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ORIGINAL ARTICLE

Eicosapentaenoic Acid-Enriched Phosphatidylcholine Attenuated Hepatic Steatosis Through Regulation of Cholesterol Metabolism in Rats with Nonalcoholic Fatty Liver Disease

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Abstract Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the world. Disturbed cholesterol metabolism plays a crucial role in the development of NAFLD. The present study was conducted to evaluate the effects of EPA-PC extracted from sea cucumber on liver steatosis and cholesterol metabolism in NAFLD. Male Wistar rats were randomly divided into seven groups (normal control group, model group, lovastatin group, low- and high-dose EPA groups, and low- and high-dose EPA-PC groups). Model rats were established by administering a diet containing 1% orotic acid. To determine the possible cholesterol metabolism promoting mechanism of EPA-PC, we analyzed the transcription of key genes and transcriptional factors involved in hepatic cholesterol metabolism. EPA-PC dramatically alleviated hepatic lipid accumulation, reduced the serum TC concentration, and elevated HDLC levels in NAFLD rats. Fecal neutral cholesterol excretion was also promoted by EPA-PC administration. Additionally, EPA-PC decreased the mRNA expression of hydroxymethyl glutaric acid acyl (HMGR) and cholesterol 7α-hydroxylase (CYP7A), and increased the transcription of sterol carrying protein 2 (SCP2). Moreover, EPA-PC stimulated the transcription of peroxisome proliferators-activated receptor α (PPARα) and adenosine monophosphate activated protein kinase (AMPK) as well as its modulators, liver kinase B1 (LKB1) and Ca^{2+}/calmodulin-dependent kinase kinase (CAMKK). Based on the results, the promoting effects of EPA-PC on NAFLD may be partly associated with the suppression of cholesterol synthesis via HMGR inhibition and the enhancement of fecal cholesterol excretion through increased SCP2 transcription. The underlying mechanism may involve stimulation of PPARα and AMPK.

Keywords Eicosapentaenoic acid-enriched phosphatidylcholine · Hepatic steatosis · Nonalcoholic fatty liver disease · Cholesterol metabolism

Abbreviations

AMPK	Adenosine monophosphate activated protein kinase
CAMKK	Ca^{2+}/calmodulin-dependent kinase kinase
CYP7A	Cholesterol 7α-hydroxylase
HMGR	Hydroxymethyl glutaric acid acyl CoA reductase,
LKB1	Liver kinase B1
NAFLD	Nonalcoholic fatty liver disease
PPARα	Peroxisome proliferators-activated receptor α
SCP2	Sterol carrying protein 2
TC	Total cholesterol
HDLC	High-density lipoprotein cholesterol
TG	Triacylglycerol
PC	Phosphatidylcholine
EPA-PC	Eicosapentaenoic acid-enriched phosphatidylcholine
EPA	Eicosapentaenoic acid
NASH	Nonalcoholic steatohepatitis
OA	Orotic acid

Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by the accumulation of triacylglycerol (TG) and cholesterol

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in the liver, and it currently affects 20–30% of adults and 10% of children in industrialized countries [1]. Owing to its increasing prevalence, NAFLD has emerged as a worldwide public health problem [2]. The severe NAFLD is nonalcoholic steatohepatitis (NASH), and its pathogenesis is still unclear. However, growing evidence has shown a connection to altered cholesterol homeostasis and hepatic cholesterol accumulation, but not TG [3]. NAFLD is also associated with cardiovascular disease, which contributes more to the mortality of patients with NAFLD than the actual liver disease [4, 5]. Clinical evidence showed that this association was related to dysregulated cholesterol metabolism and was independent of NAFLD-related comorbidities, such as diabetes, hypertension, and hyperlipidemia [6, 7]. Thus, dysregulated cholesterol metabolism is a crucial risk factor in NAFLD.

Sea cucumber is a traditional tonic in China, and it contains various bioactive components, such as collagen polypeptides, polysaccharides, vanadium-binding protein and lipids [8–10]. Eicosapentaenoic acid-enriched phosphatidylcholine (EPA-PC), the focus of the present study, was isolated from the sea cucumber *Cucumaria frondosa*, which contains abundant EPA. Preliminary studies showed that EPA was enriched in EPA-PC at the *sn*-2 position and that EPA-PC possessed the dual activities of EPA and phosphatidylcholine [11]. Multiple reports have shown that EPA and phosphatidylcholine exhibit notable effects against NAFLD [12–14]. However, to date, few bioactivities of EPA-PC from sea cucumber have been reported, except its anti-diabetic effects [15, 16]. Here, we evaluated the effects of EPA-PC on cholesterol metabolism in model rats with NAFLD induced by orotic acid administration. To determine the underlying mechanism of the effect of EPA-PC, we measured the mRNA expression levels of crucial genes involved in hepatic cholesterol synthesis, transformation, and excretion, and analyzed the transcription of their regulators, including PPARα, AMPK, and their upstream genes.

Materials and Methods

Preparation and Characterization of EPA-PC

Dry sea cucumber *C. frondosa* was purchased from a marketplace (Qingdao, China) and authenticated by Professor Yulin Liao from the Institute of Oceanology, Chinese Academy of Science (Qingdao, China). After grinding, the body walls of the sea cucumber were soaked in distilled water. Total lipids were extracted according to an optimized Folch's method [17], and subsequently mixed with 0.15 M NaCl solution (5/1, v/v). After settling into layers, lipids were isolated from the chloroform layer by evaporating the chloroform in a vacuum. EPA-PC was then concentrated and purified by the method described by Lou [11].

Table 1 Fatty acid compositions of EPA-PC

Fatty acid	Proportion (%)
C16:0	2.78 ± 0.18
C16:1	2.13 ± 0.11
C17:0	2.16 ± 0.12
C18:0	7.35 ± 0.39
C18:1	4.73 ± 0.21
C18:2	5.06 ± 0.19
C18:3	1.61 ± 0.10
C20:1	1.61 ± 0.12
C20:3	1.82 ± 0.08
C20:4	6.86 ± 0.39
C20:5	59.3 ± 1.50
C22:6	1.85 ± 0.11
C23:1	2.68 ± 0.21

Briefly, the extracted lipids were continuously eluted with an acetone, chloroform, and methanol-chloroform mixture using a silica gel column. The crude EPA-PC was obtained by evaporating the methanol-chloroform eluant. The purification of EPA-PC was conducted using HPLC (1200, Agilent, Santa Clara, CA, USA) using a quartz column (HP-INNOWAX, Agilent) and the purity of EPA-PC was ~96%. The EPA concentration of EPA-PC was analyzed by GC (6980 N, Agilent). Data (Table 1) showed that the ratio of EPA in EPA-PC's total fatty acids was 59.3% [16].

Preparation of the Liposome

The EPA-PC and EPA were intragastrically administrated in form of liposome, which was prepared using the modified method as Hossain described [18]. Concisely, equimolar agent and cholesterol were dissolved in chloroform and evaporated to a thin film in a vacuum. Then the lipid film was mixed with normal saline. The liposome suspension was obtained after sonicating the mixture for 30 min within a lucifugal nitrogen environment.

Animal Experiment

Male Wistar rats, aged 5 weeks, were purchased from the Vital River Laboratory Animal Center (Beijing, China; Licensed ID: SCXK2007-0001). All the rats were housed in an individual container maintained at a temperature of 23 ± 1 °C and with a 12–12 h light–dark cycle, with free access to distilled water. All animal experiments were conducted in accordance with internationally valid guidelines and experimental protocols, which received prior approved by the animal ethics committee according to the guidelines of the Standards for Laboratory Animals of China (GB 14922-94, GB 14923-94, and GB/T 14 925-94).

Table 2 Ingredients of the rats' forage

Composition	Control (%)	Orotic acid (%)
Cornstarch	49.95	48.95
Sucrose	10	10
Corn oil	10	10
Casein	20	20
Mineral mix[a]	3.5	3.5
Vitamin mix[a]	1.0	1.0
Choline bitartrate	0.25	0.25
Cellulose	5.0	5.0
L-Cystine	0.3	0.3
Orotic acid	–	1.0

[a] Mineral mix and vitamin mix were prepared according to the AIN-93 recipe

The rats were randomly divided into 7 groups of 10 animals each: normal control group, model group, lovastatin group (5 mg/kg bw), low dose (40 mg/kg bw) and high dose (80 mg/kg bw) of EPA-PC group [EPA-PC (L), EPA-PC (H)], and low dose (15 mg/kg bw) and high dose (30 mg/kg bw) of EPA group [EPA (L) and EPA (H)]. The doses of EPA (Sciphar Natural Product, Xian, China) were administrated equally as EPA-PC, calculated according to the concentration of EPA. Normal and model group were administrated with normal saline with the same volume as the liposome described previously. The normal group was supplied with an ordinary diet. All the other groups were fed with a diet containing 1% orotic acid [19]. The diets were established according to AIN93 recipe, and the compositions are summarized in Table 2. Animals in each group were administered for 3 weeks. Rat's feces were collected for 24 h before sacrifice to detect neutral steroid and bile acid excretion. After the last treatment, blood samples were collected to detect TC and HDLC concentrations. The livers were excised to analyze the hepatic TC and TG levels and mRNA expressions of genes relevant to cholesterol metabolism and their upstream modulators.

Determination of Serum and Hepatic Parameters

Serum TC and HDLC concentrations were determined using reagent kits from Biosino (Beijing, China). Total lipids in liver were extracted with a chloroform-methanol mixture (2/1, v/v). The extracting solutions were evaporated and redissolved using Triton X-100. Hepatic TC and TG concentrations were also determined using reagent kits (Biosino, Beijing, China).

Detection of Fecal Neutral Cholesterol and Bile Acid Excretion

Approximately 100 mg of dried feces was soaked in 95% ethanol in 75 °C for 1 h and then centrifuged. The upper and lower layers were collected to extract neutral steroid and bile acid, respectively. The upper layer was evaporated, mixed with 3 ml of 1.25 M NaOH solution and maintained at 120 °C for 3 h. After saponification, the neutral steroid was extracted with petroleum ether. The lower layer was acidified with a 6 M HCl solution using Congo red as the indicator. Petroleum ether was then added to extract the bile acid. Both of the two kinds of extracting solutions were evaporated and dissolved with Triton X-100. The fecal neutral steroid and bile acid excretion were detected using different reagent kits, respectively (Biosino, Beijing, China).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from liver tissues with TRIzol reagent, and 1 μg of RNA was reversed transcribed to cDNA with random primer (Sangog, Shanghai, China) and M-MLV. PCR was conducted by amplifying 0.5 μg of cDNA using an MJ Research thermocycler (TC-96/G/H(b) A; Hangzhou, China). The heating procedure consisted of an initial denaturation at 95 °C for 10 min, followed by cycles of 94 °C for 45 s, annealing for 30 s, 72 °C for 45 s and a final stretching at 72 °C for 10 min. The amplification parameters were listed in Table 3. PCR products were electrophoresed on 1% agarose gel in Tris–acetate-EDTA buffer, and stained with ethidium bromide. Bands were quantitated using the Image J program (National Institutes of Health, Bethesda, USA). Data were presented as the ratios of target gene gray values to those of β-actin.

Statistical Analysis

Data were presented as means ± SD. Statistical comparisons were completed by SPSS version 17.0. Statistical comparison of means among individual groups was done using one-way ANOVA followed by Tukey's test. Probability values were considered significant when $P < 0.05$ or $P < 0.01$.

Results

Body Weight Gain and Food Intake

Significant differences between the EPA-PC groups and the model group concerning body weight, liver-to-body weight ratio are shown in Table 4. Food intake in all rats had no significant difference in all groups [Normal, Model, Lovastatin, EPA-PC (L), EPA-PC (H), EPA (L) and EPA-PC (H)] in the experiment. And intergroup differences in body weight were not significant, except a slight decrease observed in the model group.

Table 3 Basic parameters applied in RT-PCR

Gene	Primer sequence	Annealing temperature (°C)	Number of cycle	Product length (bp)
HMGR	5′–3′: TCTGGCGGTCAGTGGTAA 3′–5′: GGCAGGCTTGCTGAGGTA	57	36	413
CYP7A	5′–3′: AGCCAAGTCAAGTGTCCC 3′–5′: TTCGCTTCTTCCAACCAC	56	34	120
SCP2	5′–3′: TGGTTCAGGGAGGTTTAG 3′–5′: CATCTTGGAACTGGGAAT	52	32	288
PPARα	5′–3′: ACGCTGGGTCCTCTGGTT 3′–5′: GTCTTGGCTCGCCTCTAA	60	36	147
AMPK	5′–3′: GCCCAGATGAATGCTAAG 3′–5′: AGAACGGTTGAGATACTCC	55	32	276
LKB1	5′–3′: CCCCAGAAGCACTTTATGTC 3′–5′: TGTGGTTGGTCGTGTTGTC	58	36	268
CAMKK	5′–3′: TCTGTGGTCGTCTGGCTAT 3′–5′: GTTGGGTTGAAATGGGAG	54	34	383

Hepatic Lipid Parameters

OA notably increased the liver-to-body weight ratio and serum and hepatic TG levels of the model group ($P < 0.01$), indicating that the NAFLD model was successfully established. It is well known that hepatic TG and TC accumulation are the main manifestations of NAFLD and disturbed cholesterol metabolism. After 3 weeks of treatment with high-dose EPA-PC, the three hepatic parameters, TG levels, TC levels, and the liver to body weight ratio, were significantly lowered by 75.30, 40.41 and 20.20%, respectively ($P < 0.01$). We also observed deceased hepatic TG and TC content after treatment with EPA, which was a markedly greater difference compared to that observed following administration of an equivalent dose of EPA-PC ($P < 0.05$).

Serum Cholesterol Profile

Previous studies showed that an orotic acid diet could reduce serum TC levels in NAFLD rats [19]. As shown in Table 4, the serum TC levels of the model group were significantly reduced by 25.0% compared with the normal group ($P < 0.01$). Note that neither EPA-PC nor EPA increased serum TC levels, but instead lowered the levels. In our study, serum HDLC levels in NAFLD rats were also notably decreased ($P < 0.01$). High-dose EPA-PC remarkably improved serum HDLC/TC levels by 53.85% ($P < 0.01$). A similar condition was observed in HDLC/TC levels of EPA (L), EPA (H) when compared with the Model group. HDLC/TC levels in EPA (L), EPA (H) group were also notably decreased by 23.07 and 69.23%. However, no significant differences were noted in the HDLC/TC levels between EPA and EPA-PC group.

Fecal Neutral Cholesterol and Bile Acid Excretion

To further clarify how EPA-PL affects cholesterol metabolism, The fecal neutral cholesterol and bile acid excretion in the rats were analyzed. As shown in Table 4, orotic acid caused a significant reduction in fecal neutral cholesterol and bile acid excretion in comparison to that observed in rats fed a normal diet ($P < 0.01$). High-dose EPA-PC treatment promoted fecal neutral cholesterol excretion from NAFLD rats by 103.12% ($P < 0.01$). However, low- and high-dose EPA-PC exhibited inconsistent effects on fecal bile acid excretion. When compared to the model group, this parameter increased by 28.01% in the low-dose EPA-PC group ($P < 0.05$) and decreased by 41.52% in the high-dose EPA-PC group ($P < 0.01$). A similar phenomenon was observed in the EPA groups, while the effect was significantly less than EPA-PC group ($P < 0.01$).

Relative mRNA Expression of Genes Relevant to Hepatic Cholesterol Metabolism

HMGR, CYP7A, and SCP2 are the key components in hepatic cholesterol metabolism that control the biosynthesis, transformation, and excretion of cholesterol. As shown in Fig. 1, the mRNA expression of HMGR and CYP7A were significantly increased in the model group ($P < 0.01$), while the transcription of SCP2 was inhibited in the model group ($P < 0.01$). Treatment with EPA-PC markedly reversed these changes. Particularly, high-dose EPA-PC reduced the transcription of HMGR and CYP7A by 80.22% ($P < 0.01$) and 79.41% ($P < 0.01$), respectively, and increased the transcription of SCP2 by 92.7% ($P < 0.01$). A similar phenomenon in the HMGR and CYP7A expression was also observed from the rat treated with EPA, which

Table 4 Effects of EPA-PC on body weight, food intake, liver to body weight ratio, serum and hepatic parameters in NAFLD rats

Group	Normal	Model	Lovastatin	EPA-PC (L)	EPA-PC (H)	EPA (L)	EPA (H)
Body weight (g)	304.6 ± 20.5	284.0 ± 24.2	295.0 ± 22.8	299.0 ± 23.6	288.6 ± 28.3	294.2 ± 30.9	298.4 ± 19.3
Food intake (g)	20.0 ± 1.12	19.8 ± 1.04	19.3 ± 1.19	20.2 ± 1.28	19.2 ± 1.44	18.2 ± 1.37	19.1 ± 1.26
Liver to body weight ratio (%)	3.78 ± 0.35^{a}	5.99 ± 0.89^{f}	5.65 ± 0.71^{e}	5.33 ± 0.78^{d}	4.78 ± 0.50^{b}	5.06 ± 0.38^{c}	4.92 ± 0.44^{bc}
Hepatic TG level (mg/g)	13.8 ± 3.6^{a}	126.4 ± 9.6^{f}	76.2 ± 16.3^{e}	59.5 ± 6.8^{d}	31.2 ± 5.2^{b}	76.0 ± 17.2^{e}	42.4 ± 8.3^{c}
Hepatic TC level (mg/g)	2.14 ± 0.20^{a}	7.77 ± 1.57^{g}	7.17 ± 0.58^{f}	5.85 ± 0.48^{d}	4.63 ± 0.37^{b}	6.22 ± 0.39^{e}	5.57 ± 0.40^{c}
Serum TC level (mmol/L)	5.28 ± 0.49^{d}	3.96 ± 0.49^{c}	3.14 ± 0.54^{a}	3.95 ± 0.29^{c}	3.59 ± 0.39^{b}	3.88 ± 0.59^{c}	3.50 ± 0.56^{b}
Serum HDLC level (mmol/L)	0.95 ± 0.08^{e}	0.52 ± 0.14^{a}	0.69 ± 0.15^{bc}	0.64 ± 0.10^{b}	0.73 ± 0.10^{cd}	0.63 ± 0.09^{b}	0.76 ± 0.11^{d}
Serum HDLC/TC	0.18 ± 0.01^{bc}	0.13 ± 0.03^{a}	0.24 ± 0.05^{e}	0.16 ± 0.01^{b}	0.20 ± 0.05^{cd}	0.16 ± 0.03^{b}	0.22 ± 0.05^{d}
Fecal neutral cholesterol excretion (mg/day)	7.60 ± 0.34^{g}	3.22 ± 0.28^{a}	5.16 ± 0.35^{c}	5.29 ± 0.19^{d}	6.50 ± 0.37^{f}	4.43 ± 0.32^{b}	5.41 ± 0.67^{e}
Fecal bile acid excretion (μmol/day)	8.24 ± 0.91^{f}	4.07 ± 0.50^{d}	3.31 ± 0.56^{c}	5.21 ± 0.90^{e}	2.38 ± 0.20^{a}	5.27 ± 0.77^{e}	3.15 ± 0.46^{b}

Data are presented as means ± SD, $n = 10$

Within each experiment, values with different superscript letters are significantly different ($P \leq 0.05$; *post hoc* Tukey's multiple range test)

reveals that there is no significant difference in EPA group with EPA-PC group.

Transcription Levels of PPARα, AMPK, and Their Upstream Genes

PPARα and AMPK are the main regulators of HMGR and CYP7A. To further explore the mechanism underlying the effects of EPA-PC on NAFLD, we analyzed the mRNA expression of PPARα, AMPK, and its upstream modulators, including LKB1 and CAMKK. As shown in Fig. 2, the orotic acid diet had no effect on the transcription of PPARα, but markedly reduced the expression of genes in the AMPK pathway. Nevertheless, high-dose EPA-PC treatment upregulated the transcription of PPARα, AMPK, LKB1 and CAMKK by 137.79, 194.74, 193.94, and 81.48%, respectively. The expression of PPARα was increased in the EPA group compared to the Model groups (Fig. 2a), similar with EPA-PC ($P > 0.05$). However, The expression of AMPK, CAMKK and LKB1 were increased markedly in the EPA-PC group compared to the EPA groups ($P < 0.05$).

Discussion

In the present study, we investigated the effect of EPA-PC isolated from the sea cucumber *C. frondosa* on cholesterol metabolism in NAFLD rats. Results from our study clearly demonstrated that the orotic acid diet successfully induced NAFLD and impaired cholesterol homeostasis in the model animals. We found that EPA-PC dramatically alleviated hepatic lipid accumulation, reduced the hepatic TG levels, TC levels, and the liver:body weight ratio in NAFLD rats. Some evidence has suggested that EPA-PC prevented an increase in the cholesterol levels in NAFLD rats. Dietary EPA-PC-induced reduction in the serum TC concentration and HDLC/TC level in NAFLD rats implied that EPA-PC increased cholesterol catabolism. Obviously, dose-dependence existed and a similar effect to that of EPA. Therefore, in our search for a possible explanation for these phenomena, we analyzed cholesterol excretion and transformation. And we found that fecal neutral cholesterol excretion could be promoted by EPA-PC administration. These results showed that EPA-PC may lower hepatic and serum cholesterol levels by enhancing cholesterol excretion.

The liver is the most crucial organ in mammalian cholesterol metabolism, and four major metabolic pathways maintain cholesterol homeostasis [20]. The pathways supplying cholesterol to the liver are the endogenous synthetic pathway and the exogenous absorptive pathway. The other two pathways are the major cholesterol disposal pathways: a transforming pathway that converts cholesterol into bile acid and a direct excretion pathway [21]. In our study,

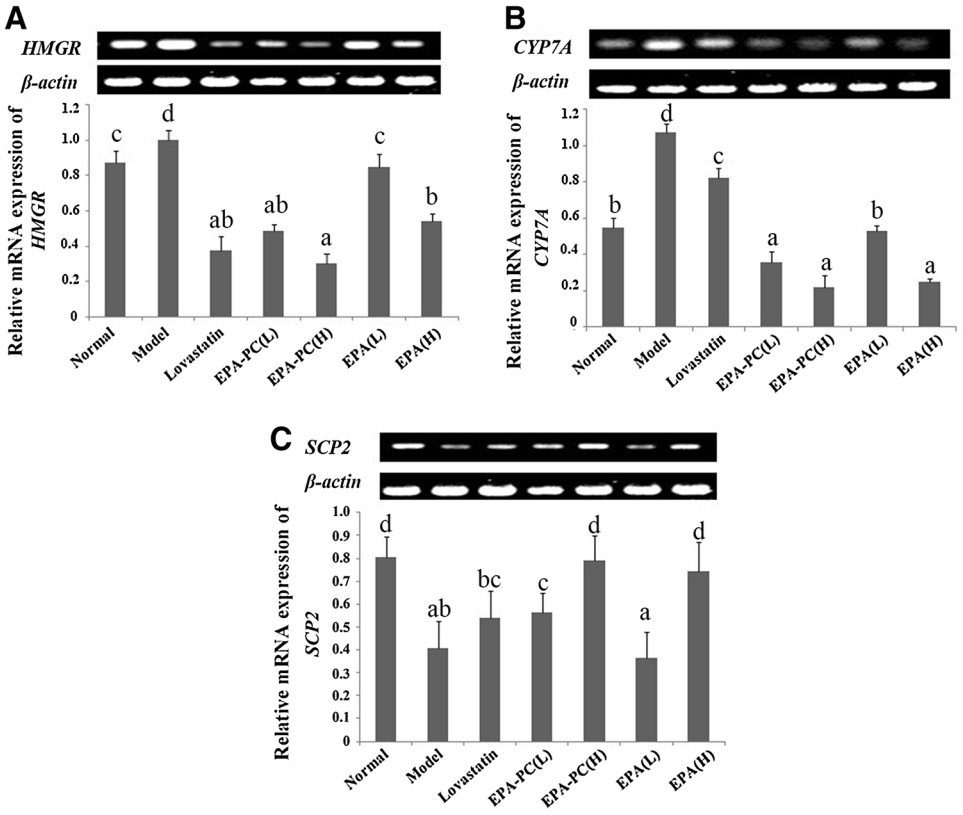

Fig. 1 Effects of EPA-PC on crucial genes relevant to cholesterol metabolism in liver of NAFLD rats. **a**, **b** and **c** are representative mRNA expression levels of *HMGR*, *CYP7A* and *SCP2*, respectively. Results were normalized by *β-actin*. Data are presented as means ± SD ($n = 10$/group). Within each experiment, *bars* topped by *different letters* are significantly different ($P \leq 0.05$; *post hoc* Tukey's multiple range test)

EPA-PC administration decreased both serum and hepatic TC levels and elevated the fecal cholesterol level. However, the two different doses of EPA-PC showed opposite effects on bile acid excretion. Besides excess cholesterol absorption, aberrant biosynthesis is another possible mechanism leading to hepatic cholesterol accumulation.

Thus, to determine the underlying mechanism, the mRNA expression of HMGR, CYP7A, and SCP2, the key components in biosynthesis, transformation, and excretion of cholesterol, was analyzed. In the present study, EPA-PC reduced the transcription of HMGR and CYP7A by 80.22% ($P < 0.01$) and 79.41% ($P < 0.01$), respectively, and increased the transcription of SCP2 by 92.7% ($P < 0.01$). Our data suggested that supplementation with EPA-PC could effectively regulate the anomalous expressions of the abovementioned genes. It is well known that HMGR is the rate-limiting enzyme in cholesterol biosynthesis [22]. Multiple studies have shown that the expression of HMGR was stimulated in both humans and rodents with NAFLD [23–26]. EPA-PC attenuated the elevated transcription induced by the orotic acid diet, indicating that EPA-PC could reduce the cholesterol synthesis. The degradation and excretion of cholesterol are two other aspects of cholesterol homeostasis. CYP7A is the key modulator of cholesterol degradation. CYP7A is a member of the cytochrome P450 family, and it catalyzes the rate-limiting step of bile acid synthesis [27]. CYP7A expression is upregulated by PPARα. Hunt's study showed that the mRNA expression of CYP7A in mice increased two to threefold after supplying mice with a PPARα agonist [28]. Additionally, there is a feedback regulation system between hepatic cholesterol and CYP7A. In rodents, the metabolic intermediates of cholesterol are ligands for the liver X receptor, which can consequently stimulate the transcription of CYP7A [29, 30]. In contrast, it has been reported that bile acid can disrupt the stability of CYP7A mRNA via bile acid response elements located in the 3′-untranslated region [31, 32]. In our study, the mRNA expression of CYP7A in NAFLD rats was increased in response to elevated hepatic TC levels. The direct excretion of cholesterol relies on specific transport mechanisms responsible for the transfer of cholesterol from the endoplasmic reticulum to the cellular membrane. SCP2 is an important carrier in this transport process [33]. Murphy *et al*. reported that SCP-2 increases cholesterol uptake and transport throughout the cell while inhibiting the flux of cholesterol from the cell through the HDL receptor [34]. Further studies showed that SCP-2 enhanced the transfer of cholesterol from plasma membranes for esterification in the endoplasmic reticulum in intact cells [35]. Puglielli *et al*. reported that the rapid transport of de novo synthesized cholesterol to the plasma membrane was reduced after treatment with SCP2 antisense RNA, suggesting the existence of an SCP2-dependent mechanism that mediates transport of the major fraction of newly synthesized cholesterol [34, 35].

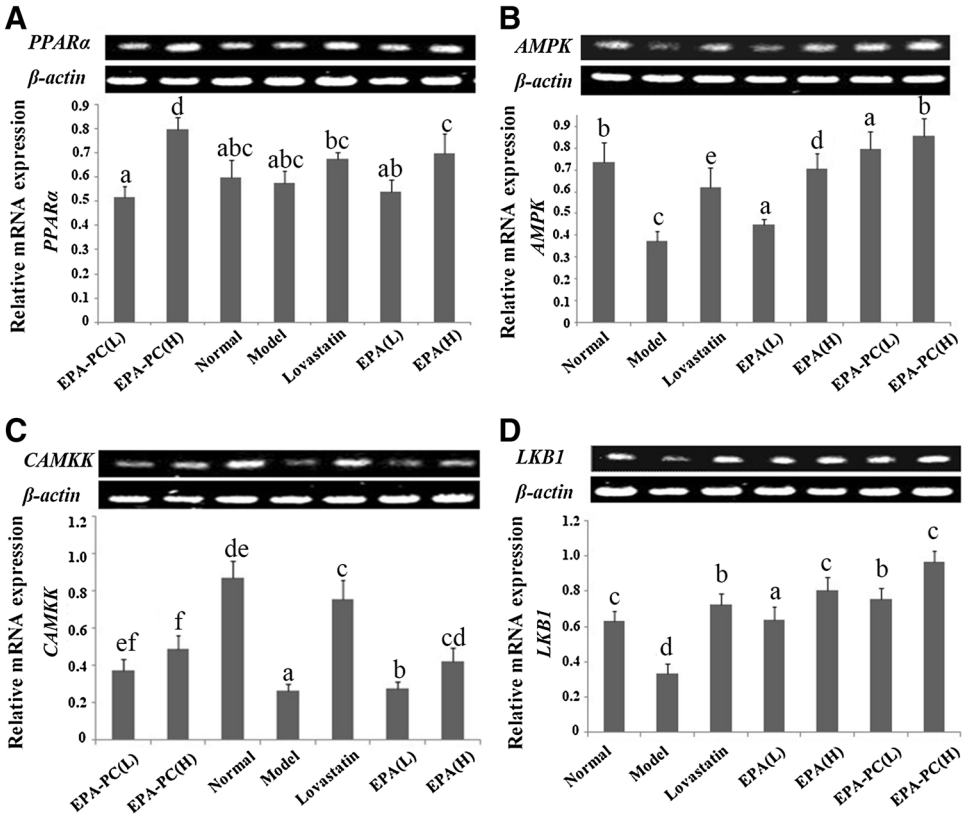

Fig. 2 Effects of EPA-PC on PPARα, AMPK and its modulators in liver of NAFLD rats. **a**, **b, c** and **d** are representative mRNA expression levels of *PPARα*, *AMPK*, *LKB1* and *CAMKK*, respectively. Results were normalized by *β-actin*. Data are presented as means ± SD ($n = 10$/group). Within each experiment, *bars* topped by *different letters* are significantly different ($P \leq 0.05$; *post hoc* Tukey's multiple range test)

Moreover, SCP2 can also accelerate cholesterol cycling by reducing the availability of cholesterol for cholesterol ester synthesis and suppressing the activity of CYP7A to inhibit conversion into bile acid [36]. In our study, EPA-PC notably upregulated the mRNA expression of SCP2. This upregulation may be responsible for the increased cholesterol excretion and reduced levels of fecal bile acid after EPA-PC treatment and a similar effect to that of EPA.

It is well established that EPA could improve lipid metabolism by enhancement of fatty acid β-oxidation. Thus, we examined the effects of dietary EPA-PC on the expression of genes involved in fatty acid formation and β-oxidation in different tissues. In our study, EPA-PC stimulated the mRNA expression of PPARα, AMPK, and its upstream modulators, including LKB1 and CAMKK. PPARα and AMPK are the main regulators of HMGR and CYP7A [37]. AMPK is present in all tissues as a heterotrimeric complex consisting of a catalytic α subunit and regulatory β and γ subunits [38]. LKB1 and CAMKK are two upstream kinases that have been shown to activate AMPK via phosphorylation at Thr172 in the subunit [39]. AMPK plays a crucial role in reducing cholesterol synthesis by inhibiting the activity of HMGR through phosphorylation of Ser872 [40]. Additionally, AMPK may affect the expression of CYP7A by activating PPARα. However, the positions of AMPK and PPARα within the pathway are still controversial. Hsu *et al*. reported that a toxin from *Monascus* could activate PPARα as an agonist of AMPK [41]. In contrast, Chen *et al*. showed that fenofibrate could facilitate the phosphorylation of AMPK as a classical agonist of PPARα [42]. In the present study, the transcriptional upregulation of the PPARα, AMPK and its modulators, indicating that the mechanism underlying the regulatory effects of EPA-PC on cholesterol metabolism involved the activation of these upstream transcription factors. High-dose EPA-PC reduced the transcription of CYP7A, thus ameliorated cholesterol accumulation and consequently lowered fecal bile acid excretion, despite the expression of PPARα was increased. Moreover, there is no difference between the EPA and EPA-PC groups on the expression of PPARα. Previous studies revealed that PPARα is a dominant mediator for the effects of dietary fatty acids on gene expression. Furthermore, the findings of show that in healthy individuals neither the lipid structure nor the overall fatty acid composition of supplements that contained EPA and DHA significantly influence their bioavailability during dietary supplementation [43]. It seems that the significantly upregulation of PPARα by EPA-PC is related to activation of PPARα by EPA. One possible explanation is that no changes in EPA bioavailability during longer-term supplementation in rats with nonalcoholic fatty liver disease. Considering the fact that low-dose EPA-PC showed a contrary effect on bile acid excretion and similar effects were observed at the equivalent dose of EPA, the mechanism

may involve the activation of PPARα, in part. On the contrary, the effect of EPA-PC on the mRNA expression of AMPK and LKB1 was superior to that of high-dose EPA ($P < 0.05$). The result showed that EPA-PC seemed to act on the AMPK, a transcription factor playing a major role in cholesterol metabolism treated with EPA-PC. Thus, we discovered that EPA-PC increased the transcription of both AMPK and its upstream kinases, suggesting that the high expression of AMPK may underlie the promoting effects of EPA-PC on cholesterol metabolism in NAFLD.

Conclusions

In conclusion, the present study demonstrated that EPA-PC could significantly ameliorate hepatic TC accumulation in NAFLD. The underlying bio-mechanisms involved the enhancement of AMPK, PPARα, and SCP2 transcription and the inhibition of HMGR and CYP7A transcription, which suppressed cholesterol synthesis and enhanced excretion. Therefore, these results suggest the application of EPA-PC as a complementary therapy for NAFLD.

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Compliance with Ethical Standards

Conflict of interest The authors report no conflict of interest.

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