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Influence of a Dietary Vegetable Oil Blend on Serum Lipid Profiles in Large Yellow Croaker (*Larimichthys crocea*)

Si Zhu,[†] Peng Tan,[†] Renlei Ji,[†] Xiaojun Xiang,[†] Zuonan Cai,[†] Xiaojing Dong,[†] Kangsen Mai,[†,‡] and Qinghui Ai*[,†,‡]

[†]Key Laboratory of Aquaculture Nutrition and Feed (Ministry of Agriculture) & Key Laboratory of Mariculture (Ministry of Education), Ocean University of China, 5 Yushan Road, Qingdao, Shangdong 266003, China

[‡]Laboratory for Marine Fisheries and Aquaculture, Qingdao National Laboratory for Marine Science and Technology, Qingdao, Shangdong China

Supporting Information

ABSTRACT: Serum lipid metabolic responses are associated with certain metabolic disorders induced by dietary habits in mammals. However, such associations have not been reported in fish. Lipidomic analyses were performed to investigate fish lipid metabolic responses to a dietary vegetable oil (VO) blend and to elucidate the mechanism of how the dietary VO blend affects serum lipid profiles. Results showed that the dietary VO blend strongly affects serum lipid profiles, especially the ratio of triglyceride/phosphatidylcholine (TAG/PC), via inhibiting hepatic PC biosynthesis and facilitating hepatic and intestinal lipoprotein assembly. Studies in vitro suggested that changes of serum TAG/PC ratio may be partially attributed to altered fatty acid composition in diets. Additionally, the reduction of 16:0/18:1-PC induced by the dietary VO blend may play a role in abnormal lipid deposition through inhibiting PPARA-mediated activation of β-oxidation. These findings suggested that the serum TAG/PC ratio might be a predictive parameter for abnormal lipid metabolism induced by dietary nutrition in fish.

KEYWORDS: *vegetable oil blend, serum lipid profiles, phosphatidylcholine, triglyceride, β-oxidation, large yellow croaker*

INTRODUCTION

Substitution of fish oil by various other lipid sources has become an imperative issue since the rise and continued expansion of aquaculture and the decreasing production of fish oil.[1,2] Vegetable oils, with relatively acceptable prices, large production volumes, and relatively low organic contaminants, are promising alternatives to fish oil. Considerable success in partially or totally replacing fish oil with vegetable oils without inhibiting fish growth performance have been reported in some commercial fish species.[3−6] However, long-term intake of vegetable oils often leads to changes in lipid metabolism of fish. Such changes include induced abnormal lipid deposition in the liver and decreased long chain polyunsaturated fatty acid (LC-PUFA) levels in tissues, which diminish the health benefits of fish for human consumption.[3−6] The effects of replacing fish oil with vegetable oils on gene expression related to lipid metabolism in fish tissues have been widely investigated, although studies were mainly focused on the regulation of fatty acids and triglyceride (TAG) mechanism by dietary vegetable oils.[7−9] Possible effects of dietary vegetable oils on other lipid classes such as phospholipids and sphingolipids, and, further, the mechanisms in regulating these lipid classes are poorly studied. Only a few reports have investigated whether fatty acid distribution of phospholipid fractions reflects dietary nutrition.[10,11] It is well-known that phospholipids and sphingolipids play a pivotal role in regulating lipid, lipoprotein, and energy metabolism.[12−14] In addition, despite the extensive studies on the liver and intestine, the effects of dietary vegetable oils on lipid metabolic responses in the blood of fish are still quite fragmentary.

Blood is a major carrier of lipids which bathes tissues and organs in the entire body and functions as a liquid highway for lipids that are being secreted, excreted, and discarded from different tissues. Studies have reported that the levels of lipids in blood and other organs are closely correlated, and changes in blood lipid profiles reflect the metabolic perturbations in response to metabolic alteration at a systemic level.[15−17] In mammals, the associations between serum lipid metabolic responses and certain metabolic disorders under excessive dietary nutrition are well demonstrated, and gene expressions related to lipid metabolism were detected to explain changes in response to diets.[18,19] However, such associations have not been reported in fish, where comprehensive data are still quite deficient.

The large yellow croaker (*Larimichthys crocea*) is an economically important marine fish species that is widely cultured in southeast China due to its delicious taste and high nutritional value.[20] The production of this fish species was 165496 t in China in 2016, earning it first place in mariculture output, which it continuous to hold.[21] Vegetable oils have been commonly used in the culture of large yellow croaker due to the limited production of fish oil. Our previous study showed that partially or totally replacing fish oil with a vegetable oil blend (soybean oil/linseed oil = 1:1) induced abnormal lipid metabolism in large yellow croaker in the form of decreased

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tissue levels of LC-PUFA and increased hepatic lipid accumulation.[22] Additionally, it was found that this fish species shares a strikingly similar regulation in lipid metabolism with that of other fish species and mammals,[23,24] which suggested that large yellow croaker is an appropriate fish species to investigate the mechanism of how dietary vegetable oils influence serum lipid profiles.

In the present study, our main objective was to investigate the lipid metabolic responses in the serum of fish under the replacement of fish oil by a vegetable oil blend (soybean oil/linseed oil = 1:1) and to elucidate the mechanism of how dietary vegetable oil blend affects serum lipid profiles. The knowledge of biochemical changes of fish will be helpful in seeking effective measures to prevent and alleviate abnormal lipid metabolism induced by long-term use of vegetable oils.

MATERIALS AND METHODS

Materials. Acetonitrile, isopropanol, tetrahydrofuran, and formic acid (LC-MS grade) were purchased from Sigma-Aldrich (St. Louis, Mo). Fatty acids (purity ≥99%) including docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), linoleic acid (LA, 18:2n-6), and linolenic acid (LNA, 18:3n-3) were obtained from Sigma-Aldrich (St. Louis, Mo). Lipid standards including phosphatidylcholine (PC; 18:1/14:0), phosphatidylethanolamine (PE; 16:0/18:1), lysophosphatidylcholine (LPC; 20:0), lysophosphatidylethanolamine (LPE; 17:1), sphingomyelin (SM; d18:1/18:0), and diacylglycerol (DAG; 16:0/18:1) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). TAG (14:0/18:2/18:1) was purchased from Larodan (Limhamn, Sweden).

Diet Preparation and Feeding Trial. Diet preparation and fish rearing were reported in our previous studies.[22] In brief, three isoproteic (41% crude protein) and isolipidic diets (12% crude lipid) were formulated with 0%, 50%, and 100% of fish oil replaced by a vegetable oil blend (soybean oil/linseed oil = 1:1) named FO (fish oil), FV (fish oil/vegetable oil blend = 1:1), and VO (vegetable oil blend). The formula and fatty acid composition of the experimental diets are shown in Supporting Information, Tables 1 and 2. Disease-free and similar sized large yellow croakers were obtained and reared at Xihu harbor (Ningbo, China). A total of 270 fish with an average initial weight of 8.93 ± 0.20 g were fasted for 24 h and randomly allocated to nine floating net cages (1.5 × 1.5 × 2.0 m) corresponding to triplicate cages of the three dietary treatments. Fish were fed twice per day to apparent satiation for 70 days (5th of August to 15th of October). Water temperature ranged between 23.5 and 30.0 °C, and dissolved oxygen ranged between 6.0 and 7.0 mg/L during the feeding trial. All diets were stored at −20 °C until used to maintain good quality on the farm. The protocols used for fish husbandry and handling in this study were approved by the Institutional Animal Care and Use Committee of Ocean University of China.

Sample Collection. At the end of the experiment, fish were fasted for 24 h to empty their guts before sampling. Nine fish per treatment (three fish per cage) were randomly selected and anesthetized with MS222 (Sigma, USA). One milliliter of blood from each individual fish was phlebotomized and stored at 4 °C for 3−4 h. After centrifugation (3000*g* for 10 min at 4 °C), the serum was collected and stored at −80 °C before analysis. The liver and intestine samples were collected from those bloodless fish, immediately frozen in liquid nitrogen, and stored at −80 °C prior to analysis.

Primary Hepatocyte Culture and Treatment. Cultivation of primary hepatocytes was performed according to our previous report.[25] Briefly, livers were isolated from large yellow croakers and washed in cold sterile PBS. Liver tissue was then dissected into 1 mm^3 pieces and digested with 0.25% Trypsin-EDTA (Gibco) at room temperature for 10 min. The cell suspension was filtered and centrifuged at 800*g* for 10 min. Primary hepatocytes were resuspended and cultured (1 × 10^6 cells/mL) in six-well plates with complete medium (DMEM/F12 medium containing 10% FBS, 1 × streptomycin, and 1 × penicillin) and then maintained at 28 °C in 5% CO_2. After 2 d of cultivation, primary hepatocytes were starved with FBS free DMEM/F12 overnight. In consideration of the major fatty acid composition in fish oil and vegetable oil blend, the cells were incubated with 100 μM DHA, EPA, LA, and LNA conjugated with 2% fatty acid free bovine serum albumin (BSA) (Equitech-Bio, USA) in DMEM/F12 for 6 h. Cells in the control group were incubated with 2% BSA in DMEM/F for 6 h.

UPLC Analysis. Chromatographic separation was carried out on an Acquity UPLC BEH C8 analytical column (2.1 × 100 mm, 1.7 μm) using an Acquity Ultra Performance LC system (Waters). The serum samples were dissolved in 1000 μL water/acetonitrile (1:4, by vol) and centrifuged at 12,000 *g* for 10 min at 4 °C. The supernatant was filtered through a 0.22 μm ultrafiltration membrane (Millipore, USA). An aliquot (3 μL) of each sample was injected into the column for analyzing. The elution was performed with mobile phase A composed of acetonitrile/isopropanol/tetrahydrofuran (1:1:1, by vol) and mobile phase B composed of water/acetonitrile (1:2, by vol), both containing 0.1% formic acid and 0.01% lithium acetate. The gradient elution was programmed as follows: 0−3 min, 2% A; 3−5 in, 2−30% A; 5−12 min, 30−50% A; 12−22 min, 50−80% A; 22−25 min, 80% A; 25−27 min, returning to initial 2% A; 27−30 min, maintaining at 2% A. The total gradient duration was 30 min at a flow rate of 0.5 mL/min.

Mass Spectrometric Analysis. Mass spectrometric analysis was conducted on Q-TOF Premier (Waters) operating in both positive and negative ion modes. For positive mode, the capillary voltage was to 3.0 kV and the sampling cone voltage was set to a ramp of 25−40 V. For negative mode, the capillary voltage was to 2.6 kV, and the sampling cone voltage was set to a ramp of 25−40 V. MS^E analysis was performed on a mass spectrometer at 6 kV for low collision energy and a ramp of 25−50 kV for high collision energy. Data were collected in centroid mode from 100 to 1200 *m*/*z*. The instrument was previously calibrated with sodium formate, and the lock mass spray for precise mass determination was set by leucine encephalin.

Identification and Semiquantitative Analysis of Lipids. The precise molecular weight (the mass difference <5 ppm) of each lipid species was obtained by a Q-TOF MS system. Structural identification of lipids was performed by comparison of the retention time, molecular compositions, and specific fragmentation behaviors with corresponding lipid standards. The fragmentation patterns of PC, PE, TAG, DAG, and SM have been previously described in our and others' reports.[26−29] A database search was also performed using METLINE (http://metlin.Scripps.edu), HMDB (http://www.hmdb.ca), and MAPS (http://www.lipidmaps.org) to elucidate the putative structure of lipids. The internal lipid standards (5 nmol/mL) were added into samples for semiquantitative analysis of lipids in large yellow croaker according to previous work.[30,31] It is noted that these lipid standards were not detected in the serum of large yellow croaker during pre-experiments. Extracted ion chromatogram of each lipid species was obtained and integrated with the corresponding area in the low collision energy scan (6 V). After variable ionization efficiencies at different proportions of the mobile phase were calibrated, semiquantitative analysis was conducted based on the area ratio of each lipid species to the corresponding internal standard.

Quantitative Real-Time Polymerase Chain Reaction. RNA extraction, cDNA synthesis, and quantitative real-time PCR were performed based on the procedures described by Zuo et al.[32] The primer sequences of phosphate cytidylyltransferase 1, choline, alpha (*pcyt1a*), choline phosphotransferase 1 (*chpt1*), phosphatidylethanolamine *N*-methyltransferase (*pemt*), diacylglycerol *O*-acyltransferase 1 (*dgat1*), diacylglycerol *O*-acyltransferase 2 (*dgat2*), microsomal triglyceride transfer protein (*mttp*), apolipoprotein B (*apoB*), carnitine palmitoyltransferase 1A (*cpt1a*), peroxisome proliferator-activated receptor alpha (*ppara*), and *β-actin* were listed in Supporting Information, Table 3. 18S rRNA, GAPDH, β-actin, and ubiquitin were selected to test for normalization of expression levels in the liver and intestine of large yellow croaker. Their stability and suitability as reference genes were validated by geNorm (version 3.5) and NormFinder algorithms.[33,34] No significant differences in β-actin expression were observed among all treatments, indicating that β-actin

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can be used as a reference gene in this study. The fluorescence data acquired were normalized to β-actin based on the $2^{-\Delta\Delta t}$ method.[35] For in vivo study, the relative mRNA expression of target genes in fish fed the FO diet was selected as the calibrator; for in vitro study, the relative mRNA expression of target genes in primarily hepatocytes incubated with 2% BSA (the control group) was selected as the calibrator.

Immunoblotting Analysis. Total protein in liver tissues was extracted and quantified using a Bradford Protein Assay Kit (Beyotime Institute of Technology, China). An equal amount of protein (15 μg) from each sample was separated on 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred to activated polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) at room temperature for 1 h, followed by incubation overnight with PCYT1A (4454, Cell Signaling Technology), GAPDH (2118, Cell Signaling Technology), CPT1A (12252, Cell Signaling Technology), CHPT1 (sc-515577, Santa Cruz), PPARA (ab8934, Abcam), or DGAT2 (ab96094, Abcam) antibody at 4 °C. After washing with TBST three times, membranes were incubated with horseradish peroxide (HRP)-conjugated secondary antibody (A0208, Beyotime Institute of Biotechnology) for 1 h. Finally, immune complexes were visualized using an Electrochemiluminescence (ECL) Kit (Beyotime Institute of Technology, China) and scanned by Epson Perfection V33 (China). Protein band density was calculated using NIH Image 1.63 software. At a minimum, triplicates were performed for each data point.

Data Processing. The serum metabolic profiles acquired by UPLC-Q-TOF-MS system were processed by MassLynx 4.1 (Waters, USA) to produce a multivariate data matrix. MarkerLynx matrices including peak numbers (according to the retention time and m/z), sample names, and normalized peak intensity were exported and input into SIMCA-P$^+$ 13.0 software (Umetrics, Sweden) for multivariate data analysis.[36] SPSS 20.0 was used to perform the statistical analysis. Results were presented as the means ± SD from at least nine fish per treatment. Data were processed using one-way ANOVA, followed by Tukey's Test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Global Lipidome Changes in the Serum of Large Yellow Croaker. A total of 1806 signals and 1324 signals were detected in ESI$^+$ and ESI$^-$ mode, respectively. In the principal components analysis (PCA) score plots, fish fed three different diets were clearly separated by the first two components in both ion scan modes, which explain 63.5% (ESI$^+$) and 56.8% (ESI$^-$) of all variances (Figure 1). These results indicated that the serum lipid profiles were obviously different among the fish fed three different diets. The distance between the experiment groups (FV/VO) and the control group (FO) in the PCA score plot revealed that serum lipid metabolites in fish fed the VO diet altered more than fish fed the FV diet compared with those fed the FO diet as well.

Significantly Different Lipid Species in the Serum of Large Yellow Croaker in Response to Different Diets. Using the criteria of VIP value (>1.0) and P value (<0.05), 41 species from 7 major lipid classes, including PC, PE, LPC, LPE, DAG, TAG, and SM were identified as the key lipid metabolites for the separation of the fish fed different diets. As is shown in the heat map (Figure 2), the dietary vegetable oil blend modified a number of lipid species in the serum, with its greatest effects on TAG and PC species. LC-PUFA containing PC and TAG decreased with the replacement of fish oil by a vegetable oil blend. PC and TAG, with relative short chain fatty acids such as C18:2 and C18:3, increased with the replacement of fish oil by a vegetable oil blend. However, one

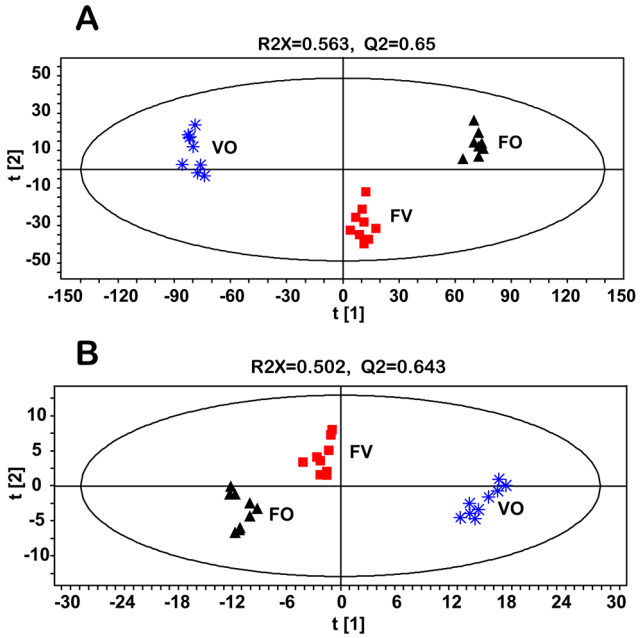

Figure 1. Principal components analysis (PCA) scatter plot in positive mode (A) and negative mode (B) for the serum lipid metabolites in large yellow croaker fed three different diets. FO, fish oil; FV, fish oil and vegetable oil blend; VO, vegetable oil blend.

species of PC with C16:0 and C18:1 fatty acids showed the opposite trend, which significantly decreased with the replacement of fish oil by a vegetable oil blend.

Altered Levels of Significantly Differential Lipid Species in the Serum of Large Yellow Croaker in Response to Different Diets. The contents of 41 significantly different lipid species are shown in Table 1. PC and TAG were the major lipid classes, which accounted for 84.96%−91.75% of all the selected significantly differential lipids. Furthermore, the relative contents of PC and TAG in serum showed a different change in trend with increasing amounts of the dietary vegetable oil blend (Figure 3). In detail, compared with the FO group, relative contents of TAG in the FV and VO groups increased approximately 42.30% and 141.30%, respectively. On the contrary, the relative contents of PC in the FV and VO groups decreased by approximately 4.06% and 25.28%, respectively. Consequently, the ratio of TAG/PC in the serum dramatically elevated from 0.13 to 0.42 with the replacement of fish oil by a vegetable oil blend.

Dietary Vegetable Oil Blend Inhibited Hepatic PC Biosynthesis in Both the CDP-Choline Pathway and the PEMT Pathway. To determine the mechanism underlying these results, we further investigated the effects of dietary vegetable oil blend on PC and TAG metabolisms in the liver and intestine. Since the majority of PC biosynthesis occurs in the organs, particularly the liver, the expression of genes related with hepatic PC biosynthesis via two pathways (CDP-choline pathway, PCYT1A and CHPT1; alternative pathway, PEMT) was assessed. The mRNA levels of *pcyt1a* and *chpt1* in the VO group were significantly lower than those in the FO and FV groups ($P < 0.05$) (Figure 4A,B). Correspondingly, PCYT1A and CHPT1 protein concentrations in the VO group was lower than in those the FO and FV groups ($P < 0.05$) (Figure 5A,B). Additionally, mRNA expression of *pemt*, a key enzyme in catalyzing the conversion of PE to PC, was inhibited in the

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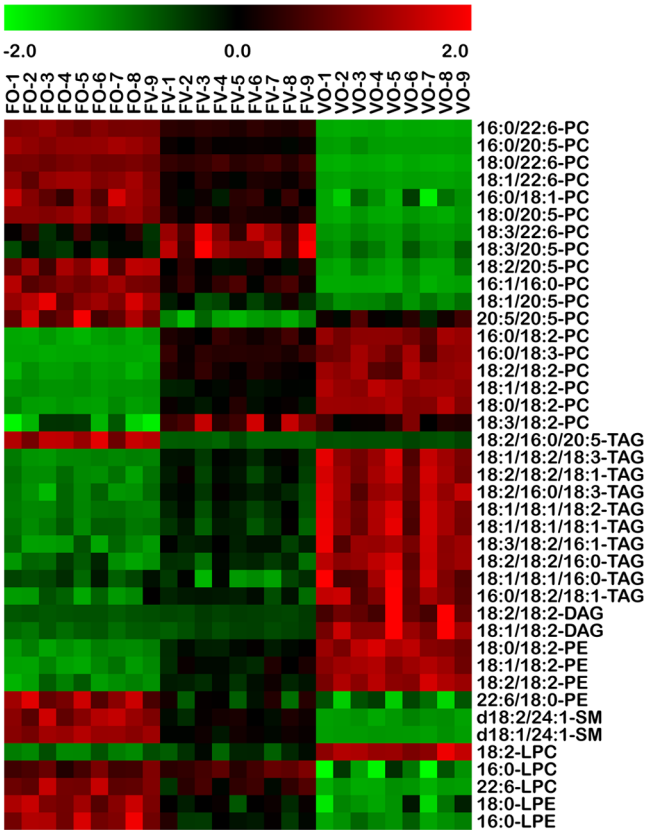

Figure 2. Heat map of significantly differential lipid species in the serum of large yellow croaker response to different diets. Red indicates a high level of lipids, whereas green indicates a low level. The axis stands for the samples divided into three groups (FO as the control group; FV and VO as the experimental groups). The vertical axis stands for variations (41 differential lipid species). DAG, diglyceride; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; TAG, triglyceride; FO, fish oil; FV, fish oil and vegetable oil blend; VO, vegetable oil blend.

VO group compared with that in the FO and FV groups ($P < 0.05$) (Figure 4C).

Dietary Vegetable Oil Blend Affected TAG Biosynthesis and Lipoprotein Assembly in the Liver and Intestine. TAG in the serum mainly existed in the form of lipoprotein, very low density lipoprotein (VLDL), which is responsible for transporting hepatic TAG from the liver, and chylomicron (CM), which is responsible for transporting exogenous TAG from the intestine. Therefore, effects of the dietary vegetable oil blend on hepatic/intestinal TAG biosynthesis and lipoprotein assembly were investigated to explain the increase of serum TAG. In the present study, the mRNA level of hepatic *dgat2*, catalyzing the final step of TAG synthesis, was not significantly affected by the dietary vegetable oil blend, though an increasing trend was found among the three treatments ($P > 0.05$) (Figure 4D). Consistent with results obtained at the transcriptional level, no statistical difference in protein amount of DGAT2 was found in fish fed with the FO, FV, and VO diets ($P > 0.05$) (Figure 5C). Additionally, the mRNA expression of *mttp*, a key protein involved in VLDL assembly, was significantly higher in fish fed the VO diet than in those fed the FO and FV diets ($P < 0.05$) (Figure 4E). However, no significant difference in *apoB100* (a structural protein of VLDL) mRNA expression level was found among the FO, FV, and VO groups ($P > 0.05$) (Figure 4F). In the intestine, the transcriptional levels of *dgat1* increased in the FV and VO groups compared with that in FO group ($P < 0.05$) (Figure 6A). Also, the mRNA level of *mttp*, was significantly elevated with the increase of the dietary vegetable oil blend ($P < 0.05$) (Figure 6B), but transcription of *apoB48* was unaffected by dietary lipids ($P > 0.05$) (Figure 6C).

Dietary Vegetable Oil Blend Inhibited PPARA-Mediated Activation of β-Oxidation Pathway. PC (16:0/18:1), which has been identified as a potent endogenous PPARA ligand, was significantly decreased in the serum of fish fed the vegetable oil blend ($P < 0.05$) (Figure 7). To test whether the reduction of PC (16:0/18:1) reflects blocking in PPARA mediated activation of the β-oxidation pathway in the liver, the expression of genes involved in the fatty acid β-oxidation were measured. Results showed that the levels of PPARA and CPT1A mRNA (Figure 8A,B) and protein (Figure 9A,B) in the FV and VO groups were significantly lower than those in the FO group ($P < 0.05$).

Effects of Different Fatty Acids on PC and TAG Metabolism Related Gene Expression in Primary Hepatocytes of the Large Yellow Croaker. An in vitro study was performed to further elucidate the mechanism of how the dietary vegetable oil blend affected hepatic PC and TAG metabolism in the large yellow croaker. Compared with the control group, DHA and EPA treatments stimulated the expressions of hepatic PCYT1A and CHPT1 both at transcriptional and post-transcriptional levels ($P < 0.05$), whereas LA and LNA treatments had no significant effects on PCYT1A and CHPT1 expressions ($P > 0.05$) (Figure 10A,B; Figure 11A,B). In addition, the *pemt* mRNA levels were significantly higher in DHA and EPA groups than those in the other groups ($P < 0.05$) (Figure 10C). Hepatocytes incubated with DHA and EPA displayed significantly lower *mttp* mRNA levels as compared to those in other groups ($P < 0.05$) (Figure 10D). However, the expressions of DGAT2 at both mRNA and protein levels, and the expression of *apoB100* at the mRNA level did not show any significant changes among all treatments ($P > 0.05$) (Figure 10E,F; Figure 11C).

DISCUSSION

In the present study, large yellow croakers underwent significant serum lipidomic changes with the replacement of fish oil by a vegetable oil blend. Differences in lipids belonging to the TAG and PC classes contributed the most to separating fish fed the FO, FV, and VO diets. Particularly, the ratio of TAG to PC in the serum dramatically elevated with the increase of dietary vegetable oil blend. Alteration of the ratio of TAG to PC has also been reported by Yeo et al., showing that rats fed sunflower oil displayed higher hepatic TAG/PC ratios than those fed fish oil.[37] However, the physiological mechanisms involved in these changes are still unknown. PC, the most abundant phospholipid class, is the principle component of cellular membranes and the precursor of signaling molecules. TAG mainly acted as a store for energy, which can be released rapidly on demand. Maintenance of TAG and PC homeostasis plays an important role in regulating lipid and energy metabolism. Studies in mammals have shown that alteration of the TAG/PC ratio is implicated in metabolic disorders associated with certain dietary habits.[19,38] Therefore, understanding how the dietary vegetable oil blend elevated serum TAG/PC ratio may contribute to increased knowledge

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Table 1. Contents of Significantly Different Lipid Species in the Serum of Large Yellow Croaker Response to Different Diets (nmol/mg Dry Total Lipids)[a]

					content (nmol/mg)		
RT (min)	adduct ion	*m/z*	VIP	compound name	FO	FV	VO
7.88	$[M + H]^+$	806.5	12.1547	16:0/22:6-PC	33.96 ± 2.66 a	21.02 ± 1.02 b	1.93 ± 0.07 c
7.44	$[M + H]^+$	780.5	9.90225	16:0/20:5-PC	27.48 ± 1.73 a	13.81 ± 0.77 b	1.40 ± 0.07 c
9.04	$[M + H]^+$	834.5	7.2255	18:0/22:6-PC	11.01 ± 0.79 a	7.79 ± 0.36 b	0.80 ± 0.02 c
8.14	$[M + H]^+$	832.5	6.72161	18:1/22:6-PC	13.37 ± 0.89 a	6.84 ± 0.16 b	0.49 ± 0.01 c
9.11	$[M + H]^+$	760.5	6.55482	16:0/18:1-PC	8.80 ± 0.42 a	7.33 ± 0.20 b	4.39 ± 0.17 c
8.56	$[M + H]^+$	808.5	5.8556	18:0/20:5-PC	10.33 ± 0.62 a	6.32 ± 0.30 b	0.89 ± 0.03 c
6.66	$[M + Li]^+$	834.5	4.0676	18:3/22:6-PC	0.55 ± 0.11 b	0.78 ± 0.07 a	0.09 ± 0.01 c
6.28	$[M + H]^+$	802.5	3.61018	18:3/20:5-PC	0.23 ± 0.03 b	0.54 ± 0.07 a	0.14 ± 0.01 b
6.9	$[M + H]^+$	804.5	2.85906	18:2/20:5-PC	2.35 ± 0.19 a	1.25 ± 0.08 b	nd[b]
7.95	$[M + H]^+$	732.5	2.66549	16:1/16:0-PC	2.16 ± 0.13 a	1.49 ± 0.04 b	0.33 ± 0.01 c
7.68	$[M + H]^+$	806.5	2.43415	18:1/20:5-PC	8.40 ± 0.56 a	3.93 ± 0.07 b	0.52 ± 0.02 c
6.24	$[M + H]^+$	826.5	2.34405	20:5/20:5-PC	0.34 ± 0.04 a	0.11 ± 0.01 b	nd
8.21	$[M + H]^+$	758.5	10.6848	16:0/18:2-PC	17.76 ± 0.78 c	23.70 ± 1.04 a	21.26 ± 0.92 b
7.42	$[M + H]^+$	756.5	9.02633	16:0/18:3-PC	1.99 ± 0.13 b	8.47 ± 0.54 a	8.05 ± 0.42 a
7.57	$[M + H]^+$	782.5	8.9109	18:2/18:2-PC	1.43 ± 0.11 c	8.24 ± 0.35 b	10.99 ± 0.55 a
8.43	$[M + H]^+$	784.5	8.33704	18:1/18:2-PC	5.33 ± 0.45 c	12.47 ± 0.94 b	15.18 ± 0.41 a
9.35	$[M + H]^+$	786.5	7.05093	18:0/18:2-PC	6.21 ± 0.38 b	11.18 ± 0.38 a	12.18 ± 0.51 a
6.88	$[M + H]^+$	780.5	5.64222	18:3/18:2-PC	nd	2.50 ± 0.11 b	4.42 ± 0.27 a
16.66	$[M + Li]^+$	883.7	4.76413	18:2/16:0/20:5-TAG	1.72 ± 0.11 a	0.95 ± 0.04 b	0.24 ± 0.01 c
16.9	$[M + Li]^+$	885.7	3.92789	18:1/18:2/18:3-TAG	1.30 ± 0.08 c	2.80 ± 0.09 b	5.29 ± 0.11 a
17.49	$[M + Li]^+$	887.7	3.78364	18:2/18:2/18:1-TAG	1.98 ± 0.08 c	3.07 ± 0.11 b	5.08 ± 0.19 a
16.82	$[M + Li]^+$	859.7	3.48175	18:2/16:0/18:3-TAG	1.14 ± 0.07 c	3.18 ± 0.13 b	4.12 ± 0.13 a
18.06	$[M + Li]^+$	889.7	3.44651	18:1/18:1/18:2-TAG	2.06 ± 0.08 c	2.91 ± 0.11 b	4.19 ± 0.24 a
18.63	$[M + Li]^+$	891.7	3.21677	18:1/18:1/18:1-TAG	2.26 ± 0.11 c	2.62 ± 0.12 b	3.43 ± 0.12 a
16.2	$[M + Li]^+$	857.7	2.68155	18:3/18:2/16:1-TAG	0.19 ± 0.02 c	1.12 ± 0.08 b	2.22 ± 0.05 a
17.43	$[M + Li]^+$	861.7	2.67359	18:2/18:2/16:0-TAG	2.33 ± 0.12 c	3.63 ± 0.12 b	4.10 ± 0.10 a
18.57	$[M + Li]^+$	865.7	2.58059	18:1/18:1/16:0-TAG	3.31 ± 0.09 a	2.80 ± 0.09 b	2.27 ± 0.03 c
18	$[M + Li]^+$	863.7	2.31799	16:0/18:2/18:1-TAG	3.27 ± 0.10 a	3.43 ± 0.23 a	3.63 ± 0.11 a
11.32	$[M + Li]^+$	623.5	3.6849	18:2/18:2-DAG	nd	0.59 ± 0.08 b	4.37 ± 0.22 a
12.26	$[M + Li]^+$	625.5	3.51251	18:1/18:2-DAG	0.50 ± 0.03 b	0.87 ± 0.08 b	4.59 ± 0.39 a
9.65	$[M - H]^-$	742.5	1.74221	18:0/18:2-PE	nd	1.45 ± 0.06 b	3.04 ± 0.17 a
8.7	$[M - H]^-$	740.5	1.68854	18:1/18:2-PE	nd	0.97 ± 0.08 b	2.01 ± 0.07 a
7.81	$[M - H]^-$	738.5	1.55678	18:2/18:2-PE	nd	0.50 ± 0.03 b	1.19 ± 0.04 a
9.32	$[M - H]^-$	790.5	1.84996	22:6/18:0-PE	5.79 ± 0.25 a	2.66 ± 0.38 b	1.69 ± 0.04 c
10.05	$[M + H]^+$	811.5	2.59086	d18:2/24:1-SM	1.19 ± 0.11 a	0.61 ± 0.04 b	0.14 ± 0.01 c
10.84	$[M + H]^+$	813.5	2.53297	d18:1/24:1-SM	1.62 ± 0.17 a	1.09 ± 0.06 b	0.34 ± 0.03 c
0.96	$[M + H]^+$	520.3	2.48434	18:2-LPC	0.49 ± 0.05 c	0.80 ± 0.08 b	1.33 ± 0.11 a
1.14	$[M + HCOO]^-$	540.3	1.67047	16:0-LPC	2.63 ± 0.24 a	2.33 ± 0.12 a	0.80 ± 0.05 b
1.03	$[M + HCOO]^-$	612.3	1.11454	22:6-LPC	1.10 ± 0.12 a	0.53 ± 0.04 b	0.14 ± 0.01 c
1.66	$[M - H]^-$	480.3	1.0873	18:0-LPE	2.83 ± 0.12 a	1.71 ± 0.07 b	1.18 ± 0.03 c
1.14	$[M - H]^-$	452.3	1.03385	16:0-LPE	1.58 ± 0.10 a	0.76 ± 0.04 b	nd

[a]Values are the means ± SD, $n = 9$. Different lowercase letters within the same row represent statistical significant difference ($P < 0.05$). RT, retention time; PC, phosphatidylcholine; TAG, triglyceride; SM, sphingomyelin; PE, phosphatidylethanolamine; DAG, diglyceride; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; FO, fish oil; FV, fish oil and vegetable oil blend; VO, vegetable oil blend. [b]nd: Content of certain lipid species was too low to be detected.

about the causes of abnormal lipid metabolism induced by the replacement of fish oil.

In the present study, an excessive dietary vegetable oil blend inhibited hepatic PC biosynthesis related genes PCYT1A and CHPT1 expression in vivo. Additionally, primary hepatocytes incubated with DHA and EPA showed significantly higher PCYT1A and CHPT1 expressions than those in the control group in vitro. Similar results were reported by Mallampalli et al., who found that increased availability of DHA elevated PC synthesis via a direct stimulatory effect of DHA on the PCYT1A.[39] Even though no direct evidence was available about the regulation of PCYT1A and CHPT1 expressions by LC-PUFA in fish, Vegusdal et al. reported that Atlantic salmon hepatocytes incubated with DHA significantly increased secretion of PC.[40] In addition, excessive dietary vegetable oil blend decreased the expression of *pemt*, which indicated an inhibition of PC biosynthesis via the methylation of PE. This was consistent with the elevated serum level of PE in fish fed the VO diet. In vitro, significantly higher mRNA levels of *pemt* were detected in primary hepatocytes incubated with DHA and EPA than in other groups. Previous studies have indicated that PEMT prefers certain species of PE that contain LC-PUFA, such as DHA and EPA, thereby forming LC-PUFA containing PC species.[41,42] One possible explanation for our results might

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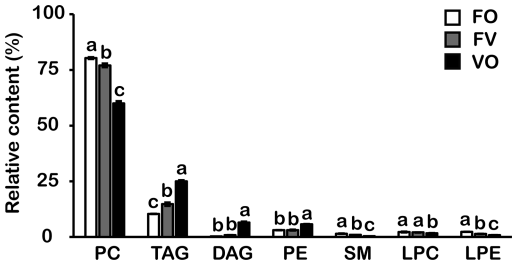

Figure 3. Relative content of each significantly differential lipid classes in the serum of large yellow croaker fed three different diets (% of all the selected significantly differential lipids). Values (means ± SD, n = 9) in bars that have the same superscript letter are not significantly different (P > 0.05, Tukey's test). DAG, diglyceride; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; TAG, triglyceride; FO, fish oil; FV, fish oil and vegetable oil blend; VO, vegetable oil blend.

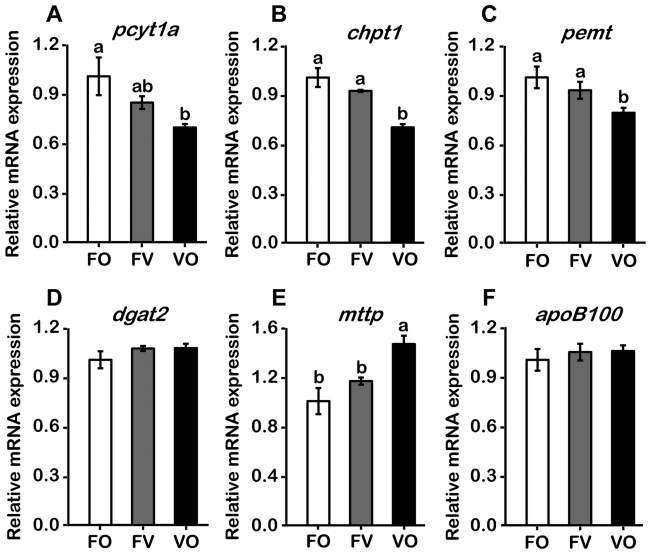

Figure 4. Phosphatidylcholine (PC) and triglyceride (TAG) metabolism related gene expression in the liver of large yellow croakers fed three different diets, including *pcyt1a*, *chpt1*, *pemt*, *dgat2*, *mttp* and *apoB100* (A–F). Values (means ± SD, n = 9) in bars that have the same superscript letter are not significantly different (P > 0.05, Tukey's test), and the relative mRNA expression of target genes in the FO group was selected as the calibrator. *pcyt1a*, phosphate cytidylyltransferase 1, choline, alpha; *chpt1*, choline phosphotransferase 1; *pemt*, phosphatidylethanolamine *N*-methyltransferase; *dgat2*, diacylglycerol *O*-acyltransferase 2; *mttp*, microsomal triglyceride transfer protein; *apoB100*, apolipoprotein B100; FO, fish oil; FV, fish oil and vegetable oil blend; VO, vegetable oil blend.

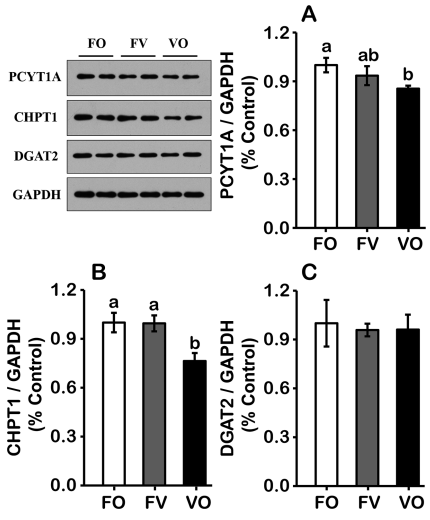

Figure 5. Protein amounts of PCYT1A (A), CHPT1 (B), and DGAT2 (C) in the liver of large yellow croakers fed three different diets. Protein band densities were quantified by NIH Image 1.63 and normalized to GAPDH. Values (means ± SD, n = 9) in bars that have the same superscript letter are not significantly different (P > 0.05, Tukey's test). PCYT1A, phosphate cytidylyltransferase 1, choline, alpha; CHPT1, choline phosphotransferase 1; DGAT2, diacylglycerol *O*-acyltransferase 2; FO, fish oil; FV, fish oil and vegetable oil blend; VO, vegetable oil blend.

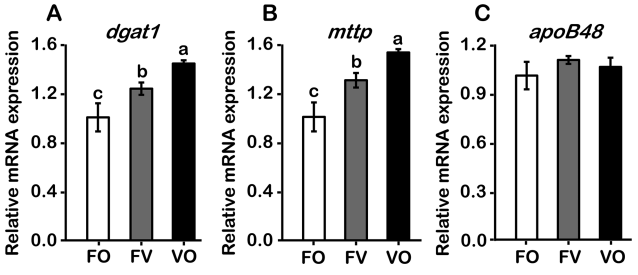

Figure 6. Triglyceride (TAG) metabolism related gene expression in the intestine of large yellow croakers fed three different diets, including *dgat1*, *mttp*, and *apoB48* (A–C). Values (means ± SD, n = 9) in bars that have the same superscript letter are not significantly different (P > 0.05, Tukey's test), and the relative mRNA expression of target genes in the FO group was selected as the calibrator. *dgat1*, diacylglycerol *O*-acyltransferase 1; *mttp*, microsomal triglyceride transfer protein; *apoB48*, apolipoprotein B48; FO, fish oil; FV, fish oil and vegetable oil blend; VO, vegetable oil blend.

be that the excessive dietary vegetable oil blend decreased the concentrations of LC-PUFA containing PE and that insufficient substrate availabilty inhibited the activity of PEMT. Taken together, these findings suggested that the excessive dietary vegetable oil blend inhibited PC biosynthesis in both the CDP-choline pathway and the PEMT pathway and that the dampening effect of the dietary vegetable oil blend on PC biosynthesis may be attributed to the deficiency of LC-PUFA in the diets.

In the liver, DAG is a common substrate for both TAG and PC synthesis, and a corresponding study has demonstrated the diversion of DAG into TAG when the CDP-choline pathway for PC synthesis is compromised.[43] In this study, despite the inhibition of PCYT1A and CHPT1 expressions, no significant increasing trends were observed in the expression of DGAT2 at mRNA and protein levels among the groups, which seems to suggest that the dietary vegetable oil blend may have no effect on hepatic TAG biosynthesis. However, significantly higher TAG content was detected in the liver of fish fed the FV and VO diets than those fed the FO diet.[44] One possible reason for these conflicting results could be that the expression of DGAT2 was not only regulated at the mRNA and protein levels. A study has reported that DGAT2 enzymatic activity can be efficiently regulated by nutritional states in mice.[45] Further studies are needed to determine if the enzyme activity of DGAT2 can be affected by the dietary vegetable oil blend.

The export of newly synthesized TAG into blood is mainly achieved by lipoprotein, and apoB and MTP are the key factors involved in the assembling and secretion of lipoprotein. No significant difference was detected in *apoB100* mRNA

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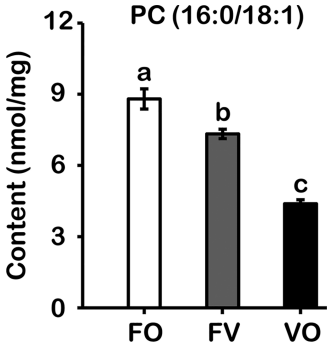

Figure 7. Content of serum PC (16:0/18:1) in large yellow croakers fed three different diets. Values (means ± SD, $n = 9$) in bars that have the same superscript letter are not significantly different ($P > 0.05$, Tukey's test). PC, phosphatidylcholine; FO, fish oil; FV, fish oil and vegetable oil blend; VO, vegetable oil blend.

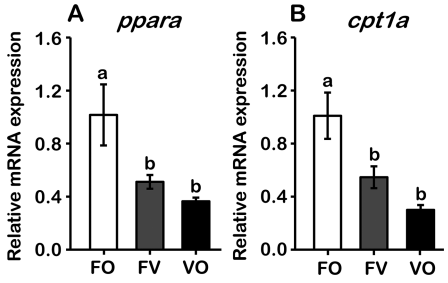

Figure 8. β-Oxidation related gene expression in the liver of large yellow croakers fed three different diets, including *ppara* (A) and *cpt1a* (B). Values (means ± SD, $n = 9$) in bars that have the same superscript letter are not significantly different ($P > 0.05$, Tukey's test), and the relative mRNA expression of target genes in the FO group was selected as the calibrator. *ppara*, peroxisome proliferator-activated receptor alpha; *cpt1a*, carnitine palmitoyltransferase 1A; FO, fish oil; FV, fish oil and vegetable oil blend; VO, vegetable oil blend.

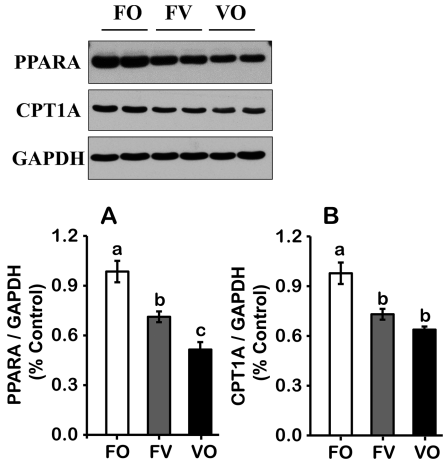

Figure 9. Protein amounts of hepatic PPARA (A) and CPT1A (B) in large yellow croakers fed three different diets. Protein band densities were quantified by NIH Image 1.63 and normalized to GAPDH. Values (means ± SD, $n = 9$) in bars that have the same superscript letter are not significantly different ($P > 0.05$, Tukey's test). PPARA, peroxisome proliferator-activated receptor alpha; CPT1A, carnitine palmitoyltransferase 1A; FO, fish oil; FV, fish oil and vegetable oil blend; VO, vegetable oil blend.

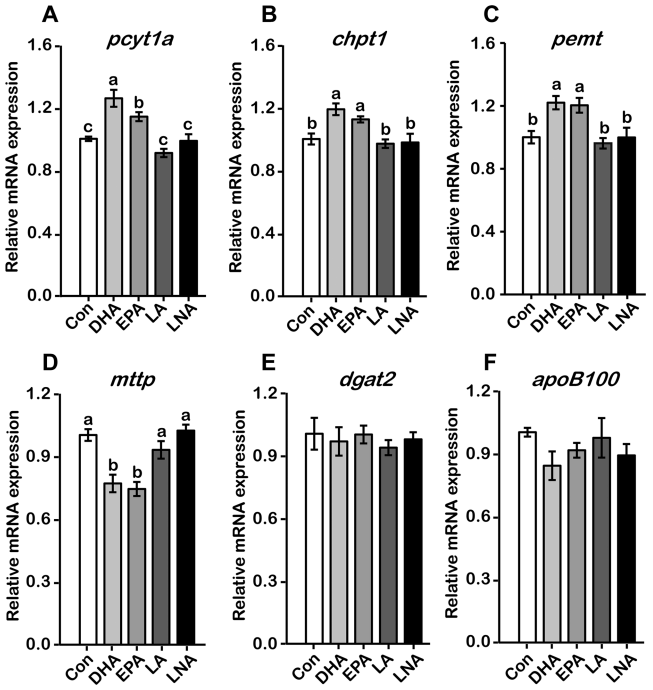

Figure 10. Phosphatidylcholine (PC) and triglyceride (TAG) metabolism related gene expression in primary hepatocytes of large yellow croakers incubated with different fatty acids, including *pcyt1a*, *chpt1*, *pemt*, *dgat2*, *mttp*, and *apoB100* (A–F). Values (means ± SD, $n = 9$) in bars that have the same superscript letter are not significantly different ($P > 0.05$, Tukey's test), and the relative mRNA expression of target genes in the control group was selected as the calibrator. *pcyt1a*, phosphate cytidylyltransferase 1, choline, alpha; *chpt1*, choline phosphotransferase 1; *pemt*, phosphatidylethanolamine *N*-methyltransferase; *mttp*, microsomal triglyceride transfer protein; *dgat2*, diacylglycerol *O*-acyltransferase 2; *apoB100*, apolipoprotein B100; FO, fish oil; FV, fish oil and vegetable oil blend; VO, vegetable oil blend.

expression among all treatments in this study. These results agreed well with previous studies in mammals, which demonstrated that the *apoB100* gene is constitutively synthesized and that the levels of *apoB100* mRNA do not regulate *apoB100* secretion under most metabolic conditions.[46] However, the mRNA level of hepatic *mttp* significantly increased after the replacement of fish oil by a vegetable oil blend, which to a certain extent indicated that the dietary vegetable oil blend facilitated the assembly of VLDL. Similar results were obtained by Peng et al., who reported that the *mttp* mRNA level significantly elevated with the replacement of fish oil by soybean oil in juvenile turbot.[8] In vitro, the *mttp* mRNA levels in primary hepatocytes treated with DHA and EPA were significantly lower than those in the control group. Although the mechanism by which LC-PUFA inhibited MTTP expression in fish is unclear, studies in hepatocytes of mammals have indicated that LC-PUFA reduces MTTP expression partially by the down-regulation of HNF-4α.[47,48] Therefore, high levels of *mttp* mRNA expression in the FV and VO groups may partially be attributed to the deficiency of DHA and EPA in the vegetable oil blend groups. In the intestine, the dietary vegetable oil blend significantly stimulated *dgat1* mRNA expression, and the same trends were observed between intestinal *mttp*/*apoB48* and hepatic *mttp*/*apoB*100 mRNA expressions in response to the dietary vegetable oil blend. These results indicated that the dietary vegetable oil blend might enhance serum TAG concentrations by facilitating

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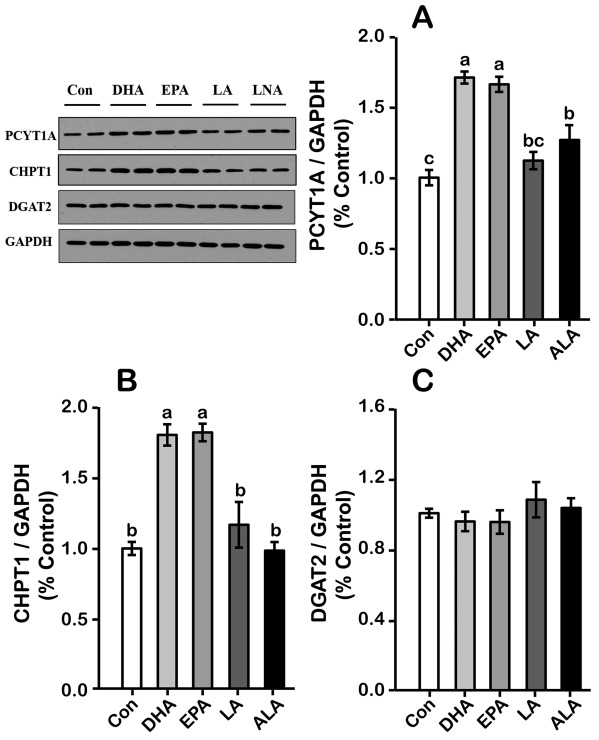

Figure 11. Protein amounts of PCYT1A (A), CHPT1 (B), and DGAT2 (C) in primary hepatocytes of large yellow croakers incubated with different fatty acids. Protein band densities were quantified by NIH Image 1.63 and normalized to GAPDH and then normalized to GAPDH. Values (means ± SD, $n = 9$) in bars that have the same superscript letter are not significantly different ($P > 0.05$, Tukey's test). PCYT1A, phosphate cytidylyltransferase 1, choline, alpha; CHPT1, choline phosphotransferase 1; DGAT2, diacylglycerol *O*-acyltransferase 2; FO, fish oil; FV, fish oil and vegetable oil blend; VO, vegetable oil blend.

VLDL and CM assembly and secretion in the liver and intestine.

Of particular interest was the significantly lower level of PC (16:0/18:1) observed in the serum of fish fed the FV and VO diets. This lipid species is a common PC biosynthesized in the liver and was confirmed as a PPARA endogenous hepatic ligand.[49] Further, studies have reported that change in serum PC (16:0/18:1) profiling reflects alteration of hepatic lipid metabolism under different dietary habits.[50,51] In consideration of the key role of PPARA in governing hepatic β-oxidation, we tested whether the reduction of serum PC (16:0/18:1) induced by dietary vegetable oil blend reflects blocking in PPARA mediated activation of the β-oxidation pathway in the liver. The expressions of PPARA and its target gene CPT1A in fish fed the FV and VO diets were obviously lower compared with that in those fed the FO diet at both protein and mRNA levels, indicating that the dietary vegetable oil blend decreased the transport of fatty acids into the mitochondria for β-oxidation. A decrease in the fatty acid β-oxidation may partially explain the abnormal lipid deposition in the liver of large yellow croaker to some degree. Although it remains to be proven if PPARA mediated activation of the β-oxidation pathway is regulated by PC (16:0/18:1) in fish, our data suggest that change in this serum PC species may be involved in the hepatic abnormal lipid deposition associated with the excessive dietary vegetable oil blend. Further studies in vitro will be carried out to test this hypothesis.

In summary, the dietary vegetable oil blend strongly affects serum lipid profiles in fish, especially the ratio of TAG to PC in the serum, which significantly increased by consuming FV and VO diets. Serum TAG and PC reportedly exert various physiological functions and are associated with certain metabolic disorders, suggesting that an alteration in serum TAG/PC ratio may be involved in abnormal lipid metabolism induced by long-term use of the vegetable oil blend in fish. In addition, the reduction of serum PC (16:0/18:1) induced by the dietary vegetable oil blend may potentially play a role in increasing hepatic lipid accumulation through inhibited PPARA mediated activation of the β-oxidation pathway.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b03382.

Formulation of the experimental diets; contents of different fatty acids in the experiment diets; and primer pair sequences for quantitative real-time PCR (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: qhai@ouc.edu.cn.

ORCID

Qinghui Ai: 0000-0002-3958-474X

Author Contributions

K.M. and Q.A. designed the research; S.Z., P.T., X.D., and R.J. conducted the research; S.Z., X.X., and Z.C. analyzed the data; and S.Z. wrote the paper.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

APOB48, apolipoprotein B48; APOB100, apolipoprotein B100; CHPT1, choline phosphotransferase 1; CPT1A, carnitine palmitoyltransferase 1A; CM, chylomicron; DAG, diglyceride; DGAT1, diacylglycerol *O*-acyltransferase 1; DGAT2, diacylglycerol *O*-acyltransferase 2; FO, fish oil; FV, fish oil and vegetable oil blend; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MTTP, microsomal triglyceride transfer protein; PC, phosphatidylcholine; PCYT1A, phosphate cytidylyltransferase 1, choline, alpha; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltransferase; PPARA, peroxisome proliferator-activated receptor alpha; SM, sphingomyelin; TAG, triglyceride; VLDL, very low density lipoprotein; VO, vegetable oil blend

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