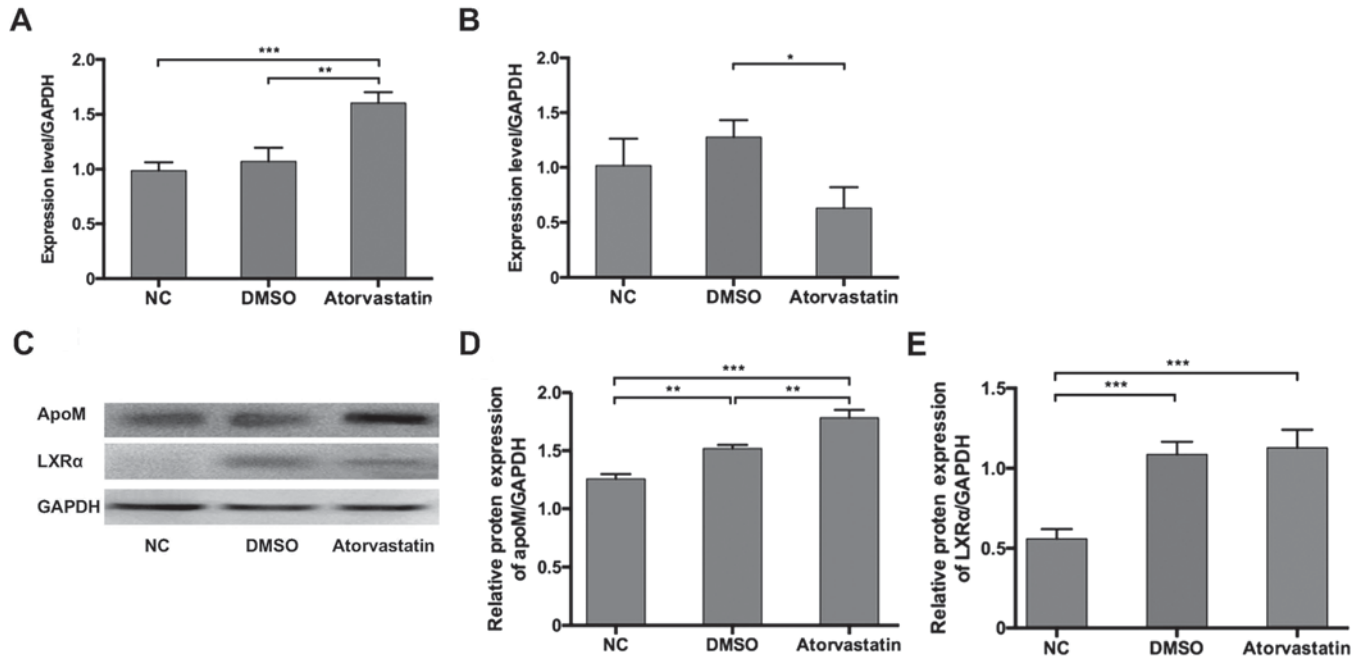

Figure 3. mRNA and protein expression was measured in HepG2 cells following atorvastatin administration. Relative expression of (A) apoM and (B) LXRα mRNA as measured using reverse transcription-quantitative polymerase chain reaction. (C) Expression of apoM and LXRα protein in HepG2 cells as determined using western blotting. Quantified (D) apoM and (E) LXRα expression. Data are presented as the mean ± standard deviation. n=4. *P<0.05, **P<0.01 and ***P<0.001. ApoM, apolipoprotein; LXRα, liver X receptor α; NC, negative control.

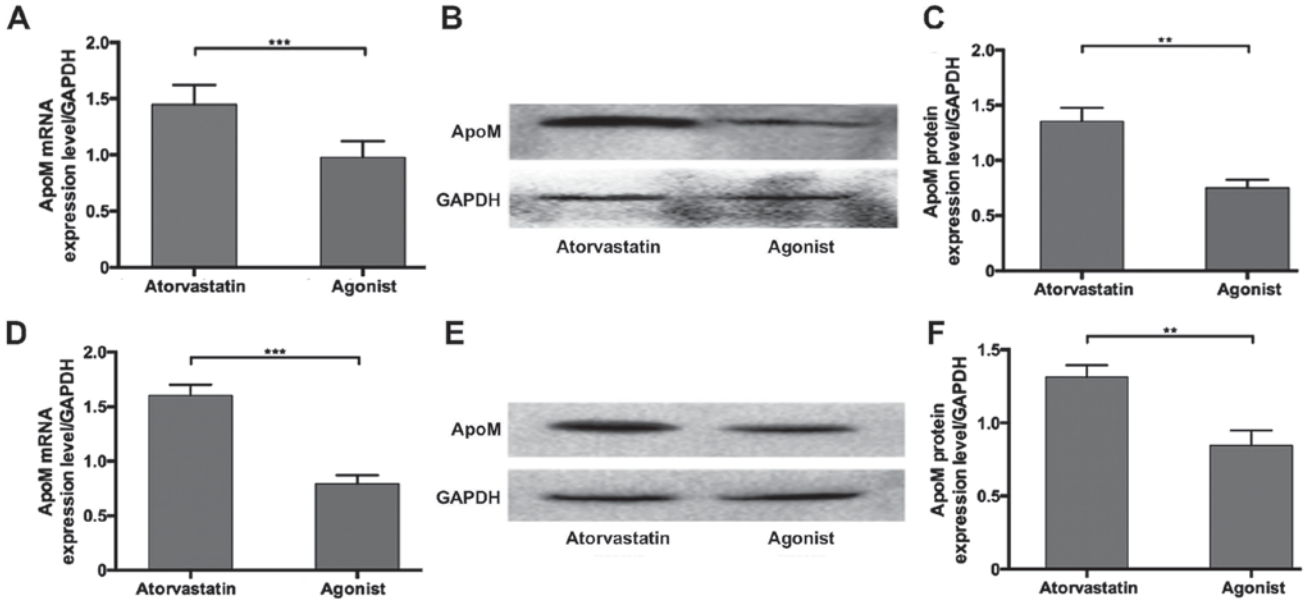

Figure 4. *In vivo* and *in vitro* apoM expression levels following the administration of atorvastatin and T0901317. ApoM (A) mRNA and (B) protein expression in mouse liver samples. (C) Quantified ApoM protein expression. ApoM (D) mRNA and (E) protein expression in HepG2 cells. (F) Quantified ApoM protein expression. Data are presented as the mean ± standard deviation. n=4. **P<0.01 and ***P<0.001. ApoM, apolipoprotein; LXRα, liver X receptor α.

blotting. No significant differences in apoM mRNA expression were observed between the control, apoE$^{-/-}$ and vehicle control groups; however, apoM mRNA expression levels were significantly increased in the statin group compared with all other groups (Fig. 2A). LXRα mRNA expression was downregulated in apoE$^{-/-}$ and vehicle control mice compared with the control group, however no statistical significance was recorded (Fig. 2B). Mice in the statin group exhibited a significant decrease in LXRα mRNA expression compared with the control, apoE$^{-/-}$ and vehicle control groups (Fig. 2B). The expression of apoM was significantly increased in the statin group compared with the vehicle control group (Fig. 2C and D), consistent with the results of RT-qPCR. However, LXRα protein expression decreased in the statin group compared with the vehicle control group (Fig. 2C and E). These results suggest that atorvastatin is able to upregulate

apoM expression in the liver of hyperlipidemic mice while simultaneously downregulating LXRα.

Effects of atorvastatin on apoM and LXRα expression in HepG2 cells. The *in vivo* study indicated that atorvastatin was able to downregulate serum lipid levels in hyperlipidemic mice by regulating liver apoM and LXRα expression at the mRNA and protein levels. In order to further study the mechanism underlying mechanism, an *in vitro* cell model was employed. RT-qPCR results suggested that apoM mRNA expression was significantly increased 1.6-fold compared with the DMSO group following statin treatment (Fig. 3A). Furthermore, LXRα was significantly downregulated at the mRNA level in response to statin treatment compared with the DMSO group (Fig. 3B). Western blotting results revealed that apoM protein was overexpressed following statin treatment compared with the DMSO group (Fig. 3C and D). Although LXRα mRNA expression levels were reduced following statin treatment, no significant differences were observed in LXRα protein expression between the DMSO and atorvastatin groups (Fig. 3C and E).

Effects of T0901317 and atorvastatin on apoM expression in vivo and in vitro. To investigate whether apoM upregulation may be mediated by the attenuation of LXRα, the LXR agonist T0901317 was used. T0901317 was administered in combination with atorvastatin to hyperlipidemic mice and HepG2 cells. The results revealed that apoM mRNA and protein expression was significantly decreased by combined treatment with the agonist compared with atorvastatin alone *in vivo* and *in vitro* (Fig. 4). These results suggest that T0901317 is able to inhibit atorvastatin-induced apoM upregulation.

Discussion

Statins are a class of drugs that inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (28). Previous investigations into statins have revealed a number of mechanisms underlying its anti-atherosclerotic effects: i) Inhibition of HMG-CoA conversion to mevalonic acid with consequential decreases in cholesterol biosynthesis and reductions in serum TC and LDL-C (29); ii) increased biosynthesis of nitric oxide (NO) and amelioration of endothelial function (30); iii) inhibition of the inflammatory reaction and the formation of foam cells in atheromatous plaques (31,32); and iv) regulation of the platelet membrane composition and inhibition of platelet aggregation (33).

ApoM was first identified as a novel apolipoprotein by Xu *et al* (34) in 1999. ApoM is primarily synthesized in the liver and secreted into the plasma where it participates in the formation of HDL and serves a role in lipid metabolism (35). Richter *et al* (36) determined that apoM serves a pivotal role in the formation of pre-β HDL. They reported that both pre-β HDL and normal HDL expression was increased following the recovery of hepatocyte nuclear factor-1α (HNF-1α) and apoM expression in an HNF-1α-deficient mouse model (36). The current hypothesis is that apoM is not essential for HDL to mobilize cholesterol, however it facilitates this action via enhancing pre-β HDL formation (37). In brief, apoM may increase the formation of pre-β HDL and facilitate cholesterol mobilization of pre-β HDL from macrophages via its interaction with ATP-binding cassette transporter member 1 (38). ApoM expression may be regulated by multiple factors *in vivo* and *ex vivo*, including HNF-1α 4α, liver receptor homolog-1, forkhead box A2 and platelet activating factor, which are able to upregulate apoM expression (5). Furthermore, LXR, retinoid X receptor, farnesoid X receptor and small heterodimer partner may downregulate apoM (5). A number of studies have investigated the effects of statins on apoM; however, the results are controversial (6,16,17). Thus, whether statins are able to regulate apoM expression and its underlying mechanisms remains unclear.

In the present study, hyperlipidemic apoE-deficient mice were treated with atorvastatin and it was demonstrated that serum HDL and pre-β HDL levels were elevated, while LDL expression was decreased. Considering that of apoM overexpression in mice increases serum HDL-C concentrations, apoM deficiency may be associated with reduced serum HDL-C concentrations (39). As apoM is associated with the formation of pre-β HDL (37), it was next investigated whether the cholesterol-lowering effects of statins are associated with its effect on apoM and other cholesterol efflux-associated genes. ApoM upregulation in the liver was revealed to be accompanied by LXRα downregulation, while serum pre-β HDL expression was also increased in the statin treated group. These results indicate that statin treatment enhances cholesterol efflux, which may be mediated by pre-β HDL and facilitated by apoM. Based on this, the effect of statin treatment *in vitro* was investigated to further explore the mechanism responsible. ApoM and LXRα expression were measured in HepG2 cells and the results were similar to those of *in vivo* analysis; statin-induced apoM upregulation in HepG2 cells was accompanied by LXRα downregulation. Importantly, co-treatment with atorvastatin and T0901317 *in vivo* and *in vitro* significantly inhibited statin-induced apoM overexpression.

In conclusion, the results of the present study indicate that atorvastatin treatment is able to upregulate apoM expression and attenuate LXRα expression, which may enhance RCT in the mouse liver. Additionally, T0901317 may block statin-induced apoM upregulation. These results suggest that atorvastatin may upregulate apoM via mediating LXRα in mouse models. Future studies may futher elucidate the mechanism behind atorvastatin-induced apoM upregulation.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JL, HH, SS, XW, YY, YH, JS and CR were responsible for performance of experiment, data analysis, and manuscript preparation. JY and ZS were responsible for manuscript writing and revision, and experimental design.

Ethical approval and consent to participate

The experiment protocols were approved by the Ethic Committee of Soochow University (reference number: SZUM2008031233).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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