Niacin regulates apolipoprotein M expression via liver X receptor-α

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Abstract. Niacin is currently the most effective drug that increases HDL-C levels. Apolipoprotein M (ApoM) in humans is mainly found in plasma high-density lipoprotein (HDL). Little is known about the role played by niacin in ApoM expression. In this study, the effects of niacin on ApoM expression were assessed as well as the associated mechanism. Human liver cancer cell line HepG2 was treated with niacin alone or with liver X receptor-α (LXRα) inhibitor at multiple concentrations. The mRNA and protein expression of ApoM were assessed by qRT-PCR and western blotting. Specific LXRα shRNA was transfected into HepG2 cells to further evaluate the regulatory effects of LXRα on ApoM. An *in vivo* model was also established to investigate the LXRα inhibitor on the mouse ApoM levels. The comparisons among groups were evaluated using one-way ANOVA and Student-Newman-Keuls test. It was revealed that in HepG2 cells, niacin dose-dependently increased ApoM gene and protein expression levels. Niacin-induced upregulation of ApoM was attenuated by an LXRα inhibitor or LXRα shRNA, indicating that LXRα mediated this effect. Moreover, niacin treatment resulted in increased LXRα mRNA levels, *in vivo* and *in vitro*; niacin treatment resulted in increased ApoM gene and protein expression levels in mice. In conclusion, niacin upregulates ApoM expression by increasing LXRα expression *in vivo* and *in vitro*.

Introduction

High-density lipoprotein cholesterol (HDL-C) levels are negatively correlated with coronary heart disease (CHD) occurrence (1,2). HDL-C exerts anti-atherosclerotic effects via its critical function in reverse cholesterol transport (RCT) (3). Apolipoprotein M (ApoM), a novel apolipoprotein associated with HDL-C, has a critical function in HDL-C metabolism (4,5). ApoM is predominantly found in hepatocytes and renal tubular epithelial cells (6). Reducing hepatic ApoM expression by siRNA decreases HDL-C amounts, increases HDL levels, and suppresses pre-β HDL (7). In addition, ApoM-deficient HDL exhibited reduced efficacy compared with normal HDL in promoting cholesterol export from macrophages (7). It is known that ApoM impacts RCT essentially by regulating pre-β-HDL synthesis. ApoM is therefore crucial for HDL-C biosynthesis and RCT.

Hepatic ApoM expression is controlled by transcription factors regulating critical steps in liver fat and glucose metabolism. Liver X receptor-α (LXRα) belongs to nuclear receptors which respond to elevated levels of intracellular cholesterol (8). LXRα is known to enhance transcription of genes that control cholesterol efflux and fatty acid biosynthesis (8). Recent studies have revealed new functions of LXRα as an essential nuclear receptor that regulates ApoM expression (8), suggesting that targeting the LXRα-mediated ApoM expression may be applied for anti-atherosclerosis therapy.

Niacin is widely used clinically as an antihyperlipidemic drug. It increases HDL-C amounts and improves RCT; however, the exact underlying mechanism is largely unknown. The aim of this study was to investigate whether niacin increases ApoM via LXRα in *in vitro* and *in vivo* models. The present research potentially contributes to further investigating the molecular mechanisms of niacin increasing HDL and development of therapeutic strategies against atherosclerosis disease.

Materials and methods

Cell lines, mice and reagents. Eight-week-old male C57BL/6N mice were obtained from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. Human liver cancer cell line HepG2 was obtained from the Cell Center of Xiangya School of Medicine, Central South University.

The following reagents and kits were used in the present study: Quantitative PCR kit (cat. no. A6101; Promega

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Corporation), anti-ApoM antibody (cat. no. ab66379; Abcam), secondary antibody goat anti-mouse horseradish peroxidase (HRP)-conjugated IgG (cat. no. A0216, Beyotime Institute of Biotechnology), niacin (cat. no. 1461003, Sigma-Aldrich; KGaA), DMEM (cat. no. 10569044; Gibco; Thermo Fisher Scientific, Inc.), TRIzol reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.), cell protein extraction kit (cat. no. P0027, Beyotime Institute of Biotechnology); LXRα inhibitor (ECHS; Wako Pure Chemicals Inc.). ApoM and LXRα specific primers were obtained from AuGCT Biotechnology.

Cell culture and treatment. DMEM supplemented with 10% fetal bovine serum (FBS) and an antibiotic cocktail (1.0×10^5 U/l penicillin and 1.0×10^5 U/l streptomycin) was employed for liver cancer cell line HepG2 culture in a humid environment containing 5% CO_2 at 37°C. The cells were passaged every other day.

Liver cancer cell line HepG2 cells were treated with niacin at concentrations of 0, 0.25, 0.5, 1.0 and 2.0 mmol/l for 24 h. For LXRα inhibition assays, cells were treated with niacin (0.5 mmol/l) and ECHS (LXRα inhibitor; 100 μmol/l) for 24 h. ApoM and LXRα mRNA levels were determined with specific primers.

Animal experiments. A total of 80 mice were used in this experiment. Eight-week-old male C57BL/6N mice were housed in single cages under a 12-h light/dark cycle at 24-28°C, with 60-75% relative humidity. The animals were allowed to adapt for one week before treatments. After 12 h of fasting, fasting lipid levels (week 0) were determined, and used as a baseline value for the experimental animals. A normal diet (15 kJ/g feed; proteins, carbohydrates, and fats contributed to 23, 65, and 12% of total energy, respectively) was provided by Xiangya Hospital, Central South University Animal Laboratory. The animals were randomized into control and niacin (1% w/w niacin, and 0.5 g niacin added in every 100-g feed, for 12 weeks) groups. The average intake of each mouse was 4.2-4.5 g/day. At 12 weeks later, fasting blood lipid levels were determined. Liver tissues were harvested at weeks 0, 3, 6, 9 and 12 (n=8/group at each time-point) after the mice were euthanized and death was confirmed when the eyes turned white. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (2%, 40 mg/kg) and sacrificed by cardiac puncture. The Ethics Committee of the Xiangya Hospital of Central South University reviewed and approved this study.

Fasting plasma lipids. At time zero and 12 weeks, 0.2 ml of tail vein blood was obtained under fasting conditions. After 30 min of incubation at room temperature, serum was obtained by centrifugation at 1,000 x g for 15 min. Serum lipid levels were determined by investigators blinded to treatment regimens. Measurements included total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) amounts. Serum TC and TG amounts were assessed by standard enzymatic methods using the kits from Beijing Solarbio Science & Technology Co., Ltd. (cat. nos. BC1985 and BC0625) according to the manufacturer's instructions. LDL-C and HDL-C concentrations were evaluated by the chemical shielding method using kits from Beijing Solarbio Science &

Technology Co., Ltd. (cat. nos. BB-47437-1 and BB-47438-1) according to the manufacturer's instructions.

Short hairpin RNA (shRNA) transfection. The pLKO.1-GFP-LXRα shRNA Plasmids were purchased from Santa Cruz Biotechnology, Inc. (cat. no. sc-38829-SH). The transfected shRNA plasmid is a pool of 3 target-specific lentiviral vector plasmids. The three shRNA sequences are: Sequence #1, 5'-CCGGGATCTGGGATGTGCACGAAT GCTCGAGCATTCGTGCACATCCCAGATCTTTTTTG-3'; sequence #2, 5'-CCGGAGTTCTCCAGGGCCATGAATGCT CGAGCATTCATGGCCCTGGAGAACTTTTTTTG-3'; and sequence #3, 5'-CCGGGCAACTCAATGATGCCGAGTTCT CGAGAACTCGGCATCATTGAGTTGCTTTTT-3'. The control pLKO.1-GFP-shRNA plasmid encodes a scrambled shRNA sequence that will not lead to the specific degradation of any cellular message. shRNA transfection was performed using the Lipofectamine® 3000 (cat. no. L3000008; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, transfection was performed in a six-well tissue culture plate, at a density of 6×10^5 cells with 50-70% confluency in antibiotic-free normal growth medium supplemented with FBS. Plasmid (1 μg) was transfected into 4×10^5 cells/well. Subsequently, 72 h later, the cells were collected for downstream assays. The transfection efficiency was determined by RT-qPCR.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA extraction was performed from cells and mice liver tissues using TRIzol according to the manufacturer's instructions. RNA purity and amounts were assessed on a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Inc.). First strand cDNA was synthesized with reverse transcriptase (AMV; Beijing Aoke Biotechnology Co., Ltd.) as directed by the manufacturer. The primers used for RT-qPCR were: ApoM forward, 5'-CTGACAACTCTGGGCGTGGA-3' and reverse, 5'-CAGAGCCAGCAGCCATATTGAA-3'; LXRα forward, 5'-AGAACAGATCCGCCTGAAGA-3' and reverse, 5'-AGCCTCTCCACCTGGAGCTGGT-3'. GAPDH was used as a housekeeping gene for normalization with the following primers: Forward, 5'-CCATGTTCGTCATGGGTGTGAAC CA-3' and reverse, 5'-GCCAGTAGAGGCAGGGATGAT GTTC-3'. SYBR green was employed for RT-qPCR at 50°C (2 min) and 95°C (10 min), followed by 40 cycles of amplification at 95°C (15 sec), 61°C (45 sec) and 61°C (10 sec). Data analysis was performed using the $2^{-\Delta\Delta Cq}$ method (9).

Immunoblotting. Cell lysates were submitted to centrifugation for 10 min at 4°C and 15,000 x g. Proteins were extracted using RIPA lysis buffer (Shanghai Biyuntian Biotechnology Co., Ltd.) from cultured cells or mouse livers. Protein amounts in the supernatant were assessed by the BCA method. Equal amounts (50 μg) of total protein were resolved by 6% SDS-PAGE and subsequently electro-transferred onto PVDF membranes. After blocking with 5% milk for 2 h at room temperature, the membranes were probed with anti-ApoM primary antibody (1:500, molecular weight: 21 kDa), at 4°C overnight, and washed in TBST. Then, HRP-conjugated secondary antibodies (1:2,000) were added for 1 h at room temperature. Immunoreactive bands were detected using
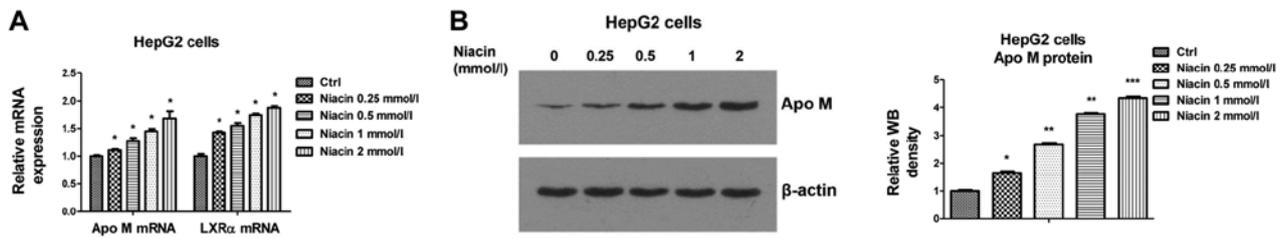
Figure 1. Niacin treatment dose-dependently affects ApoM protein expression. The effects of various niacin concentrations on (A) ApoM and LXRα mRNAs were detected by reverse transcription-quantitative PCR analysis, and (B) ApoM protein levels by western blotting. β-actin was a loading control. *P<0.05; **P<0.01; ***P<0.001 vs. control group. ApoM, apolipoprotein M; LXRα, liver X receptor-α.
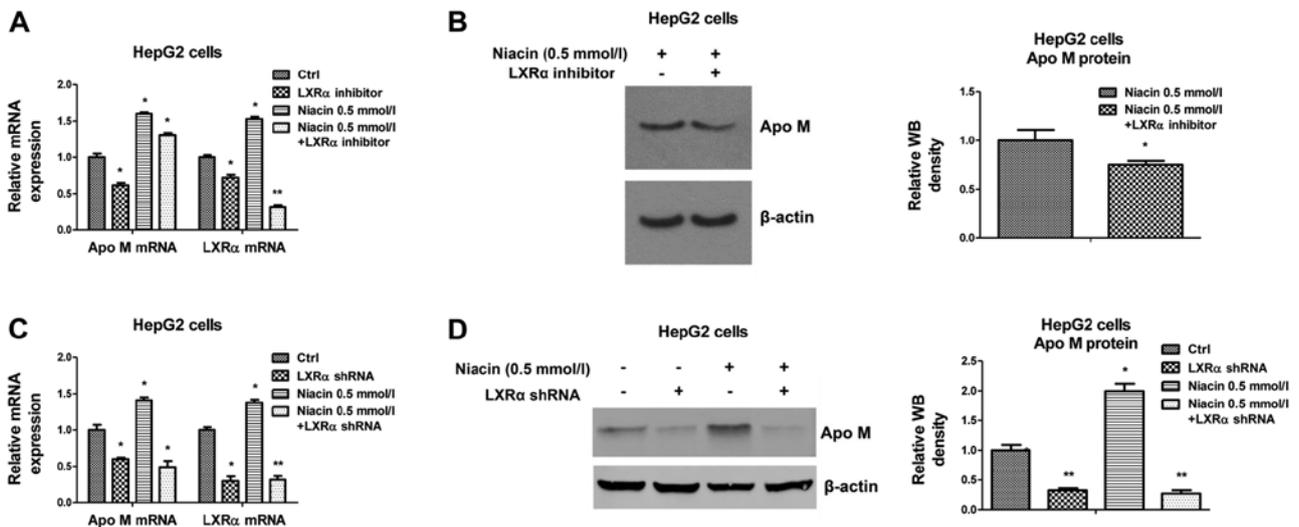

Figure 2. LXRα inhibitor ECHS inhibits niacin-associated upregulation of ApoM protein expression in HepG2 cells. (A) Control, niacin (0.5 mmol/l) alone, LXRα inhibitor alone or in combination with the LXRα inhibitor ECHS (100 µmol/l) was administered to cells for 24 h. ApoM and LXRα mRNAs were detected by RT-qPCR. (B) HepG2 cells were treated with niacin (0.5 mmol/l) alone or in combination with the LXRα inhibitor ECHS (100 µmol/l) for 24 h. ApoM protein amounts were assessed by immunoblotting. (C) HepG2 cells were treated with control plasmid, LXRα shRNA alone, niacin (0.5 mmol/l) alone, or niacin in combination with the LXRα shRNA for 24 h. mRNA expression of ApoM and LXRα was detected by RT-qPCR. (D) Cells that underwent the same treatment as those detected by RT-qPCR were subjected to western blotting analysis. β-actin was a loading control. *P<0.05; **P<0.01 vs. control group. ApoM, apolipoprotein M; LXRα, liver X receptor-α; RT-qPCR, reverse transcription-quantitative PCR; shRNA, short hairpin RNA.

an ECL western blotting detection kit (Pierce; Thermo Fisher Scientific, Inc.) and assessed with TINA 2.09 image processing software (http://www.tina-vision.net/index.php). Semi-quantitative analysis of band intensities was performed against the control group.

Statistical analysis. All experimental data are presented as the mean ± standard deviation, and analyzed with the software SPSS 15.0 (SPSS, Inc.). Comparisons among groups were evaluated using one-way ANOVA and Student-Newman-Keuls test. Experiments were performed in triplicate and repeated three times. P<0.05 was considered to indicate a statistically significant difference.

Results

Niacin induces ApoM and LXRα expression in HepG2 cells. Multiple studies have indicated that the liver cancer cell line HepG2, derived from hepatoblastoma (10), is a good model for evaluating cholesterol and lipid metabolism in the liver (11-13). Therefore, HepG2 cells were adopted to assess the effects of niacin on hepatic apolipoprotein M expression.

HepG2 cells were administered various concentrations (0.25-2.0 mmol/l) of niacin for 24 h. Notably, niacin treatment resulted in higher ApoM and LXRα mRNA amounts compared to the control (Fig. 1A). Likewise, ApoM protein expression increased with increasing niacin concentrations (Fig. 1B).

Effects of the LXRa inhibitor ECHS on niacin-associated ApoM upregulation in HepG2 cells. To explore the mechanism by which niacin upregulates ApoM, HepG2 cells were administered 0.5 mmol/l niacin in the presence of the LXRα inhibitor ECHS. As revealed in Fig. 2 and Table I, ECHS inhibited niacin-associated ApoM upregulation, both at the mRNA and protein levels (Fig. 2A and B). In addition, the LXRα inhibitor ECHS significantly inhibited the expression of LXRα mRNA (Fig. 2A and Table II). To assess the specificity of LXRα inhibitor, LXRα expression was knocked down by transfection of LXRα shRNA into HepG2 cells. As anticipated, knockdown of LXRα protein expression by shRNA significantly inhibited the basal level of ApoM and the niacin-stimulated ApoM (Fig. 2C and D). Collectively, these results consistently demonstrated LXRα positively regulated ApoM.

Table I. LXRα inhibitor, ECHS, inhibits ApoM mRNA expression induced by niacin.

Group	Niacin (0.5 mmol/l) group	Niacin (0.5 mmol/l) + LXRα inhibitor
ApoM mRNA	1.60±0.02	1.37±0.06[a]

[a]P<0.05 vs. control group. ApoM, apolipoprotein M; LXRα, liver X receptor-α.

Table II. LXRα inhibitor, ECHS, inhibits LXRα mRNA expression.

Group	Control group	Niacin (05 mmol/l)	Niacin (0.5 mmol/l) + LXRα inhibitor
LXRα mRNA	1.00±0.03	1.53±0.03[a]	0.31±0.02[a,b]

[a]P<0.05 vs. control group; [b]P<0.05 vs. niacin group. LXRα, liver X receptor-α.

Table III. Fasting blood lipid levels in mice after niacin treatment.

Group	TG	TC	LDL	HDL
Control group (n=10)	0.85±0.02	1.47±0.04	0.55±0.03	0.57±0.03
Niacin group (n=10)	0.73±0.02[a]	1.31±0.03[a]	0.23±0.04[a]	0.85±0.05[a]

[a]P<0.05 vs. control group. TG, total triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Fasting blood lipid levels are reduced in mice treated with niacin. In comparison with control values, TG, TC, and LDL-C levels in niacin-treated animals were significantly decreased (P<0.05), whereas HDL-C amounts were markedly increased (P<0.05) (Table III).

During the total 12-week niacin treatment, ApoM and LXRα mRNA levels in the murine liver were assessed by real-time RT-PCR at weeks 0, 3, 6, 9 and 12. ApoM protein expression was determined by immunoblotting at week 12. ApoM mRNA and protein levels were significantly increased in the niacin group compared with the control group (Fig. 3A and B, Table IV). In addition, LXRα mRNA amounts were significantly elevated after treatment with niacin (Fig. 3C, Table V).

Discussion

Multiple epidemiological findings indicate that HDL-C levels are negatively associated with risk of CHD (13). Elevated HDL-C and RCT induction are considered to be crucial for CHD prevention and treatment. ApoM, a novel lipid transfer protein, is a major component of HDL. Plasma HDL-C levels are increased in mice overexpressing ApoM. Conversely, ApoM gene silencing results in a 25% decrease of plasma HDL-C levels. Lack of ApoM leads to complete loss of pre-β-HDL, which significantly reduces the amounts of cholesterol flowing from macrophages to HDL. This affects the RCT function of HDL (7); thus, ApoM plays an important role in RCT and HDL formation.

Table IV. Effect of niacin on the ApoM mRNA expression in mice liver.

Group	Control group (n=10)	Niacin group (n=10)
ApoM mRNA	1.00±0.03	1.39±0.04[a]

[a]P<0.05 vs. control group. ApoM, apolipoprotein M.

Table V. Effect of niacin on the LXRα mRNA expression in mice liver.

Group	Control group (n=10)	Niacin group (n=10)
LXRα mRNA	1.00±0.02	1.45±0.03[a]

[a]P<0.05 vs. control group. LXRα, liver X receptor-α.

Niacin is currently the most effective drug that increases HDL-C amounts (14), resulting in reduced coronary events (15,16). The ARBITER 2 (17) study demonstrated that combination of niacin with statins could reverse atherosclerosis in CHD patients with reduced HDL-C amounts, who mainly benefit from the inductive effect of niacin on HDL-C amounts. Niacin may increase HDL-C primarily by preventing the liver from removing ApoA1, which increases
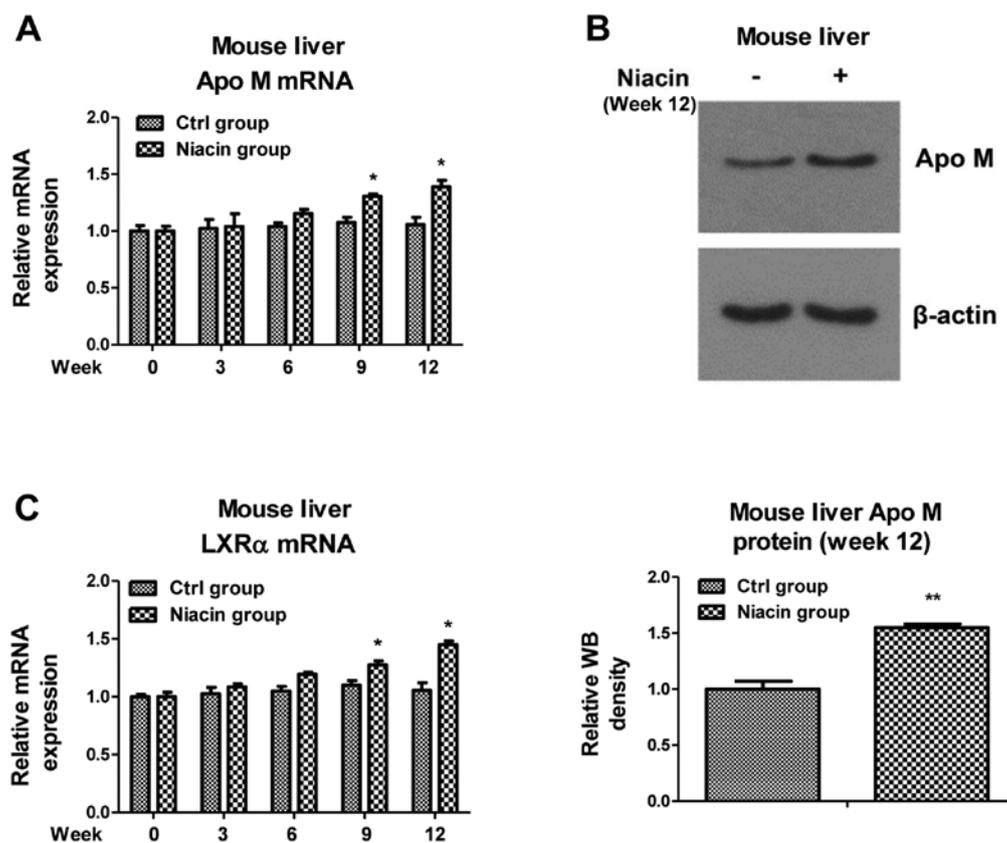
Figure 3. Effects of niacin on ApoM levels in mice. (A) ApoM mRNA was detected in the control group and the Niacin group by RT-qPCR at weeks 0, 3, 6, 9 and 12 of niacin treatments. (B) ApoM protein expression at week 12 was measured in the control group and the niacin group by western blotting. (C) The LXRα mRNA expressions were detected in the control group and the niacin group by RT-qPCR at weeks 0, 3, 6, 9 and 12 of niacin treatments. *P<0.05; **P<0.01 vs. control group. ApoM, apolipoprotein M; LXRα, liver X receptor-α; RT-qPCR, reverse transcription-quantitative PCR.

ApoA1 concentrations as well as the amounts of large ApoA1 containing HDL particles (18). In addition, niacin preserves the ability to retain ApoA1 and augment RCT. Furthermore, niacin promotes cholesterol efflux from fat cells to ApoA1 by activating PPAR-γ-LXRα-ABCA1 signaling (19). No reported studies have investigated whether niacin increases HDL-C and promotes cholesterol efflux by regulating ApoM.

The present study firstly demonstrated that niacin increased ApoM mRNA and protein levels *in vivo* and *in vitro*, indicating that niacin may control HDL-C levels and promote cholesterol efflux via ApoM upregulation. These findings provide novel insights into the mechanism by which niacin augments HDL-C levels, revealing that ApoM is one of the niacin targets in lipid regulation.

LXRα is a ligand-activated transcription factor that regulates lipid metabolism and inflammation (20,21). LXRα induced by a specific ligand forms a heterodimer with retinol receptor, and binds to the promoter of target genes for regulation (22). LXRα is an intracellular cholesterol sensor that regulates genes that control cholesterol absorption, secretion, degradation, and efflux, and has a critical function in the maintenance of cholesterol homeostasis in cells (23). Target genes of LXRα include ATP binding cassette transporter A1 (ABCA1), ABCG5, ABCG8, lipoprotein, cholesterol ester transfer protein (CETP), lipoprotein lipase (LPL), fatty acid synthase (FAS) and element binding protein 1C (SREBP-1C). LXRα is an essential factor in the regulation of lipid and cholesterol metabolism (24-28). A recent study revealed that ApoM is a target gene of LXRα, which is recruited to the proximal ApoM promoter region -241/+42; in addition, LXRα ligands (oxysterols) overtly induced human ApoM gene expression and promoter activity in HepG2 cells (29). Niacin promotes cholesterol efflux via induction of LXRα expression, which is considered a possible mechanism behind the elevated HDL-C levels (18). The present study demonstrated that niacin increased LXRα expression *in vivo* and *in vitro*. As aforementioned, the LXRα inhibitor ECHS reduced ApoM expression induced by niacin, and knocking down LXRα protein expression by shRNA significantly inhibited the level of niacin-stimulated ApoM, suggesting that LXRα signaling is involved in niacin-associated regulation of ApoM metabolism.

Previous findings indicate that niacin affects PPAR-γ, which is a regulator of LXRα (19). Whether niacin regulates ApoM through the PPAR-γ-LXRα-ApoM pathway remains unknown. Recently, ApoM was reported to serve as a carrier for the bioactive lipid sphingosine-1-phosphate (S1P) on HDL particles. S1P induces five distinct G-protein-coupled receptors (S1P-receptors 1-5), affecting multiple biological processes, including lymphocyte trafficking, lipid metabolism, angiogenesis, and atherosclerosis (29). The ApoM-S1P axis induces S1P-receptor-1, and is responsible for several HDL-associated functions (30-32). Additionally, evidence indicates that niacin affects S1P levels in plasma, red blood cells and platelets (33).

Whether niacin impacts lipid metabolism and atherosclerosis through the ApoM-S1P axis requires further investigation.

In summary, it was first demonstrated that ApoM is a niacin target in lipid regulation. The mechanism by which niacin upregulates ApoM may involve LXRα regulation. ApoM regulation may constitute a novel mechanism for increasing HDL levels and promoting RCT. The present findings provide novel insights into the anti-atherosclerotic mechanism of niacin.

Acknowledgements

Availability of data and materials

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

Authors' contributions

LY, TL, SPZ and SDZ conceived and designed the experiments. LY and TL performed the experiments, collected and analyzed the data. LY and TL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of Xiangya Hospital of Central South University reviewed and approved the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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