Oleic and palmitic acids induce hepatic angiopoietin-like 4 expression predominantly via PPAR-γ in *Larimichthys crocea*

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Abstract

Angiopoietin-like 4 (ANGPTL4) is a potent regulator of TAG metabolism, but knowledge of the mechanisms underlying ANGPTL4 transcription in response to fatty acids is still limited in teleost. In the current study, we explored the molecular characterisation of ANGPTL4 and regulatory mechanisms of ANGPTL4 in response to fatty acids in large yellow croaker (*Larimichthys crocea*). Here, croaker *angptl4* contained a 1416 bp open reading frame encoding a protein of 471 amino acids with highly conserved 12-amino acid consensus motif. *Angptl4* was widely expressed in croaker, with the highest expression in the liver. *In vitro*, oleic and palmitic acids (OA and PA) treatments strongly increased *angptl4* mRNA expression in croaker hepatocytes. Moreover, *angptl4* expression was positively regulated by PPAR family (PPAR-α, β and γ), and expression of PPARγ was also significantly increased in response to OA and PA. Moreover, inhibition of PPARγ abrogated OA- or PA-induced *angptl4* mRNA expression. Beyond that, PA might increase *angptl4* expression partly via the insulin signalling. Overall, the expression of ANGPTL4 is strongly upregulated by OA and PA via PPARγ in the liver of croaker, which contributes to improve the understanding of the regulatory mechanisms of ANGPTL4 in fish.

Key words: Angiopoietin-like 4: Oleic acids: Palmitic acids: Mechanism: PPARγ: Large yellow croaker

Angiopoietin-like 4 (ANGPTL4) is a secreted protein that plays an important role in the regulation of lipid metabolism, making it a promising pharmacological target for treating hyperlipidaemia[1,2,3]. ANGPTL4 is widely expressed in mice, with the highest levels in the white and brown adipose tissue, while ANGPTL4 is mainly expressed in the liver of humans[4,5]. ANGPTL4 interacts with lipoprotein lipase (LPL) and inhibits plasma LPL activity, resulting in increased plasma TAG levels[6,7]. ANGPTL4 knockout studies showed a dramatic reduction in TAG levels in mice[8,9]. Meanwhile, mice with systemic or liver-specific overexpression of ANGPTL4 exhibited increased levels of plasma TAG[2]. Moreover, the expression of *angptl4* is regulated by metabolic states and fatty acids in various tissues[10,11,12,13]. Liver-derived ANGPTL4 plays a critical role in the regulation of whole-body metabolism[14,15]. However, the regulatory mechanism underlying fatty acids-induced ANGPTL4 expression in the liver is yet to be well elucidated.

The regulation of ANGPTL4 expression has been extensively studied in a variety of tissues and is under the positive transcriptional control of PPAR -α, -β and -γ[5,16,17]. Although ANGPTL4 is identified as a target of PPAR, the main PPAR isotypes involved in ANGPTL4 regulation are dependent on cell types. PPAR-α and -γ have been shown to upregulate *angptl4* expression in the liver and adipose tissue, respectively. In the skeletal muscle, fatty acids-induced ANGPTL4 expression via PPARβ/δ, but not PPAR-α and -γ[18]. In addition, it has also been reported that *angptl4* gene expression is negatively regulated by insulin in glial cells, 3T3-L1 adipocytes and epididymal adipose tissue[19,20,21]. In H4IIE hepatoma cells, treatment with insulin could attenuate fatty acids-induced *angptl4* mRNA expression[22]. However, whether and how PPAR and insulin signalling regulate hepatic ANGPTL4 expression in response to different fatty acids in the liver remains unclear.

Abbreviations: ALA, α-linolenic acid; ANGPTL4, angiopoietin-like 4; LA, linoleic acid; LPL, lipoprotein lipase; OA, oleic acid; PA, palmitic acid.

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Fish are the most diverse and species-rich group of vertebrates. Given the unique position in the evolutionary spectrum, there is an increasing interest in deep understanding of lipid metabolism in fish[23,24,25,26]. The long-term inclusion of high levels of vegetable oils often leads to increased plasma TAG content and abnormal hepatic lipid deposition in cultured aquatic animals[27,28,29]. In fish, LPL is an important modulator of lipid partitioning to different organs and plays a pivotal role in the regulation of hepatic lipid accumulation[30,31,32]. Moreover, dietary lipid levels and species have shown a significant impact on the expression and activity of LPL[33,34]. Therefore, targeting ANGPTL4, the negative regulator of LPL, will provide a theoretical basis for the treatment of hyperlipidaemia and fatty liver diseases in fish. Large yellow croaker (*Larimichthys crocea*) is an economically and nutritionally important marine fish in China. In addition, the regulation of lipid metabolism in large yellow croaker is evolutionarily conserved compared with mammals[23,35,36]. Hence, the main objective of the current study is to investigate the molecular characterisation of ANGPTL4 and the regulatory mechanism of *angptl4* expression in response to different fatty acid in large yellow croaker.

Materials and methods

Animal experiments

The present study was performed strictly according to the Management Rule of Laboratory Animals (Chinese Order No. 676 of the State Council, revised 1 March 2017) and approved by the Institutional Animal Care and Use Committee of the Ocean University of China. The diet formulation and feeding trial protocol have been described in the previous work[37]. In brief, three diets contained 43 % crude protein and 12 % crude fat with fish oil, palm oil and olive oil and then labelled as fish oil, palm oil and olive oil, respectively. Juveniles of large yellow croaker with similar size ($10 \cdot 05 \pm 0 \cdot 03$ g) were randomly distributed into nine floating cages (1 m × 1 m × 1·5 m) and divided into three groups. Fish were fed twice a day for 10 weeks. At the end, samples were collected and stored at $-80°C$ for further analysis after fasted for 24 h.

RNA extraction and cDNA synthesis

Total RNA extraction was conducted using TransZol (TransGen Biotech) according to the manufacturer's protocol, and RNA quality was examined by Nanodrop (NanoDrop Technologies). Residual DNA contaminants were removed by DNase, and cDNA was performed with the PrimeScriptTM RT reagent kit (Takara).

Gene cloning and sequence analysis

Primers (online Supplementary Table S1) for the amplification of coding DNA sequences were designed according to the predicted sequence of large yellow croaker *angptl4* (Genebank number: XM_010733377.3). The coding DNA sequences were converted to amino acid sequences, and the amino acid sequence was analysed using DNAMAN software (Lynnon-Biosoft). Multiple-sequence alignment of the protein sequences was conducted in MAFFT version 7[38]. The best fit model was selected with Bayesian information criterion in ModelFinder[39]. Bayesian inference phylogenies were performed with MrBayes 3.2.6[40].

Quantitative RT-qPCR

RT-qPCR primer sequences for target genes were designed by Primer Premier 5.0 software (online Supplementary Table yS1). RT-qPCR was performed on a CFX96 Touch real-time PCR detection system (Bio-Rad) using a SYBR Premix Ex Taq kit (TaKaRa) according to manufacturer instructions. The total volume for RT-PCR was 20 µl (1 µl cDNA, 1 µl each primer, 10 µl SYBR qPCR Master Mix and 7 µl DEPC water). For regular RT-PCR amplification, the programme was performed as follows: 95°C for 2 min, afterwards 39 cycles of 95°C for 10 s, 58°C for 15s and 72°C for 10 s. A melting curve (from 58°C to 95°C) was performed after the amplification phase. β-actin, glyceraldehyde-3-phosphate dehydrogenase, 18S rRNA, elongation factor 1α (ef1α) and ubiquitin were selected to test for normalisation of expression. NormFinder algorithms, BestKeeper and geNorm were further used to verify the stability and suitability of these genes. The β-actin gene was used as the reference gene in the current study. Relative mRNA expression was calculated via the $2^{-\Delta\Delta Ct}$ method[41].

Cell culture and treatment

Hepatocytes of large yellow croaker were isolated after digestion with 0·25 % trypsin and obtained according to our previous methods[37]. Hepatocytes were plated in six-well plates (2×10^6 cells/ml) in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 media containing 15 % fetal bovine serum (BI, Israel) at 27°C. In order to explore the underlying mechanism of ANGPTL4 expression in response to FFA, we analysed the effects of 200 µm oleic acid (OA), palmitic acid (PA), α-linolenic acid (ALA), linoleic acid (LA), DHA or EPA on hepatocytes. To investigate the regulatory mechanism of ANGPTL4 expression, several inhibitors and activators were used, including Rosiglitazone (an PPARγ agonist, HY-17386; MCE), GW9662 (an PPARγ inhibitor, HY-16578; MCE), Fenofibrate (an PPARα agonist, HY-17356; MCE), Seladelpar sodium salt (an PPARδ agonist, HY-19522A; MCE), MK-2206 (an AKT inhibitor, S1078, Selleck Chemicals), GSK2033 (an LXR inhibitor, HY-108688; MCE), T0901317 (an LXR agonist, HY-10626; MCE). The control cells were treated with 1 % fatty acid-free bovine serum albumin or the corresponding concentrations of dimethyl sulfoxide.

Plasmid construction and dual-luciferase reporter assay

ANGPTL4 promoter (2248 bp genomic fragment, GenBank Accession No: LT972183.1) was amplified from large yellow croaker genome and cloned into the luciferase reporter vector, pGL6-TA, to construct pGL6-ANGPTL4 plasmid. The plasmid pGL6-TA was purchased from Beyotime Biotechnology (Shanghai, China). For transcription factor plasmids, CCAAT-enhancer-binding protein family (C/EBP α, β, δ), peroxisome proliferator-activated receptor (PPAR α, β, γ), liver X receptors

(LXRα), retinoic X receptor (RXRα), carbohydrate response element-binding protein (ChREBP) and cAMP-responsive element-binding protein were previously obtained[37].

Dual-luciferase reporter assays were performed in HEK-293T cells. Briefly, HEK-293T cells were transfected with pGL6-ANGPTL4 reporter plasmid, transcription factor expression plasmids and pRL-TK renilla luciferase plasmid. Whole-cell lysates were collected after 48 h transfection and performed using the Dual Luciferase Reporter Assay System (TransGen Biotech Co., Ltd.).

Western blot analysis

The protocol for western blot was performed as previously described[42]. Briefly, total protein from cells and tissues was harvested using RIPA lysis buffer (Solarbio) with protease and phosphatase inhibitor cocktails (Roche). Protein concentrations were determined by BCA protein assay and volumes were adjusted to equal protein concentrations. Equal amount of protein samples was load and separated in 10 % SDS-PAGE gel. After electrophoresis, the protein band was transferred onto 0·45 μm activated polyvinylidene fluoride membranes, which were blocked with 5 % non-fat milk and incubated with the following primary antibodies overnight at 4°C: anti-ANGPTL4 (1:1000, ab196746, Abcam), anti-AKT (1:2000, 9272S, Cell Signaling Technology), anti-Phospho-Akt (Ser473) (1:2000, 4060S, Cell Signaling Technology), anti-glyceraldehyde-3-phosphate dehydrogenase (R001, Goodhere). Species-matched horseradish peroxide-conjugated secondary antibodies were incubated at room temperature for 120 min in Tris Buffered Saline + 1% Tween 20 (TBST). Target protein bands were visualised by an enhanced chemiluminescence (ECL) method.

Statistical analysis

All results were presented as mean values ± standard error of mean (SEM). Data were analysed using one-way ANOVA and Tukey's test by SPSS 22.0 software. Comparisons between two groups were determined by Student's t-test. Equality of variances between groups was first evaluated by the F test. Statistical significance was set at $P < 0.05$.

Results

Molecular characterisation and bioinformatics analysis of large yellow croaker angiopoietin-like 4

ANGPTL4 contained an open reading frame of 1416 bp that encoded a protein of 471 amino acids (online Supplementary Fig. S1). Conserved and semi-conserved amino acid residues were highlighted in red and purple (Fig. 1). In addition, a highly conserved 12-amino acid consensus motif was marked by black rectangles in the deduced amino acid sequences of ANGPTL4 (Fig. 1). The phylogenetic tree was constructed based on protein sequences of ANGPTL family members and revealed that the cloned croaker ANGPTL4 belonged to the ANGPTL4 gene family and formed an independent clade (Fig. 2).
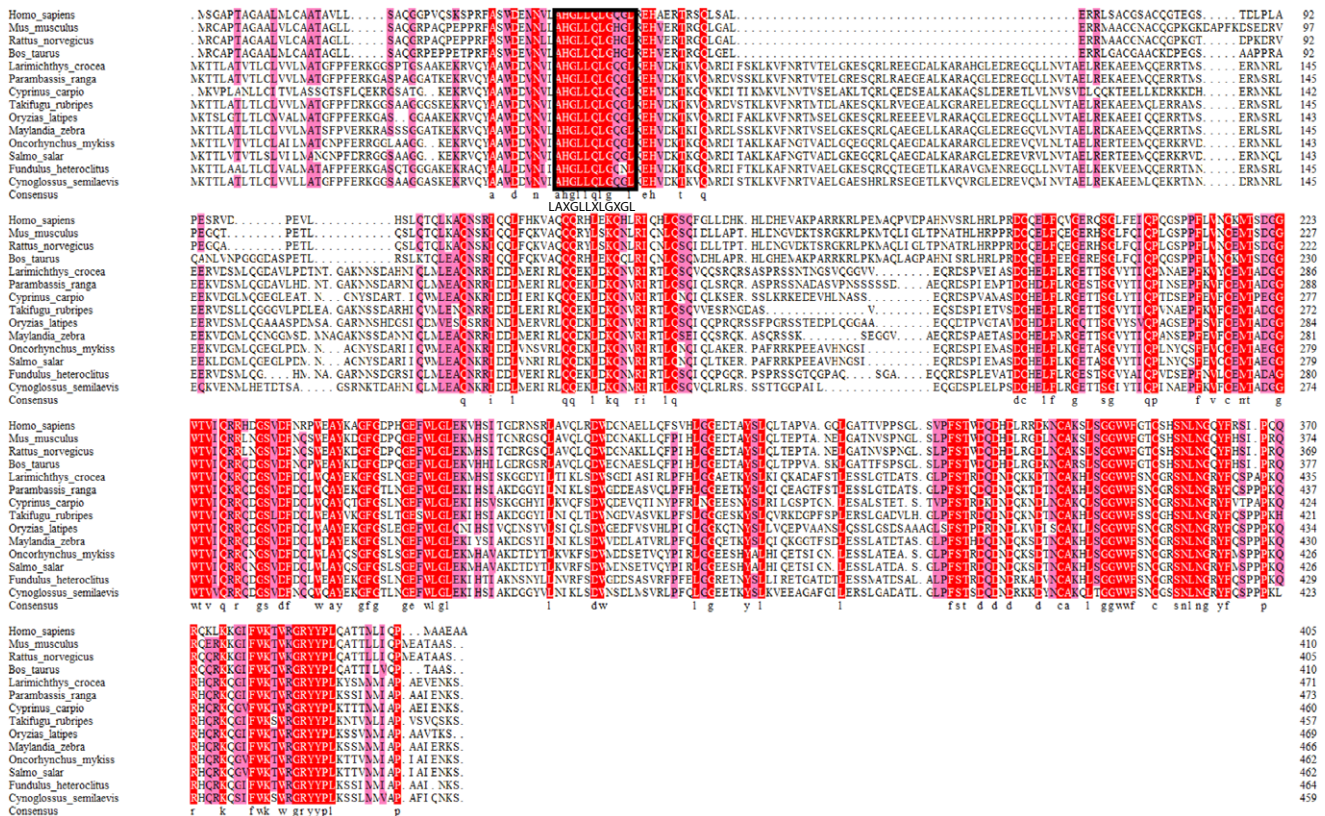

Fig. 1. Bioinformatics analysis of angiopoietin-like 4 (ANGPTL4) amino acid sequence in large yellow croaker. The conserved 12-amino acid consensus motif within the ANGPTL4 had been highlighted.
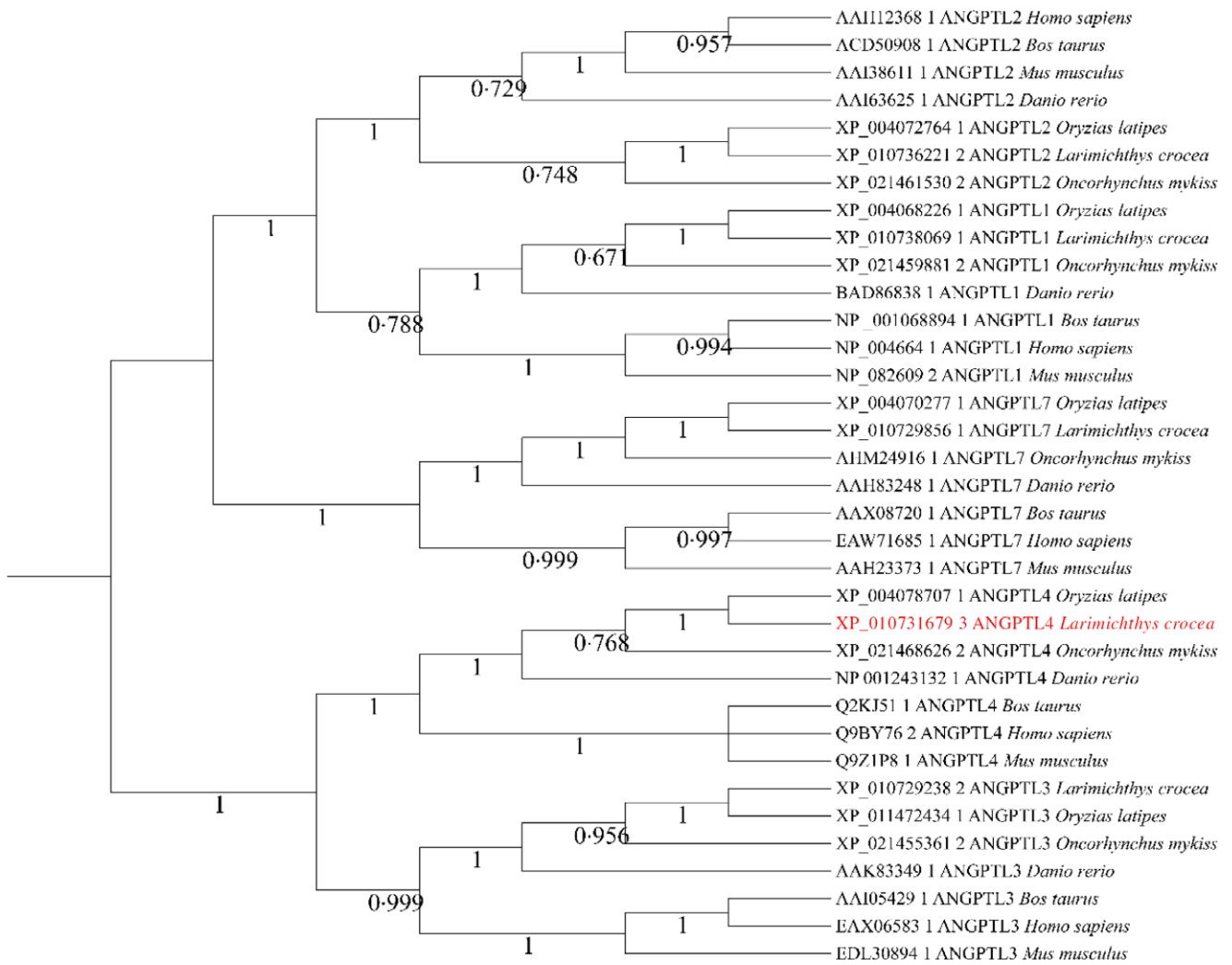
Fig. 2. Bayesian phylogenetic tree of large yellow croaker angiopoietin-like 4 (ANGPTL4) and its homologs in other species.

Tissue distribution of angptl4 *mRNA in large yellow croaker*

The differential expression analyses were carried out in multiple tissues, indicating that expression levels of *angptl4* varied widely in different tissues (Fig. 3). *Angptl4* mRNA was detected in all tissues and has the highest expression in the liver and brain. Moreover, *angptl4* expression was lowest in the kidney (Fig. 3).

Effects of different fatty acids on angiopoietin-like 4 expression in vitro *and* in vivo

Results showed that OA and PA significantly increased the mRNA expression of *angptl4* ($P < 0.05$) (Fig. 4(a)). Hepatocytes were further incubated with OA and PA at different time points, and OA and PA treatments induced strong and sustained increase of *angptl4* expression from 4 to 24 h ($P < 0.05$) (Fig. 4(b) and (c)). In addition, ANGPTL4 protein levels were increased in hepatocytes after incubation with OA or PA for 24 h (Fig. 4(d)
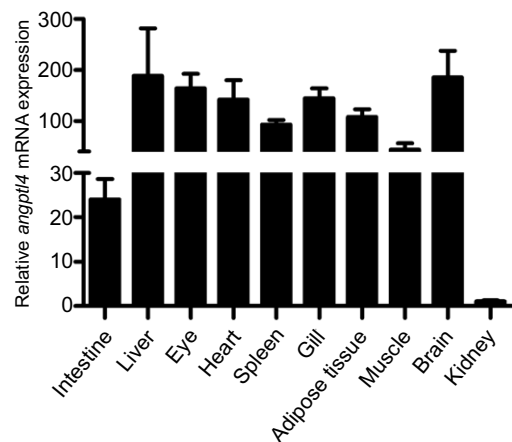

Fig. 3. Differential expression of *angptl4* among different tissues of large yellow croaker. Expression of *angptl4* in the kidney was used as normalisation. β-Actin was used as an internal reference. Data were presented as means with SEM (n 3).
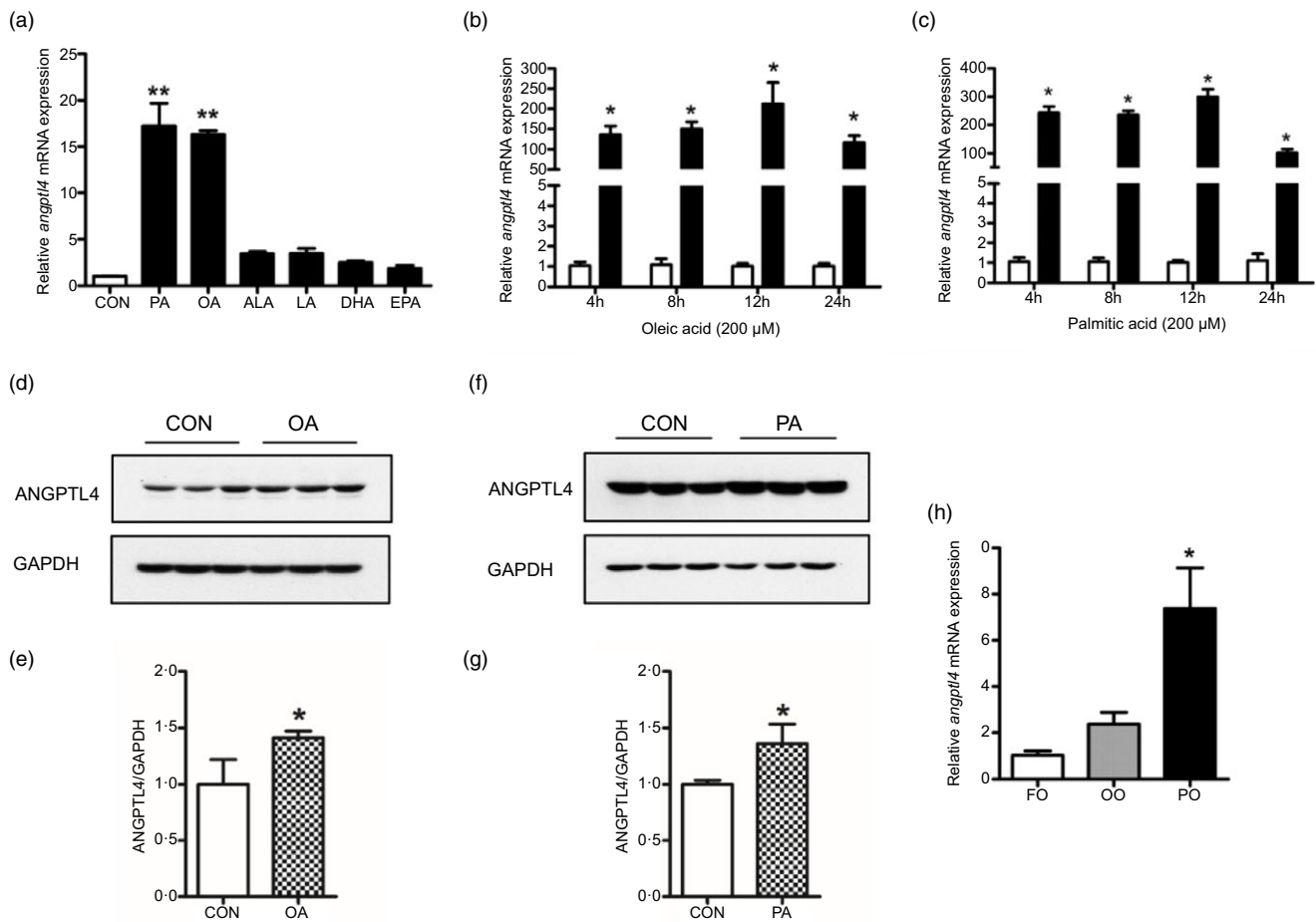
Fig. 4. Oleic and palmitic acids induced angiopoietin-like 4 (ANGPTL4) expression *in vitro* and *in vivo*. (a) Effects of different fatty acids on *angptl4* expression *in vitro* (*n* 3). Primary hepatocytes from croaker were incubated with 200 μm fatty acid for 12 h. PA, palmitic acid; OA, oleic acid; ALA, α-linolenic acid; LA, linoleic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid. CON: control group, hepatocytes of croaker were treated with 1 % bovine serum albumin (BSA). (b–c) Effects of different incubation times of oleic acid (OA) and PA on *angptl4* expression in hepatic cell line of croaker (*n* 3). (b) □, CON; ■, OA. (c) □, CON; ■, PA (d–g) Effects of OA and PA on ANGPTL4 protein expression in hepatic cell line of croaker. The levels of ANGPTL4 were examined by western blot analysis and quantitated (*n* 3). (h) A*ngptl4* expression in the liver of large yellow croaker fed the diets with fish oil (FO), olive oil (OO) and palm oil (PO) (*n* 3). Data were presented as mean ± SEM of three independent experiments. *$P < 0.05$; **$P < 0.01$.

and (e)). Furthermore, PO and OO could upregulate *angptl4* mRNA expression *in vivo* ($P < 0.05$) (Fig. 4(f)).

Identification of transcriptional factors controlling angiopoietin-like 4 gene expression

Dual luciferase reporter assays in HEK-293T cells revealed that PPAR family members (PPAR-α, -β and -γ) and cAMP-responsive element-binding protein had significantly positive effect on the promoter activity of ANGPTL4 ($P < 0.05$) (Fig. 5(a)). LXR and RXRα negatively regulated the ANGPTL4 promoter activity ($P < 0.05$) (Fig. 5(a)). In addition, CEBP family members (CEBP-α, -β and -δ) and ChREBP had no significant effect on the promoter activity of ANGPTL4 (Fig. 5(a)).

To further validate the role of PPAR in regulating ANGPTL4 expression, several selective agonists of PPAR were used to determine whether PPAR could regulate ANGPTL4 expression in hepatocytes of croaker. The results showed that expression of *angptl4* was significantly upregulated in croaker hepatocytes treated with selective agonists of PPAR-α, -β and -γ for 12 h

($P < 0.05$) (Fig. 5(b–d)). Moreover, *angptl4* expression was increased dose-dependently with increasing agonist concentration.

Expression profiles of PPAR response to oleic acid and palmitic acid

PA treatment had no significant effect on the expression of PPARα and PPARβ in hepatocytes of croaker (Fig. 6(a)). Treatment of hepatocytes with OA (200 μm) for 12 h suppressed expression of PPARα and had no effect on PPARβ expression (Fig. 6(b)). However, OA and PA treatments resulted in significant increase in PPARγ mRNA expression in a dose-dependent manner ($P < 0.05$) (Fig. 6(c)).

Oleic acid and palmitic acid-induced angiopoietin-like 4 expression mainly via PPARγ

The expression of PPARγ was significantly upregulated in hepatocytes after 6 h of incubation with rosiglitazone ($P < 0.05$) (Fig. 7(a)). Moreover, protein levels of ANGPTL4 were increased
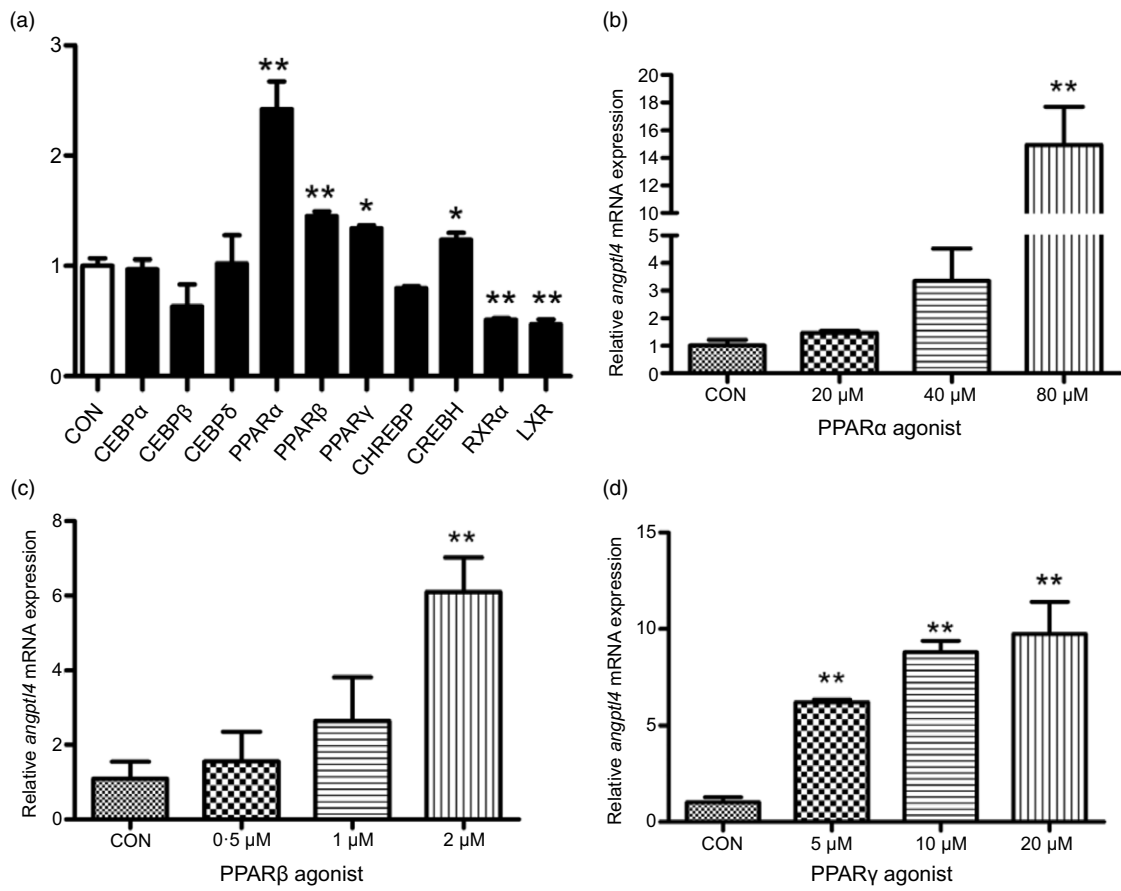
Fig. 5. Peroxisome PPAR family members positively regulated *angptl4* expression at the transcriptional level. (a) Relative luciferase activities of the angiopoietin-like 4 (ANGPTL4) promoter in large yellow croaker in HEK-293T cells. The control group (CON) was co-transfected with pGL6-ANGPTL4 plasmid and pCS2 + empty plasmid. The luciferase activity in control group was selected as normalisation (*n* 3). (b–d) Effects of selective PPAR (-α, -β and -γ) antagonists on *angptl4* mRNA expression *in vitro* (*n* 3). Data were expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$.

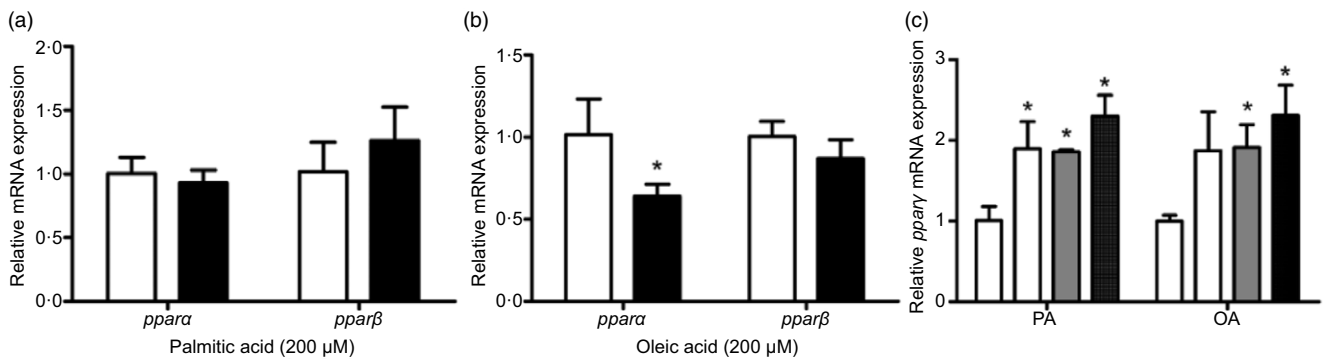**Fig. 6.** Expression of PPAR (-α, -β and -γ) in hepatocytes of large yellow croaker after 12 h of incubation with oleic acid (OA) or palmitic acid (PA). ▢, CON; ■, PA (a) Effects of PA treatment on PPARα and PPARβ expression *in vitro* (*n* 3). (b) Effects of OA treatment on PPARα and PPARβ expression *in vitro* (*n* 3). ▢, CON; ■, OA (c) Effects of different concentrate of OA and PA on PPARγ expression *in vitro* (*n* 3). Data were presented as means ± SEM. *$P < 0.05$; **$P < 0.01$. ▢, CON; ▢, 200 μM; ■, 400 μm; ▨, 800 μm

dose-dependently by rosiglitazone concentration (Fig. 7(b)). Furthermore, inhibition of PPARγ by inhibitor (GW9662) completely abrogated the effects of OA on *angptl4* expression (Fig. 7(c)). However, pre-treatment of hepatocytes with the PPARγ inhibitor partially inhibited PA-induced *angptl4* expression (Fig. 7(d)).

Palmitic acid-induced angiopoietin-like 4 expression partly through inhibiting the insulin signalling

The results revealed that OA treatment had no significant influence on Akt phosphorylation (Ser473) *in vivo* and *in vitro* (Fig. 8(a) and (b)). PA could significantly suppress the phosphorylation of AKT (Ser473) *in vivo* and *in vitro* (Fig. 8(c) and (d)).
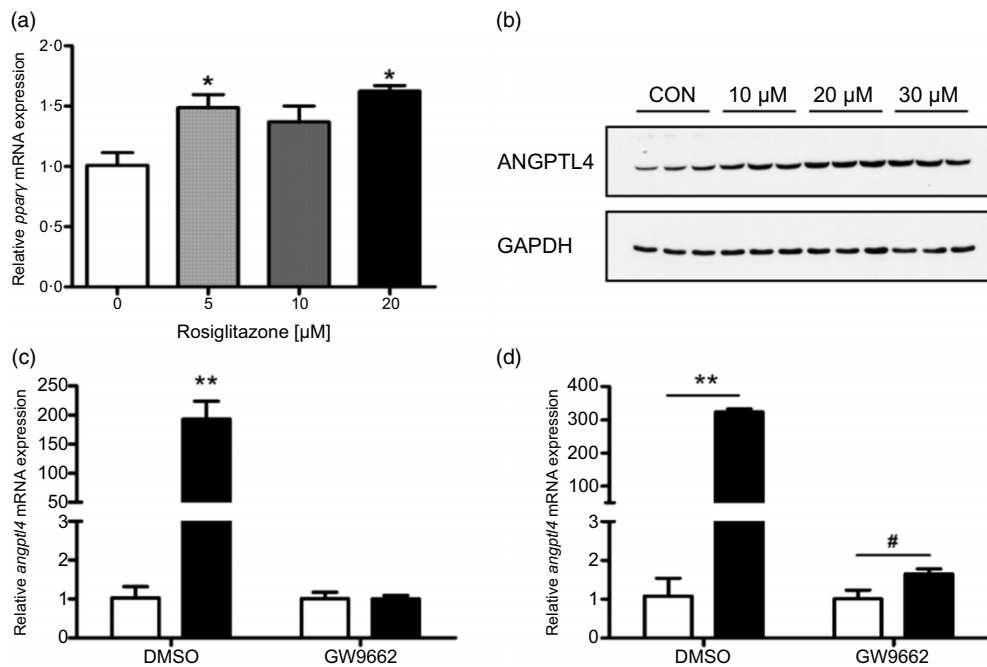
Fig. 7. Oleic acid (OA) and palmitic acid (PA)-induced ANGPTL4 expression mainly via PPARγ. (a) Expression of PPARγ in hepatocytes of large yellow croaker incubated with PPARγ agonists (rosiglitazone) for 6 h (n 3. (b) Western blot analysis for ANGPTL4 in hepatocytes of large yellow croaker incubated with PPARγ agonists (n 3). OA (c) The PPARγ inhibitor (GW9662) abolished the effect of OA on *angptl4* upregulation (n 3). ☐, CON; ■, OA (d) Inhibition of PPARγ by GW9662 abolished activation of *angptl4* (n 3). Data were presented as means ± SEM. *$P < 0.05$. ☐, CON; ■, PA

Moreover, the effects of insulin signalling on *angptl4* expression were subsequently determined. Insulin significantly inhibited *angptl4* expression in hepatocytes of large yellow croaker ($P < 0.05$) (Fig. 8(e)). Meanwhile, Akt inhibitor (MK-2206) dramatically increased the mRNA expression of *angptl4* in croaker hepatocytes ($P < 0.05$) (Fig. 8(f)).

Discussion

In fish, changes in activities and mRNA expression of LPL have a significant influence on hepatic lipid metabolism[43,44]. ANGPTL4 serves as an endogenous inhibitor of LPL and is involved in regulation of lipid metabolism, glucose homoeostasis and insulin sensitivity[45,46]. In the present study, croaker ANGPTL4 protein sequence possessed the 12-amino acid consensus motif within the conserved coiled-coil domain, which is a typical feature for ANGPTL4[47]. It was consistent with previous observations that characteristics of ANGPTL family proteins in fish are thought to be conserved[48,49]. The results suggested that role of ANGPTL4 in regulating LPL activity may be conserved between croaker and other species. Meanwhile, expression patterns of *angptl4* in croaker showed that ANGPTL4 is predominantly expressed in the liver, similar with results in human[4]. In mammals, ANGPTL4 expression is subject to complex cell type-specific regulation and might have important functional consequences on vertebrate physiology[50,51,52]. It suggested that ANGPTL4 might be mainly secreted by the liver in croaker and play an important role in regulation of hepatic lipid metabolism. However, the research on ANGPTL4 in other fish species has not been reported yet, and it needs to be further explored.
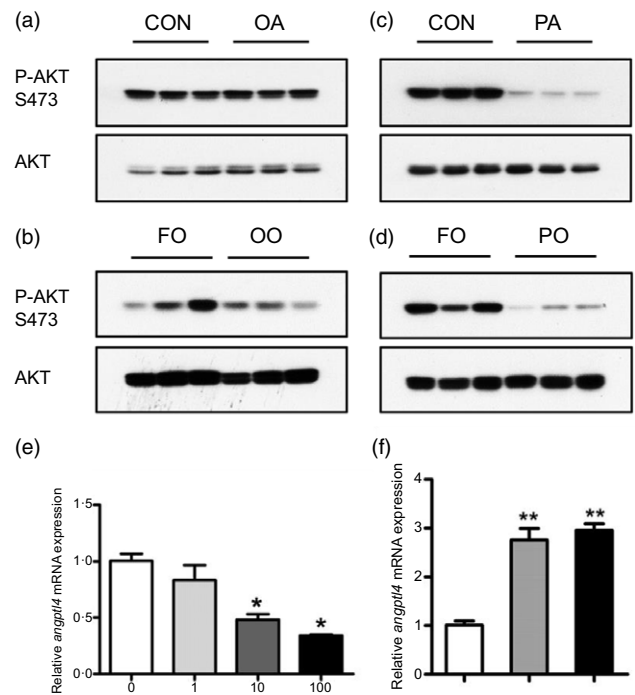

Fig. 8. Insulin signalling was involved in regulation of palmitic acid (PA)-induced *angptl4* expression. (a, c) Western blot analysis for Akt phosphorylated at serine 473 (p-Akt) and total Akt in hepatocytes of large yellow croaker treated with olive oil (OA) (a) or PA, (c) (n 3). (b) Western blot analysis for p-Akt and total Akt in the liver of large yellow croaker fed the diets with fish oil (FO) and olive oil (OO) (n 3). (d) Western blot analysis for p-Akt and total Akt in the liver of large yellow croaker fed the diets with FO and palm oil (PO) (n 3). (e–f) The mRNA expression levels of *angptl4* were analysed after treatment with insulin (e) or AKT inhibitor (f) (n 3). Data were presented as means ± SEM. *$P < 0.05$. **$P < 0.01$. ☐, CON; ▨, AKTi (5 μM); ■, AKTi (10 μM)

It is known that expression of *angptl4* is stimulated by fatty acids in various tissues and is dependent on the species of fatty acids and cell types in mammals. DHA has the highest potency to induce *angptl4* in rat hepatoma cells[11]. Expression of *angpltl4* was upregulated by PA, OA, EPA and arachidonic acid in human adipocytes[10]. To our knowledge, the response mechanism of ANGPTL4 to fatty acids in fish remains unclear. However, in the current study, our results showed that OA and PA strongly induce hepatic *angptl4* expression *in vitro*, indicating that the ANGPTL4 of croaker in response to fatty acids is different from mammals. Hence, the diets enriched with SFA (PA) and MUFA (OA) may stimulate the expression of *angptl4* to further inhibit LPL activities, which may induce the disorder of lipid metabolism in croaker[27,34]. Interestingly, in contrast to *in vitro* results, we found that changes of *angptl4* mRNA levels in the liver induced by PO and OO are much less than that achieved in hepatocytes, indicating that the regulation of ANGPTL4 *in vivo* is more complex and might be influenced by nutritional and healthy status.

To investigate the mechanisms involved in the regulation of *angptl4* expression, we identified several transcription factors that might regulate its promoter activity. Consistent with fatty acids being potent activators of PPAR, numerous studies have shown that ANGPTL4 is under transcriptional control of PPAR in mammals[5,53,54]. In fish, the transcriptional activity of PPAR was highly conserved, and expression of PPAR was significantly regulated by dietary fatty acid[55,56,57]. In addition, individual PPAR isotypes have different roles in different tissues in mammals and fish[17,58,59,60]. Here, PPAR (α, β and γ) were able to activate ANGPTL4 expression in hepatocytes of croaker. PPARγ plays a critical role in OA- and PA-induced ANGPTL4 expression in the liver of croaker. These results were agreed well with previous studies in fish that PPAR family members are significantly involved in regulating lipid metabolism in livers of fish fed with vegetable oil-based diets[61,62].

Furthermore, insulin signalling played an important role in the regulation of ANGPTL4 expression. To delve further into the potential mechanism behind the upregulation of *angptl4* expression, the results revealed that insulin signalling was also involved in the regulation of *angptl4* expression in hepatocytes of croaker after PA treatment. In accordance with the present results, treatment with insulin in H4IIE cells attenuated the elevated expression of *angptl4* induced by PA treatment[63]. The present study suggested that PPARγ pathway and insulin signalling were all involved in PA-induced *angptl4* expression. The related studies about the effect of fatty acids on *angptl4* expression are rare, more corresponding work should be performed in the future.

In conclusion, our data indicated that the upregulation of ANGPTL4 expression in response to different fatty acids is distinct in the liver of large yellow croaker and PPARγ might play a key role in regulating OA- or PA-induced hepatic ANGPTL4 expression in fish. These results may contribute to improve multiple pathologies in fish and ensure the quality of aquatic products.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S000711452100386X

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