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RESEARCH ARTICLE

Atorvastatin remodels lipid distribution between liver and adipose tissues through blocking lipoprotein efflux in fish

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Abstract

The regulation of cholesterol metabolism in fish is still unclear. Statins play important roles in promoting cholesterol metabolism development in mammals. However, studies on the role of statins in cholesterol metabolism in fish are currently limited. The present study evaluated the effects of statins on cholesterol metabolism in fish. Nile tilapia (*Oreochromis niloticus*) were fed on control diets supplemented with three atorvastatin levels (0, 12, and 24 mg/kg diet, ATV0, ATV12, and ATV24, respectively) for 4 wk. Intriguingly, the results showed that both atorvastatin treatments increased hepatic cholesterol and triglyceride contents mainly through inhibiting bile acid synthesis and efflux, and compensatorily enhancing cholesterol synthesis in fish liver ($P < 0.05$). Moreover, atorvastatin treatment significantly inhibited hepatic very-low-density lipoprotein (VLDL) assembly and thus decreased serum VLDL content ($P < 0.05$). However, fish treated with atorvastatin significantly reduced cholesterol and triglycerides contents in adipose tissue ($P < 0.05$). Further molecular analysis showed that atorvastatin treatment promoted cholesterol synthesis and lipogenesis pathways, but inhibited lipid catabolism and low-density lipoprotein (LDL) uptake in the adipose tissue of fish ($P < 0.05$). In general, atorvastatin induced the remodeling of lipid distribution between liver and adipose tissues through blocking VLDL efflux from the liver to adipose tissue of fish. Our results provide a novel regulatory pattern of cholesterol metabolism response caused by atorvastatin in fish, which is distinct from mammals: cholesterol inhibition by atorvastatin activates hepatic cholesterol synthesis and inhibits its efflux to maintain cholesterol homeostasis, consequently reduces cholesterol storage in fish adipose tissue.

atorvastatin; cholesterol metabolism; lipid metabolism; Nile tilapia

INTRODUCTION

Cholesterol is an essential cellular structural component, which serves as a precursor for biosynthesis of bile acids, vitamin D, and steroid hormones in animals (1). However, high cholesterol accumulation in the body usually leads to a series of diseases such as cardiovascular disease (2), nonalcoholic fatty liver disease, and obesity (3, 4). Therefore, to maintain cellular cholesterol homeostasis, mammals tightly control cholesterol biosynthesis, uptake, transport, efflux, and esterification (5). The available limited studies (6, 7) have indicated a similar cholesterol metabolism in fish and its homeostasis regulation based on cellular sterol levels as in mammals. Evidently, Atlantic salmon (*Salmo salar*) upregulated cholesterol biosynthesis-related genes and their transcriptional factors after feeding dietary vegetable ingredients (without cholesterol inclusion) as replacement for fishmeal or fish oil (6, 8). On the contrary, high dietary cholesterol intake inhibited a key gene involved in cholesterol synthesis (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, *hmgcr*) in juvenile turbot (9). Indeed, the plasma cholesterol concentrations of most fish are remarkably higher than those of mammals (10), consequently, salmonids are prone to coronary atherosclerotic lesions (11, 12). Therefore, fish were previously believed to grow normally without the need for exogenous dietary cholesterol supplementation (13, 14). The regulation of cholesterol metabolism in fish was thought to meet body cholesterol requirements compared with mammals. This notion leads to little scientific studies exploring cholesterol metabolism in fish. Therefore, further studies are required to reveal the regulation of cholesterol metabolism in fish. Such knowledge may enrich our understanding of the evolutional cause of cholesterol-related diseases in human considering that fish is an evolutionally older vertebrate group than human.

Statins are a class of drugs used to lower cholesterol due to their ability to inhibit a crucial step during cholesterol biosynthesis in which HMGCR, 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase converts HMG CoA to mevalonate, a cholesterol precursor (15). In humans, cholesterol

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depletion by statins induced sterol regulatory element-binding protein 2 activity (SREBP2), thus further promoted the expression of genes related to cholesterol synthesis and uptake such as *HMGCR* and low-density lipoprotein receptor (*LDLR*) (16, 17). Therefore, the mechanisms used by statins to lower plasma cholesterol level are not clear because the HMGCR gene and protein were dramatically induced by in vitro or in vivo studies after statins treatments. A recent study in mice revealed the increased cholesterol biosynthesis rates (predominantly occurred in the liver) after persistent exposure to statins, accompanied with the reduced cholesterol efflux into plasma (18). Indeed, the atorvastatin-treated mice promoted biliary cholesterol excretion and elimination to alleviate cholesterol accumulation in the liver (18). Moreover, atorvastatin therapy also reduced triglycerides (TGs) levels in humans (19) because cholesterol plays an important function in regulating TG metabolism. Such an effect was linked to the inhibition of very-low-density lipoprotein (VLDL) assembly and secretion, rather than lipid synthesis in the liver (20). These findings call for more studies to understand the mechanisms used by statins in cholesterol metabolism.

Statins exist in wild aquatic environment at low concentrations ranging from 1 to 117 ng/L (21, 22). Accordingly, concerns have been expressed about their impacts on nontarget species because fish in the wild aquatic environment show hypercholesterolemia (23). Therefore, the plasma cholesterol concentrations in most fish are remarkably higher than in mammals, which might be related to the off-target effects of statins in wild aquatic environment (10, 23). However, limited studies have reported the role of statins in cholesterol metabolism in fish. The available studies show that the mRNA transcript abundance of *hmgcr* and *ldlr* were induced by atorvastatin exposure likely through the upregulation of *srebp2* in rainbow trout hepatocytes (24) and vivo zebrafish (25). Furthermore, the atorvastatin treatment disrupted lipid dynamics in fish. For example, atorvastatin exposure induced peroxisome proliferator-activated receptor γ (*pparγ*) and sterol regulatory element-binding protein 1 (*srebp1*) expressions in female adult zebrafish liver (25) and trout hepatocytes (24). However, it remains unclear on the mechanisms used by statins in reducing cholesterol in fish.

Given the distinct regulation of cholesterol metabolism might exist between fish and mammals, and statins as powerful tools play important role in promoting the research development of cholesterol metabolism in mammals, the present study investigated the role of atorvastatin in cholesterol metabolism in fish. Therefore, Nile tilapia (*Oreochromis niloticus*), which is a widely used fish in metabolic studies (26, 27), were fed on control diets containing different levels of atorvastatin (0, 12, or 24 mg/kg diet) for 4 wk. Afterward, we determined the cholesterol and TG metabolism in different tissues. The obtained results are useful to understand the mechanism of atorvastatin on lipid metabolism in fish and the evolutional cause of cholesterol-related diseases in human.

MATERIALS AND METHODS

Animal studies were conducted strictly under the Guide for the Care and Use of Laboratory Animals in China, and approved by the Committee on Ethics of Animal Experiments of East China Normal University (Approval No. F20190101).

Experimental Animals and Diets

Nile tilapia were purchased from Guangzhou Huihai Aquatic Technology, Co., Ltd. (Guangzhou, China), and acclimated into an indoor-culture system (200-L tanks) for 2 wk. During this period, fish were fed by using a commercial diet (Tongwei, Co. Ltd., Chengdu, China). Three experimental diets were formulated as control diet (ATV0) and control diets containing two levels of atorvastatin (12 mg/kg diet, ATV12 and 24 mg/kg diet, ATV24; Table 1). The doses of atorvastatin (99.00% purity obtained from Glpbio, CA) were based on a previous in vivo fish study (25). The control diet was formulated to contain ~39% protein, 32% carbohydrate, and 13% lipid levels according to previous studies (28–31). All dry ingredients were finely ground through a 60-mesh screen and thoroughly mixed, and then soybean oil and atorvastatin-dissolved water were added to blend thoroughly for producing 2-mm diameter pellets with a twin-screw extruder (South China University of Technology, Guangzhou, China). All the experimental diets were air-dried and stored at −20°C until used.

After acclimatization, visually healthy Nile tilapia fingerlings (average weight 5.14 ± 0.1 g) were randomly divided into three treatments in tanks (each tank with 30 fish in three replicates). The fish were fed with the experimental diets twice daily (0900 and 1700) at 4% of their body weight per day for 4 wk. Fish in each tank were bulk weighed to adjust feed amount each week. During the entire feeding

Table 1. *The formulation and proximate analysis of experimental diets*

	ATV0	ATV12	ATV24
Ingredients, g/kg			
Casein	320.00	320.00	320.00
Gelatin	90.00	90.00	90.00
Corn starch	320.00	320.00	320.00
Soybean oil	130.00	130.00	130.00
Vitamin[a]	15.00	15.00	15.00
Minerals[b]	25.00	25.00	25.00
CMC	24.75	24.75	24.75
Cellulose	60.00	60.00	60.00
Choline chloride	5.00	5.00	5.00
Dimethyl-β-propiothetin	0.25	0.25	0.25
$Ca(H_2PO_4)_2$	10.00	10.00	10.00
Atorvastatin	0	0.012	0.024
Total	1,000	1,000	1,000
Proximate composition (g/kg dry matter)			
Crude protein	388.00	391.00	394.00
Crude lipid	133.00	128.00	135.00
Ash	61.00	63.00	62.00

ATV, atorvastatin. [a]Vitamin premix (mg or IU/kg): 500,000 international units (IU); vitamin A, 50,000 IU; vitamin D3, 2,500 mg; vitamin E, 1,000 mg; vitamin K3, 5,000 mg; vitamin B1, 5,000 mg; vitamin B2, 5,000 mg; vitamin B6, 5,000 μg; vitamin B12, 25,000 mg; inositol, 10,000 mg; pantothenic acid, 100,000 mg; cholin, 25,000 mg; niacin, 1,000 mg folic acid; 250 mg biotin; 10,000 mg vitamin C; [b]Mineral premix (g/kg): 314.0 g $CaCO_3$; 469.3 KH_2PO_4; 147.4 g $MgSO_4 \cdot 7H_2O$; 49.8 g NaCl; 10.9 g Fe(II) gluconate; 3.12 g $MnSO_4 \cdot H_2O$; 4.67 g $ZnSO_4 \cdot 7H_2O$; 0.62 g $CuSO_4 \cdot 5H_2O$; 0.16 g; 0.08 g $CoCl_2 \cdot 6H_2O$; 0.06 g NH_4 molybdate; 0.02 g $NaSeO_3$.

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trial, dissolved oxygen, total ammonia-nitrogen, and water temperature ranged from 5.00–6.50 mg/L, 0–0.20 mg/L, and 27.00–29.00°C, respectively.

Sample Collection

At the end of feeding trial, all fish in each tank were starved overnight, euthanized with MS-222 (0.1 g/L, Sigma, St. Louis, MO), and then bulk weighed for growth performance determination. Four fish were randomly selected from each tank and individually weighed and measured for their total length for calculation of condition factor (CF). Then they were used for blood collection. Blood was drawn from the caudal vein by using 2-mL syringes (Klmedical, China). Blood samples were immediately centrifuged at 2,000 rpm for 10 min at 4°C, the serum was placed into polypropylene tubes. The remaining fish were dissected to obtain carcass, mesenteric fat, and liver tissues, which were further weighed. The weights of tissues and individual fish were used for calculation of organ indices. Intestinal content was squeezed out of intestine and then collected in tube. Serum, liver, adipose, and intestinal content samples were immediately frozen into liquid nitrogen and stored at −80°C for further analysis.

Biochemical and Histological Analyses

Liver, adipose tissues, and intestinal content were homogenized in phosphate buffer saline (PBS) at a ratio of 1:10 (wt:vol), and then centrifuged (4°C, 3,000 rpm, 10 min). The supernatant was collected and used for biochemical assays. The total cholesterol (TC), TG, total bile acids (TBA), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), very-low-density lipoprotein cholesterol (VLDL-C), free fatty acids (FFA) in different tissues (serum, liver, and adipose), and intestinal content were measured by using specific commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The liver tissues were fixed in 4% paraformaldehyde solution and embedded in paraffin wax followed by neutral lipid staining according to the standard procedure (32) for histological analysis.

Quantitative Real-Time PCR

The quantitative real-time PCR was conducted according to our previous study (33). Total RNA from liver and adipose tissues (~50 mg) was isolated by using TRIzol reagent (Takara, Dalian, China) according to the manufacturer's instructions. The integrity and purity of RNA were determined by using 1% agarose gel electrophoresis and spectrophotometer (NanoDrop 2000, Thermo), respectively. The reverse transcription of mRNAs was performed by using PrimeScript RT reagent kit (with gDNase, Tiangen, China) following the manufacturer's instructions. The quantitative real-time PCR was conducted in the CFX96 Real-Time RCR system (Bio-Rad, CA) with ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech, Co., Ltd., Nanjing, China) according to the manufacturer's protocols. The amplification condition applied to qPCR was as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s. The reference and specific gene primers used in this study are given in Table 2. A single PCR product was evaluated by using melting curves analysis at the end of each PCR reaction. The amplification efficiencies of the candidate genes ranged from 95 to 105%, and the correlation coefficient was more than 0.98 for each gene. The relative mRNA expression of target genes was measured by the $2^{-\Delta\Delta Ct}$ method (34).

Western Blotting

The Western blotting was performed according to the method described previously in our laboratory (35). Total

Table 2. *Primer sequences used in the experiment*

Gene	Accession No.	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)
p62	XM_005463795.4	CCCTTCTAAACCTGCTGCTGA	TCACCTTGGTCCGTTGGC
atg5	XM_003450274.5	ACAGCGTCTTACCCTGGAGCA	TCACAGAGCTGGATGGGCAGT
atg12	XM_025911479.1	TCATATCTCGCTTCCTCAAGC	CCCTACTTCTTGATCCGGTGA
hmgcr	XM_003437610.5	TGGTTAGGTCACGATGCCAC	GGGCCGGATAACCTCATCTTT
sqle	XM_003453510.5	GCCTGTCTTTGTTACCTGTT	GCCCGTTTCCACTCTGTTC
msmo1	XM_003449688.4	AGTGCTTCGGCTGTGCT	ATGGTGGACTTTGTGGATGT
lss	XM_025903774.1	CACAGACGCTCGGAGTT	AGATGAATAAGGGAGGTTTC
abcg8	XM_019366916.2	GTCGGGATGAAGGAGGC	TCGGACGGTGAGGTGAG
cyp7a1	XM_003456729.5	GGGATAAGACACAGGCAACCA	TGCGGAGGAATTGAAGTGGG
abcb11	XM_005452962.4	GCTCATCCGAAACCCCAAGA	AGAAATCGTCGTCCTGCCTG
abcg1	XM_005462721.3	GGGGATGAAGGGCGAGAT	TGGACACTGAGGTGAGGC
abca1	XM_005454754.4	ACACCAGACACTCCAGCAA	ACCTCCTCCCACATCCC
ldlr	XM_003443172.5	CACTGCAAGGCCATTGGAAC	GGCGAGGGATTACTCTCGTG
fasn	GU433188	TCATCCAGCAGTTCACTGGCATT	TGATTAGGTCCACGGCCACA
acca	XM_005471970	TAGCTGAAGAGGAGGGTGCAAGA	AACCTCTGGATTGGCTTGAACA
acly	XM_003442027	AAAAGCTTTGATGAGCTTGGGG	TACAGTGGGAGGAGGCAACTCTT
dgat	XM_003458972	GCTTGAATTCTGTCACCCTGAAGA	ACCTGCTTGTAGGCGTCGTTCT
srebp1	XM_005471970	TGCAGCAGAGAGACTGTATCCGA	ACTGCCCTGAATGTGTTCAGACA
pparγ	KF918712	TGGACTACACAAACATGCACAGC	CACGGGACTATCTGAGTACTGTGGA
atgl	XM_003440346.5	GACACATGCTGCAAAGCACT	ACCAGGACGTTTTCTCCGTC
hsl	XM_005463937.4	AGTTCACTCCAGCCATTCGG	TGGCTGCTACCCCTATTCCT
mgl	XM_005478351.4	GGGCTCCATCGAGTCCAAAT	AATGATACTCGCATCCCGCC
mttp	XM_005458713.4	TGCATGTGGCTTTGCTTCAC	CTTGACCTGCTCCACGGTAG
cpt1a	XM_003440552	TTTCCAGGCCTCCTTACCCA	TTGTACTGCTCATTGTCCAGCAGA
β-actin	KJ126772.1	AGCCTTCCTTCCTTGGTATGGAAT	TGTTGGCGTACAGGTCCTTACG
ef1a	KJ123689.1	ATCAAGAAGATCGGCTACAACCCT	ATCCCTTGAACCAGCTCATCTTGT

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proteins from liver tissues were extracted by using the radio immunoprecipitation assay (RIPA) lysis buffer with 1 mM phenylmethanesulfonyl fluoride (Beyotime Biotechnology, China). The total protein concentration was measured by using protein quantification kit (New Cell & Molecular Biotech, China). The supernatant protein (30 μg of total protein) was loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for separation, and then transferred to nitrocellulose membranes that were further incubated with 5% milk in buffered saline with Tween 20 (TBST) buffer. The target proteins of membrane were incubated overnight at 4°C with primary antibodies (anti-SREBF1, 1:1,000, A13049, ABclonal and anti-GAPDH, 1:3,000, AB0036, Abways), which shared high homology with fish. After being washed with TBST buffer, membranes were incubated with secondary antibodies. The protein bands were visualized by using an Odyssey CLx Imager (Li-Cor), and were proved to be corresponding position of molecular weight.

Proteomics Analysis

Proteomics analysis of liver tissue from ATV0 and ATV24 treatments was performed by Shanghai Applied Protein Technology, Co., Ltd. Briefly, liver samples were homogenated with lysis buffer (4% SDS, 100 mM Tris·HCl, 1 mM DTT, pH7.6) for protein extraction. Total protein concentration was quantified by using a protein quantification kit (Bio-Rad) according to the manufacturer's protocol. Protein digestion by trypsin was conducted with filter-aided sample preparation and desalted on C18 Cartridges (Empore SPE Cartridges C18). The proteins were loaded onto 12.5% SDS-PAGE gel, and their images were obtained by Coomassie Blue R-250 staining. Next, 100 μg of lyophilized protein samples were labeled using Tandem Mass Tag (TMT) reagent following the manufacturer's protocol (Thermo Scientific). The labeled peptides were analyzed by liquid chromatography tandem-mass spectrometry (LC-MS/MS) on a Q Exactive mass spectrometer coupled to Easy nLC (Thermo Fisher Scientific). The peptide mixtures were first loaded onto a reversed-phase trap column (Acclaim PepMap100, 100 μm·2 cm, nanoViper C18, Thermo Scientific), and then separated by using a mobile phase (0.1% formic acid and 84% acetonitrile) at a flow rate of 300 nL/min. The mass spectrometry raw data obtained ranged from 300 to 1,800 m/z in the survey scan searched against the Uniprot database (*Oreochromis niloticus*) by using the MASCOT engine (Matrix Science, London, UK) embedded into Proteome Discoverer 1.4 software for finding homolog sequences.

Statistical Analyses

The obtained data were tested for normality and homogeneity of variances by using Shapiro–Wilk test and Levene's test, respectively. Significant differences between control and atorvastatin groups were determined using one-way ANOVA followed by Duncan's test in Fig. 1 or a two-tailed independent t test in Figs. 2, 3, 4, and 5. Results with $P \leq 0.05$ were considered statistically different. All the obtained experimental data were analyzed by using the SPSS Statistics 23.0 software (IBM). All values in the results are presented as the means with their standard deviation.

RESULTS

Atorvastatin Induced Hepatic Lipid Deposition, but Reduced the Adipose Tissue Mass of Fish

After the feeding trial, although different atorvastatin levels did not affect body weight (Fig. 1*A*) and length (Fig. 1*B*) of Nile tilapia ($P > 0.05$), both atorvastatin levels (ATV12 and ATV24) significantly decreased condition factor (CF, $P < 0.05$, Fig. 1*D*). Accordingly, both atorvastatin treatments increased carcass ratio (CR) compared with the control fish (ATV0, $P < 0.05$, Fig. 1*C*). Although no obvious change in hepatosomatic index (HSI) was found in fish among treatments ($P > 0.05$, Fig. 1*E*), atorvastatin (ATV24) treatment significantly reduced mesenteric fat index (MFI) compared with the control group ($P < 0.05$, Fig. 1*F*). However, Oil Red staining analysis showed that both atorvastatin treatments induced lipid deposition in the liver of fish (Fig. 1*G*). Detailed biochemical analysis showed that, both atorvastatin, especially ATV24 treatment remarkably increased hepatic TC (Fig. 1*H*) and TG (Fig. 1*I*) contents compared with the control fish ($P < 0.05$). Overall, atorvastatin treatment induced lipid deposition in the liver, but reduced adipose tissue mass of fish. In the following analyses, the atorvastatin (ATV24) treatment was chosen to explore its mechanisms by which atorvastatin induced the hepatic lipid deposition, but alleviated it in the adipose tissue of fish.

Atorvastatin Induced Hepatic Lipid Accumulation Associated with Enhancing Cholesterol Biosynthesis

To further investigate the mechanisms, which contributed to the lipid accumulation in the liver of fish treated with atorvastatin, we performed proteomics analysis between fish in ATV0 and ATV24 groups. We found 375 significantly changed proteins ($|\log_2 FC| > 1.2$, $P < 0.05$) in the liver for both treatments (Fig. 2, *A* and *B*). Subsequent Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of proteomics showed that lipid metabolism, including steroid biosynthesis, synthesis, and degradation of ketone bodies and sphingolipid metabolism, were significantly upregulated in the liver of fish treated with ATV24 ($P < 0.05$, Fig. 2*C*). The fish treated with ATV24 significantly promoted cholesterol biosynthesis pathway, including acetyl-CoA acetyltransferase 2 (Acat2), 3-hydroxy-3-methylglutaryl-CoA synthase 1 (Hmgcs), squalene epoxidase (Sqle), methylsterol monooxygenase 1 (Msmo1), mevalonate diphosphate decarboxylase 1 (Mvd1), sterol-C5-desaturase (Sc5d), and cytochrome P450 2R1 (Cyp2r1) proteins ($P < 0.05$, Fig. 2*D*). On the contrary, the fish treated with ATV24 significantly downregulated protein synthesis, including transcription (RNA polymerase), translation (ribosome), and amino acid metabolism (glycine, serine, and threonine metabolism) ($P < 0.05$, Fig. 2*C*). In general, atorvastatin treatment induced hepatic lipid accumulation associated with the compensatory enhancement of cholesterol biosynthesis in fish.

Atorvastatin Promoted Cholesterol Synthesis and Inhibited Hepatic Bile Acid Synthesis and Efflux

Furthermore, we investigated the mechanism of atorvastatin to induce cholesterol accumulation in the liver of fish. We found that atorvastatin treatment enhanced the cholesterol synthesis-related genes (*hmgcr*; squalene epoxidase,

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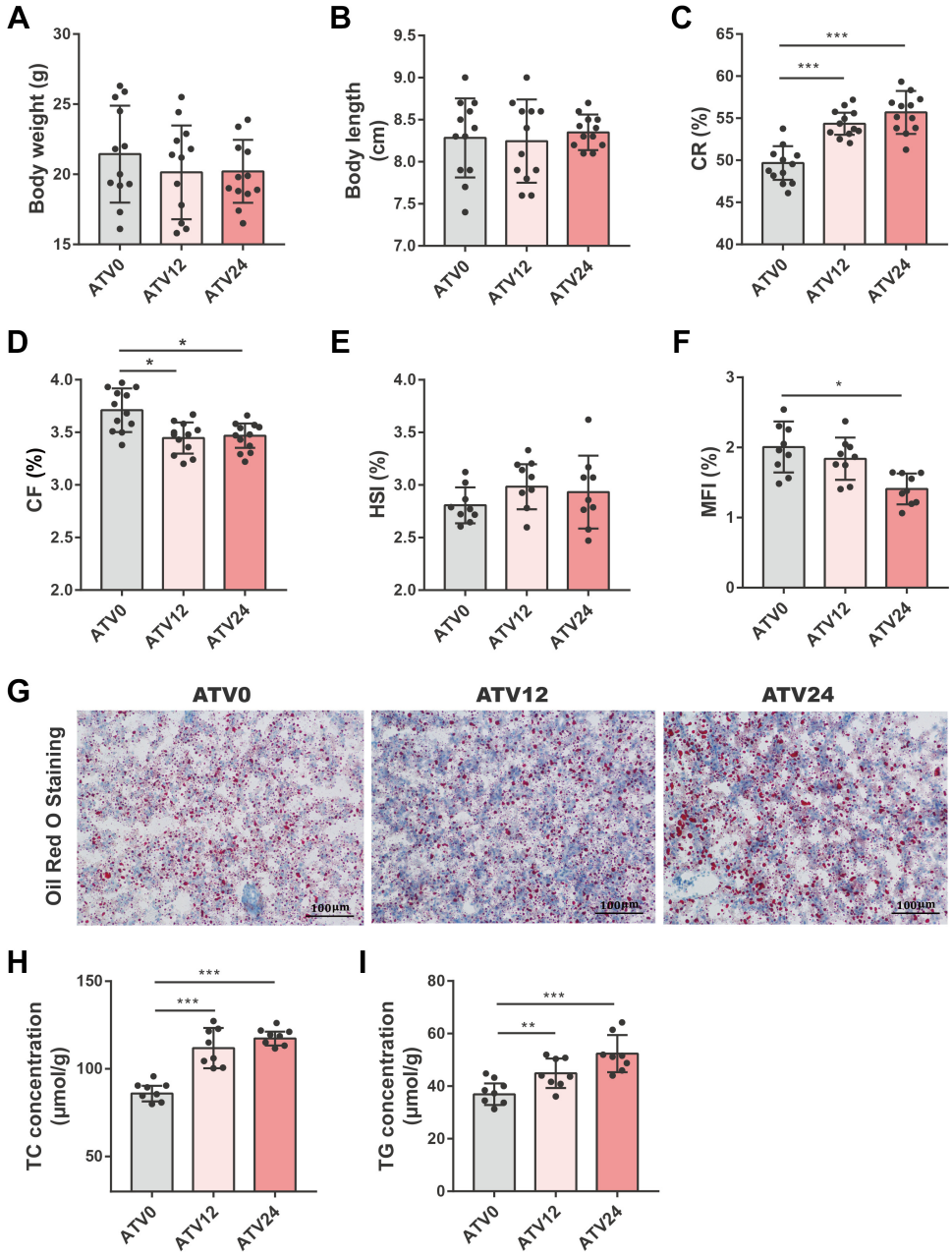


Figure 1. Atorvastatin induced hepatic lipid deposition, but reduced the adipose tissue mass of Nile tilapia. Body weight (n = 12) (*A*); body length (n = 12) (*B*); condition factor (CF, n = 12) (*C*); Carcass ratio (CR, n = 12) (*D*); hepatosomatic index (HSI, n = 9) (*E*); mesenteric fat index (MFI, n = 9) (*F*); Oil O Red staining of liver, scale bars = 100 μm (n = 4) (*G*); triglyceride (TG) and total cholesterol (TC) in the liver (n = 8) (*H* and *I*). Data are represented as mean values with their standard deviation. n, number of animals measured. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ significant difference was analyzed among various atorvastatin levels group (one-way ANOVA); ATV0, ATV12, or ATV24, control diet supplemented with 0, 12, or 24 mg/kg atorvastatin. CF (%), individual fish body weight ×100/individual fish body length^3; CR (%) = 100 × carcass weight/body weight; HSI (%) = 100 × liver weight/individual fish body weight; MFI (%) = 100 × mesenteric fat weight/individual fish body weight.

sqle; lanosterol synthase, *lss*; methylsterol monooxygenase 1, *msmo1*) ($P < 0.05$, Fig. 3*A*) and their transcription factor protein (Srebp2) expression ($P < 0.05$, Fig. 3*B*), but inhibited the cholesterol efflux genes (ATP-binding cassette subfamily G member 5, *abcg5*) expression in the liver of fish ($P < 0.05$, Fig. 3*H*). Accordingly, total cholesterol content was significantly reduced in the intestinal content of fish treated with atorvastatin compared with the control fish ($P < 0.05$, Fig. 3*C*). In addition, the atorvastatin treatment significantly reduced bile acids content in the liver and intestinal content ($P < 0.05$, Fig. 3, *D* and *E*) and inhibited hepatic bile acid synthesis and efflux-related genes (cholesterol 7α-hydroxylase, *cyp7a1* and ATP-binding cassette subfamily B member 11, *abcb11*) expression in fish ($P < 0.05$, Fig. 3*H*). On the contrary, although the atorvastatin treatment did not affect serum HDL-C content ($P > 0.05$, Fig. 3*F*) and hepatic cholesterol efflux-related genes (ATP-binding cassette subfamily A member 1, *abca1* and ATP-binding cassette subfamily G member 1, *abcg1*) ($P > 0.05$, Fig. 3*H*), but it significantly decreased serum LDL-C content and *ldlr* gene expression in fish liver ($P < 0.05$, Fig. 3, *G* and *H*). Altogether, atorvastatin treatment induced cholesterol accumulation mainly through inhibiting bile acid synthesis and efflux, and compensatorily enhancing cholesterol synthesis in the liver of fish.

Atorvastatin Increased TG Accumulation via Inhibiting Hepatic VLDL Efflux

Apart from cholesterol accumulation in liver, atorvastatin treatment also significantly elevated TG (Fig. 1*I*) and FFA (Fig. 4*B*) contents in the liver of fish ($P < 0.05$). However, we found that atorvastatin treatment significantly suppressed the expression of lipid synthesis-related genes such as ATP citrate

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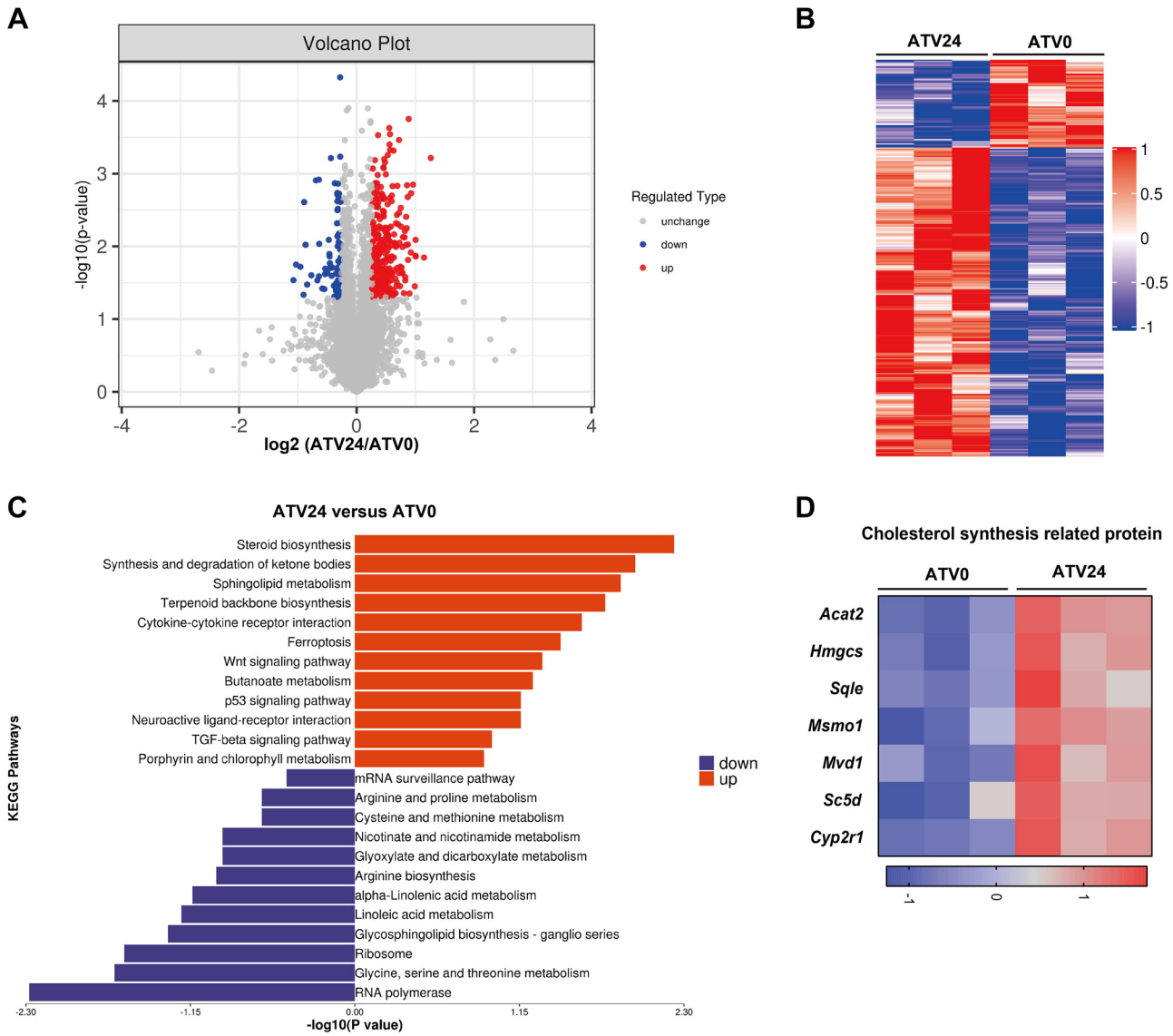


Figure 2. Atorvastatin induced hepatic lipid accumulation associated with promoting cholesterol biosynthesis in Nile tilapia. Volcano plot showing the up- (red) and downregulated (blue) proteins (n = 3) (*A*); hierarchical clustering of the differentially expressed proteins (n = 3) (*B*). The color scale bar locates in the right, and blue and red indicate decreased and increased levels of the identified proteins, respectively (n = 3); KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis of the differentially expressed proteins between treatments (n = 3) (*C*). The ordinate shows the names of the KEGG pathways. The abscissa is the difference significance. Red corresponds to upregulation, and blue corresponds to downregulation; the differentially expressed proteins involved in steroid biosynthesis (n = 3) (*D*). The ordinate is the protein name. The abscissa is the color scale bar. Red corresponds to upregulation, and blue corresponds to downregulation; ATV0 or ATV24, control diet supplemented with 0 or 24 mg/kg atorvastatin. Acat2, acetyl-CoA acetyltransferase 2; Cyp2r1, cytochrome P450 2R1; Hmgcs, 3-hydroxy-3-methylglutaryl-CoA synthase 1; Msmo1, methylsterol monooxygenase 1; Mvd1, mevalonate diphosphate decarboxylase 1; Sc5d, sterol-C5-desaturase; Sqle, squalene epoxidase; n, number of animals measured.

lyase (*acly*), fatty acid synthase (*fasn*), and diacylglycerol acyltransferases (*dgat*) and their transcription factors (*srebp1* and *ppary*) ($P < 0.05$, Fig. 4*C*), and promoted lipid catabolism-related genes such as monoglycerol lipase (*mgl*), autophagy-related genes 12 (*atg12*), sequestosome 1 (*p62*), and carnitine palmitoyl transferase 1a (*cpt1a*) expression ($P < 0.05$, Fig. 4*D*) in the liver of fish. Notably, atorvastatin treatment significantly reduced serum VLDL content ($P < 0.05$, Fig. 4*E*), which is loaded with a large amount of partial TG. Accordingly, atorvastatin treatment inhibited hepatic VLDL assembly-related gene microsomal triglyceride transfer protein (*mttp*) expression ($P < 0.05$, Fig. 4*G*), thus accumulated VLDL content in the liver of fish ($P < 0.05$, Fig. 4*F*). These results indicated that atorvastatin treatment increased TG accumulation possibly via inhibiting VLDL efflux from the liver of fish.

Atorvastatin Reduced Cholesterol and TG Accumulation via Inhibiting LDL Uptake in Adipose Tissue

Given atorvastatin treatment reduced the adipose tissue mass of fish, we further determined its lipid deposition and found that atorvastatin treatment significantly reduced cholesterol (Fig. 5*A*) and TG accumulation (Fig. 5*C*) in the adipose tissue ($P < 0.05$). Further molecular analysis showed that atorvastatin treatment significantly enhanced cholesterol synthesis limited rate enzyme gene (*hmgcr*) expression, but unaltered cholesterol efflux-related gene (*abca1* and *abcg1*) in the

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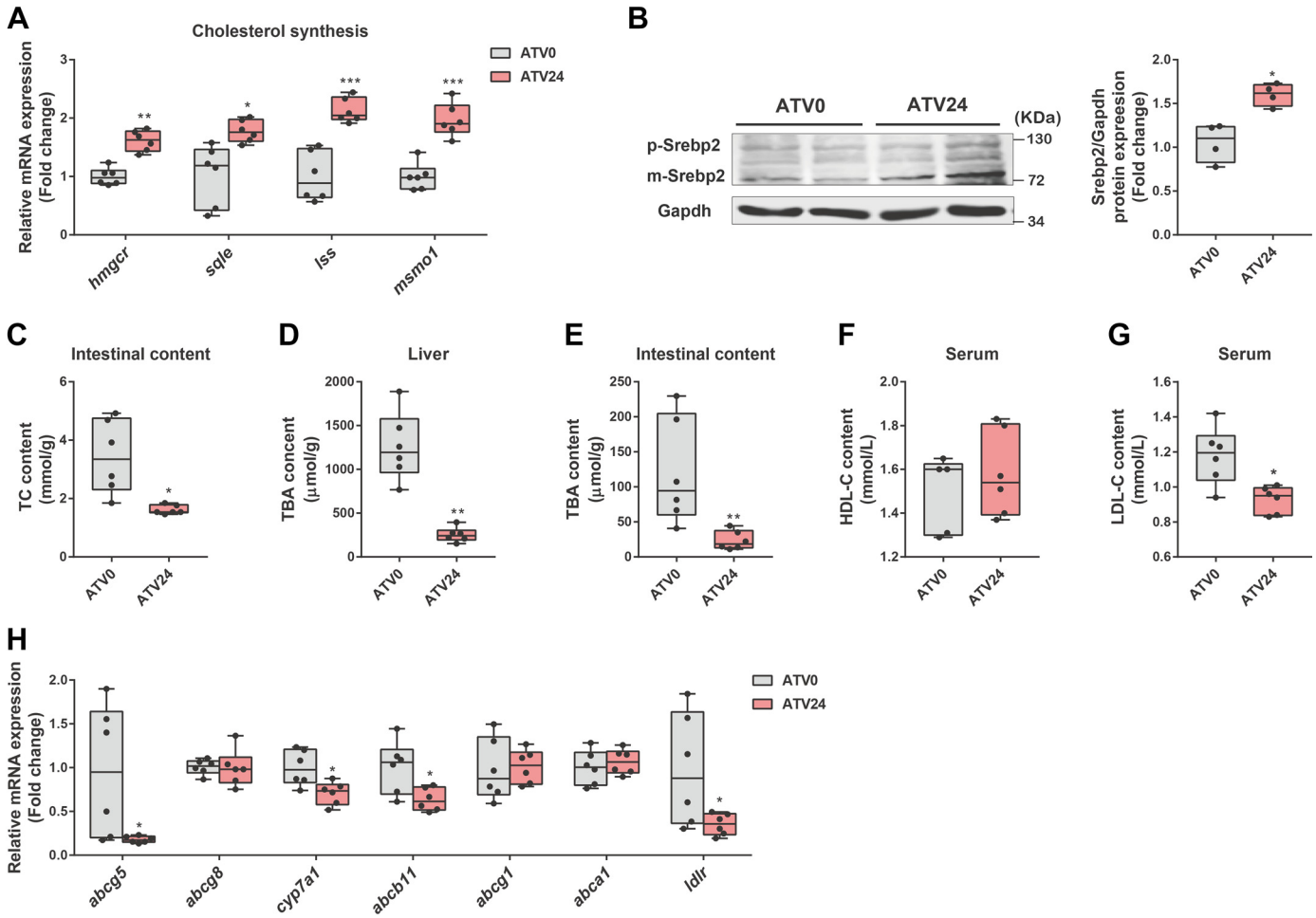


Figure 3. Atorvastatin inhibited bile acid synthesis and efflux, and promoted cholesterol synthesis in the liver of Nile tilapia. mRNA expression of genes related to cholesterol synthesis in the liver ($n = 6$) (*A*); protein expressions of Srebp2 in the liver ($n = 4$) (*B*); total cholesterol (TC) content in the intestinal content ($n = 6$) (*C*); total bile acids (TBA) content in the liver and intestinal content ($n = 6$) (*D* and *E*); high-density lipoprotein cholesterol (HDL-C) in the serum ($n = 6$) (*F*); low-density lipoprotein cholesterol (LDL-C) in the serum ($n = 6$) (*G*); mRNA expression of genes related to cholesterol efflux and uptake and bile acid metabolism in the liver ($n = 6$) (*H*). Data are represented as mean values with their standard deviation; *n*, number of animals measured. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ significant difference was analyzed between ATV24 and ATV0 (two-tailed independent *t* test). *abcb11*, ATP-binding cassette subfamily B member 11; *abca1*, ATP-binding cassette subfamily A member 1; *abcg1*, ATP-binding cassette subfamily G member 1; *abcg5*, ATP-binding cassette subfamily G member 5; *abcg8*, ATP-binding cassette subfamily G member 8; ATV0 or ATV24, control diet supplemented with 0 or 24 mg/kg atorvastatin; *cyp7a1*, cholesterol 7α-hydroxylase; *hmgcr*, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; *lss*, lanosterol synthase; *ldlr*, low-density lipoprotein receptor; *msmo1*, methylsterol monooxygenase 1; *sqle*, squalene epoxidase; Srebp2, sterol regulatory element-binding transcription factor 2.

adipose tissue (Fig. 5*B*). In addition, atorvastatin treatment significantly ($P < 0.05$) promoted the expressions of the lipogenesis-related genes (*acly*, *acca*, *fasn* and *ppary*; Fig. 5*D*), and inhibited lipid catabolism-related genes (*mgl*, *atg5*, *atg12* and *cpt1a*; Fig. 5*E*) and lipoprotein uptake-related gene (*ldlr*, Fig. 5*B*) in the adipose tissue. Therefore, atorvastatin treatment reduced cholesterol and TG accumulation possibly via inhibiting LDL uptake, which contains cholesterol ester and partial TG amounts in fish adipose tissue.

DISCUSSION

Atorvastatin Increased Cholesterol and TG Accumulations in Fish Liver

Atorvastatin is a common lipid-lowering pharmaceutical drug, which decreases cholesterol level via inhibiting HMGCR enzyme involved in cholesterol biosynthesis (19, 36, 37). In humans, cholesterol depletion by statins induced SREBP2 activity, thus further promoted expression of genes related to cholesterol synthesis and uptake such as HMGCR and LDLR (16, 17). Liver is the major site for cholesterol biosynthesis and storage. Also, liver eliminates cholesterol mainly through direct cholesterol and indirect bile acids excretion into gallbladder and intestine (38). A previous study in mice reported that statins increased hepatic cholesterol synthesis and stimulated fecal cholesterol elimination, thus excess synthesized cholesterol did not accumulate in plasma, because its concentration decreased (18). In the present study, although atorvastatin treatment decreased the condition factor, it did not affect hepatosomatic index and serum cholesterol level in fish. Intriguingly, we found that atorvastatin treatment induced lipid deposition in fish liver. Further proteomics analysis between fish treated with ATV0 and ATV24 revealed that lipid metabolism, particularly steroid biosynthesis pathway was upregulated in the liver of

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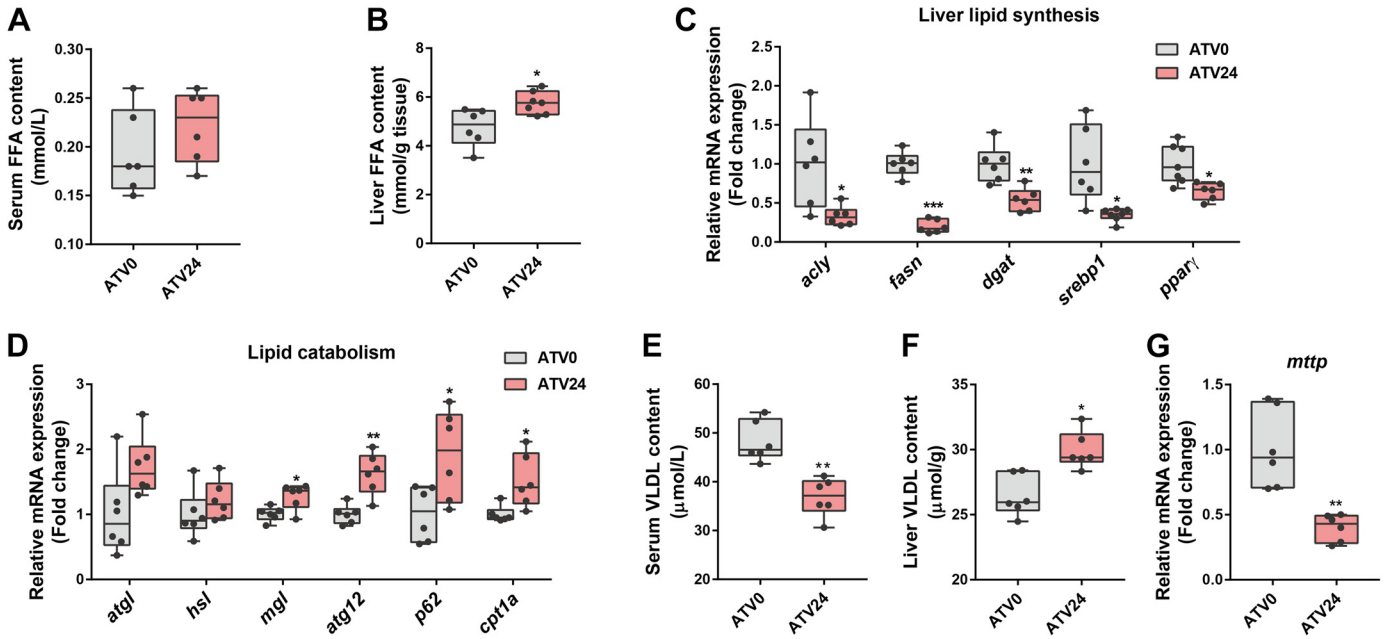


Figure 4. Atorvastatin increased triglycerides (TG) accumulation via inhibiting very-low-density lipoprotein (VLDL) efflux from the liver of Nile tilapia. Free fatty acids (FFAs) in the serum and liver (n = 6) (*A* and *B*); mRNA expression of genes related to lipid synthesis in the liver (n = 6) (*C*); mRNA expression of genes related to lipid catabolism in the liver (n = 6) (*D*); very-low-density lipoprotein (VLDL) contents in the serum and liver (n = 6) (*E* and *F*); mRNA expression of genes related to VLDL assembly in the liver (n = 6) (*G*). Data are represented as mean values with their standard deviation; n, number of animals measured. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{**}P < 0.001$ significant difference was analyzed between ATV24 and ATV0 (two-tailed independent t test); ATV0 or ATV24, control diet supplemented with 0 or 24 mg/kg atorvastatin; *acly*, ATP citrate lyase; *atgl*, adipose triglyceride lipase; *atg12*, autophagy-related genes 12; *cpt1a*, carnitine palmitoyl transferase 1a; *dgat*, diacylglycerol acyltransferases; *fasn*, fatty acid synthase; *hsl*, hormone-sensitive lipase; *mgl*, monoglycerol lipase; *p62*, sequestosome 1; *mttp*, microsomal triglyceride transfer protein; *ppary*, peroxisome proliferator-activated receptor γ; *srebp1*, sterol regulatory element-binding protein 1.

fish treated with ATV24. The increased cholesterol levels in the liver of fish treated with atorvastatin were contributed by the compensatory enhancement of cholesterol biosynthesis-related proteins including Acat2, Hmgcs, Sqle, Msmo1, Mvd1, Sc5d, and Cyp2r1. Moreover, atorvastatin treatment stimulated cholesterol synthesis including *hmgcr* gene and Srebp2 protein but inhibited liver cholesterol efflux into intestine. Also, atorvastatin treatment reduced hepatic bile acid synthesis and efflux into the intestine of fish. In general, atorvastatin treatment induced cholesterol accumulation mainly via compensatorily promoting cholesterol synthesis, and inhibiting bile acids synthesis and efflux. These results suggested that the cholesterol-lowering property of atorvastatin did not appear in fish liver. We hypothesize that fish possess a more delicate and strong cholesterol homeostasis regulation, which meets the body requirement compared with mammals. This might be true because fish are cold-blooded animals. A stable cholesterol amount in fish body helps to maintain normal physiological functions by promoting cell membrane fluidity and avoiding conversion from liquid to solid states. Therefore, fish possesses distinct physiological features that allow them to tolerate higher cholesterol level than mammals.

In addition, statin treatment has been reported to disrupt lipid metabolism. Elevation of TG was observed in human treated with fluvastatin (another statin drug) (39). Similarly, in HepG2 cells, different statins (including ATV) led to obvious increase in TG content (40). In fish, atorvastatin exposure induced *pparα* (transcription regulator of fatty acid catabolism), *pparγ*, and *srebp1* (transcription regulator of adipogenesis) gene expressions in female adult zebrafish liver (25) and trout hepatocytes (24). In addition, statins showed the property of autophagy induction related to the depletion of cellular geranylgeranyl pyrophosphate (GGPP, an isoprenoid metabolite), which is converted from HMG-CoA (41). In the present study, atorvastatin treatment increased TG content in the liver of fish, which was not associated with the findings of the inhibited lipid synthesis (such as *acly*, *fasn*, *dgat*, *srebp1*, and *pparγ*) and promoted lipid catabolism, including lipolysis (*mgl*) and autophagy (*atg12* and *p62*), in the same group. In fact, among many causes of liver lipid deposition, the compromised formation and secretion of TG-rich VLDL is also increasingly considered as prevalent factor to the lipid disorder. The assembly and secretion of VLDL primarily occur in liver. This biochemical process involves the following two steps: microsomal triglyceride transfer protein (MTTP) transfers lipid to apoB, and then they are modified to form mature VLDL for secretion (42). Atorvastatin has also been reported to decrease plasma TG levels in human via inhibiting VLDL assembly and secretion in liver (19, 20, 43). Therefore, atorvastatin exerts lipid-lowering effect mainly through the inhibited VLDL biogenesis and their secretion into the circulatory system by liver. In the present study, atorvastatin treatment elevated VLDL content in the liver, but reduced serum VLDL content and the expression of *mttp* gene related to hepatic VLDL assembly. Therefore, atorvastatin treatment increased TG content via inhibiting VLDL efflux to plasma from the liver of fish

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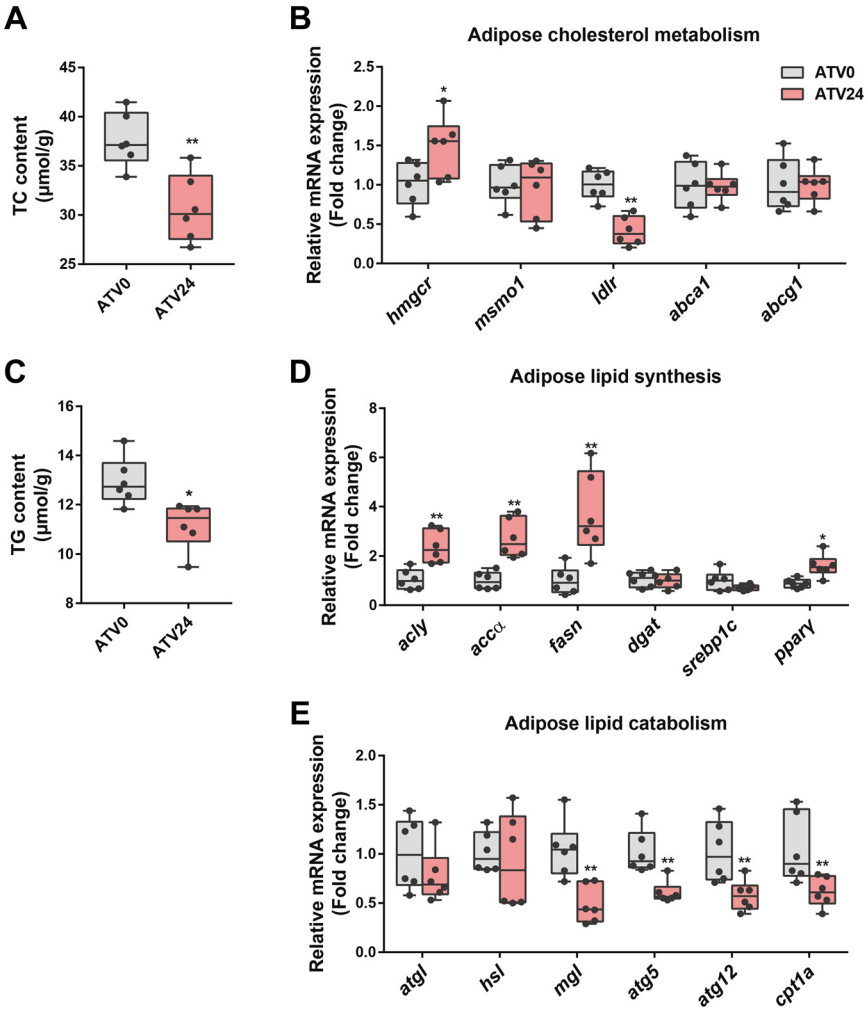


Figure 5. Atorvastatin reduced cholesterol and triglycerides (TGs) accumulation via inhibiting low-density lipoprotein (LDL) uptake in the adipose tissue of Nile tilapia. Total cholesterol (TC) and triglyceride (TG) in adipose tissue (n = 6) (*A* and *C*); mRNA expression of genes related to cholesterol metabolism in adipose tissue (n = 6) (*B*); mRNA expression of genes related to lipid synthesis in adipose tissue (n = 6) (*D*); mRNA expression of genes related to lipid catabolism in adipose tissue (n = 6) (*E*). Data are represented as mean values with their standard deviation; n, number of animals measured. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{**}P < 0.001$ significant difference was analyzed between ATV24 and ATV0 (two-tailed independent t test); ATV0 or ATV24, control diet supplemented with 0 or 24 mg/kg atorvastatin; *abca1*, ATP-binding cassette subfamily A member 1; *abcg1*, ATP-binding cassette subfamily G member 1; *acca*, acetyl-CoA carboxylase alpha; *acly*, ATP citrate lyase; *atgl*, adipose triglyceride lipase; *atg5/12*, autophagy-related genes 5/12; *cpt1a*, carnitine palmitoyl transferase 1a; *dgat*, diacylglycerol acyltransferases; *fasn*, fatty acid synthase; *hmgcr*, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; *hsl*, hormone-sensitive lipase; *ldlr*, low-density lipoprotein receptor; *mgl*, monoglycerol lipase; *msmo1*, methylsterol monooxygenase 1; *ppary*, peroxisome proliferator-activated receptor γ; *srebp1*, sterol regulatory element-binding protein 1.

like that in mammals. However, the regulatory mechanism of the compromised VLDL assembly by statins has not been reported in animals.

Atorvastatin Reduced Cholesterol and TG Accumulation through Inhibiting LDL Uptake in Adipose Tissue

During obesity progression, hypertrophic expansion occurs in adipocyte accompanied with lipid remodeling (44). Adipose tissue is an important storage site for large amounts of cholesterol even though possessing a relatively low cholesterol biosynthesis capacity (<10% of total cholesterol production) (45–47). Previous studies revealed that cholesterol in adipocytes mainly originated from plasma lipoproteins, via cholesterol uptake coupled with TG accumulation through lipoprotein receptors (48–50). In addition, statins exhibited an inhibitory effect on adipocyte differentiation (51, 52). For example, the histology analysis of the adipocyte tissue in obese mice treated with statins revealed that the size of the adipocytes was decreased, accompanied by the declined adipose tissue mass, whereas the fatty acid-binding protein, PPARγ, and SREBP-1c gene expressions were upregulated (53). In the present study, atorvastatin treatment also decreased the mesenteric fat index, along with the reduced cholesterol and TG accumulations in the adipose tissue of fish. The decreased cholesterol content in fish treated with atorvastatin was not linked to the promoted cholesterol synthesis and unaltered cholesterol efflux in the adipose tissue. Furthermore, atorvastatin treatment enhanced lipogenesis pathway, but suppressed lipid catabolism in the adipose tissue. Therefore, we speculate that the enhanced lipogenesis and reduced lipid catabolism might be compensatory responses for the decreased LDL uptake, which contains cholesterol ester and partial TG in the adipose tissue of fish. Expectedly, LDL uptake-related gene was downregulated in the adipose tissue of fish treated with atorvastatin. Since liver produces VLDL, which is further converted into LDL by lipoprotein lipase and triglyceride lipase, we hypothesize that atorvastatin reduces cholesterol and TG accumulation through inhibiting LDL uptake in the adipose tissue. This might be associated with the reduced VLDL efflux into plasma from the liver of fish. It is more likely that cholesterol inhibition by atorvastatin in the liver activated its synthesis and inhibited its elimination to maintain cholesterol homeostasis, and simultaneously reduced its storage in the adipose tissue of fish. Therefore, atorvastatin exerted the lipid-lowering effect in fish adipose tissue, which is attributed to the remodeling of lipid distribution between the liver and adipose tissues (see Fig. 6). These novel findings were not reported before in mammals.

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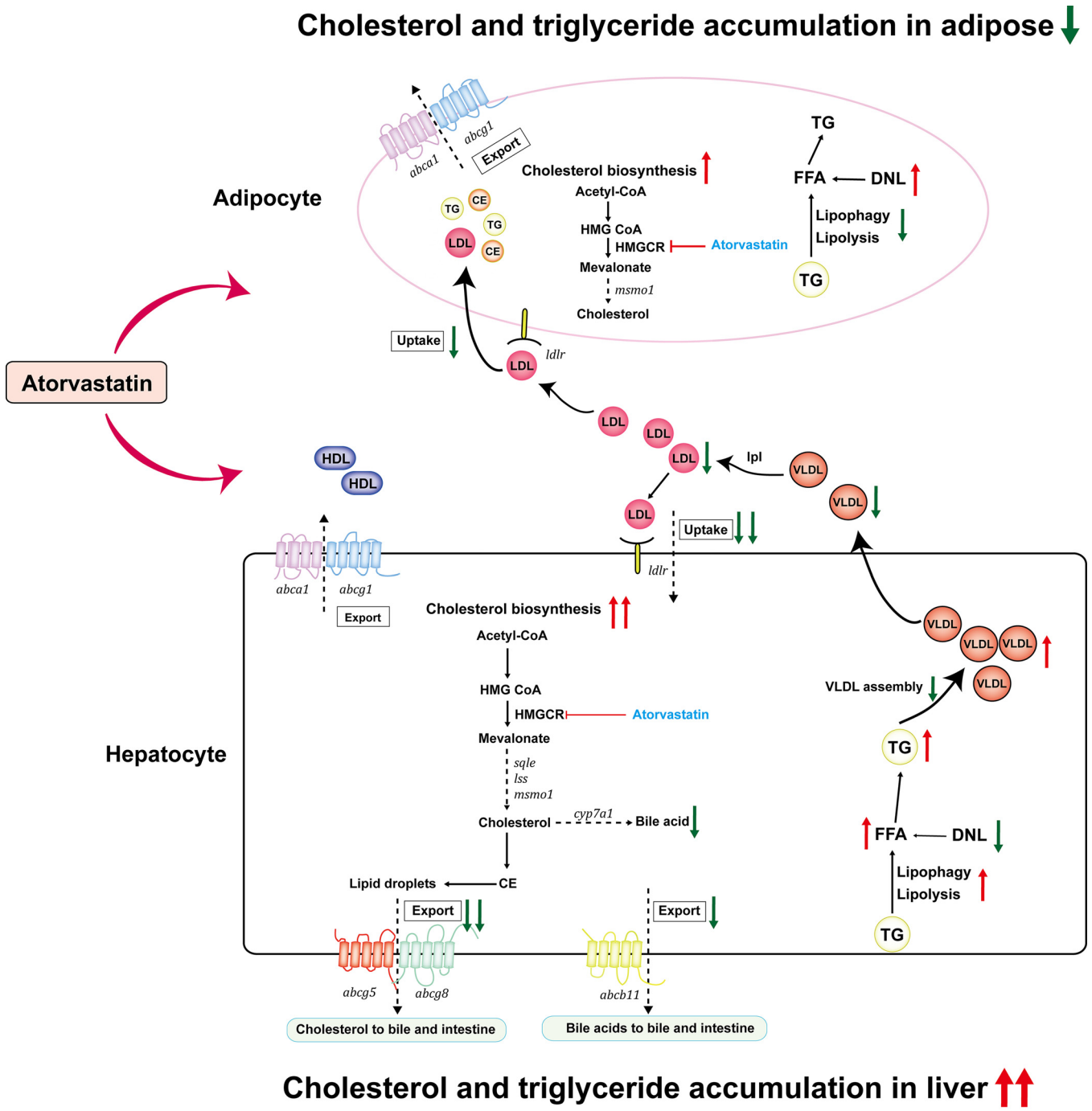


Figure 6. A hypothetical diagram showing atorvastatin treatment remodels lipid distribution between liver and adipose tissues probably through the inhibition of very-low-density lipoprotein (VLDL) efflux from the liver to adipose tissue of fish. In general, atorvastatin increased lipid deposition including cholesterol and triglycerides (TGs) in the liver, but reduced these parameters in the adipose tissue of fish. Furthermore, atorvastatin treatment induced hepatic cholesterol accumulation via inhibiting bile acids synthesis and efflux and promoting cholesterol synthesis, and increased TG accumulation owing to the hepatic VLDL efflux inhibition. In addition, atorvastatin treatment reduced cholesterol and TG accumulation via inhibiting LDL uptake in the adipose tissue of fish. CE, cholesterol ester; DNL, de novo lipogenesis; FFA, free fatty acids; HDL, high-density lipoprotein; LDL, low-density lipoprotein; lpl, lipoprotein lipase; VLDL, very-low-density lipoprotein; *abcb11*, ATP-binding cassette subfamily B member 11; *abcg1*, ATP-binding cassette subfamily G member 1; *abcg5*, ATP-binding cassette subfamily G member 5; *abca1*, ATP-binding cassette subfamily A member 1; *cyp7a1*, cholesterol 7alpha-hydroxylase; *hmgcr*, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; *ldlr*, low-density lipoprotein receptor; *lss*, lanosterol synthase; *msmo1*, methylsterol monooxygenase 1; *sqle*, squalene epoxidase.

Perspectives and Significance

In summary, atorvastatin induced lipid deposition including cholesterol and TG in the liver of fish but reduced them in the adipose tissue unlike in mammals. Furthermore, atorvastatin treatment induced hepatic cholesterol accumulation via inhibiting bile acids synthesis and efflux and compensatorily promoting cholesterol synthesis. Moreover,

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atorvastatin treatment reduced cholesterol and TG contents possibly via inhibiting LDL uptake in the adipose tissue of fish, which is associated with VLDL efflux blockade from the liver to adipose tissue in fish. Therefore, by using atorvastatin, we found that fish might have a different regulatory mechanism from mammals for maintaining cholesterol homeostasis. This knowledge enriches our understanding of the evolutional cause of cholesterol-related diseases in humans, and it is interesting research to further explore the unique regulation mechanism of cholesterol metabolism in fish.

DATA AVAILABILITY

Data will be made available upon reasonable request.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.-X.L., Z.-Y.D., and Y.L. conceived and designed research; R.-X.L., L.-Y.C., B.Y., and W.-H.Z. performed experiments; R.-X.L., L.-Y.C., and B.Y. analyzed data; R.-X.L. interpreted results of experiments; R.-X.L. prepared figures; R.-X.L. drafted manuscript; S.M.L., Y.-F.Q., L.-Q.C., F.Q., M.-L.Z., Z.-Y.D., and Y.L. edited and revised manuscript; Z.-Y.D. and Y.L. approved final version of manuscript.

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