



Original Research Article

DHA induces adipocyte lipolysis through endoplasmic reticulum stress and the cAMP/PKA signaling pathway in grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

Docosahexaenoic acid (DHA) is a biologically active fatty acid that reduces the accumulation of lipids. However, the molecular mechanism underlying this process, particularly in fish, is not well understood. Recent studies show that endoplasmic reticulum (ER) stress triggers the activation of the unfolded protein response, which has been revealed to play an essential role in lipid metabolism. In this study, we explored the effect of DHA on ER stress and investigated the potential molecular mechanisms underlying DHA-induced adipocyte lipolysis in grass carp (*Ctenopharyngodon idella*) both in vivo and in vitro. We found that DHA remarkably reduced the triglyceride content, increased the secretion of glycerol, promoted lipolysis in adipocytes and evoked ER stress, whereas inhibiting ER stress using 4-phenyl butyric acid (4-PBA) inhibited the effects of DHA ($P < 0.05$). These results implied that ER stress potentially participates in DHA-induced adipocyte lipolysis. Additionally, STF-083010, a specific inositol-requiring enzyme 1 α (IRE1 α)-inhibitor, attenuated the effects of DHA on lipolysis, demonstrating that IRE1 α and X-box binding protein 1 potentially participate in DHA-induced lipolysis. DHA also activated the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) pathway by increasing the level of cAMP and activating the PKA enzyme ($P < 0.05$). Nevertheless, H89, a PKA inhibitor, weakened DHA-induced lipolysis by inhibiting the cAMP/PKA signaling pathway. Furthermore, inhibiting ER stress using 4-PBA also inhibited lipolysis and alleviated DHA-induced activation of the cAMP/PKA signaling pathway, suggesting that ER stress may participate in DHA-induced lipolysis through the activation of the cAMP/PKA signaling pathway. Our data illustrate that DHA supplementation can be a promising nutritional strategy for ameliorating lipid accumulation in grass carp. The present study elucidated the molecular mechanism for DHA-induced lipolysis in grass carp adipocytes and emphasized the importance of ER stress and the cAMP/PKA pathway in DHA-induced lipolysis. These results deepen our understanding of ameliorating lipids deposition in freshwater fish by targeting DHA.

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1. Introduction

Imbalanced nutrition or excessive energy intake by some cultured fish species cause excessive fat deposition in the abdominal adipose tissue. Adipose tissue is the main reservoir of neutral lipids

(such as cholesteryl ester, triglyceride [TG], and wax ester) and plays an important role in regulating energy homeostasis and lipid metabolism through lipogenesis, lipolysis and secretion of several cytokines (leptin, adiponectin and resistin) (Wang et al., 2008; Koc et al., 2015). Excessive fat deposition and adipocyte dysfunction cause metabolic syndrome (Wang et al., 2016). In fish, excessive fat deposition in the abdominal cavity is not only a waste of energy but also promotes unhealthy deposition of fats in the liver and reduces resistance to diseases (Du et al., 2006). Adipose tissue is a vital regulatory target for improving the health and quality of fish (Salmerón, 2018). Thus, exploring the molecular mechanisms of alleviating lipid deposition in fish adipose tissue is highly necessary.

Endoplasmic reticulum (ER) stress is a key contributory factor in the dysfunction of adipose tissue and lipid metabolism (Lemmer

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et al., 2021; Menikdiwela et al., 2021). The ER is a sensor for nutrients (e.g., lipid) responsible for lipid synthesis, protein translocation and folding and Ca^{2+} homeostasis (Mandl et al., 2009; Han and Kaufman, 2016; Schwarz and Blower, 2016). Disrupting the ER (e.g., the accumulation of lipids as well as unfolded or misfolded proteins in the lumen of the ER) causes ER stress and a complex signaling network called the unfolded protein response (UPR) is activated to maintain ER function (Han and Kaufman, 2016; Stevenson et al., 2016). The UPR signaling pathway comprises three canonical branches: inositol-requiring enzyme 1 α (IRE1 α) and X-box binding protein 1 (IRE1 α -XBP1), protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) and eukaryotic initiation factor 2 α (PERK-eIF2 α), and activating transcription factor 6 (ATF6) (Hetz et al., 2020). ER stress is associated with numerous diseases caused by lipid metabolism disorders (Fu et al., 2012; Han and Kaufman, 2016). Mice models have revealed that empagliflozin attenuates non-alcoholic fatty liver disease (NAFLD) by reducing ER stress and mitigating lipogenesis (Nasiri-Ansari et al., 2021). A separate study revealed comparable findings in the epithelial cells of yellow catfish (*Pelteobagrus fulvidraco*) (Ling et al., 2019). Inhibiting ER stress significantly reduced lipolysis in white adipose tissue of rats with chronic kidney disease (Zhu et al., 2014).

Lipolysis is the catabolism of TG (Grabner et al., 2021), in which TG are hydrolyzed into glycerol (GL) and free fatty acids (FFA) catalyzed by lipolytic enzymes (Kim et al., 2016). Lipolysis is a complex process regulated through numerous signaling pathways (Grabner et al., 2021). Lipolysis in response to ER stress is mediated via the cyclic AMP (cAMP)-dependent protein kinase A (PKA) signaling pathway (Wang et al., 2016; Deng et al., 2012). The cAMP/PKA signaling pathway is a classical mechanism for lipolysis activity (Londos et al., 1999) which involves the plasma membrane hormone receptors, GTP-binding proteins, the alpha subunit of the G-protein (guanine nucleotide-binding protein G (s) subunit α [GANS]), activation of adenylyl cyclase (ADCY), and increases the intracellular cAMP content (Carmen and Victor, 2006; Larsson et al., 2016). cAMP promotes the catabolism of TG and diglycerides by activating PKA, which activates lipolytic enzymes (such as hormone-sensitive lipase HSL), promoting the release of GL and FFA (Grabner et al., 2021). Most reports on the mechanism of ER stress in lipid metabolism have mainly focused on lipogenesis in mammals (Gentile et al., 2011; Basseri and Austin, 2012). However, studies on the potential relationship between ER stress and lipolysis, particularly in fish, remain scant (Brasaemle and Wolins, 2012). Therefore, the mechanism underlying lipolysis needs further investigation.

Fatty acids (n-3) are essential dietary nutrients that promote good health and regulate lipid metabolism (Kuda et al., 2018). Docosahexaenoic acid (DHA) (22:6n-3) is a type of biologically active fatty acid (FA) in the family of n-3 long-chain polyunsaturated fatty acids (n-3 LC PUFA-), which plays a critical role in reducing lipid accumulation (Soni et al., 2019). Our previous study reported that DHA induced lipolysis by upregulating the level of GL and the transcription of messenger RNA (mRNA) for adipose triglyceride lipase (ATGL) while reducing the TG content (Liu et al., 2014). On the other hand, the dietary fatty acid composition influences the composition of fatty acids in the ER membrane of the liver. This affects the integrity, fluidity and function of the ER, causing the UPR (Hammer and Wills, 1978; Volmer and Ron, 2015). A study using a female C57BL/6J mice model revealed that DHA ameliorates NAFLD by alleviating ER stress induced by a high-fat diet (Yang et al., 2021). The findings of this study demonstrated the relationship between ER stress and DHA-regulated lipid metabolism.

Grass carp (*Ctenopharyngodon idella*) is the largest cultivated freshwater herbivorous fish in China (China Society of Fisheries, 2022). To increase yield and shorten maturity, grass carp are fed

on high-energy feeds. Unfortunately, this causes lipid metabolism disorders (Zhao et al., 2019; Tang et al., 2019). In addition, grass carp can accumulate high lipid content in adipose tissue under natural conditions, making them a potential model for exploring lipid metabolism in adipose tissue (Du et al., 2006). Our previous study found that supplementation with 0.5% n-3 LC PUFA in feed significantly promoted growth performance and decreased the intra-peritoneal fat (IPF) content in grass carp (Ji et al., 2011). As a member of n-3 PUFAs, we hypothesized that DHA might play an important role in reducing lipid accumulation. In the present study, grass carp were fed on a diet containing three levels of DHA content (0%, 0.5% and 1%) to investigate the role of DHA on lipolysis in the adipocytes of this fish. Our data illustrated the important role of ER stress and the cAMP/PKA pathway in DHA-induced lipolysis and suggested that DHA supplementation is a promising nutritional strategy for ameliorating lipid accumulation in the adipocytes of grass carp.

2. Materials and methods

2.1. Ethics statement

The protocols for the animal experiments were approved by the Animal Care and Use Committee of Northwest A&F University (NWAUFU-DKXC-20200602).

2.2. Reagents

Oil containing 50% DHA was purchased from Xunda Marine Biological Products Co., Ltd (Jiangsu, China). DHA and the ER stress inhibitor 4-phenyl butyric acid (4-PBA) (Sigma-Aldrich, St. Louis, Missouri, USA) were prepared as described previously (Huang et al., 2022; Bian et al., 2022). The PKA inhibitor H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide), and the specific IRE1 α inhibitor STF-083010 (N-[(2-Hydroxy-1-naphthalenyl)methylene]-2-thiophenesulfonamide, shortened to STF), were purchased from MedchemExpress (Monmouth Junction, NJ, USA). The concentrations of 4-PBA (15 μM), STF (30 μM), and H89 (20 μM) were selected based on our pilot experiment.

2.3. Experimental design

2.3.1. Experiment 1: the effects of DHA on adipocyte lipolysis and ER stress in vivo

Three purified (isonitrogenous and isoenergetic) experimental diets were formulated with DHA concentrations of 0% (control group), 0.5% (0.5% DHA group) and 1% (1% DHA group), respectively. It should be noted that the commercial feeds used in aquaculture contain a certain amount of DHA, usually 0.5% and 1% DHA, but rarely 0%. The experimental feeds contained about 36% crude protein and 6.0% crude fat. The fat was mainly soybean oil, linseed oil, lard oil and DHA-enriched oil. DHA was added as a substitute for lard oil. The experimental feeds were prepared as described by Jin et al. (2018). Briefly, the ingredients were finely ground and sieved through a 40-mesh screen, weighed depending on the formulation, thoroughly mixed, and made into 2-mm pellets. The preparations were dried under compressed air to a moisture content of less than 10% and stored at $-20\text{ }^{\circ}\text{C}$ until use. The measured values of experimental feed components for each group were as follows: crude lipid (5.42%, 5.66%, 5.60%), crude protein (36.48%, 36.12%, 36.0%), moisture (9.80%, 9.98%, 9.56%) and ash (5.83%, 5.86%, 5.89%). The measured values of DHA were 0.06%, 0.46% and 1.01%, respectively. The feed formulation (Supplemental Table S1) and fatty acid composition (Supplemental Table S2) have been published in Huang et al. (2022).

The management and feeding of grass carp were performed in a recirculation system (225 L/tank) according to our previously published protocol (Shi et al., 2017). Briefly, a total of 108 healthy fish (initial body weight of 29.76 ± 2.34 g) were divided into 3 groups, each group with 3 replicates of 12 fish. The fish were hand-fed to apparent visual satiation 3 times a day (08:30, 12:30 and 16:30). During the 8 wk feeding trial, the fish were maintained at a photoperiod of 12 h/12 h (light/dark), a water temperature of 28.2 ± 0.20 °C, dissolved oxygen concentration of 9.35 ± 0.31 mg/L, ammonia content of 0.14 ± 0.30 mg/L and PH of 7.43 ± 0.16 .

After 8 wk, all the fish were fasted for 24 h before sampling and euthanized using immersion in tricaine methane sulfonate (MS-222) (100 mg/L) (Sigma, St. Louis, MO, USA). Two randomly selected fish from each group were stored at -20 °C for whole body crude lipid analysis. Blood samples (4 fish in each tank) were collected from the caudal vein with a syringe, incubated for 6 h at 4 °C and centrifuged at $825 \times g$, 4 °C for 10 min to obtain serum, which was stored at -80 °C for TG and total cholesterol (TC) content analysis. After blood sampling, the fish were dissected on ice and the adipose tissues were quickly stripped along the intestine with forceps and weighed. The adipose tissues were fixed in 4% paraformaldehyde (Biosharp, Hefei, China) or 2.5% glutaraldehyde solution (Leagene biotech Co., Ltd., Beijing, China) for histological and morphological assessment, including hematoxylin & eosin (H&E) staining, immunohistochemistry and ultrastructural observation. The adipose tissue of the remaining fish was immediately extracted, weighed, and then stored in liquid nitrogen for further analysis of the TG content and the mRNA expression of genes related to lipolysis and ER stress.

IPF index (IPFI) was calculated using the following formula:

$$\text{IPFI (\%)} = \text{IPF weight} \times 100/\text{body weight}.$$

2.3.2. Experiment 2: the effects of DHA on adipocyte lipolysis and ER stress in vitro

Isolation and culture of grass carp pre-adipocytes were performed according to a previously described method (Bian et al., 2022). In detail, healthy grass carp (approximately 1 kg) were purchased from the market (Yangling, China) and kept in 300 L aquariums for at least a week to acclimatize. The fish were fed on commercial feeds 3 times a day. Before the experiment, grass carp were euthanized with 100 mg/L methane sulfonate (MS-222) and the gill arches were cut with scissors. The mucus on the fish was washed using running water. The fish were dissected and the abdominal adipose tissue was carefully isolated with forceps and washed 4 times in phosphate-buffered saline (PBS, pH 7.4) under aseptic conditions. The adipose tissue was minced in a clean beaker and its volume was measured. Then the adipose tissue was digested with 0.1% type I collagenase (Sigma Aldrich, USA) and 2% bovine serum albumin (Sigma Aldrich, USA) at room temperature. Dulbecco's modified Eagle's/F12 (DME/F12) medium was added after 30 min to stop the digestion. The mixture was centrifuged in a 50-mL centrifuge tube at $590 \times g$ for 10 min. The supernatant was discarded. Erythrocyte lysing buffer was added to the pellet, incubated for 6 min, and filtered through a 200-purposse nylon mesh. The filtrate was centrifuged in a 50-mL centrifuge tube ($380 \times g$, 6 min) and the cell pellet was resuspended and washed with DME/F12. The centrifugation process ($380 \times g$, 6 min) was repeated twice. Subsequently, the cells were seeded in 24-well plates at a density of 15 mL tissue/32 cm². The growth medium (GM) was changed every 2 days (DME/F12 supplemented with a mixture of 100 U/mL streptomycin and penicillin and 10% fetal bovine serum). The cells were grown at 37 °C in humidified incubators under 5% CO₂ and 95% air. After confluence, the GM was replaced with adipogenic medium (10 µg/mL insulin, 10 nM triiodothyronine, 1 µM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine and GM), which was changed every 2 days. In the late phase of differentiation (about differentiation day 6),

the adipocytes were randomly divided into 3 groups and treated with 0, 100 and 200 µM DHA for 48 h. The cells were stained with Nile red (Sigma, St. Louis, MO, USA) and 4',6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO, USA) and observed under a fluorescent microscope (Dhyan 400DC, Tucsen) to assess the amount of neutral lipid. TG and GL content and the expression of genes involved in lipolysis and ER were also determined.

2.3.3. Experiment 3: the role and mechanism of ER stress in DHA-induced lipolysis in vitro

To explore whether ER stress mediated DHA-induced lipolysis, ER stress was inhibited using 4-PBA as previously described (Wang et al., 2016). Three groups, including control, 200 µM DHA and 200 µM DHA +15 µM 4-PBA, were designed. Adipocytes were preincubated with ER stress inhibitor (4-PBA) for 4 h before DHA treatment. The cells were collected after 48 h.

To explore the effect of IRE1 α -XBP1 (the classical signaling pathway of ER stress) in DHA-induced lipolysis, the adipocytes were incubated with STF (an inhibitor of IRE1 α) (Song et al., 2017). Three groups were designed: control, 200 µM DHA and 200 µM DHA +30 µM STF. The adipocytes were preincubated with STF for 2 h before DHA treatment for 48 h. Nile red and DAPI staining were then performed to assess the neutral lipids. TG and GL content and the expression of genes, proteins and enzymes that participate in lipolysis and ER stress were determined.

2.3.4. Experiment 4: the potential role of the cAMP/PKA signaling pathway in DHA-induced lipolysis

The role of the cAMP/PKA signaling pathway in DHA-induced lipolysis in adipocytes was explored using H89 (a PKA inhibitor) (Deng et al., 2012; Song et al., 2017). Three groups were designed: control, 200 µM DHA and 200 µM DHA+20 µM H89 group. The adipocytes were preincubated with PKA inhibitor (H89) for 3 h before DHA treatment for 48 h. Nile red and DAPI staining were also performed. TG and GL content and the expression of mRNAs for genes and enzymes that participate in lipolysis and regulate PKA signaling pathway were determined.

2.4. Sample analysis

2.4.1. Fatty acid composition in adipose tissue

The fatty acid composition in the adipose tissue was measured using Gas chromatography (GC) as previously described (Shi et al., 2017).

2.4.2. Crude lipid content in the whole fish body

The amount of crude lipid in the whole fish body was determined by the Soxhlet method, as previously described (Xu et al., 2021b).

2.4.3. TG, TC and GL content assays on adipose tissue and serum

Serum biochemical parameters, including TG (mM) and TC (mM), were analyzed by an automatic biochemical analyzer (Hitachi 7180, Tokyo, Japan). Adipose tissue TG (mmol/g protein) and serum GL (µM) content was measured using a triglyceride assay kit and liquid sample glycerase assay kit (Applygen Technologies Inc., Beijing, China), respectively.

2.4.4. Histological and morphological analysis of adipose tissue

The adipose tissue of grass carp was washed 3 times with normal saline, and immediately fixed in 4% paraformaldehyde for 24 h, washed with running water for 12 h, dehydrated, equilibrated, embedded in paraffin, sliced into 5 µm sections and stained with H&E as previously described (Jin et al., 2018). The histological samples were observed and photographed using an upright

microscope (Nikon Biosystems, model of Ni–U, Japan). The adipocyte size was estimated using Image J. An average value across 7 non-overlapping images (7/section × 2 fish × 3 tanks) was calculated for each group.

2.4.5. Nile red and DAPI staining

Nile red and DAPI staining was performed to observe neutral lipids and the nucleus of adipocytes, respectively, as previously described (Bian et al., 2022).

2.4.6. Ultrastructural observation

The ultrastructure of adipocytes was observed using transmission electron microscopy (TEM) (Song et al., 2013). For fixation, fresh adipose tissue was cut into 1 to 3 mm³ cubes (cell pellets did not need to be cut) and fixed in 2.5% glutaraldehyde solution for 24 h. The samples were washed 3 times with phosphate-buffered saline (PBS) (pH 7.2) for 10 min each time. The samples were fixed with 1% osmium acid (Ted Pella, Inc., Redding, CA, USA) for 4 h, washed 3 times with PBS, 8 min each time, and dehydrated twice in each ethanol concentration (30%, 50%, 70%, 90% and 100%) (Kermel Chemical Reagent Co., Ltd., Tianjin, China) for 15 min each time. The samples were infiltrated in a mixed solution of alcohol and London resin (LR) white (HEAD Biotechnology Co., Ltd., Beijing, China) at the ratio of 3:1 for 2 h; 1:1 for 8 h; 1:3 for 12 h, and infiltrated in LR white for 24 h. This process was repeated twice before embedding the samples in LR white. The samples were then polymerized for 48 h at 55 °C and cut into thin sections using an ultrathin microtome (Leica Biosystems, EMUC7 model, Germany) equipped with a diamond knife, double-stained with uranyl acetate-lead citrate and uranyl acetate (Ted Pella, Inc., Redding, CA, USA) for 30 min in a dark vacuum, rinsed with distilled water for 6 min, stained with lead citrate for 5 min in a dark vacuum, and rinsed again with distilled water for 6 min. Finally, the ultrathin sections were observed under a TEM (FEI Tecnai G2 Spirit Bio, USA).

2.4.7. Immunohistochemical and Western blot analysis

Immunohistochemistry analysis was performed using the citrate antigen retrieval method (Wang et al., 2018). First, the paraffin sections were immersed in citrate buffer (pH 6.0, Biosharp, Hefei, China) and heated for 15 min at 100 °C in a water bath to release antigens. The sections were then soaked in 3% hydrogen peroxide solution for 15 min to deactivate endogenous catalase. The tissue sections were blocked with serum for 2 h, incubated with primary antibody overnight at 4 °C, washed 3 times, 5 min each time with tris-buffered saline (TBS, pH 7.2) and incubated with the secondary antibody for 30 min in the dark. The tissues were washed 3 times again with TBS after 30 min. 3,3'-Diaminobenzidine tetrahydrochloride (Biosharp, Hefei, China) was used as the chromogenic reagent. Staining was complete when the reagent darkened. At this stage, the sections were washed 3 times with distilled water for 5 min each time. Subsequently, the sections were counterstained with hematoxylin for 1 min, dehydrated, covered with a transparent coverslip and observed under a microscope (Nikon, Ni–U model, Japan) and photographed. The primary antibodies against PERK, C/EBP homolog protein (CHOP), Glucose-regulated protein 78 (GRP78) and EIF2 α were purchased from Cell Signaling Technology (Inc., USA). The antibodies were diluted to a ratio of 1:1000 (Wang et al., 2018).

The protein levels in the adipocytes were measured as previously described (Jin et al., 2018). The primary antibodies against PERK, CHOP and GRP78 were purchased from Cell Signaling Technology (Inc., USA) and β -actin was purchased from Prosci (Inc., USA). The goat anti-rabbit secondary antibody was conjugated to horseradish peroxidase enzyme (Abbkine Scientific Co., Ltd., CA, USA). The antibodies were diluted to a ratio of 1:1000.

2.4.8. cAMP and PKA levels

cAMP and PKA levels were measured using ELISA kits (FANKWEL Industrial Co., Ltd., Shanghai, China) following the manufacturer's instructions.

2.4.9. Quantitative real-time PCR (qPCR)

The transcription level of genes related to lipolysis, ER stress and cAMP/PKA pathways was analyzed using qPCR as previously described (Jin et al., 2018). The specific primers used are shown in Supplemental Table S3. The gene expression level was based on the Ct values and was calculated using the 2^{- $\Delta\Delta$ Ct} method (Pfaffl, 2001).

2.5. Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS) software, V. 25.0 (SPSS, Chicago, IL, USA). Except for fatty acid composition, which was presented as mean \pm standard deviation (SD), the rest of the normally distributed data were presented as mean \pm standard error of the mean (SEM). The normality of data distribution was analyzed by the Shapiro–Wilk test and the homogeneity of variances was assessed using Levene's test (Wang et al., 2021). Percentage data were subjected to logarithmic (log 10) transformation before analysis (Wang et al., 2021). Differences among groups were assessed by one-way analysis of variance (ANOVA) and Duncan's test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. DHA activated lipolysis and induced ER stress in adipocytes in vivo

The DHA content in adipose tissue was significantly increased with the increasing of dietary DHA level (Table 1, $P < 0.05$). DHA intake also significantly increased the proportion of n-3 PUFA, PUFA and n-3/n-6 PUFA (Table 1, $P < 0.05$). However, compared with

Table 1

The fatty acid composition of adipose tissue in grass carp fed on DHA for 8 wk (% total fatty acid)¹.

Components	Group		
	Control	0.5% DHA	1% DHA
14:0	2.03 \pm 0.15 ^a	1.84 \pm 0.04 ^b	1.93 \pm 0.09 ^{ab}
16:0	22.02 \pm 0.78 ^a	21.6 \pm 2.17 ^{ab}	19.60 \pm 1.85 ^b
18:0	4.17 \pm 0.14 ^a	4.30 \pm 0.84 ^a	3.37 \pm 0.34 ^b
SFA	28.22 \pm 0.98 ^a	27.74 \pm 3.02 ^{ab}	24.91 \pm 2.03 ^b
16:1n-7	8.55 \pm 0.28	8.10 \pm 0.77	8.65 \pm 1.12
18:1n-9c	43.19 \pm 0.86 ^a	40.82 \pm 2.91 ^{ab}	38.16 \pm 1.76 ^b
MUFA	51.74 \pm 1.11 ^a	48.92 \pm 3.59 ^{ab}	46.81 \pm 1.2 ^b
18:2n-6	10.44 \pm 1.20	10.62 \pm 0.60	10.91 \pm 3.12
18:3n-6	0.34 \pm 0.09	0.33 \pm 0.03	0.28 \pm 0.08
20:4n-6	0.71 \pm 0.05	0.77 \pm 0.07	0.82 \pm 0.10
22:4n-6	0.53 \pm 0.14 ^b	0.92 \pm 0.42 ^a	1.12 \pm 0.16 ^a
n-6 PUFA	12.38 \pm 1.27	12.90 \pm 1.07	13.39 \pm 3.31
18:3n-3	6.28 \pm 0.41 ^c	6.57 \pm 0.40 ^b	7.69 \pm 0.59 ^a
20:5n-3	0.42 \pm 0.03 ^c	0.62 \pm 0.09 ^b	0.92 \pm 0.07 ^a
22:6n-3	0.95 \pm 0.12 ^c	3.24 \pm 0.31 ^b	6.29 \pm 0.51 ^a
n-3 PUFA	7.65 \pm 0.36 ^c	10.44 \pm 0.72 ^b	14.90 \pm 1.01 ^a
PUFA	20.04 \pm 1.22 ^c	23.34 \pm 0.93 ^b	28.28 \pm 2.72 ^a
n-3/n-6 PUFA	0.62 \pm 0.08 ^c	0.81 \pm 0.10 ^b	1.16 \pm 0.26 ^a

DHA = docosahexaenoic acid; MUFA = monounsaturated fatty acid; n-3PUFA = n-3 polyunsaturated fatty acid; n-3PUFA/n-6PUFA = the ratio of n-3 polyunsaturated fatty acids to n-6 polyunsaturated fatty acids; n-6 PUFA = n-6 polyunsaturated fatty acid; PUFA = polyunsaturated fatty acid; SFA = saturated fatty acid.

¹ Values are mean \pm SD, $n = 6$ (2 fish/tank). Labeled means without a common letter differ based on 1-factor ANOVA followed by Duncan's test, $P < 0.05$.

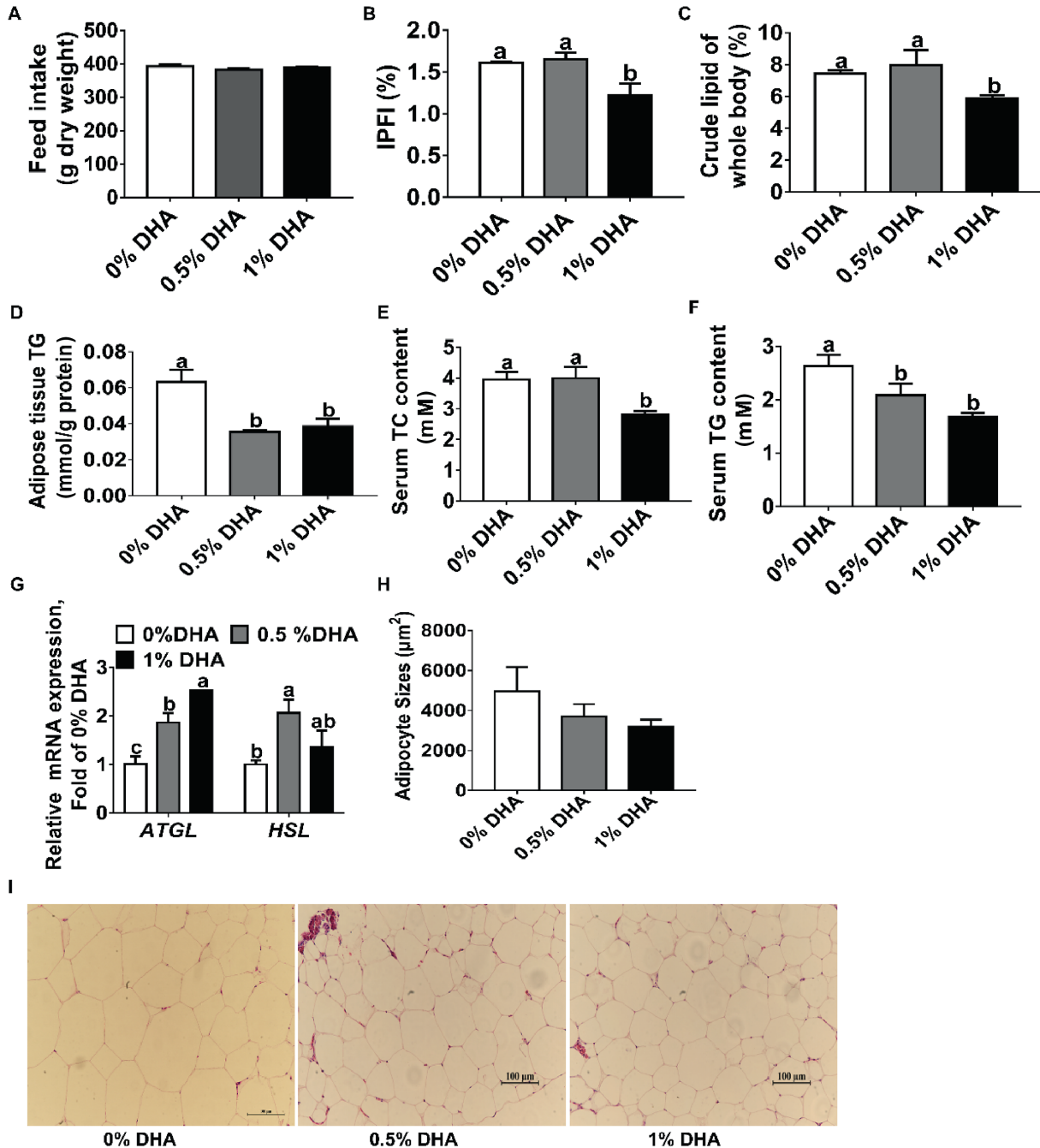


Fig. 1. Docosahexaenoic acid (DHA) ameliorated lipid deposition and activated lipolysis in adipose tissue of grass carp (Experiment 1). (A) Feed intake. (B) Intraperitoneal fat index (IPFI). (C) Crude lipid of whole body. (D) Triglyceride (TG) level of adipose tissue. (E–F) The serum content of total cholesterol (TC) and TG. (G) Relative mRNA expression of lipolysis (triglyceride lipase [*ATGL*] and hormone-sensitive lipase [*HSL*]) in adipose tissue. (H) Adipocyte sizes. (I) Hematoxylin & Eosin (H&E) staining of adipose tissue sections. Data are means \pm SEM, $n = 6$ fish (2 fish/tank). Labeled means without a common letter differ based on 1-factor ANOVA followed by Duncan's test, $P < 0.05$.

controls, saturated fatty acids (SFA) and monounsaturated fatty acid (MUFA) levels were significantly lower in the 1% DHA group (Table 1, $P < 0.05$). Compared with the control group, the content of DHA in adipose tissue increased 7-fold in the 1% DHA group, whereas the content of SFA and MUFA decreased 0.12-fold and 0.10-fold, respectively (Table 1).

The effects of DHA on adipose tissue lipolysis were evaluated in vivo. There was no difference in food intake among the groups (Fig. 1A). However, compared with the control group, 1% DHA in the diet significantly reduced IPFI, whole body crude lipids, adipose tissue TG content and serum TC and TG content (Fig. 1B–F,

$P < 0.05$). In addition, DHA increased the transcription of mRNA for *ATGL* and *HSL* genes (Fig. 1G, $P < 0.05$). Although DHA had no significant effect on the size of adipocytes, it slightly decreased the size of these cells (Fig. 1H and I).

The effects of DHA on ER stress were also investigated in vivo. We found that 1% DHA in the diet significantly increased the expression of ER stress-related genes such as *GRP78* and *CHOP* (Fig. 2A, $P < 0.05$). Further analysis revealed that 1% DHA disrupted the arrangement of the ER and caused the lumen of the ER to swell (Fig. 2B). In addition, 1% DHA increased the expression of PERK, CHOP, GRP78 and EIF2 α proteins (Fig. 2C).

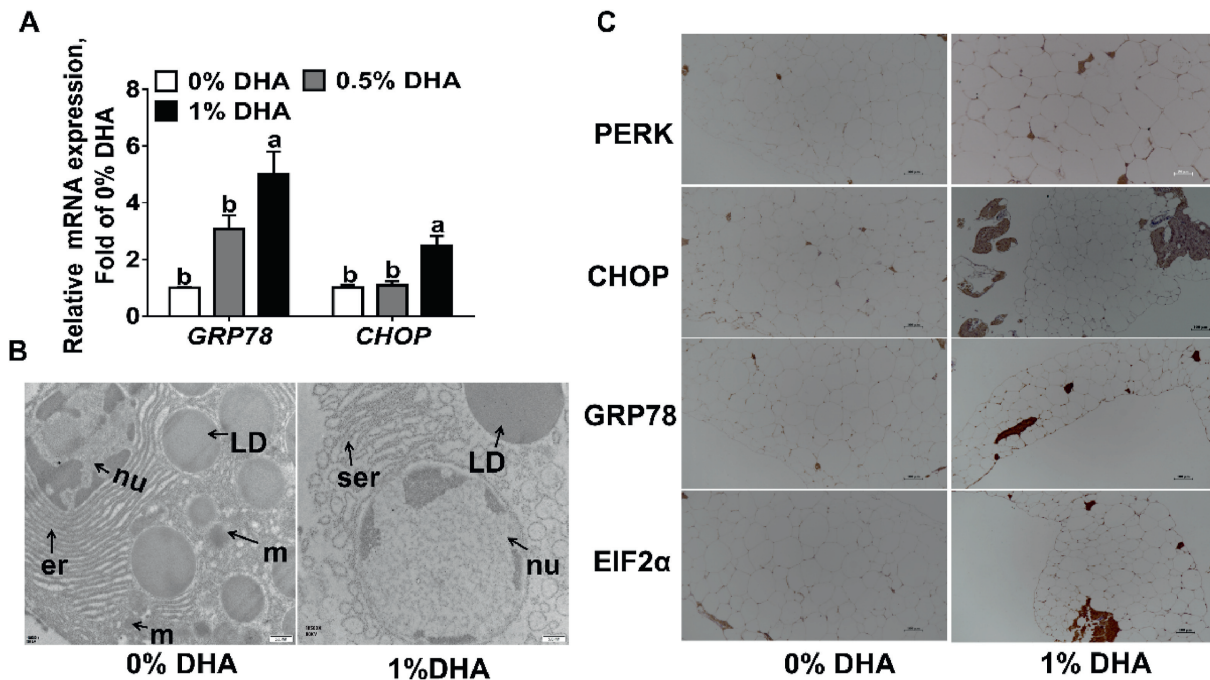


Fig. 2. Docosahexaenoic acid (DHA) induced endoplasmic reticulum (ER) stress in adipose tissue of grass carp (Experiment 1). (A) Relative mRNA expression of ER stress in adipose tissue. (B) Ultrastructure of adipose tissue (transmission electron microscopy [TEM], original magnification 13,000 \times , scale bar = 1 μ m). (C) Immunohistochemical (IHC) staining in adipose tissue (200 \times). Data are means \pm SEM, n = 6 fish (2 fish/tank). Labeled means without a common letter differ based on 1-factor ANOVA followed by Duncan's test, P < 0.05. GRP78 = glucose-regulated protein 78; CHOP = C/EBP homologous protein; LD = lipid droplet; m = mitochondria; nu = adipocyte nucleus; ser = swelling of endoplasmic reticulum; PERK = protein kinase RNA-like ER kinase; EIF2 α = eukaryotic initiation factor 2 α .

3.2. DHA promoted lipolysis and induced ER stress in vitro

The in vitro model revealed that 200 μ M DHA treatment reduced TG accumulation (Fig. 3A and B, P < 0.05) and increased GL release from adipocytes and the transcription of *ATGL* and *HSL* mRNA in a dose-dependent manner (Fig. 3C and D, P < 0.05).

DHA altered the ultrastructure of the ER. As shown in Fig. 3E, the ER in the control group was well arranged, but 200 μ M DHA caused the ER to swell. Also, a similar dose of DHA increased the expression of mRNA ER stress-related genes, including *GRP78*, *CHOP*, *EIF2 α* , *PERK* and *ATF6* (Fig. 3F, P < 0.05).

3.3. ER stress participates in DHA-induced lipolysis in adipocytes

To explore the role of ER stress in DHA-induced lipolysis, ER stress was inhibited using 4-PBA. As shown in Fig. 4A, compared with the control group, the expression of mRNA for genes related to ER stress increased significantly in the 200 μ M DHA group. However, 15 μ M 4-PBA (blocking ER stress) ameliorated the effect of DHA (P < 0.05). The expression of *PERK*, *GRP78* and *CHOP* proteins followed a similar pattern (Fig. 4B). Nile red and DAPI staining and TG content results further showed that inhibiting ER stress by 4-PBA reversed the effect of DHA on modulating the content of TG (Fig. 4C and D, P < 0.05). Additionally, 200 μ M DHA increased GL content and upregulated the expression of mRNA related to lipolysis. However, 4-PBA treatment inhibited the effects of DHA (Fig. 4E and F, P < 0.05).

The effect of the IRE1-XBP1 signaling axis on DHA-induced lipolysis of adipocytes was explored using a specific IRE1 α inhibitor (STF). As shown in Fig. 5A and B, 30 μ M STF treatment significantly reduced the expression of mRNA for the *IRE1 α* and *XBP1s* genes, lipolysis-related genes (*ATGL*, *HSLa* and *HSLb*) and those related to the PKA signaling pathway (*GNAS*, *ADCY5*, *PRKACAB* and *PRKACBa*) induced by 200 μ M DHA (P < 0.05). DHA treatment

decreased the cell TG content, whereas pretreatment with STF significantly reversed this phenomenon (Fig. 5C, P < 0.05). STF markedly diminished the activity of *ATGL* and *HSL* enzymes enhanced by 200 μ M DHA (Fig. 5D, P < 0.05).

3.4. The activation of the cAMP/PKA signaling pathway participates in DHA-induced adipocyte lipolysis in vitro

Grass carp adipocytes were pre-incubated with H89 (PKA pathway inhibitor) for 3 h and then treated with DHA to explore the role of the cAMP/PKA pathway in DHA-induced lipolysis. We examined the mRNA expression levels of the PKA catalytic subunit (*PRKACAA*, *PRKACAB*, *PRKACBA* and *PRKACBB*). As shown in Fig. 6A and 200 μ M DHA dramatically elevated the transcription of mRNA for *PRKACAB* and *PRKACBA* (Fig. 6A, P < 0.05). Nile red and DAPI staining and TG content detection revealed that H89 reversed the DHA-induced reduction of TG level (Fig. 6B and C, P < 0.05). Compared with the 200 μ M DHA group, H89 significantly decreased the GL level, inhibited the expression of genes related to the cAMP/PKA signaling pathway (*PRKACBa*, *GNAS* and *ADCY5*) and lipolysis (*HSL*, *Perilipin 1* and *ATGL*) (Fig. 6D–F, P < 0.05). H89 also inhibited the activity of *ATGL* and *HSL* upregulated by DHA (Fig. 6G, P < 0.05).

The relationship between ER stress and the cAMP/PKA signaling pathway in DHA-induced lipolysis was also investigated. As shown in Fig. 7A–C, compared with the 200 μ M DHA group, 4-PBA pretreatment effectively decreased the expression of genes related to the cAMP/PKA pathway (*PRKACBA*, *GNAS* and *ADCY5*) and reduced the content of cAMP and PKA (P < 0.05).

4. Discussion

Although DHA is known to regulate lipid metabolism (Xu et al., 2021a), the molecular mechanism underlying this process in fish

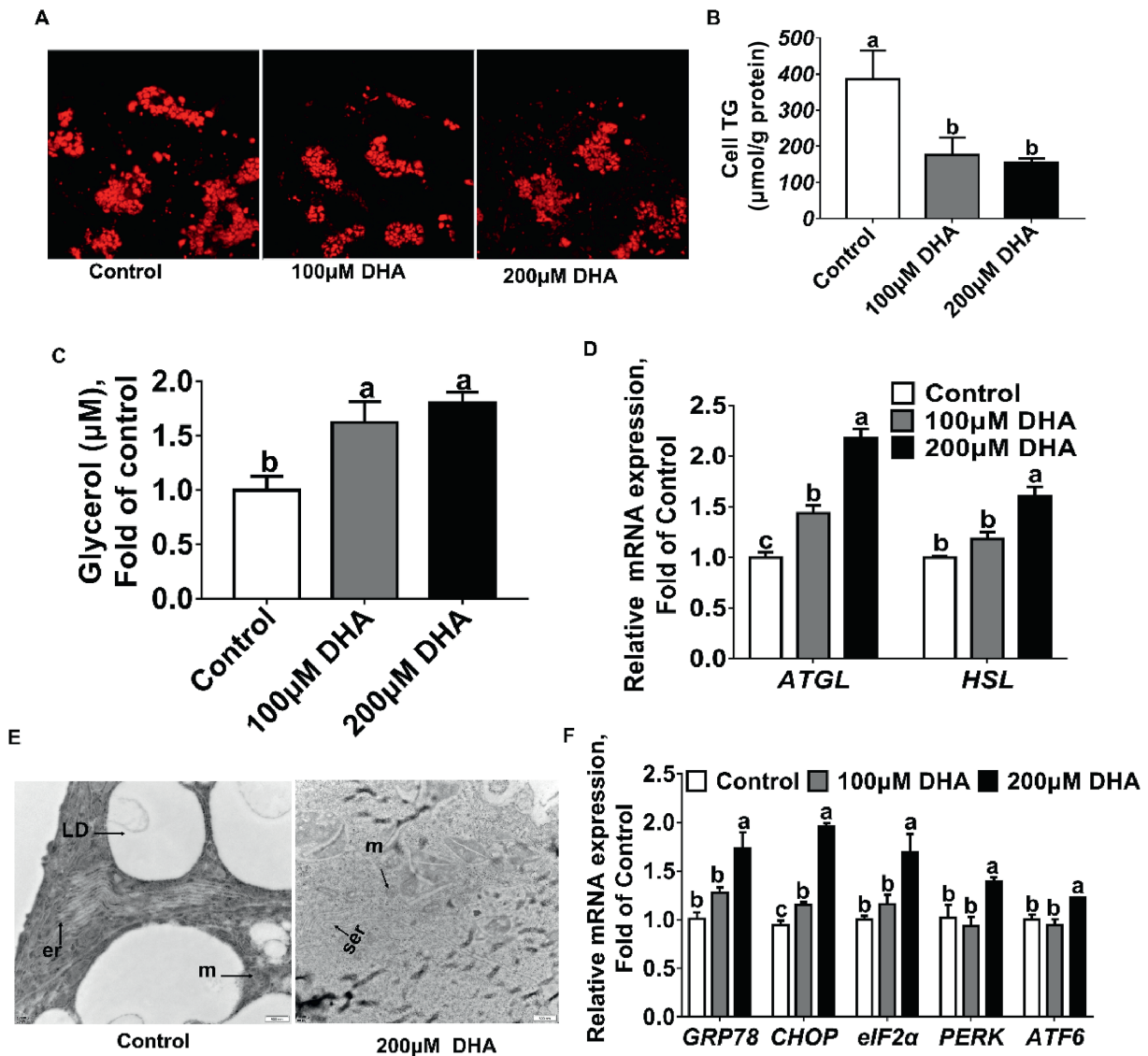


Fig. 3. Docosahexaenoic acid (DHA) ameliorated lipid deposition and activated adipocyte lipolysis and endoplasmic reticulum (ER) stress in vitro (Experiment 2). (A) Neutral lipid Nile red staining. (B) Cell triglyceride (TG) content. (C) The release of glycerol content. (D) Relative mRNA expression of lipolysis. (E) Ultrastructure (transmission electron microscopy [TEM], original magnification 18,500 \times , bar = 500 nm). (F) Relative mRNA expression of ER stress. Data are means \pm SEM, $n = 3$. Labeled means without a common letter differ based on 1-factor ANOVA followed by Duncan's test, $P < 0.05$. *ATGL* = triglyceride lipase; *HSL* = hormone-sensitive lipase; LD = lipid droplet; m = mitochondria; nu = adipocyte nucleus; ser = swelling of endoplasmic reticulum; *GRP78* = glucose-regulated protein 78; *CHOP* = C/EBP homologous protein; *eIF2 α* = eukaryotic initiation factor 2 α ; *PERK* = protein kinase RNA-like ER kinase; *ATF6* = activating transcription factor 6.

adipocytes is poorly understood. In this study, we found that DHA stimulates ER stress and the cAMP/PKA signaling pathway and regulates the transcription and activity of *ATGL* and *HSL* enzymes, promoting lipolysis in adipocytes of grass carp. We found that IRE1 α -XBP1 is a key pathway in DHA-induced lipolysis in the adipocytes of grass carp.

As a type of n-3 LC-PUFA, DHA is associated with health benefits (Buckley and Howe, 2010), such as modulation of lipidosis and prevention metabolic syndrome induced by excessive lipid accumulation (Lombardo et al., 2007). In this study, the SFA and MUFA content in adipose tissue decreased as the level of DHA increased, because DHA was added at the expense of lard oil, and lard oil contains more than 90% SFA and MUFA. These results suggested that the fatty acid composition in adipose tissue is closely related to the experimental feeds. Although the SFA and MUFA in adipose tissue decreased, we believe that several changes in the adipocytes, including activation of lipolysis and a decrease in IPFI, were mainly

mediated by DHA. A possible alternative explanation is that the DHA content in adipose tissue increased 7-fold in the 1% DHA group, but the SFA and MUFA level decreased 0.12- and 0.10-fold, respectively, indicating massive deposition of DHA in adipose tissue.

On the other hand, our previous study revealed that treating mature adipocytes with DHA for 6 h increased the GL level and the expression of *ATGL* and *HSL* (Liu et al., 2014), demonstrating the potential role that DHA plays in lipolysis in fish. Diets with lipid content higher than 6% negatively affect the growth of juvenile grass carp (Du et al., 2005). However, 1% DHA significantly increased the final body weight of grass carp (Huang et al., 2022). Moreover, in this study, 1% DHA reduced body fat accumulation and upregulated the expression of lipolysis-related genes (*ATGL* and *HSL*). These findings suggest that 1% DHA partially ameliorates lipid deposition by promoting lipolysis. Experiments in vitro further revealed that 200 μ M DHA decreased the TG content, elevated the

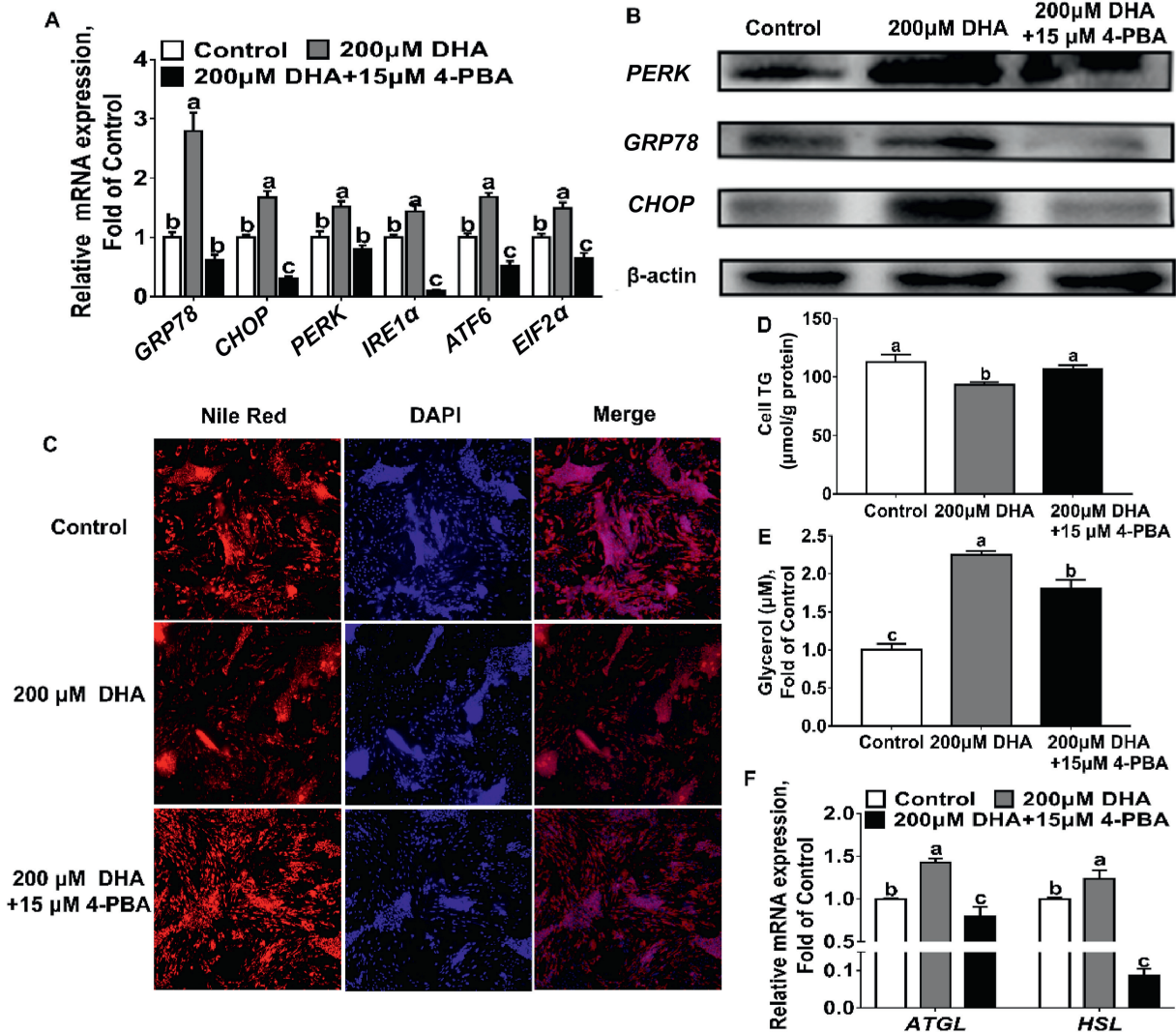


Fig. 4. Endoplasmic reticulum (ER) stress may involve in docosahexaenoic acid (DHA)-induced lipolysis in adipocytes of grass carp (Experiment 3). (A–B) The mRNA and protein expression of ER stress hallmarks. (C) Nile red and 4',6-diamidino-2-phenylindole (DAPI) staining. (D) Cell triglyceride (TG) content. (E) The release of glycerol content. (F) Relative mRNA expression of lipolysis. Data are means ± SEM, n = 3. Labeled means without a common letter differ based on 1-factor ANOVA followed by Duncan's test, P < 0.05. 4-PBA = 4-phenyl butyric acid; *GRP78* = glucose-regulated protein 78; *CHOP* = C/EBP homologous protein; *PERK* = protein kinase RNA-like ER kinase; *IRE1α* = inositol-requiring enzyme α; *ATF6* = activating transcription factor 6; *EIF2α* = eukaryotic initiation factor 2α; β-actin = beta-actin; *ATGL* = triglyceride lipase; *HSL* = hormone-sensitive lipase.

expression of genes related to lipolysis, upregulated the activity of ATGL and HSL enzymes and increased the secretion of GL, which was consistent with previous studies (Sun et al., 2011; Barber et al., 2013). In vivo and in vitro experiments confirmed that DHA reduces lipid accumulation in part by promoting lipolysis. Although several studies have reported the beneficial role of DHA in lowering TG and preventing metabolic syndrome (Rodríguez-Cruz and Serna, 2017; Petsini et al., 2019; Wei et al., 2021), DHA also plays a crucial role in adipogenesis (Fleckenstein-Elsen et al., 2016). Twenty μM DHA increased lipid accumulation and the expression of genes related to CCAAT/enhancer binding proteins alpha (C/EBPα) in differentiating human adipose stem cells (Fleckenstein-Elsen et al., 2016). Even in the same species, DHA increased the expression of ATGL in differentiating adipocytes and decreased ATGL expression in mature adipocytes, indicating that the regulatory effect of DHA on lipid metabolism varies with concentration, cell type and the growth stage of the cell (Wang et al., 2012).

ER stress interacts with a variety of physiological or pathological mechanisms, such as oxidative stress, inflammation, apoptosis, cell death, autophagy, obesity, cancer, etc (Oakes and Papa, 2015;

Fernández et al., 2015; Nakka et al., 2016). The ER is one of the largest membranous organelles in eukaryotic cells, which synthesizes proteins and TG and regulates Ca²⁺ homeostasis (Oakes and Papa, 2015; Schwarz and Blower, 2016). In addition to the accumulation of unfolded or misfolded proteins, excessive or deficient lipid content is also one of the factors that induces ER stress, in which the UPR is triggered to restore ER homeostasis (Fu et al., 2012). Generally, the UPR is activated by three ER stress sensors (*IRE1α*, *PERK* and *ATF6*). Under normal conditions, these sensors interact with the protein chaperone glucose-regulated protein 78 (*GRP78*) to remain inactive. Under ER stress, unfolded/misfolded proteins promote the dissociation between *GRP78* and the ER stress sensors by binding to *GRP78*, activating downstream signaling. Despite the initial reports that the UPR maintains protein homeostasis in the ER, increasing evidence has shown that the UPR regulates the metabolism of lipids (Han and Kaufman, 2016). A previous study revealed that incubating human colon cancer cells with DHA induced ER stress in 3 h (Jakobsen et al., 2008). In the current study, we found that 1% DHA disrupted the arrangement of the ER, caused the lumen of the ER to swell and significantly

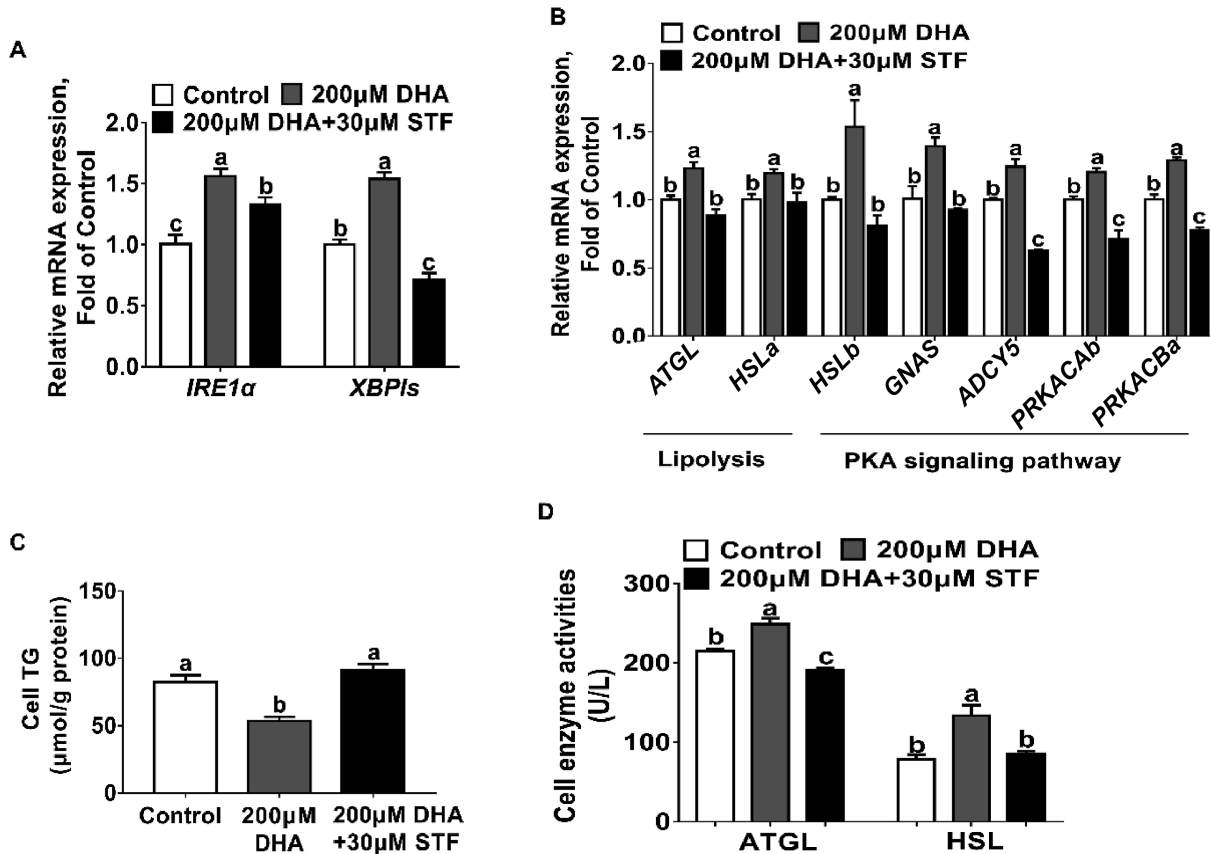


Fig. 5. Effects of inositol-requiring enzyme 1 α (*IRE1 α*)-X-box binding protein (*XBP1*) on docosahexaenoic acid (DHA)-induced adipocyte lipolysis in vitro (Experiment 3). (A–B) The mRNA expression of genes in *IRE1 α* -*XBP1s* signaling axis, lipolysis and cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) (cAMP/PKA) signaling pathway. (C) Triglyceride (TG) content. (D) The enzyme activity of triglyceride lipase (*ATGL*) and hormone-sensitive lipase (*HSL*). Data are means \pm SEM, $n = 4$. Labeled means without a common letter differ based on 1-factor ANOVA followed by Duncan's test, $P < 0.05$. *GNAS* = guanine nucleotide-binding protein G(s) subunit alpha; *ADCY5* = adenylyl cyclase 5; *PRKACAb* and *PRKACBa* = cAMP-dependent protein kinase catalytic subunit; STF = N-[(2-Hydroxy-1-naphthalenyl) methylene]-2-thiophenesulfonamide.

increased the expression of genes and proteins in the UPR signaling pathway. These findings suggest that 1% DHA activates ER stress. The role of ER stress in lipolysis has been increasingly reported (Deng et al., 2012; Wang et al., 2016). In the study, further experiments revealed that inhibiting the ER stress pathway abrogated DHA-induced effects on the expression of mRNA for genes related to lipolysis (*ATGL*, *HSL*, *PKA* and *ADCY5*) and the secretion of GL, indicating that DHA caused lipolysis in adipocytes by inducing ER stress. Similarly, inhibiting ER stress remarkably reduces lipolysis in chronic kidney disease by blocking the binding of *ATGL* to comparative gene identification-58 (*CGI-58*) (Zhu et al., 2014). In our study, we found that inhibiting the ER stress pathway abrogated the effect of DHA, including upregulating the expression of *GRP78*, *CHOP*, *PERK*, *ATF6* and *IRE1*. This is consistent with the inhibition effects of 4-PBA on ER stress induced by Zn in yellow catfish (Song et al., 2016, 2017). These results indicate that ER stress potentially participates in DHA-induced lipolysis in grass carp adipocytes.

Lipolysis is stimulated through multiple mechanisms. ER stress-induced lipolysis is mediated via the activation of the cAMP/PKA signaling pathway (Deng et al., 2012; Song et al., 2017). Similarly, 200 μ M DHA remarkably elevated the expression of the PKA catalytic subunits (*PRKACAb* and *PRKACBa*), genes in the PKA signaling pathway and the content of cAMP and PKA. These results suggest that cAMP/PKA signaling might be activated by DHA. Furthermore, inhibiting the cAMP/PKA signaling pathway reversed the effects of DHA on stimulating lipolysis and reducing

lipid accumulation, implying that the cAMP/PKA signaling pathway may participate in DHA-induced adipocyte lipolysis in grass carp. These findings are consistent with the study on kinsenoside-treated adipocytes, that H89 eliminated PKA-dependent lipid reduction by inhibiting the phosphorylation of *HSL* and perilipin activation (Lee et al., 2019). Further analyses revealed that 4-PBA treatment inhibited DHA-induced ER stress and DHA-induced activation of the cAMP/PKA signaling pathway, suggesting that the activation of ER stress promoted lipolysis might be via the cAMP/PKA signaling pathway. Based on these results, DHA acts as an ER stress agonist to promote adipocyte lipolysis by activating the cAMP/PKA signaling pathway.

Although our study found that lipolysis was accompanied by ER stress after DHA treatment, the relationship between ER stress and lipid metabolism needs further investigation. In primary mouse hepatocytes treated with fructose, the activation of ER stress promoted lipogenesis and caused hepatic steatosis (Zheng et al., 2016), consistent with a separate study of human hepatocytes in which palmitate increased the expression of genes and proteins related to lipogenesis by stimulating ER stress (Cha et al., 2020). Conversely, a study in yellow catfish revealed that Zn promoted lipolysis in the hepatocytes by activating the ER stress response (Song et al., 2017). Some related studies in adipocytes suggested that the activation of ER stress promoted lipolysis (Deng et al., 2012; Zhu et al., 2014; Wang et al., 2016). These inconsistent findings imply that ER stress-mediated regulation of lipid metabolism is a complex process that requires further investigation.

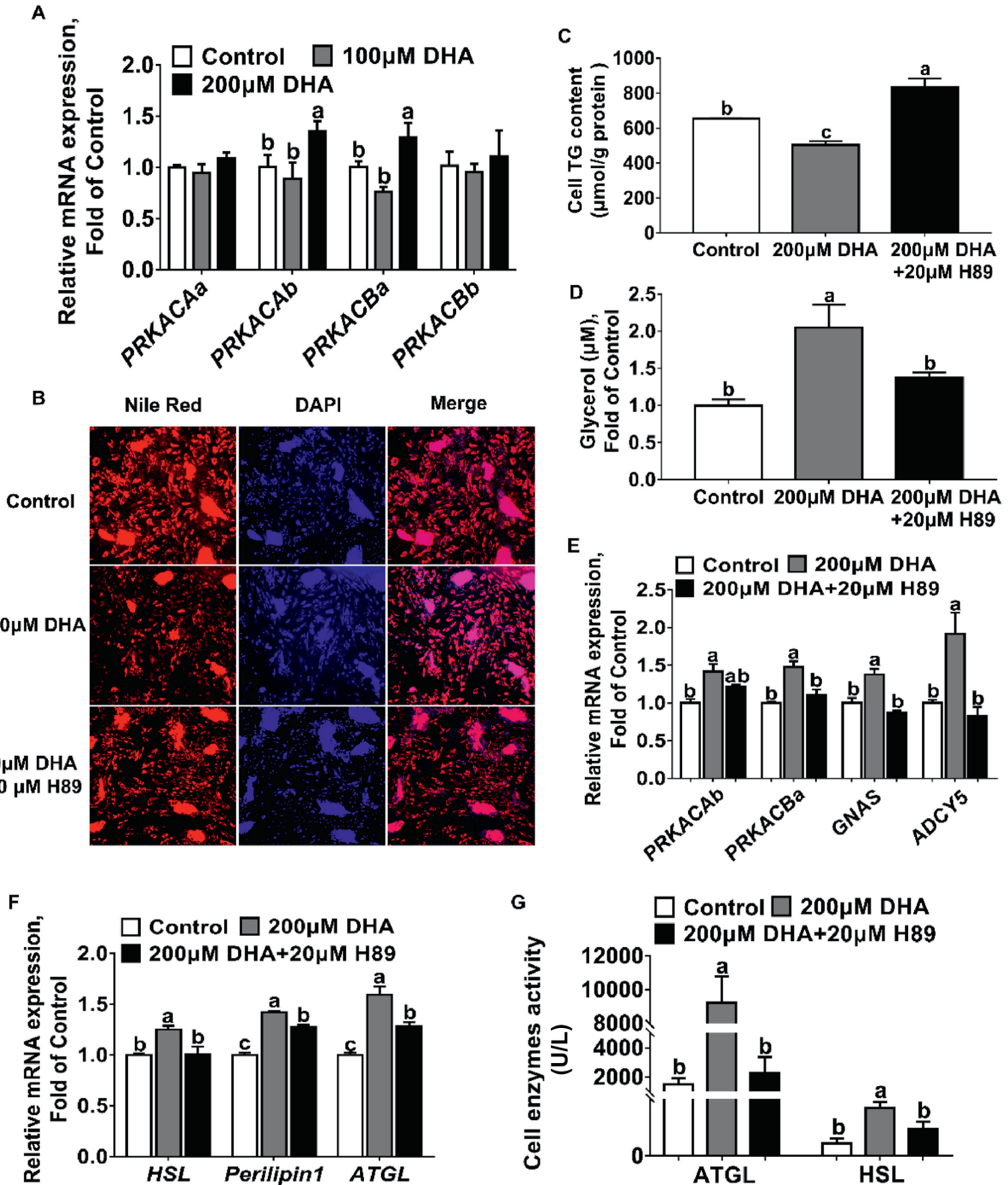


Fig. 6. The activation of the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) (cAMP/PKA) signaling pathway may be involved in docosahexaenoic acid (DHA)-induced lipolysis in adipocytes of grass carp (Experiment 4). (A) The mRNA expression of PKA catalytic subunits. (B) Cell Nile red and 4',6-diamidino-2-phenylindole (DAPI) staining. (C) Cell triglyceride (TG) content. (D) The release of glycerol content. (E-F) The mRNA expression of the cAMP/PKA signaling pathway and lipolysis. (G) The enzyme activity of triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). Data are means ± SEM, n = 3. Labeled means without a common letter differ based on 1-factor ANOVA followed by Duncan's test, P < 0.05. PRKACAa, PRKACAb, PRKACBa and PRKACBb = cAMP-dependent protein kinase catalytic subunit; GNAS = guanine nucleotide-binding protein G(s) subunit alpha; ADCY5 = adenyl cyclase 5. H89 is a PKA inhibitor.

Although 200 µM DHA upregulated 2 catalytic subunits of PKA (PRKACAB and PRKACBA), inhibiting ER stress or cAMP/PKA signaling only inhibited the expression of PRKACBA. Similar to our previous study (Ji et al., 2022), inhibiting PKA only dysregulated PRKACBA, implying that PRKACBA might play a major role in ER stress-mediated lipolysis. To the best of our knowledge, there are limited studies on the effect of PRKACBA in ER stress-mediated

lipolysis. Thus, it is necessary to explore further the role of PKA in ER stress-induced lipolysis.

In this study, we emphasized the importance of the IRE1α-XBP1 axis in DHA-induced adipocyte lipolysis in grass carp. IRE1α, one of the most conservative branches of the UPR, can sense fluctuations in ER lipid homeostasis (Smith and Wilkinson, 2017; Ho et al., 2020). IRE1α-XBP1 plays a pivotal role in lipid metabolism and regulates

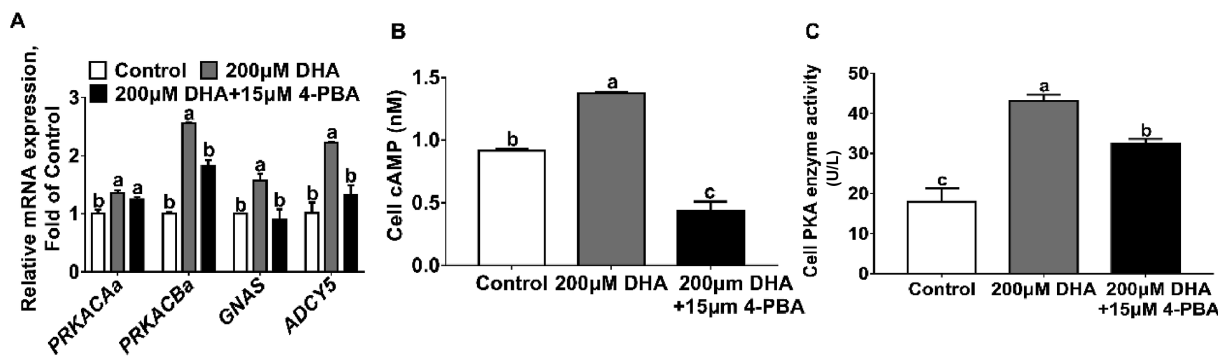


Fig. 7. Endoplasmic reticulum (ER) stress-stimulated lipolysis may be via activation of the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) (cAMP/PKA) signaling pathway in adipocytes of grass carp (Experiment 4). (A) The mRNA expression of cAMP/PKA hallmarks. (B) The level of cAMP. (C) The enzyme activity of PKA. Data are means \pm SEM, $n = 3$. Labeled means without a common letter differ based on 1-factor ANOVA followed by Duncan's test, $P < 0.05$. 4-PBA = 4-phenyl butyric acid; PRKACA and PRKACBa = cAMP-dependent protein kinase catalytic subunit; GNAS = guanine nucleotide-binding protein (G) subunit alpha; ADCY5 = adenylyl cyclase 5.

lipogenesis (Han and Kaufman, 2016). Reports on the effect of IRE1 α -XBP1 on lipolysis, especially in fish, remain scant. Our data showed that inhibiting IRE1 α not only significantly weakened the effects of DHA on increasing the transcription of lipolysis and PKA pathway-related genes but also reduced TG content. A related study revealed that scutellarin ameliorates hepatic lipid accumulation by suppressing the IRE1 α /XBP1 pathway (Zhang et al., 2022). However, inhibiting IRE1 α had no effect on the Zn-mediated increase in HSL activity and a reduction in the TG content (Song et al., 2017). Precise mechanisms underlying ER stress-mediated lipolysis, especially the similarities and differences among the three branches of the UPR in lipolysis, need further investigation.

In conclusion, our study demonstrated that DHA promotes lipolysis in grass carp adipocytes by activating ER stress and modulating lipid accumulation. We found that ER stress mediated DHA-induced lipolysis through the cAMP/PKA signaling pathway. The findings of this study provide new insights into the beneficial role of DHA in fish and underline the significance of ER stress and the cAMP/PKA pathway in DHA-induced lipolysis. Collectively, the findings of this study enrich our understanding of how DHA regulates lipid metabolism in vertebrates.

Author contributions

Xiaocheng Huang: Conceptualization, methodology, data curation, writing – original draft and writing – review & editing. **Chenchen Bian:** Methodology, data curation, writing – review & editing and funding acquisition. **Shanghong Ji and Jian Sun:** Investigation and data curation. **Hong Ji:** Conceptualization, methodology, project administration, supervision, funding acquisition and writing – review & editing. All authors: contributed to interpreting the results, and read and approved the final manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aninu.2022.10.010>.

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