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Dietary palm oil enhances Sterol regulatory element-binding protein 2-mediated cholesterol biosynthesis through inducing endoplasmic reticulum stress in muscle of large yellow croaker (*Larimichthys crocea*)

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Abstract

Sterol regulatory element-binding protein 2 (SREBP2) is considered to be a major regulator to control cholesterol homoeostasis in mammals. However, the role of SREBP2 in teleost remains poorly understand. Here, we explored the molecular characterisation of SREBP2 and identified SREBP2 as a key modulator for 3-hydroxy-3-methylglutaryl-coenzyme A reductase and 7-dehydrocholesterol reductase, which were rate-limiting enzymes of cholesterol biosynthesis. Moreover, dietary palm oil *in vivo* or palmitic acid (PA) treatment *in vitro* elevated cholesterol content through triggering SREBP2-mediated cholesterol biosynthesis in large yellow croaker. Furthermore, our results also found that PA-induced activation of SREBP2 was dependent on the stimulating of endoplasmic reticulum stress (ERS) in croaker myocytes and inhibition of ERS by 4-Phenylbutyric acid alleviated PA-induced SREBP2 activation and cholesterol biosynthesis. In summary, our findings reveal a novel insight for understanding the role of SREBP2 in the regulation of cholesterol metabolism in fish and may deepen the link between dietary fatty acid and cholesterol biosynthesis.

Keywords: **Palm oil: Cholesterol biosynthesis: Endoplasmic reticulum stress: Sterol regulatory element-binding protein 2: Large yellow croaker**

Cholesterol, an essential lipid first isolated from human gallstones over two centuries ago, plays a crucial role in the maintenance of cellular structure and function[1]. In eukaryotic cells, cholesterol principally localises to cell membrane to regulate the membrane structure and function through interplaying with the adjacent lipids and transmembrane proteins[2,3]. Moreover, cholesterol is also a precursor of some biological molecules including bile acids and steroid hormones, which participate in the regulation of a wide range of biological processes[4]. Furthermore, cholesterol can covalently modify proteins to control embryonic development and metabolic balance[5,6]. Considering the physiological importance of cholesterol, dysregulated cholesterol homoeostasis is associated with a diverse range of diseases such as cardiovascular disease (CVD), obesity and cancers[7]. Hence, intracellular cholesterol content is tightly regulated via multiple sophisticated molecular mechanisms.

All cholesterol in bodies is predominantly derived from exogenous dietary intake and cellular *de novo* synthesis[8]. Cholesterol synthesis is an energy-consuming biological process through approximately thirty steps, requiring numerous catalysing enzymes and biological molecules including acetyl-CoA, ATP and oxygen[9]. Given that the uncertainty of dietary cholesterol levels, the rate of cholesterol de novo synthesis is fluctuant and under strict regulation. Sterol regulatory element-binding protein 2 (SREBP2), which is a crucial regulator of cholesterol biosynthesis, is synthesised as an endoplasmic reticulum (ER)-anchored precursor to bind to SREBP-cleavage

Abbreviations: ALT, Alanine transaminase; AST, Aspartate aminotransferase; CAT, Catalase; CVD, cardiovascular disease; DHCR7, 7-dehydrocholesterol reductase; ER, endoplasmic reticulum; ERS, endoplasmic reticulum stress; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; PA, palmitic acid; PO, palm oil; PVDF, polyvinylidene fluoride; ORF, open reading frame; SCAP, SREBP-cleavage activating protein; SREBP2, sterol regulatory element-binding protein 2; T-AOC, Total antioxidant capacity; 4-PBA, 4-Phenylbutyric acid.

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activating protein (SCAP)[10]. When ER membrane cholesterol level is low, the SCAP–SREBP2 complex is sorted into COPII vesicle and translocated from the ER to the Golgi apparatus, where site 1 protease and site 2 protease cut SREBP2 precursor, respectively, thus releasing its N-terminal fragment from the membrane to enter the nucleus. In the nucleus, nSREBP2 binds to the sterol regulatory element sequence to activate the transcriptions of target genes including 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), a rate-limiting enzyme in cholesterol biosynthesis. When ER membrane cholesterol level is high, insulin-induced gene (INSIG) interacts with SCAP to prevent translocation of SCAP–SREBP2 complex to Golgi, resulting in the inhibition of cholesterol biosynthesis[11]. Besides cholesterol and its derivatives, fatty acids can also modulate the translocation or transcriptional activity of SREBP2[12]; however, the underling mechanisms remain poorly understand.

Fish are the largest group of vertebrates in the world, whereas the regulatory circuits and networks of cholesterol metabolism in fish are unclear. Several studies show that dietary substitution of fish oil with vegetable oils can induce cholesterol synthesis and lead to cholesterol accumulation in the liver of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*)[13,14]. Moreover, our previous work also found that olive oil and palm oil (PO) could elevate hepatic cholesterol levels through activating the long noncoding RNA lincsc5d in large yellow croaker (*Larimichthys crocea*)[15]. However, it is still unclear whether vegetable oil can influence the cholesterol synthesis in the muscle of fish and the underling mechanisms remain to be explored. In this study, we investigate the effect of dietary PO on the cholesterol homoeostasis in the muscle of large yellow croaker and highlight the role of SREBP2 in PO-induced muscular cholesterol synthesis. This findings may further deepen the understanding of cholesterol metabolism in fish and also provide a potential target for improving muscle quality of farmed aquatic animals.

Results

Dietary palm oil increased the cholesterol content and mRNA expressions of cholesterol biosynthesis-related genes in muscle of large yellow croaker

To investigate the effects of dietary PO on cholesterol metabolism of large yellow croaker, we fed juvenile fish with control diet (CON) or PO diet for 10 weeks. Compared with CON diet, PO diet significantly enhanced the cholesterol content of skeletal muscle (Fig. 1(a)). Similar, PO diet also elevated the contents of cholesterol and LDL-cholesterol in the plasma of juvenile fish (Fig. 1(b) and (c)) but had no effect on the plasma of HDL-cholesterol content (Fig. 1(d)), suggesting that dietary PO disrupted the systemic cholesterol homoeostasis of large yellow croaker. To investigate the mechanism of the increase in cholesterol content of muscle, we assayed the effect of dietary PO on the mRNA expressions of cholesterol metabolism-related genes. The results showed that dietary PO significantly increased the mRNA expressions of *hmgcr* and *dhcr7*, two crucial enzymes in cholesterol biosynthesis (Fig. 1(e) and (f)) but had no effect on the mRNA expressions of *abca1*, *abcg5*, *abcg8* and *cyp7a1* (Fig. 1(g)–(j)). Together, these results indicated that dietary PO may raise the cholesterol content of skeletal muscle through enhancing cholesterol synthesis in large yellow croaker.

Palmitic acid elevated the cholesterol content and mRNA expressions of cholesterol biosynthesis genes in croaker myocytes

To mimic the condition that dietary PO *in vivo*, we treated croaker myocytes with indicated concentrations of palmitic acid (PA) *in vitro*. Likewise, PA treatment significantly enhanced the cholesterol contents of croaker myocytes in a dose-dependent manner (Fig. 2(a)). Moreover, the mRNA expressions of *hmgcr* and *dhcr7* were significantly elevated under PA treatment (Fig. 2(b) and (c)) but had no effect on the mRNA expressions of *abca1*, *abcg5*, *abcg8* and *cyp7a1* (Fig. 2(d)–(g)). Collectively, these results suggested that PA may increase cholesterol contents through enhancing mRNA expressions of cholesterol biosynthesis-related genes *in vivo* and *in vitro*.

Molecular cloning, phylogenetic analysis and tissue distribution of sterol regulatory element-binding protein 2

Given that SREBP2 is considered to be a pivotal regulator of cholesterol biosynthesis, we cloned the complete open reading frame (ORF) of SREBP2 from *large yellow croaker*. The complete ORF contained 3543 bp and encoded a putative protein of 1180 amino acid residues (Fig. 3(a)). Multisequence alignment showed that SREBP2 of *Larimichthys crocea* shared high identity with those of *Chelmon rostratus*, 93·67 %; *Dicentrarchus labrax*, 93 % and *Takifugu rubripes*, 83·1 % (Fig. 3(b)). Moreover, the results of phylogenetic tree indicated that the *Larimichthys crocea* SREBP2 was clustered with *Takifugu rubripes* (Fig. 3(c)).

To investigate the distribution of SREBP2 in large yellow croaker, we examined the mRNA expressions of *srebp2* in different tissues including liver, muscle, heart, adipose, brain, eye, intestine, head kidney, gill and spleen. The highest mRNA expression of *srebp2* was observed in the liver, followed by the muscle, heart and adipose, while the lowest gene expression of *srebp2* was found in the spleen (Fig. 3(d)).

Hyperactivation of sterol regulatory element-binding protein 2 contributed to palm oil-induced cholesterol biosynthesis

To explore the role of SREBP2 in PO-caused induction of cholesterol biosynthesis, we assessed the expressions of SREBP2 in juvenile fish fed with CON or PO diet. The results showed that dietary PO increased the mRNA and protein expressions of SREBP2 (Fig. 4(a) and (b)). To mimic the condition *in vivo*, we also investigate the expressions of SREBP2 in croaker myocytes treated with PA and the results showed that PA treatment also boosted the mRNA and protein expressions of SREBP2 (Fig. 4(c) and (d)). To further investigate whether SREBP2 activation is associated with the induction of cholesterol biosynthesis under PA condition, we inhibited the expression of SREBP2 with a pharmacological inhibitor Fatostatin. The results showed that Fatostatin reduced the mRNA and protein expressions of SREBP2 (Fig. 4(e) and (f)). Moreover, Fatostatin treatment ameliorated the increase of *hmgcr* and *dhcr7* mRNA expressions induced by

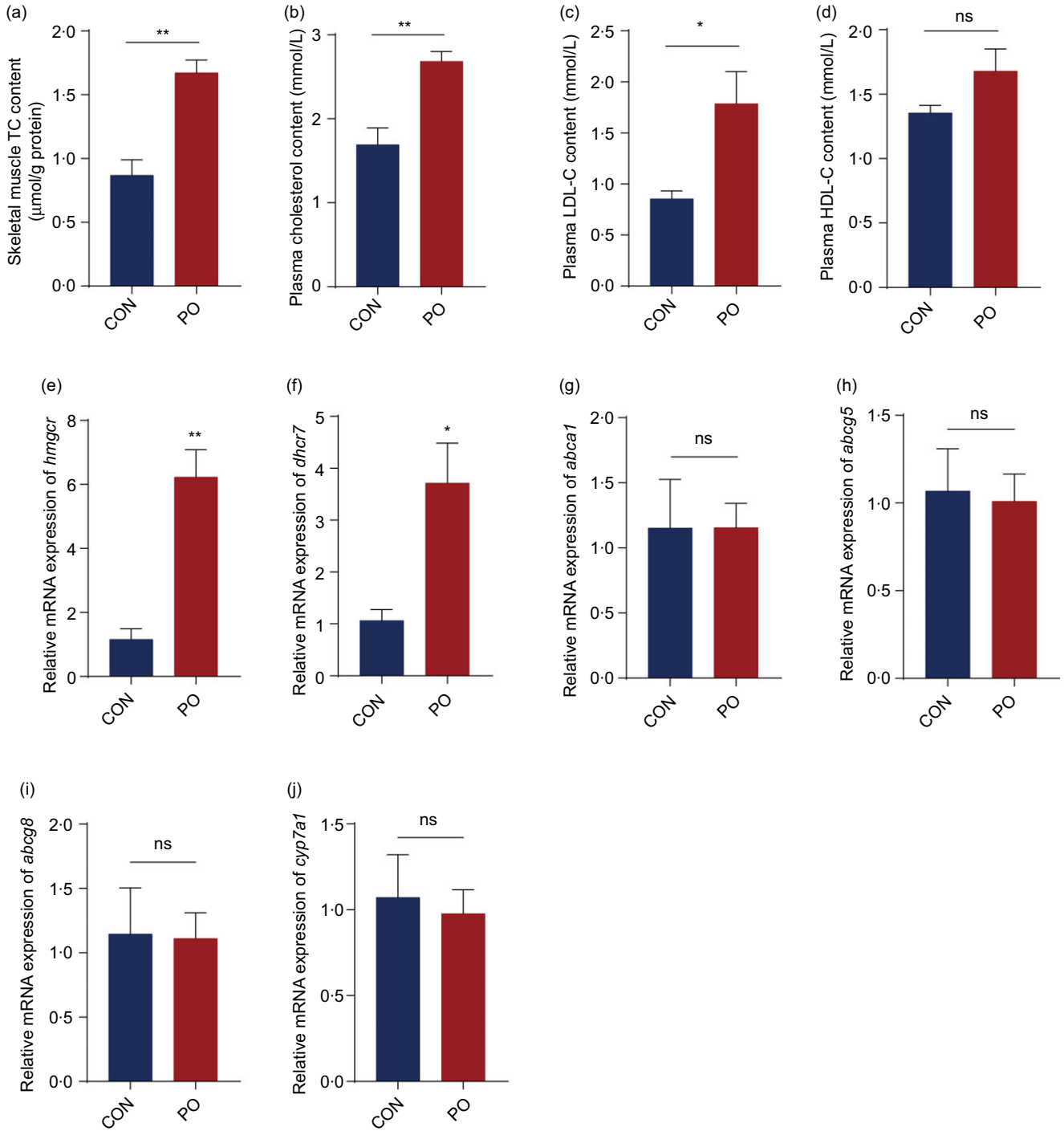

Fig. 1. Dietary PO increased the cholesterol content and mRNA expressions of cholesterol biosynthesis genes in muscle of large yellow croaker. (a) TC levels in skeletal muscle of juvenile fish fed CON or PO diet were measured (n 4). (b) TC levels in plasma were measured in juvenile fish fed CON or PO diet (n 4). (c) LDL-cholesterol and (d) HDL-cholesterol in plasma were measured in juvenile fish fed CON or PO diet (n 4). (e)–(j) Relative mRNA levels of *hmgcr* (e), *dhcr7* (f), *abca1* (g), *abcg5* (h), *abcg8* (i) and *cyp7a1* (j) were tested by quantitative PCR in muscle of juvenile fish fed CON or PO diet (n 4). The results are presented as the mean values with their standard error of means and were analysed using independent t tests (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ns, not significant). CON, control diet; PO, palm oil; TC, total cholesterol.

PA (Fig. 4(g) and (h)) and also prevented the induction of cholesterol contents induced by PA (Fig. 4(i)). Furthermore, dual luciferase experiments exhibited that SREBP2 significantly elevated the luciferase activity of HMGCR promoter and 7-dehydrocholesterol reductase (DHCR7) promoter (Fig. 4(i) and (j)). Collectively, these results suggested that the induction of SREBP2 may mediate PO-induced cholesterol biosynthesis.

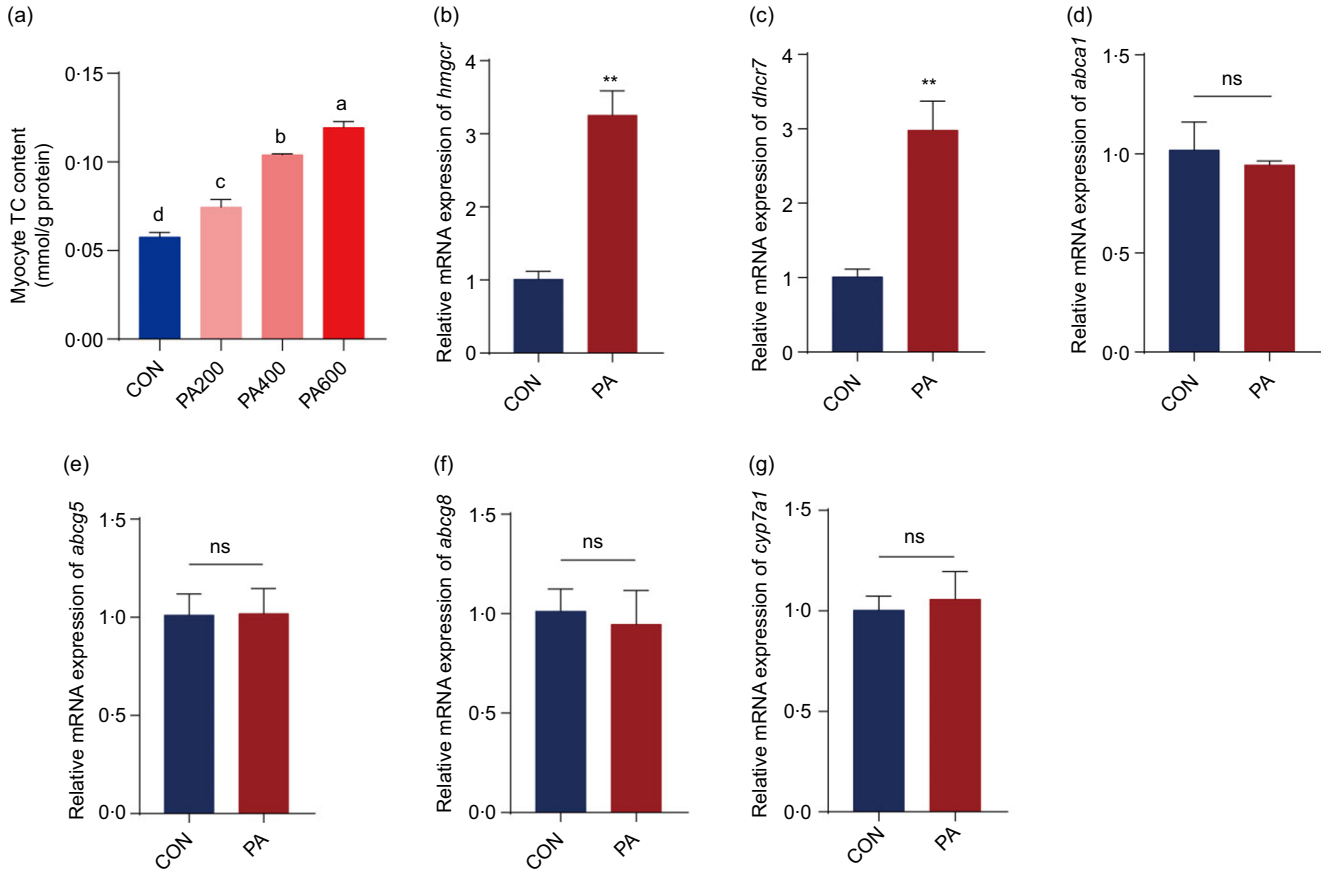

Fig. 2. PA elevated the cholesterol contents and mRNA expressions of cholesterol biosynthesis genes in croaker myocytes. (a) TC levels in croaker myocytes were measured under 0 μM, 200 μM, 400 μM and 600 μM PA treatments for 12 h (n 3). (b)–(g) Relative mRNA levels of *hmgcr* (b), *dhcr7* (c), *abca1* (d), *abcg5* (e), *abcg8* (f) and *cyp7a1* (g) were tested by quantitative PCR in croaker myocytes under control or 400 μM PA treatment for 12 h (n 3). The results are presented as the mean values with their standard error of means and were analysed using independent t tests (**$P < 0·01$) and Tukey's tests (bars bearing different letters are significantly different among treatments ($P < 0·05$)). PA, palmitic acid; TC, total cholesterol.

Dietary palm oil in vivo *or palmitic acid treatment* in vitro *triggered endoplasmic reticulum stress*

Considering that the SREBP2 precursor resides in the ER and endoplasmic reticulum stress (ERS) can affect the transport and subsequent activation of SREBP2, we analysed the effect of PO on ERS. The results exhibited that dietary PO diet significantly elevated the mRNA expressions of ERS-related genes, including *grp78*, *chop*, *atf4*, *atf6* and *xbp1s,* compared with CON diet (Fig. 5(a)–(e)). Moreover, the protein levels of GRP78 and XBP1s were increased and the phosphorylation levels of eIF2*α* was enhanced in the muscle of juvenile fish fed PO diet (Fig. 5(f)). Next, we further assessed the gene and protein levels related to ERS in croaker myocytes treated with PA. Likewise, PA treatment increased the mRNA expressions of *grp78*, *chop*, *atf4*, *atf6* and *xbp1s* significantly (Fig. 5(g)–(k)). Furthermore, western blotting analysis showed that the protein levels of GRP78 and XBP1s and the phosphorylation levels of eIF2*α* were promoted by PA treatment (Fig. 5(l)). These results indicated that dietary PO diet provoked ERS in muscle of large yellow croaker.

Inhibition of endoplasmic reticulum stress alleviated palmitic acid-induced activation of sterol regulatory element-binding protein 2 and cholesterol biosynthesis

To further investigate the role of ERS in PA-induced cholesterol biosynthesis, we treated croaker myocytes with an ERS inhibitor 4-Phenylbutyric acid (4-PBA). The results exhibited that 4-PBA treatment significantly diminished the mRNA expressions of *grp78*, *chop*, *atf4*, *atf6* and *xbp1s* (Fig. 6(a)–(e)), and reduced the protein levels of GRP78 and the phosphorylation levels of eIF2*α* (Fig. 6(f)), indicating that 4-PBA treatment effectively relieved PA-induced ERS. Notably, 4-PBA treatment ameliorated the increase of SREBP2 expression induced by PA treatment (Fig. 6(f) and (g)). Furthermore, 4-PBA treatment decreased the mRNA expressions of *hmgcr* and *dhcr7* under PA condition (Fig. 6(h) and (i)) and also prevented the induction of cholesterol content induced by PA treatment (Fig. 6(j)), suggesting that ERS may promote cholesterol biosynthesis. Taken together, these results indicated that PA-induced ERS may contribute to the activation of SREBP2 and cholesterol biosynthesis.

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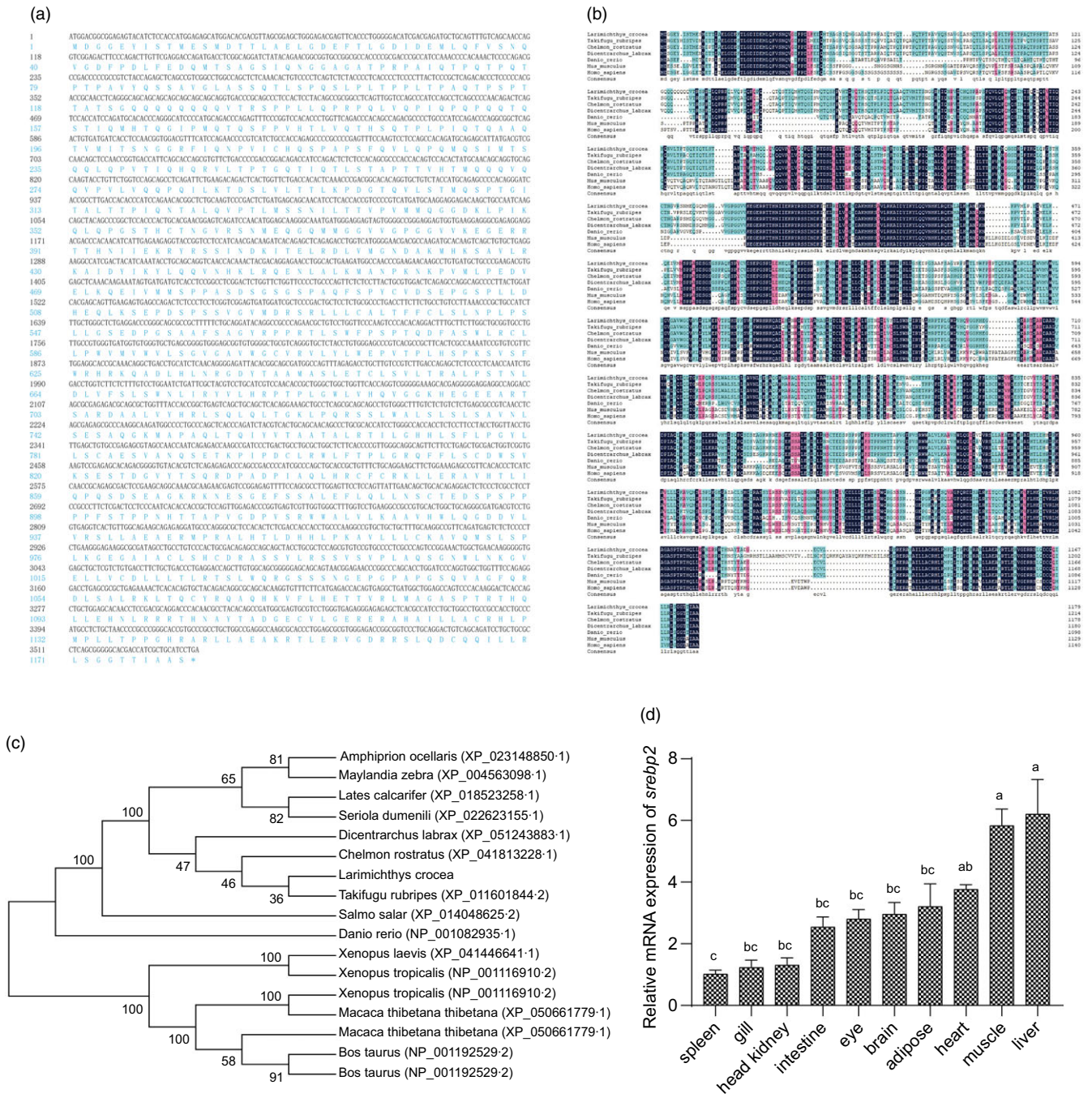

Fig. 3. Molecular cloning, phylogenetic analysis and tissue distribution of SREBP2. (a) Nucleotide and deduced amino acids sequences of *srebp2* ORF in *Larimichthys crocea*. (b) Multiple sequence alignment of SREBP2 of *L. crocea* and other species. Sequence alignment was performed using DNAMAN. Accession numbers used are: *Takifugu rubripes* (XP_011601844.2), *Chelmon rostratus* (XP_041813228.1), *Dicentrarchus labrax* (XP_051243883.1), *Danio rerio* (NP_001082935.1), *Mus musculus* (NP_150087.1) and *Homo sapiens* (NP_004590.2). (c) Phylogenetic tree of *Larimichthys crocea* SREBP2 with other vertebrates by MEGA7. The tree was performed by selecting the neighbour connection method in the software. The numbers represent the frequencies with which the tree topology presented here was replicated after 1000 bootstrap iterations. (d) Tissue distribution of *srebp2* in large yellow croaker (n 3). The results are presented as the mean values with their standard error of means and were analysed using Tukey's tests (bars bearing different letters are significantly different among treatments ($P < 0{\cdot}05$)). SREBP2, sterol regulatory element-binding protein 2.

Discussion

Cholesterol, which is the major sterol present in animal tissues, plays a vital role in the regulation of cellular homoeostasis and function[8]. However, excess cholesterol accumulation can lead to cellular toxic effect and is associated with pathogenesis of multiple diseases[16]. In the present study, we found dietary PO diet *in vivo* or PA treatment *in vitro* induced the accumulation of cholesterol in muscle or myocytes of large yellow croaker. Our previous studies had shown that dietary PO diet increased activities of plasma Alanine transaminase (ALT) and Aspartate

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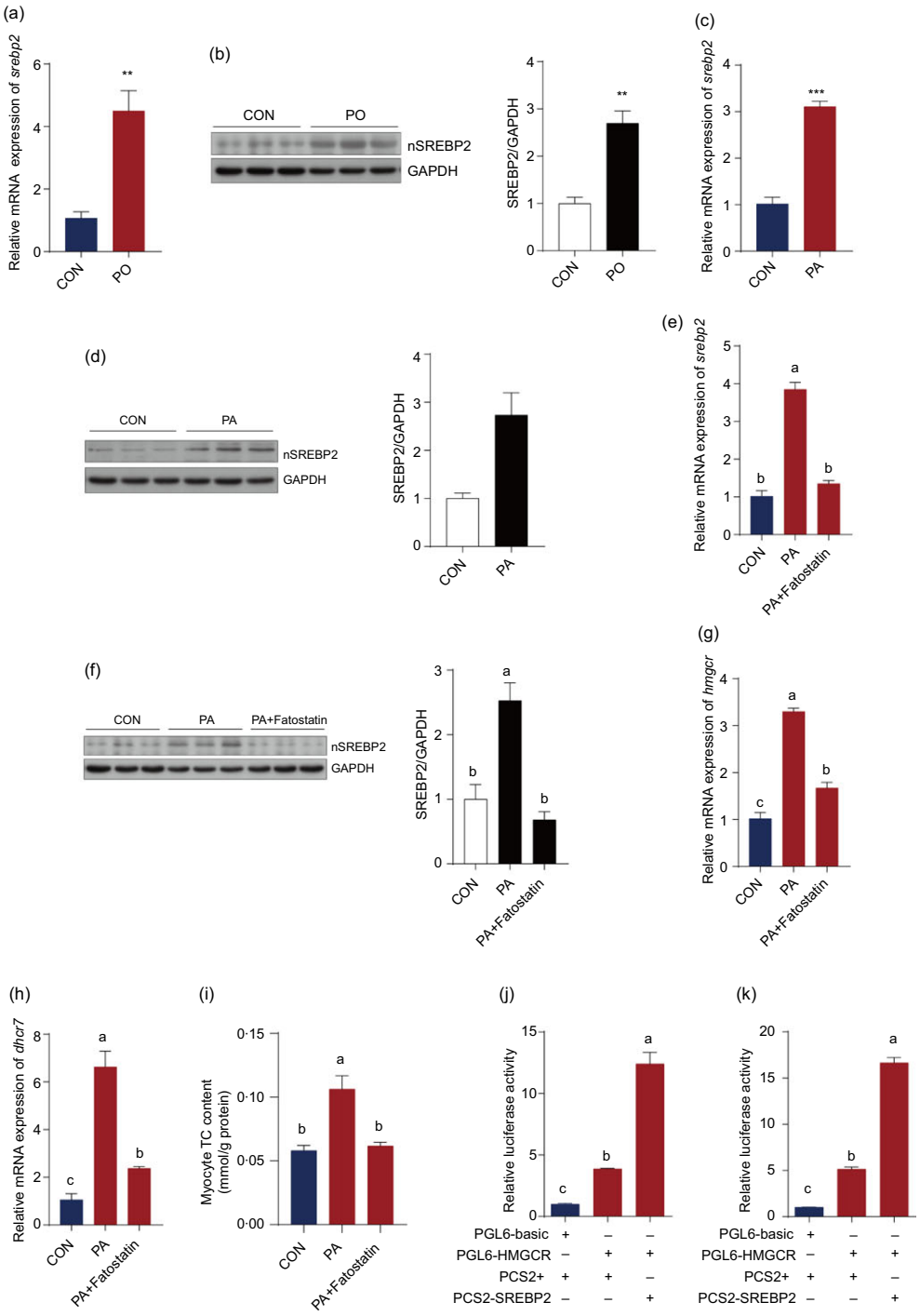

Fig. 4. Hyperactivation of SREBP2 led to PO-induced cholesterol biosynthesis. (a) Relative mRNA levels of *srebp2* were tested by quantitative PCR in muscle of juvenile fish fed CON or PO diet (n 4). (b) The protein expression levels of SREBP2 in muscle of juvenile fish fed CON or PO diet were measured by immunoblotting (n 3). (c) Relative mRNA levels of *srebp2* were tested by quantitative PCR in croaker myocytes under control or 400 μM PA treatment for 12 h (n 3). (d) The protein expression levels of SREBP2 in croaker myocytes under control or 400 μM PA treatment for 12 h were measured by immunoblotting (n 3). (e) Relative mRNA levels of *srebp2* were tested by quantitative PCR in croaker myocytes treated with Fatostatin in the presence of 400 μM PA for 12 h (n 3). (f) The protein expression levels of SREBP2 in croaker myocytes treated with Fatostatin in the presence of 400 μM PA for 12 h were measured by immunoblotting (n 3). (g)–(h) Relative mRNA levels of *hmgcr* (g) and *dhcr7* (h) were tested by quantitative PCR in croaker myocytes treated with Fatostatin in the presence of 400 μM PA for 12 h (n 3). (i) TC levels were measured in croaker myocytes treated with Fatostatin in the presence of 400 μM PA for 12 h (n 3). (j) Relative dual luciferase activity analysis was conducted to measure the effect of SREBP2 on HMGCR promoter activity in HEK293T cells (n 3). (k) Relative dual luciferase activity analysis was conducted to measure the effect of SREBP2 on DHCR7 promoter activity in HEK293T cells (n 3). The results are presented as the mean values with their standard error of means and were analysed using independent t tests (**$P < 0{\cdot}01$, ***$P < 0{\cdot}001$) and Tukey's tests (bars bearing different letters are significantly different among treatments ($P < 0{\cdot}05$)). CON, control diet; PO, palm oil; PA, palmitic acid; SREBP2, sterol regulatory element-binding protein 2; TC, total cholesterol.

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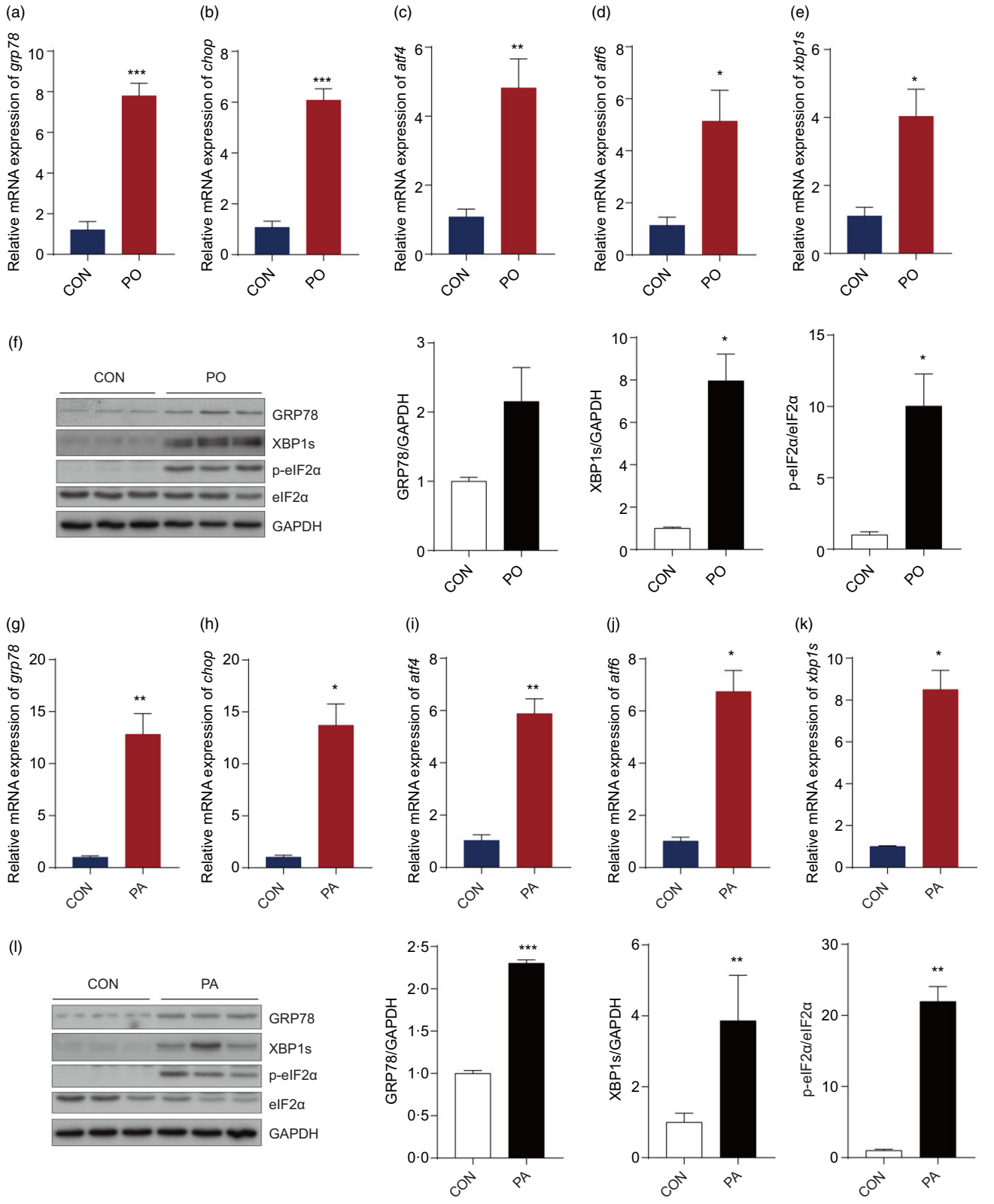

Fig. 5. Dietary PO or PA treatment triggered endoplasmic reticulum stress. (a)–(e) Relative mRNA levels of *grp78* (a), *chop* (b), *atf4* (c), *atf6* (d) and *xbp1s* (e) were tested by quantitative PCR in muscle of juvenile fish fed CON or PO diet (n 4). (f) The indicated protein expression levels in muscle of juvenile fish fed CON or PO diet were measured by immunoblotting (n 3). (g)–(k) Relative mRNA levels of *grp78* (g), *chop* (h), *atf4* (i), *atf6* (j) and *xbp1s* (k) were measured by quantitative PCR in croaker myocytes under control or 400 μM PA treatment for 12 h (n 3). (l) The indicated protein expression levels in croaker myocytes under control or 400 μM PA treatment for 12 h were measured by immunoblotting (n 3). The results are presented as the mean values with their standard error of means and were analysed using independent t tests (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$). CON, control diet; PO, palm oil; PA, palmitic acid.

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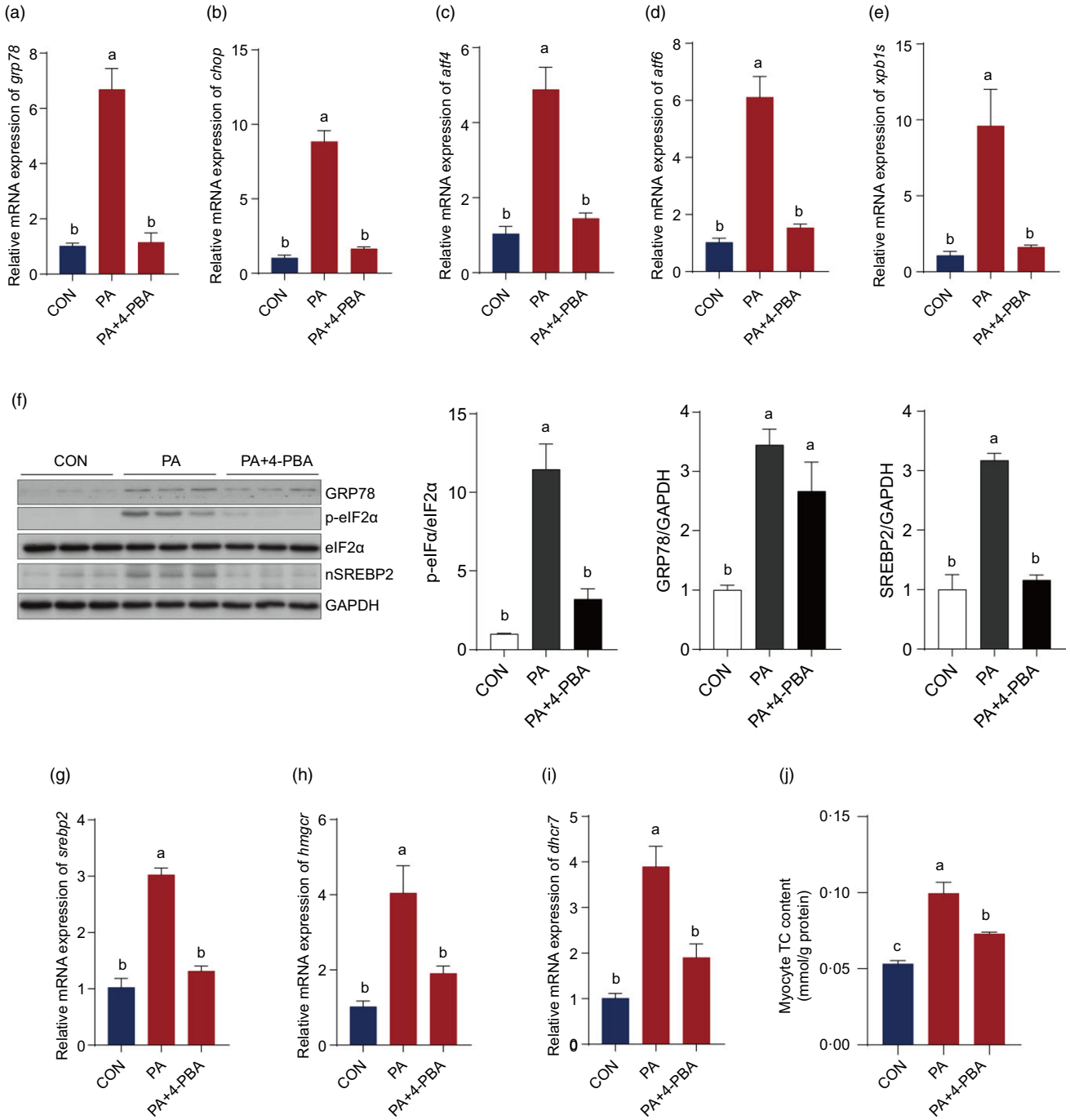

Fig. 6. Inhibition of ERS alleviated PA-induced activation of SREBP2 and cholesterol biosynthesis. (a)–(e) Relative mRNA levels of *grp78* (a), *chop* (b), *atf4* (c), *atf6* (d) and *xbp1s* (e) were measured by quantitative PCR in croaker myocytes treated with 4-PBA in the presence of 400 μM PA for 12 h (n 3). (f) The indicated protein expression levels in croaker myocytes treated with 4-PBA in the presence of 400 μM PA for 12 h were measured by immunoblotting (n 3). (g)–(i) Relative mRNA levels of *srebp2* (g), *hmgcr* (h) and *dhcr7* (i) were measured by quantitative PCR in croaker myocytes treated with 4-PBA in the presence of 400 μM PA for 12 h (n 3). (j) TC levels were measured in croaker myocytes treated with 4-PBA in the presence of 400 μM PA for 12 h (n 3). The results are presented as the mean values with their standard error of means and were analysed using Tukey's tests (bars bearing different letters are significantly different among treatments ($P < 0{\cdot}05$)). ERS, endoplasmic reticulum stress; PA, palmitic acid; SREBP2, sterol regulatory element-binding protein 2; 4-PBA, 4-Phenylbutyric acid; TC, total cholesterol.

aminotransferase (AST) but suppressed activities of Total antioxidant capacity (T-AOC) and Catalase (CAT). Moreover, dietary PO diet also led to lipid deposition and inflammatory response in liver of juvenile large yellow croaker[17]. Thus, dietary PO diet impairs the health status of juvenile large yellow croaker. Likewise, numerous studies in mammals have shown dietary PA-rich diet or PA treatment can also contribute to the disorder in cellular function and metabolism[18–20]. Therefore, we speculate that the accumulation of cholesterol induced by PA may play a crucial role in this process. There may be the

following reasons for this hypothesis. First, several studies found that PA could decrease cell membrane fluidity and impair the normal cellular structure[21]. It is known that cholesterol is a prevalent component of mammalian cell membranes and excess cholesterol could decrease membrane fluidity and disrupt membrane micro-domains. Thus, PA-induced decrease in cell membrane fluidity may be associated with the excess cholesterol accumulation. Second, multiple studies have shown that PA can cause the induction of mitochondrial damage and dysfunction[22,23]. As free cholesterol toxicity has also been reported to induce mitochondrial injury[24], we conjecture that mitochondrial damage triggered by PA may be related to the cholesterol accumulation. Third, growing evidence has shown that PA can lead to inflammatory response through various molecular mechanisms[25]. Moreover, high levels of cholesterol have been implicated in the activation of the inflammasome and pro-inflammatory cytokine secretion[26]. Therefore, the mechanism of PA-induced inflammatory response may depend in part on the accumulation of cholesterol.

In this study, we found dietary PO diet increased cholesterol content in the muscle of large yellow croaker through increasing cholesterol synthesis. However, it is well known that the liver is the main site of cholesterol synthesis and about 50 % of total synthesis occurs in the liver. Moreover, we also found that PO diet elevated plasma cholesterol content and plasma LDL-cholesterol content. Furthermore, our previous studies showed that dietary PO diet could promote the activation of SREBP2 and cholesterol biosynthesis in liver[15,27]. Thus, we suppose that PO diet-induced accumulation of cholesterol in muscle is not only associated with the induction of muscular cholesterol synthesis but also is partly related to the increase in hepatic cholesterol synthesis.

Cholesterol biosynthesis, which occurs in almost all cells, is under tightly regulated. In this study, we found that dietary PA-rich diet *in vivo* or PA treatment *in vitro* increased cholesterol biosynthesis. Similar to our results, dietary SFA are positively correlated with plasma cholesterol concentration, and SFA can also promote cholesterol biosynthesis in mammals[28]. However, unlike SFA, unsaturated fatty acids are thought to inhibit the synthesis of cholesterol. Studies in C6 glioma cells showed that oleic acid could inhibit cholesterol biosynthesis through decreasing HMGCR activities and expressions[29,30]. Moreover, an increase in linoleic acid intake may lower plasma cholesterol through inhibiting cholesterol biosynthesis[31]. Furthermore, highly purified EPA administration effectively reduced plasma and hepatic cholesterol levels in mice through reducing cholesterol biosynthesis. Together, our results further develop the understanding of the regulation in cholesterol synthesis by fatty acids.

In mammals, SREBP2 is a subclass of basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factors and is considered to be a key modulator for cellular cholesterol homoeostasis. On one hand, SREBP2 can activate the transcription of numerous enzymes involved in cholesterol biosynthesis pathway including HMGCR, HMGCS, MVK and DHCR7 to induce cholesterol *de novo* synthesis[32]. On the other hand, SREBP2 can promote the expression of the Low-density lipoprotein receptor (LDLR) to boost cholesterol uptake[33]. In this study, we cloned and characterised the ORF of SREBP2 from large yellow croaker and found that the highest gene expressions of SREBP2 were in the liver and muscle. Consistent with the studies in mammals, we also demonstrated that SREBP2 can induce the activity of HMGCR promoter and DHCR7 promoter. Moreover, inhibiting SREBP2 activity by Fatostatin reduced the mRNA expressions of *hmgcr* and *dhcr7*. Thus, these results identified SREBP2 as an evolutionarily conservative regulator for cholesterol biosynthesis in large yellow croaker.

As a crucial modulator of cholesterol metabolism, SREBP2 activity is regulated in a sophisticated and complicated manner. Accumulating evidences have shown that cholesterol and its derivatives can negatively regulate the biosynthesis of cholesterol to prevent excessive accumulation of intracellular cholesterol[34]. Moreover, several studies have shown that cholesterol or 25-hydroxycholesterol can inhibit SREBP2 activation through inducing a conformational change of Scap, which promotes Scap to combine with insig[35,36]. In addition to sterols, several studies have also shown fatty acids can regulate the expression and activation of SREBP2. Recent study finds that industrial trans fatty acids promote cholesterol biosynthesis through induction of the SCAP–SREBP2 pathway[37]. However, α-linolenic acid is reported to suppress cholesterol biosynthesis pathway via reducing the expression of transcriptional factor SREBP2[12]. In the present study, we found that dietary PO significantly increased the mRNA and protein expressions of SREBP2, which promoted the transcriptions of cholesterol biosynthesis genes, leading to induction of cholesterol biosynthesis in large yellow croaker. Moreover, we also found PA treatment could also induce the activation of SREBP2 in croaker myocytes. Together, these results may provide a novel insight for understanding the link between SFA and cholesterol metabolism.

Excess SFA intake is associated with ER dysfunction and induction of ERS. In this study, we showed that dietary PO diet or PA treatment increased expressions of genes and proteins involved in ERS, suggesting that PO diet or PA treatment induced ERS in large yellow croaker. Similar to our results, multiple studies in mammals have also shown that PA could induce ERS[38,39]. Furthermore, several studies have explored the molecular mechanisms behind PA-induced ERS. A variety of fatty acids are distributed in cellular membrane phospholipids and the degree of unsaturation of fatty acids in membrane phospholipids can affect membrane-related function. Thus, PA can trigger ERS through decreasing phospholipid unsaturation in membrane[40]. Moreover, other studies have also shown that PA-induced ERS is associated with a reduction of ER luminal Ca[41]. Besides, a recent study has found that PA can cause ERS through promoting aberrant protein palmitoylation[42]. Therefore, we speculate that ERS induced by dietary PO diet or PA treatment in large yellow croaker may be associated with variations in phospholipid unsaturation, ER luminal Ca or protein palmitoylation.

As the precursor of SREBP2 is anchored in the ER, ER homoeostasis is associated with the activity of SREBP2. Studies in HeLa and MCF7 cells have shown that thapsigargin induced proteolytic cleavage of SREBP-2 to promote cellular cholesterol and TAG biosynthesis[43]. Likewise, another study finds that homocysteine-triggered ERS activates SREBP2 to increase

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expression of genes responsible for cholesterol biosynthesis and uptake, which induces intracellular accumulation of cholesterol[44]. Similar to previous studies, we found that dietary PO or PA treatment could induce ERS which contributed to activation of SREBP2 and induction of cholesterol biosynthesis in large yellow croaker. Furthermore, we also demonstrated that the expression of XBP1s has been elevated in PO diet or PA condition. Considering the fact that unspliced XBP1 led to cholesterol biosynthesis and tumourigenesis through stimulating SREBP2 activation in hepatocellular carcinoma[45], we speculated that ERS-induced SREBP2 may be dependent on the activation of XBP1s under PA condition in large yellow croaker.

In the present study, we identified that SREBP2 as a key regulator of cholesterol biosynthesis in large yellow croaker, and found that dietary PO diet promoted cholesterol biosynthesis through inducing SREBP2 activation in muscle. Moreover, we also demonstrated that activation of SREBP2 is associated with PO diet-induced ERS. Our findings deepened the understanding of cholesterol metabolism in fish and may provide a new insight for improving fish muscle cholesterol metabolism in aquaculture.

Materials and methods

Experimental diets and feeding process

Two iso-nitrogenous (42 % crude protein) and iso-lipidic (12 % crude lipid) experimental diets were formulated, containing CON diet (fish oil as a source of dietary fat) and PO diet (PO as a source of dietary fat). The details of dietary formulations are listed in Table 1[46] and the fatty acid profiles of the experimental diets are listed in Table 2. Four-month-old large yellow croakers in similar size (mean weight 15·71 (SEM 0·12) g) were purchased from the Aquatic Seeds Farm of the Marine and Fishery Science and Technology Innovation Base. A total of 360 fish were randomly allocated into six floating sea cages with three replicates per dietary treatment under conditions of 26·3 (SEM 3)°C, 29–33‰ salinity and 5·5–7 mg/l dissolved oxygen and were fed twice a day at 05.00 and 17.00 hours for 10 weeks. In the end of the feeding trial, the dorsal muscle and plasma of these large yellow croakers were sampled for subsequent analysis. In the present study, all experimental procedures performed on large yellow croakers were conducted in strict accordance with the Management Rule of Laboratory Animals (Chinese Order No. 676 of the State Council, revised 1 March 2017).

Table 1. Formulation and chemical proximate composition of the experimental diets (% DM)[46]

Ingredient*	FO†	PO†
Casein	36·80	36·80
Gelatin	9·20	9·20
Dextrin	28·00	28·00
Microcrystalline cellulose	3·00	3·00
α-Starch	6·35	6·35
Lecithin	2·00	2·00
Vitamin premix‡	2·00	2·00
Mineral salt§	2·00	2·00
Ethoxyquin	0·05	0·05
Attractant‖	0·30	0·30
Choline chloride	0·20	0·20
Mold inhibitor¶	0·10	0·10
Fish oil	10·00	
Palm oil		10·00
Total	100·00	100·00
Proximate analysis		
Crude protein	43·38	42·89
Crude lipid	12·53	11·98
Cholesterol	0·66	0·64

* All the ingredients were purchased from Great Seven Biotechnology Co., Ltd.
† FO, fish oil; PO, palm oil.
‡ Vitamin premix (mg or g/kg diet): cholecalciferol, 5 mg; retinol acetate, 32 mg; thiamin, 25 mg; riboflavin, 45 mg; pantothenic acid, 60 mg; vitamin B_{12} (1 %), 10 mg; vitamin K_3, 10 mg; pyridoxine HCl, 20 mg; ascorbic acid, 2 g; α-tocopherol (50 %), 240 mg; inositol, 800 mg; niacin acid, 200 mg; folic acid, 20 mg; biotin (2 %), 60 mg; choline chloride (50 %), 4 g; microcrystalline cellulose, 12·47 g.
§ Mineral premix (mg or g/kg diet): Ca $(IO_3)_2 \cdot 6H_2O$ (1 %), 60 mg; $CaH_2PO_4 \cdot H_2O$, 10 g; $CuSO_4 \cdot 5H_2O$, 10 mg; $FeSO_4 \cdot H2O$, 80 mg; $CoCl_2 \cdot 6H_2O$ (1 %), 50 mg; $MgSO_4 \cdot 7H_2O$, 1200 mg; $MnSO_4 \cdot H_2O$, 45 mg; $NaSeSO_3 \cdot 5H_2O$ (1 %), 20 mg; $ZnSO_4 \cdot H_2O$, 50 mg; zeolite, 8·49 g.
‖ Attractants: glycine and betaine.
¶ Mold inhibitor: contained 50 % calcium propionic acid and 50 % fumaric acid.

Table 2. Fatty acid profiles of the experimental diets*

Fatty acid	CON	PO
C14:0	8·08	1·64
C16:0 (PA)	36·88	56·95
C18:0	6·69	5·07
C20:0	0·16	0·07
∑SFA	51·81	63·73
C16:1*n*-7	3·63	0·48
C18:1*n*-9	11·49	22·21
∑MUFA	15·12	22·69
C18:2*n*-6	9·97	11·27
C20:4n-6	0·19	0·02
∑*n*-6 PUFA	10·16	11·29
C18:3*n*-3	1·22	0·41
C20:5*n*-3	4·4	0·94
C22:6*n*-3	4·22	0·51
∑*n*-3 PUFA	9·84	1·86
n-3/*n*-6PUFA	0·97	0·16
Σ*n*-3LC-PUFA	8·62	1·45

CON, control diet; PO, PA-rich diet.
* Fatty acid content is expressed as % total fatty acids.

Cell culture and treatment

Croaker primary myocytes were isolated from skeletal muscle of large yellow croaker according to the following methods. In brief, muscle tissues were removed and cut into small pieces in a Dulbecco's Modified Eagle Medium/Ham's F12 medium (1:1) (DMEM/F12, Biological Industries). Then the tissues were digested with 0·2 % trypsin (Thermo Fisher Scientific) for 20 min and washed twice with DMEM/F12 medium. Whereafter, the cell precipitates were resuspended in DMEM complete medium composed of DMEM/F12 medium supplemented with 15 % fetal bovine serum (Biological Industries), 100 U penicillin and 100 mg/ml streptomycin. The cell suspension was inoculated into a six-well culture plate and incubated at 28°C under 5 % CO_2.

Croaker primary myocytes were incubated with the indicated concentrations of PA (Merck) for 12 h to explore the effect of PA on cholesterol biosynthesis, ERS and SREBP2 activity.

Table 3. Sequences of the primers used in this study

Target genes	Primer sequences (5' to 3')
For clone	
SREBP2-CDS-F	ATGGACGGCGGAGAGTACATCTC
SREBP2-CDS-R	TCAGGATGCAGCGATGGTCG
PCS2-SREBP2-F	CGATTCGAATTCAAGGCCTCTCGAGATGGACGGCGGAGAGTACATCTC
PCS2-SREBP2-R	CTCACTATAGTTCTAGAGGCTCGAGTCAGGATGCAGCGATGGTCG
PGL6-HMGCR-F	CTAACTGGCCGGTACCGCTAGCTTACAAGGACTTACCTGGGTTTAGCA
PGL6-HMGCR-R	CTACGCGTGAGCTCCTCGAGTCTGATGACAGGAAACACAGCCAC
PGL6-DHCR7-F	CTAACTGGCCGGTACCGCTAGCAGCCCAGTAGTGACTTCGGTGAG
PGL6-DHCR7-R	CTACGCGTGAGCTCCTCGAGACTCTTGTCTGGCGGGCACTCG
For RT-qPCR	
β-actin-F	TTATGAAGGCTATGCCCTGCC
β-actin-R	TGAAGGAGTAGCCACGCTCTGT
srebp2-F	ACGAGATGCTGCAGTTTGTCA
srebp2-R	GGGGAGTTTGTGGGGTTTG
hmgcr-F	TCCCTGCGTGTGTCTCTCTG
hmgcr-R	GGTTGGGTTTGTTGTCCTCC
dhcr7-F	GGGAAGACATCTGGACACGGG
dhcr7-R	CGCAGGCAATCACGAAGTAG
abca1-F	CTTCACCCTCTACCTGCCCTAC
abca1-R	TATTCACAGCCGAACCCGA
abcg5-F	GAGCAAAGAGTCACCCAACG
abcg5-R	CAAAGAACGCCACAAAGAGG
abcg8-F	CCAGCACAAACCGAAAGCC
abcg8-R	TCGAAGCCGTGGACCAATA
cyp7a1-F	ATGTTGCTCTGCTCTGGGCT
cyp7a1-R	AGTTTCAGTGTGGGGTCGCT
grp78-F	GGTGGCGATGACAAGCAAAC
grp78-R	CTGAGAACAGCAGCAACAAGC
chop-F	TCTGGATGTTCTGGAGAGTTGTTC
chop-R	AGGATGATGATGAGGTGTGATGC
xbp1s-F	GGTCTTCTGAGTCCGCAGCAGG
xbp1s-R	AGGATGTCCAGAATGCCCAGTAG
atf4-F	GCCGTTATTCTGCTCCATCTTCT
atf4-R	AGACCTTACCCTGAGCCCACAT
atf6-F	CAGATAATAAGGAGGCTGAGAGTGC
atf6-R	CGTAGGTATGATGAGGTGCGTAGT

To investigate the effect of SREBP2 inhibition on mRNA expression of *hmgcr* and *dhcr7*, we treated croaker primary myocytes with 20 μM Fatostatin (Med Chem Express, #HY-14452) for 12 h in the presence of PA. Moreover, to explore the role of ERS in PA-induced activation of SREBP2 and cholesterol biosynthesis, we treated croaker primary myocytes with 3 mM 4-PBA (Med Chem Express, # HY-A0281) for 12 h in the presence of PA.

HEK293T cells were cultured in DMEM supplemented with 10 % fetal bovine serum, 100 units/mL penicillin and 100 mg/ml streptomycin at 37°C with 5 % CO2.

Cloning, sequence analysis and tissue-specific expression of sterol regulatory element-binding protein 2

The cDNA of *Larimichthys crocea* SREBP2 was cloned according to our previously reported method[47]. Primers for SREBP2 cloning are designed and listed in Table 3. The multiple sequence alignment was conducted using DNAMAN (Lynnon BioSoft). A phylogenetic tree was established by MEGA 7.0 (http://www.megasoftware.net). To investigate the tissue distribution of SREBP2, we measured the mRNA expression of *srebp2* in the liver, muscle, heart, adipose tissue, brain, eye, intestine, head kidney, gill and spleen of large yellow croaker.

RNA extraction and RT-quantitative PCR

Using RNAiso Plus (Takara) to lyse muscle tissue or croaker primary myocytes, RNA precipitates were obtained by sequential centrifugation with chloroform and isopropanol, followed by centrifugation with 75 % anhydrous ethanol to remove residual organic solvent, and after evaporation of the 75 % ethanol, the RNA obtained was lysed in DEPC water without RNAase. The quality and concentration of RNA were measured using 1·2 % denaturing agarose gel electrophoresis and NanoDrop Nucleic Acid Protein Assay (Thermo Fisher Scientific), respectively, to ensure that the absorbance ratio (260/280) at 260 nm and 280 nm was between 1·8 and 2·0. The extracted RNA was reversed transcribed into first-strand cDNA using PrimeScript RT Reagent Kit (Takara) according to the manufacturer's instructions. RT-quantitative PCR was performed by SYBR qPCR Master Mix (Vazyme) according to the manufacturer's instructions. The primers used for quantitative PCR are listed in Table 3.

Western blot analysis

Total proteins were extracted from muscles or croaker primary myocytes using RIPA lysis buffer with protease inhibitors and phosphatase inhibitors. Protein concentrations were determined with a BCA Protein Assay Kit (Beyotime Biotechnology Co., Ltd.) according to the manufacturer's instructions. SDS-PAGE gels (6 %, 10 % and 12 %) were configured to suit the experimental requirements. Equal amounts of prepared protein samples were spotted into the sample wells and electrophoresed at 150 V for the appropriate time. After electrophoresis completion, the SDS-PAGE gels were trimmed and the 0·45 μm polyvinylidene fluoride (PVDF) membranes were trimmed accordingly. Then the trimmed PVDF membranes were activated in methanol for 1 min. The transfer time was adjusted according to the size of the protein. After the transfer, the PVDF membranes were closed by shaking with 5 % skimmed milk powder at room temperature for 2 h. The membranes were then incubated overnight at 4°C with different primary antibodies. After incubation with primary antibodies, secondary antibodies of the appropriate species were selected and incubated for 1 h at room temperature, followed by incubation with ECL luminescent solution (Beyotime Biotechnology Co., Ltd.) for 1 min in a dark room and development of the film using a scanner. Primary antibodies against SREBP2 (#28212-1-AP) were purchased from Proteintech. Primary antibodies against GRP78 (#3177), XBP1s (#12782), p-eIF2*α* (Ser51) (#9721) and eIF2*α* (#9722) were purchased from Cell Signaling Technology Inc. Antibodies against GAPDH (#309154) and secondary antibodies were purchased from Golden Bridge Biotechnology.

Plasmid constructs and dual-luciferase reporter assay

For expression plasmids, the *Larimichthys crocea* ORF of SREBP2 was amplified and subcloned into PCS2 vector. For reporter plasmids, the HMGCR promoter and DHCR7 promoter fragment was cloned from the *Larimichthys crocea* genomic DNA and then subcloned into the PGL6 vector. The primers used are listed in Table 3.

For dual-luciferase reporter assay, HEK293T cells were transfected with the PCS2-SREBP2 expression vector, the PGL6-HMGCR promoter reporter vector and the pRL-CMV renilla luciferase plasmid using Lipofectamine 2000 reagent (Invitrogen). After transfection for 24 h, the luciferase activity was assayed using the Dual-Luciferase Reporter Assay System Kit (TransGen Biotech Co., Ltd.) according to the manufacturer's instructions.

The content of total cholesterol, LDL-cholesterol and HDL-cholesterol assays

The total cholesterol contents of muscle, plasma and myocytes were analysed by the total cholesterol assay kit (Nanjing Jiancheng Bio-Engineering Institute) according to the manufacturer's instructions. The contents of LDL-cholesterol and HDL-cholesterol in plasma were measured by the LDL-cholesterol assay kit (Nanjing Jiancheng Bio-Engineering Institute) and the HDL-cholesterol assay kit (Nanjing Jiancheng Bio-Engineering Institute) according to the manufacturer's instructions.

Statistical analysis

The data are presented as the mean values with their standard error of means and analysed using independent *t* tests for two groups and one-way ANOVA with Tukey's test for multiple groups in SPSS 23.0 software. $P < 0{\cdot}05$ was considered to indicate statistical significance.

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The authors declare no competing interests.

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