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ORIGINAL ARTICLE

# Dietary Arachidonic Acid Has a Time-Dependent Differential Impact on Adipogenesis Modulated via COX and LOX Pathways in Grass Carp *Ctenopharyngodon idellus*

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**Abstract** In this study, we explored the function of arachidonic acid (ARA) in adipogenesis in the grass carp (*Ctenopharyngodon idellus*) using *in vivo* and *in vitro* models. An 8-week feeding trial was performed using three isonitrogenous and isoenergetic purified diets: ARA-free, ARA, and ARA + acetylsalicylic acid [ASA, a cyclooxygenase (COX) inhibitor]. Fish were sampled after 4 and 8 weeks of feeding. Results showed that ARA-fed fish had a significantly lower intraperitoneal fat index (IPFI) and smaller adipocytes; these decreases were reversed by ASA after 8 weeks of feeding. Nevertheless, at week 4, the IPFI and adipocyte size were higher in the ARA group, and they were comparable to those of fish fed ARA + ASA. To further investigate the influence of ARA on adipocyte differentiation, confluent pre-adipocytes of grass carp were incubated with ARA for 3 days. This *in vitro* experiment demonstrated that ARA promoted adipogenesis in a dose-dependent manner. Pre-treatment with the lipoxygenase (LOX) inhibitor nordihydroguaiaretic acid attenuated the pro-adipogenic function of ARA. However, after treatment with ARA for 8 days, adipocytes had a lower lipid content than cells treated with oleic acid, and ASA could suppress this effect. We thus revealed the dual function of ARA in adipogenesis in grass carp. The LOX pathway may play a key role in pro-adipogenesis after short-term treatment with ARA, whereas the COX pathway is possibly responsible for the inhibition of adipogenesis after long-term treatment.

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**Keywords** Arachidonic acid · Adipocyte · *Ctenopharyngodon idellus* · Eicosanoids · Lipolysis · Cell culture

**Abbreviations**

| | |
|---|---|
| ACC | Acetyl-coA carboxylase |
| ALA | Alpha-linolenic acid |
| ARA | Arachidonic acid |
| ASA | Acetylsalicylic acid |
| ATGL | Adipose triglyceride lipase |
| C/EBP | CCAAT enhancer binding protein |
| COX | Cyclooxygenase |
| CPT | Carnitine palmitoyltransferase |
| DHA | Docosahexaenoic acid |
| EET | Epoxyeicosatetraenoic acid |
| EPA | Eicosapentaenoic acid |
| FAME | Fatty acid methyl esters |
| FAS | Fatty acid synthase |
| HETE | Hydroxyeicosatetraenoic acid |
| IPF | Intraperitoneal fat |
| LNA | Linoleic acid |
| LOX | Lipoxygenase |
| LPL | Lipoprotein lipase |
| NDGA | Nordihydroguaiaretic acid |
| OLA | Oleic acid |
| PBS | Phosphate-buffered saline |
| PG | Prostaglandin |
| PMA | Palmitic acid |
| PPAR | Peroxisome proliferator-activated receptor |
| PPOH | 6-(2-Propargyloxyphenyl) hexanoic acid |
| PUFA | Polyunsaturated fatty acid |
| SCD | Stearoyl-coA desaturase |
| SREBP | Sterol regulatory element-binding protein |
| TAG | Triacylglycerol |

## Introduction

Adipose tissue is the major repository of excess energy stored in the form of triacylglycerol (TAG) under conditions of nutritional excess [1]. During energy deficiency, adipocyte lipolysis involving the hydrolysis of TAG releases free fatty acids to different tissues for energy production [2]. Adipose tissue is also a well-characterized endocrine organ that secretes hormones and regulates lipid homeostasis throughout the body [3]. Adipogenesis, the process of adipocyte differentiation from pre-adipocytes to mature adipocytes, is regulated by a transcription factor network consisting of peroxisome proliferator-activated receptor (PPAR) γ, CCAAT enhancer binding protein (C/EBP) α, and sterol regulatory element-binding protein (SREBP) 1c [1]. These transcription factors regulate a series of lipid biosynthesis and metabolism-related genes, such as lipoprotein lipase (LPL), acetyl-CoA carboxylase (ACC) 1, and fatty acid synthase (FAS) [1].

Arachidonic acid (ARA), the main n-6 long-chain polyunsaturated fatty acid (LC-PUFA), plays important roles in modulating cell membrane composition, signaling pathways, and gene expression [4, 5]. Moreover, ARA serves as a precursor of one of the most important groups of bioactive lipid mediators, the eicosanoids [6]. There are three major enzymatic routes for the synthesis of eicosanoids: the cyclooxygenase (COX) pathways that lead to the formation of prostaglandins (PGs) and thromboxane; the lipoxygenase (LOX) pathways that result in the formation of leukotrienes (LTs) and hydroxyeicosatetraenoic acid (HETE) with different regioisomers; and the cytochrome P (CYP) 450 pathways, which generate HETE and epoxyeicosatetraenoic acid (EET) [6–8]. These eicosanoids are involved in numerous and complex homeostatic and physiological processes in an autocrine and paracrine fashion. In mammals, eicosanoids have various effects on adipogenesis. In the COX pathway, $PGE_2$ and $PGF_{2\alpha}$ inhibit, whereas $PGI_2$ and 15-deoxy-$\Delta^{12,14}$ $PGJ_2$ promote adipocyte differentiation [9–12]. In the LOX pathway, $LTB_4$ was shown to inhibit, whereas hepoxilins were shown to promote adipocyte differentiation [13–15]. Additionally, a metabolite of ARA in the CYP450 pathway, EET, has been found to inhibit lipid accumulation in mice [16]. However, studies of this type have not yet been conducted on fish.

The grass carp, a typical herbivorous freshwater fish species that is widely cultured for food in China, readily accumulates excess lipids in the abdominal cavity during aquaculture [17]. In a previous study, we found that moderate dietary ARA levels (0.30 %) suppressed intraperitoneal fat (IPF) accumulation in grass carp, and some adipogenic genes, such as those for PPARγ, FAS, fatty acid desaturase, and fatty acid elongase, showed lower expression levels compared to fish fed a control diet [18]. However, the mechanism by which ARA or its metabolites function in lipid regulation has not been investigated. Because most PGs have been shown to have an anti-adipogenesis function in mammals [19], we hypothesized that ARA inhibits lipid accumulation through the COX pathway. However, our feeding results demonstrated a more complex role for ARA. Thus, in the present study, we employed both *in vivo* and *in vitro* methods with a variety of inhibitors in order to identify the regulatory role of ARA in grass carp adipogenesis.

## Materials and Methods

### Experimental Diets

Three isonitrogenous and isoenergetic semi-purified diets containing 36.0 % crude protein and 6.0 % crude lipid were formulated, based on the method of Lovell (1989) with some modifications [20]. Soybean oil (Kerry Oils & Grains Co., Shenzhen, China) and linseed oil (Hoval Seasons Bio-Sci Co., Changchun, China) were added to satisfy the essential fatty acid requirements [1.0 % linoleic acid (LNA) and 1.0 % alpha-linolenic acid (ALA)] of grass carp [21] (Table 1). The control diet was ARA-free, whereas the other two treatments contained ARA-enriched oil (ARA content, 40.8 % of total fatty acid, in the form of ARA-methylester; Hubei Fuxing Biotechnology Co., Ltd., China) instead of lard oil (purchased at Kangle market, Yangling, China) to meet 0.30 % ARA content (dry weight). The second diet was not supplemented with acetylsalicylic acid (ASA, defined as the ARA group), and the third diet was supplemented with 0.1 % ASA (Sigma-Aldrich, USA; defined as the ARA + ASA group). The concentration of ASA was based on that used in a previous study on tilapia with some modifications [22]. Butylated hydroxytoluene (0.1 %; Sigma-Aldrich, USA) was added as an antioxidant (Table 1). The fatty acid compositions of the experimental diets were determined by gas chromatography and are shown in Table 2. On the basis of the fatty acid composition and levels of lipid, the corresponding levels of dietary ARA were 0.00 % (the control diet), 0.30 % (the ARA diet), and 0.31 % (the ARA + ASA diet), respectively. All of the powdered ingredients were mixed thoroughly by hand, and then the appropriate oil mixture was added and mixed until a homogeneous mixture was obtained. Approximately 70 % water was added to prepare a dough, and the dough was extruded into noodle-like pellets with a diameter of 2 mm. The pellets were dried under forced air at room temperature for 24 h and then stored at −20 °C until use.

**Table 1** Formulation and chemical composition of the experimental diets (g/kg dry matter)

| Components | Groups | | |
|---|---|---|---|
| | Control | ARA | ARA + ASA |
| Casein | 320 | 320 | 320 |
| Gelatin | 80 | 80 | 80 |
| Dextrin | 280 | 280 | 280 |
| Cellulose | 189 | 189 | 188 |
| Soybean oil | 11 | 11 | 11 |
| Linseed oil | 19 | 19 | 19 |
| ARA-enriched oil | 0 | 7.5 | 7.5 |
| Lard oil | 30 | 22.5 | 22.5 |
| Carboxymethylcellulose | 20 | 20 | 20 |
| Mineral mix[a] | 40 | 40 | 40 |
| Vitamin mix[b] | 10 | 10 | 10 |
| BHT | 1 | 1 | 1 |
| ASA | | | 1 |
| Total | 1000 | 1000 | 1000 |
| Chemical composition | | | |
| Moisture (%) | 9.82 | 10.16 | 10.25 |
| Crude protein (%, $N\% \times 6.25$) | 35.19 | 35.46 | 35.25 |
| Crude fat (%) | 5.96 | 5.97 | 6.03 |
| Ash (%) | 5.48 | 5.37 | 5.60 |

[a] The mineral mix contained (g/100 g of the total mineral): $KAl(SO_4)$ 0.159, $CaCO_3$ 18.101, $Ca(H_2PO_4)_2$ 44.601, CoCl 0.070, $MgSO_4$ 5.216, $MnSO_4 \cdot H_2O$ 0.070, KCl 16.553, KI 0.014, $ZnCO_3$ 0.192, $NaH_2PO_4$ 13.605, $Na_2SeO_3$ 0.006,$CuSO4 \cdot 5H_2O$ 0.075, and ferric citrate 1.338

[b] The vitamin mix contained (mg/1000 g of diet): vitamin C, 200; thiamine, 10; riboflavin, 20; vitamin A, 3000 IU; vitamin E, 50 IU; vitamin D3, 1500 IU; menadione, 10; pyridoxine HCl, 10; cyanocobalamin, 0.02; biotin, 1.0; calcium pantothenate, 40; folic acid, 5; niacin, 20; inositol, 400; choline chloride, 2000; cellulose was used as a carrier

**Table 2** Fatty acid composition of the experimental diets (% total fatty acids)

| Fatty acids | Groups | | |
|---|---|---|---|
| | Control | ARA | ARA + ASA |
| 14:0 | 1.37 | 1.35 | 1.31 |
| 16:0 | 18.61 | 16.73 | 16.70 |
| 18:0 | 12.33 | 10.79 | 10.82 |
| SFA | 32.31 | 28.87 | 28.84 |
| 16:1n-7 | 0.85 | 0.73 | 0.74 |
| 18:1n-9 | 30.31 | 26.65 | 26.28 |
| 18:1n-7 | 1.38 | 1.23 | 1.22 |
| 20:1n-9 | 0.26 | 0.29 | 0.33 |
| MUFA | 32.79 | 28.89 | 28.57 |
| 18:2n-6 | 17.50 | 17.30 | 17.34 |
| 18:3n-6 | 0.18 | 0.51 | 0.48 |
| 20:4n-6 | 0.00 | 5.19 | 5.36 |
| 22:4n-6 | 0.00 | 1.55 | 1.77 |
| n-6 PUFA | 17.68 | 24.55 | 24.95 |
| 18:3n-3 | 17.21 | 17.69 | 17.64 |
| 20:5n-3 | 0.00 | 0.00 | 0.00 |
| 22:6n-3 | 0.00 | 0.00 | 0.00 |
| n-3 PUFA | 17.21 | 17.69 | 17.64 |
| PUFA | 34.89 | 42.24 | 42.59 |
| n-3/n-6 PUFA | 0.97 | 0.72 | 0.71 |

*SFA* saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid

## Experimental Feeding Procedure

Juvenile grass carp were obtained from the Ankang Fisheries Experimental and Demonstration Station of the Northwest A&F University (Ankang, China). To acclimatize the fish to the experimental conditions, they were reared in aquaria and fed a commercial diet (Huaqin 601; protein 36.54 % and fat 4.99 % dry weight; soybean oil as a lipid source) for 2 weeks.

Before the feeding experiment, fish were fasted for 24 h. A total of 114 fish (27.65 ± 3.05 g body weight) were randomly divided into 6 aquaria (0.73 × 0.46 × 0.60 m). Each diet was then randomly assigned to two aquaria. Fish were hand-fed to apparent satiation twice daily (at 09:00 and 16:00) for 8 weeks. Satiation feeding was achieved by allowing fish to eat until feeding activity stopped, with no feed remaining in the tank. The feed intake was recorded. During the feeding experiment, water was renewed to maintain acceptable water quality. Water temperature was controlled at 28 °C. The dissolved oxygen content of the aquarium water was maintained at approximate saturation through continuous aeration. The photoperiod was approximately 12-h light/12-h dark (from 08:00 to 20:00) [17].

The fish were not sampled until they had been fasted for 24 h. Two different time points were selected for sampling (4 and 8 weeks). Fish were sampled after being anesthetized with tricaine methanesulfonate (MS222) (0.06 mg/l). At week 4, all of the fish were weighed, and then nine fish/aquarium were randomly selected and sampled. The remaining ten fish/aquarium continued to be used in the feeding experiment until week 8. After sampling, fish were killed and dissected after weighing the body weight and measuring the body length. The IPF was then stripped and weighed. Thereafter, these IPF samples from three or four fish/aquarium were stored at −20 °C for fatty acid composition analyses. IPF samples from a further four fish/aquarium were frozen in liquid nitrogen and then stored at −80 °C for eicosanoid and gene expression analyses. IPF samples from the remaining dissected fish (two fish/aquarium) were fixed in paraformaldehyde solution for histological analysis. All experimental animal procedures were approved by the

institutional animal care and use committee and performed in accordance with national and institutional regulations on the care and use of experimental animals.

Specific growth rate (SGR), feed conversion ratio (FCR), survival rate (SR), and IPF index (IPFI) were calculated using the following formulae:

$$\text{Specific growth rate (SGR)} = (\text{Ln final weight} - \text{Ln initial weight}) \times 100/28 \text{ or } 56 \text{ days},$$

$$\text{Feed conversion ratio (FCR)} = \text{amount of feed given/weight gain (g)},$$

$$\text{Survival rate (SR \%)} = \text{final number of fish} \times 100/\text{initial number of fish},$$

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

### Proximate Composition Analysis

The proximate composition of the diets was determined in accordance with the Association of Official Analytical Chemists (AOAC) Procedures (1995) [23]. Briefly, samples were dried at 105 °C to a constant weight to determine moisture. Crude protein was determined by measuring the nitrogen ($N \times 6.25$) in the samples using the Kjeldahl method. Crude lipid was measured by ether extraction using the Soxhlet method. Crude ash was determined by combustion at 550 °C in a muffle furnace.

### Fatty Acid Composition Analysis

Samples of the diets and IPF were smashed or homogenized before the fatty acid analyses. Lipid extraction was conducted based on the Folch method [24]. The preparation of fatty acid methyl esters (FAMEs) was performed based on a previously described method [18, 25]. FAMEs were determined using an Agilent 7820a Series gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector and capillary column (HP-88, length 100 m, internal diameter 0.25 mm, film thickness 0.20 μm, USA). Individual methyl esters were identified through comparison with known standards (47015-U, Sigma-Aldrich, Inc., St. Louis, MO, USA). The results were expressed as the percentage of each FA in the detected total fatty acids.

### Eicosanoid Measurements

The IPFs were homogenized for determination of the eicosanoid contents among treatments. $PGE_2$, which is a main metabolite of ARA in the COX pathway, was measured using an enzyme-linked immunosorbent assay kit for $PGE_2$ (Cloud-Clone Corp, Houston, TX, USA). 5-HETE, which is a main metabolite of ARA in the LOX pathway, was measured using an enzyme-linked immunosorbent assay kit for 5-HETE (Cusabio Biotech Co., Ltd., Wuhan, People's Republic of China). These two eicosanoids were measured based on the kit manufacturer protocols. All assays were performed in sextuplicate. The results were expressed as picograms eicosanoid per milligram tissue.

### Histological Examinations

The samples of fixed IPF (two individuals/aquarium) were washed in tap water for 12 h, followed by a routine dehydration in a graded series of ethanol (30, 50, 70, 80, 90, 95, and 100 % twice). The samples were then equilibrated in xylene and embedded in paraffin based on standard histological techniques, as described previously [26]. Thereafter, sections were cut at 5 μm using a rotary microtome (RM2235, Leica, Germany) and mounted on glass slides, which were then stained with hematoxylin and eosin (H&E). Histological samples were observed using an upright fluorescence microscope (Leica Biosystems, Germany). The average adipocyte size per image was quantified using Photoshop, as previously described [27]. An average value across five non-overlapping images (five/section × four fish) was calculated for each group.

### Real-Time Quantitative RT-PCR

IPF from four fish per aquarium were used for the detection of gene expression. RNA extraction, cDNA synthesis, and gene expression measurements were performed as described previously [18]. The primer sequences for β-actin, PPARγ, C/EBPα, LPL, SREBP-1c, FAS, stearoyl-CoA desaturase (SCD), PPARα, adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and carnitine palmitoyltransferase (CPT) 1 are listed in Table 3. After the PCR reaction, melting curves were analyzed to confirm that single products were obtained in these reactions. A relative quantification method, the comparative CT method ($2^{-\Delta\Delta Ct}$), was used to calculate the gene expression values, as described in the literature [28, 29].

### Pre-adipocyte Cell Culture

Pre-adipocytes of grass carp were prepared by enzymatic digestion as described previously [30]. Briefly, grass carp (1–2 kg; four fish per experiment) were anesthetized with MS-222 (0.1 g/l) before extraction of adipose tissue. Subsequently, the fish were knocked dizzy by a crabstick, and the arch bows of the gills were cut and bled out. In a bacteria-free environment, adipose tissue in the abdominal cavity was carefully isolated (~30 g/fish). Collected adipose tissues were washed three times with PBS (pH 7.4) in a laminar

**Table 3** Primers used in real-time quantitative PCR

| Target gene | Accession number | Forward (5′–3′) | Reverse (5′–3′) |
|---|---|---|---|
| Peroxisome proliferator activated receptor γ (PPARγ) | EU847421 | GCATCTGTACGAGTCCTATCT | GAGACTTCATGTCGTGGATAAC |
| CCAAT enhancer binding protein α (C/EBPα) | KR071139 | ACCCACATACCACCACTCTCAACA | TTTCCCTCGATCGCCCATCTTCAT |
| Lipoprotein lipase (LPL) | FJ716100 | TACAGCGGCGTTCACACTTG | CTACATGAGCACCAAGACTG |
| Sterol regulatory element-binding protein-1c (SREBP-1c) | GU339498 | TCACTCCAAATGGTGGTCGTCAGT | AAAGTGTTCAGACAGGCTTTGGGC |
| Fatty acid synthase (FAS) | GQ466046 | CCTCAGCTTACAGCAGAATC | CTCTTCAGCAAGGGAGTTTAG |
| Stearoyl-CoA desaturase (SCD) | AJ243835 | GCCTTCCAGAATGACATCTAC | GCCGATGTGAGCAAAGAA |
| Peroxisome proliferator activated receptor α (PPARα) | FJ623265 | CGCTGAGGTTCGGATATTT | ACGTCACCTGGTCATTTAAG |
| Adipose triglyceride lipase (ATGL) | HQ845211 | TCGTGCAAGCGTGTATATG | GCTCGTACTGAGGCAAATTA |
| Hormone-sensitive lipase (HSL) | HQ446238 | TGGAACGTTACTGAGTCTGG | AAGCGCACGTTGACTGG |
| Carnitine palmitoyltransferase 1 (CPT-1) | JF728839 | GCATCCATGACACGTTTATTC | GAAGTTTCTCTTCTCTCGTCTC |
| β-Actin | DO211096 | TCCACCTTCCAGCAGATGTGGATT | AGTTTGAGTCGGCGTGAAGTGGTA |

flow and then minced and digested in 0.1 % Type I collagenase (Sigma-Aldrich, USA) containing 2 % bovine serum albumin (BSA, Sigma-Aldrich, USA) at room temperature for 1 h. The cell suspension was filtered through a 200-μm nylon net to remove the large particulate mass. The filterable cell suspension was centrifuged at 2000×*g* for 10 min. After removing the supernatant, the cell pellet was resuspended in erythrocyte lysing buffer to lyse the red cells and then centrifuged for 10 min at 2000×*g*. Subsequently, the cells were washed twice and were then resuspended in growth medium [GM, containing Dulbecco's modified Eagle's medium (DMEM), 6.7 % fetal calf serum (FBS), 3.3 % grass carp serum, 100 U/ml penicillin, and 100 U/ml streptomycin] and plated in 1 % gelatin precoated plates or flasks at a density of approximately 10 g tissue/25 $cm^2$. Pre-adipocytes were incubated at 28 °C in 5.0 % $CO_2$ atmosphere. Confluent pre-adipocytes (day 0) were treated with a differentiation cocktail (MDI) of 100 μM lipid mix [50 % oleic acid (OLA), 25 % LNA, 25 % ALA], 10 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine (IBMX) in the presence of fatty acids (100, 200, 300, 400, or 500 μM) or BSA. Prior to treatment, the cytotoxicity of fatty acids was assessed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. For the inhibitor experiments, confluent cells were pre-incubated with the respective inhibitors for 2 h followed by ARA or BSA treatments. The medium was changed every 2 days.

### Oil Red O Staining

Oil red O staining was performed based on the standard method, as previously described [31]. Cell morphology was observed using an inverted microscope (Olympus BX41, Japan) and photographed with a digital camera (Nikon DSRi1, Japan). Thereafter, the dye was extracted with isopropanol. The optical density (OD) was estimated at an absorbance of 490 nm using a microtiter plate spectrophotometer (Multiskan MK3, Thermo Labsystems, Philadelphia, PA, USA). The cellular lipid content was calculated from the OD value.

### Fatty Acid Composition Analysis (*In Vitro*)

Adipocytes were harvested after incubation with BSA or ARA for 3 days. Cells were digested and washed two times with PBS, followed by the addition of chloroform/methanol (2:1, v/v) for 0.5 h. The suspension was collected and lipids were extracted based on the Folch method [24]. The preparation and analysis of FAME were performed using the aforementioned method.

### Real-Time Quantitative RT-PCR (*In Vitro*)

Cells were harvested after incubation with BSA or 300 μM ARA for 0.5, 6, 12, 24, or 48 h. RNA extraction, cDNA synthesis, and gene expression measurements were performed as described previously [30]. The primer sequences for β-actin, as well as those for the adipocyte differentiation marker genes PPARγ, C/EPBα, LPL, and FAS, are listed in Table 3. PCR reactions and calculation of relative expression values were performed as described above.

### Statistical Analysis

All data are expressed as the mean ± SD (standard deviation). Percentage data were arcsine-transformed prior to

analysis. One-way analysis of variance was used to compare differences between the experimental treatments, followed by Duncan's *post hoc* test. Differences among the OD value, fatty acid, and gene expression data for BSA-treated and ARA-treated cells were determined by using an independent-samples *t* test. All analyses were conducted using PASW Statistics 18 (SPSS, Chicago, IL, USA). Statistical significances are denoted with asterisks as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

## Results

### *In Vivo*

#### *Growth Performance, Feed Utilization, and SR*

Grass carp were fed an ARA diet with or without ASA for 4 or 8 weeks. No significant differences in final weight, body length, or survival rate were observed among the treatments. Interestingly, the ARA + ASA group exhibited numerically higher FCR than the other groups (Table 4).

#### *Fatty Acid Composition of the IPF*

To confirm that dietary ARA was effectively incorporated into the adipose tissue of grass carp, the fatty acid composition of the IPF was measured (Table 5). Compared with fish fed the control diet, fish fed the ARA and ARA + ASA diets both had significantly higher ARA proportions in their IPF after 4 or 8 weeks of feeding. Interestingly, at week 8, the inclusion of ASA decreased the ARA content by a significant amount in comparison to the ARA-only group, a change that was not apparent at week 4. Other aspects of fatty acid composition were also influenced by dietary ARA with or without ASA supplementation. At week 4, fish fed with 0.3 % ARA and 0.3 % ARA + ASA exhibited significantly higher C16:0 levels than fish fed the control diet, but no other fatty acid proportions had changed at that time point. In contrast, at week 8, the ARA + ASA group showed significantly higher C16:1n-7 content than the other groups, and fish fed ARA also exhibited lower C18:1n-9 and lower total MUFA content than the control. Moreover, the IPF of fish fed the ARA + ASA diet had significantly lower LNA, n-6 PUFA, n-3 PUFA, and PUFA levels than fish fed the ARA-only diet.

**Table 4** Growth performance, feed utilization, and survival rate in grass carp fed with arachidonic acid (ARA) diets supplemented with or without acetylsalicylic acid (ASA) for 4 and 8 weeks

| | Groups | | |
|---|---|---|---|
| | Control | ARA | ARA + ASA |
| Initial weight (g) | 27.5 ± 2.5 | 27.7 ± 0.2 | 27.7 ± 0.1 |
| 4 weeks | | | |
| Body weight (g) | 36.3 ± 4.0 | 35.2 ± 3.3 | 34.6 ± 4.1 |
| Body length (cm) | 12.7 ± 0.1 | 12.3 ± 0.3 | 12.3 ± 0.2 |
| Specific growth rate (SGR) | 1.0 ± 0.2 | 0.9 ± 0.0 | 0.8 ± 0.1 |
| Feed conversion ratio (FCR) | 1.4 ± 0.2 | 1.4 ± 0.0 | 1.8 ± 0.1 |
| Survival rate (SR, %) | 100 | 97.4 ± 3.7 | 100 |
| 8 weeks | | | |
| Body weight (g) | 49.9 ± 4.6 | 49.5 ± 7.4 | 55.3 ± 9.9 |
| Body length (cm) | 13.7 ± 0.2 | 13.7 ± 0.0 | 13.8 ± 0.1 |
| Specific growth rate (SGR) | 1.2 ± 0.2 | 1.3 ± 0.0 | 1.8 ± 0.2 |
| Feed conversion ratio (FCR) | 1.4 ± 0.0 | 1.3 ± 0.0 | 1.9 ± 0.7 |
| Survival rate (SR, %) | 100 | 97.4 ± 3.7 | 100 |

Different symbols denote significant differences ($P < 0.05$); values are mean ± standard deviation, $n = 38$ for body weight and body length of 4 weeks; $n = 20$ for body weight and body length of 8 weeks; $n = 2$ for FCR, SGR, and SR

#### *Eicosanoid Measurement*

To test the efficacy of ASA, the $PGE_2$ content of IPF was measured. No significant differences in $PGE_2$ levels among the treatments were observed at week 4 (Fig. 1a). However, after feeding for 8 weeks, ARA had increased the $PGE_2$ levels approximately onefold, but this increase was blocked when ASA was also present (Fig. 1b). Interestingly, fish fed with 0.3 % ARA exhibited significantly higher concentrations of 5-HETE at week 4 compared to fish fed the control diet, and this increase was not attenuated by ASA (Fig. 1c). However, no significant difference in 5-HETE content was observed between the control and ARA groups at week 8, although by that time point, the inclusion of ASA did markedly decrease the 5-HETE content (Fig. 1d).

#### *IPF Index and Adipocyte Size*

To ascertain the effects of ARA on adipogenesis in grass carp, IPFI and adipocyte size were measured. Unexpectedly, after feeding for 4 weeks, IPFI and adipocyte size had significantly increased in ARA-fed fish, and fish fed ARA + ASA exhibited a status comparable to that of ARA-fed fish (Fig. 2a–c). However, fish fed the ARA diet displayed decreased adipocyte development compared with fish fed the control diet, whereas this decrease did not occur in fish fed ARA + ASA (Fig. 2d–f).

#### *Real-Time Quantitative PCR (RT-PCR)*

To reveal the potential causes of decreased lipid content in IPF after 8 weeks of ARA feeding, genes related to lipid metabolism were measured. We found that ARA reduced

**Table 5** Fatty acid composition of intraperitoneal fat (IPF) in grass carp fed with arachidonic acid (ARA) diets supplemented with or without acetylsalicylic acid (ASA) for 4 and 8 weeks (% total fatty acid)

| Fatty acid | Initial sample | 4 weeks | | | 8 weeks | | |
|---|---|---|---|---|---|---|---|
| | | Control | ARA | ARA + ASA | Control | ARA | ARA + ASA |
| 14:0 | 1.4 ± 0.1 | 2.3 ± 0.2 | 2.2 ± 0.2 | 2.0 ± 0.2 | 2.2 ± 0.1 | 2.2 ± 0.3 | 2.3 ± 0.1 |
| 16:0 | 17.4 ± 0.2 | 20.6 ± 2.4$^{a}$ | 18.3 ± 1.6$^{b}$ | 18.1 ± 1.0$^{b}$ | 18.2 ± 0.4 | 17.6 ± 0.9 | 18.2 ± 0.3 |
| 18:0 | 6.4 ± 0.5 | 6.0 ± 1.4 | 5.0 ± 0.9 | 4.9 ± 0.4 | 4.2 ± 0.2 | 4.0 ± 0.3 | 3.9 ± 0.3 |
| SFA$^{b}$ | 25.2 ± 0.5 | 29.0 ± 4.0$^{a}$ | 25.6 ± 2.6$^{ab}$ | 25.0 ± 1.4$^{b}$ | 24.6 ± 0.3 | 23.7 ± 1.2 | 24.4 ± 0.5 |
| 16:1n-7 | 2.1 ± 0.1 | 5.4 ± 0.7 | 5.2 ± 0.9 | 5.3 ± 0.9 | 6.6 ± 0.8$^{b}$ | 6.6 ± 0.7$^{b}$ | 7.6 ± 0.4$^{a}$ |
| 18:1n-9 | 28.3 ± 0.8 | 33.2 ± 1.3 | 32.1 ± 2.3 | 32.5 ± 1.4 | 38.2 ± 0.8$^{a}$ | 36.0 ± 1.7$^{b}$ | 36.7 ± 1.8$^{ab}$ |
| 18:1n-7 | 3.7 ± 0.2 | 5.0 ± 1.2 | 4.7 ± 1.6 | 5.0 ± 1.1 | 3.5 ± 0.8 | 3.9 ± 0.7 | 3.9 ± 1.1 |
| 20:1n-9 | 0.6 ± 0.2 | 0.5 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.8 ± 0.6 | 0.5 ± 0.1 | 0.6 ± 0.1 |
| MUFA$^{c}$ | 34.6 ± 1.0 | 44.1 ± 2.7 | 42.3 ± 1.8 | 43.2 ± 1.1 | 49.2 ± 1.4$^{a}$ | 47.0 ± 1.5$^{b}$ | 48.8 ± 1.1$^{ab}$ |
| 18:2n-6 | 22.9 ± 1.1 | 14.3 ± 2.0 | 16.1 ± 2.5 | 15.7 ± 2.1 | 13.5 ± 1.0$^{ab}$ | 14.0 ± 0.9$^{a}$ | 12.7 ± 0.7$^{b}$ |
| 18:3n-6 | 0.5 ± 0.1 | 0.5 ± 0.0 | 0.5 ± 0.1 | 0.5 ± 0.1 | 0.4 ± 0.0 | 0.4 ± 0.1 | 0.4 ± 0.1 |
| 20:4n-6 | 0.8 ± 0.1 | 1.0 ± 0.4$^{b}$ | 2.5 ± 0.3$^{a}$ | 2.4 ± 0.5$^{a}$ | 0.9 ± 0.1$^{c}$ | 2.6 ± 0.3$^{a}$ | 2.3 ± 0.1$^{b}$ |
| 22:4n-6 | 0.7 ± 0.1 | 0.3 ± 0.2 | 0.5 ± 0.2 | 0.5 ± 0.2 | 0.3 ± 0.1 | 0.4 ± 0.1 | 0.5 ± 0.2 |
| n-6 PUFA$^{d}$ | 24.9 ± 1.2 | 16.0 ± 1.7 | 19.6 ± 2.3 | 19.1 ± 1.7 | 15.1 ± 1.1$^{c}$ | 17.5 ± 1.1$^{a}$ | 15.9 ± 0.8$^{bc}$ |
| 18:3n-3 | 12.3 ± 0.2 | 8.2 ± 1.1 | 9.8 ± 1.3 | 9.5 ± 1.5 | 8.8 ± 0.5 | 9.3 ± 0.6 | 8.7 ± 0.3 |
| 20:5n-3 | 1.1 ± 0.3 | 0.7 ± 0.2 | 1.0 ± 0.5 | 0.9 ± 0.3 | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.7 ± 0.1 |
| 22:6n-3 | 1.8 ± 0.3 | 2.0 ± 0.4 | 1.9 ± 0.3 | 2.2 ± 0.9 | 1.7 ± 0.1 | 1.9 ± 0.3 | 1.6 ± 0.2 |
| n-3 PUFA | 15.2 ± 0.1 | 11.0 ± 1.0 | 12.6 ± 1.3 | 12.6 ± 0.6 | 11.2 ± 0.5$^{ab}$ | 11.9 ± 0.7$^{a}$ | 11.0 ± 0.4$^{b}$ |
| PUFA | 40.1 ± 1.2 | 27.0 ± 0.7 | 32.2 ± 3.3 | 31.7 ± 2.3 | 26.3 ± 1.6$^{b}$ | 29.4 ± 1.7$^{a}$ | 26.9 ± 1.2$^{b}$ |
| n-3/n-6 PUFA | 0.6 ± 0.0 | 0.7 ± 0.0 | 0.7 ± 0.1 | 0.7 ± 0.0 | 0.8 ± 0.0$^{a}$ | 0.7 ± 0.0$^{b}$ | 0.7 ± 0.0$^{b}$ |

Different symbols denote significant differences ($P < 0.05$); values are mean ± standard deviation, $n = 6$ per group

*SFA* saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid

the relative expression of PPARγ, LPL, and SCD, but not FAS. Unexpectedly, although PPARγ exhibited a numerically high expression level ($P = 0.12$), the expression levels of none of these three genes were restored by ASA. In contrast, ARA promoted the mRNA expression of ATGL and CPT-1, although these increases in expression were blocked by ASA in the IPF of fish that were fed for 8 weeks (Fig. 3).

### *In Vitro*

#### *Treatment with ARA for 3 Days Induces Pre-adipocyte Differentiation*

To further examine whether ARA promotes the differentiation of adipocytes, confluent pre-adipocytes (day 0) were treated with increasing doses of ARA (0, 100, 200, 300, 400, and 500 μM) for 3 days. Lipid droplet formation increased proportionally with ARA concentration (Fig. 4a). The OD value demonstrated that ARA promoted pre-adipocyte differentiation in grass carp in a dose-dependent manner (Fig. 4b). When adipocytes were incubated with other doses of ARA, such as 5, 10, 15, 20, 25, 50, and 75 μM, the results also indicated that ARA promotes adipogenesis in grass carp (data not shown). To explore whether ARA functions like other fatty acids or plays a unique role in inducing lipid accumulation, pre-adipocytes of grass carp were treated with various fatty acids [palmitic acid (PMA), OLA, LNA, ALA, ARA, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)] in equimolar concentrations (300 μM) for 3 days. The OD values demonstrated that all the fatty acids except PMA effectively promoted adipocyte differentiation. However, compared with the other fatty acids, ARA and EPA played different and distinctive roles in promoting adipocyte differentiation (Fig. 4c). Furthermore, fatty acid analysis showed that the ARA proportion significantly increased in ARA-treated cells compared with BSA-treated cells (Fig. 4d). The mRNA expression levels of PPARγ were significantly higher at 24 h, and C/EBPα exhibited apparently higher expression levels at 12 h. Importantly, the expression levels of LPL and FAS showed an upward trend, but only after 24 h was there a marked increase in these levels (Fig. 4e).

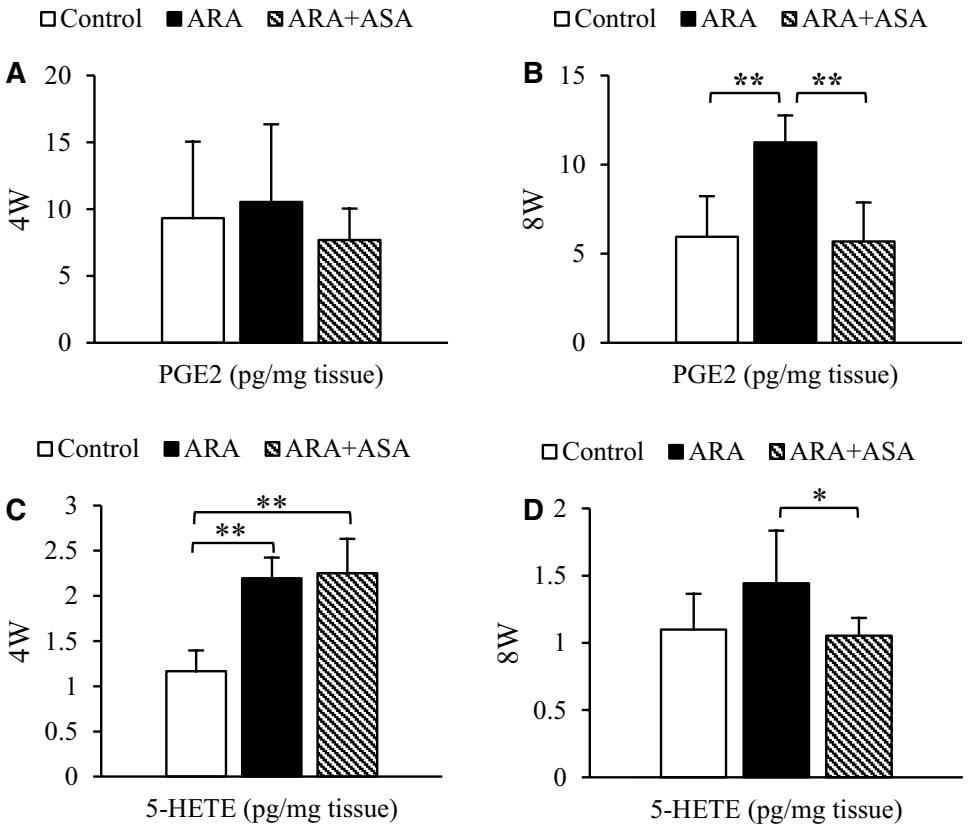


**Fig. 1** Prostaglandin $E_2$ ($PGE_2$) and 5-hydroxyicosatetraenoic acid (5-HETE) contents of intraperitoneal fat (IPF) in grass carp fed an arachidonic acid (ARA) diet supplemented with or without acetylsalicylic acid (ASA) for 4 and 8 weeks. All results are presented as the mean ± SD (*error bars*) ($n = 8$). Statistical significances are denoted with *asterisks* as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

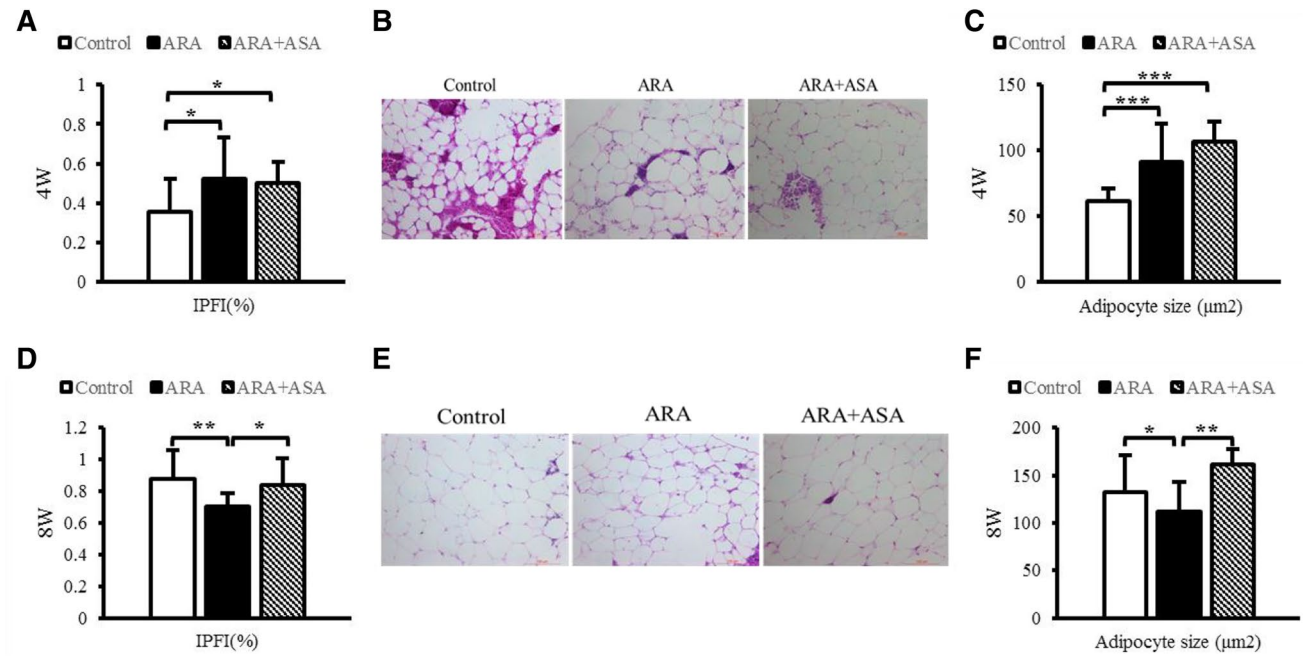


**Fig. 2** Intraperitoneal fat (IPF) index, morphology, and adipocyte sizes in grass carp fed an arachidonic acid (ARA) diet supplemented with or without acetylsalicylic acid (ASA) for 4 and 8 weeks. All results are presented as the mean ± SD (*error bars*) ($n = 8$). Statistical significances are denoted with *asterisks* as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

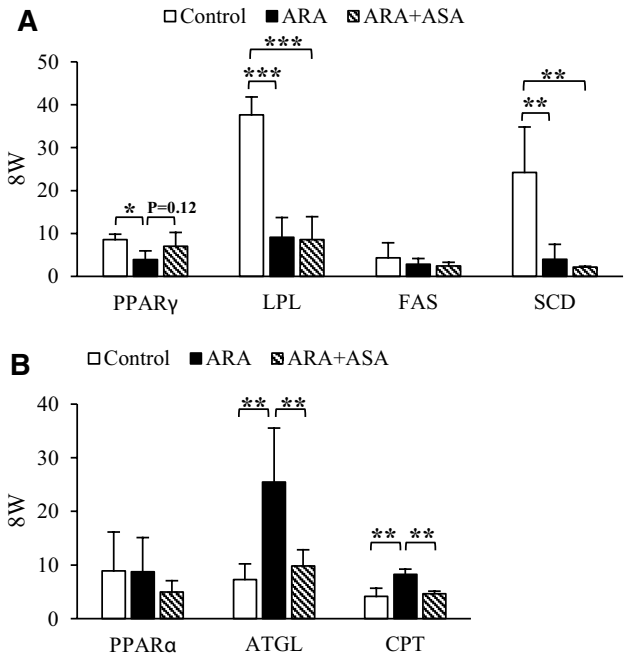


**Fig. 3** The relative expression of genes involved in lipid metabolism of intraperitoneal fat (IPF) in grass carp fed an arachidonic acid (ARA) diet supplemented with or without acetylsalicylic acid (ASA) for 8 weeks. All results are presented as the mean ± SD (*error bars*) ($n = 8$). Statistical significances are denoted with *asterisks* as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

### *The LOX Pathway Acts as a Key Regulator of Adipocyte Differentiation and is Influenced by Short-Term Treatment with ARA*

ARA is a precursor of the eicosanoids through three main pathways: the COX, LOX, and CYP450 pathways. To examine whether these derivatives of ARA play a role in promoting adipocyte differentiation, the pre-adipocytes of grass carp were pre-treated with indomethacin (a general COX inhibitor), nordihydroguaiaretic acid (NDGA; a LOX inhibitor), or 6-(2-propargyloxyphenyl) hexanoic acid (PPOH; a CYP450 inhibitor) at various concentrations (1, 10, and 100 μM) before being subjected to ARA incubation. NDGA significantly blocked the ARA-dependent induction of adipocyte differentiation in a dose-dependent manner (Fig. 5a, b). However, the effect of ARA was not blocked by the COX or CYP450 inhibitors (Fig. 5a–c).

### *The COX Pathway Contributes to Inhibition of Adipocyte Differentiation After Treatment with ARA for 8 Days*

To confirm that long-term treatment with ARA inhibits adipocyte differentiation in grass carp, pre-adipocytes were incubated with BSA, OLA (300 μM), ARA (300 μM), or ARA + ASA (100 μM) for 8 days. OLA significantly promoted adipogenesis compared with BSA, whereas cells treated with ARA exhibited significantly lower lipid content than cells treated with OLA, but were comparable to those treated with BSA. Interestingly, ASA suppressed the inhibition of adipogenesis by ARA (Fig. 6a). With the exception of SREBP-1c, there were no obvious differences in the adipogenic genes, such as PPARγ, LPL, and FAS, between BSA- and OLA-treated cells. However, ARA significantly suppressed the mRNA levels of PPARγ and FAS, and these decreases were not reversed by ASA (Fig. 6b). With respect to lipolytic genes, ARA significantly increased the levels of PPARα, ATGL, and HSL, whereas these were blocked by ASA (Fig. 6b).

## Discussion

Many studies have shown that ARA and ARA-derived eicosanoids play important roles in adipogenesis in mammals [9–16, 19, 32, 33]. However, these functions remain poorly understood in fish. In the present investigation, we revealed the complex role of ARA in adipogenesis in grass carp using *in vivo* and *in vitro* models. We showed that ARA inhibited lipid accumulation in grass carp adipose tissue after 8 weeks of feeding, and ASA, an inhibitor of COX, blocked the effects of ARA. However, ARA promoted lipid accumulation in adipose tissue after feeding for 4 weeks. The *in vitro* study also demonstrated that 3 days of treatment with ARA facilitated primary adipocyte differentiation in grass carp. Furthermore, the LOX inhibitor NDGA suppressed this phenomenon. Interestingly, 8 days of treatment with ARA inhibited adipocyte differentiation, and ASA effectively prevented this inhibition. Collectively, these results suggest that ARA plays a dual role in adipogenesis during different treatment periods and that eicosanoids might act as key regulators (Fig. 7).

It is known that the tissue fatty acid composition in fish reflects that of the diet [21]. Consistent with this observation, we found that ARA was effectively incorporated into the adipose tissue. Although fed with 1 % LNA (a substrate of ARA biosynthesis), fish in the control group nevertheless exhibited apparently limited ARA content (approximately one-third of that observed in the ARA-treated groups). This could be attributable to the lower capacity for desaturation and elongation of adipose tissue [25]. Moreover, we have also shown that ASA treatment slightly reduced the proportional ARA content in fish after feeding for 8 weeks, possibly because of a higher overall lipid content that effectively diluted the ARA. In this study, the ASA dosage was 30 mg/kg body weight (based on consumption of an approximately 30 g/kg body weight diet each day), and this was less than the dosages used in previous studies on tilapia and gilthead seabream (100 mg/kg) [22, 34]. However, ASA

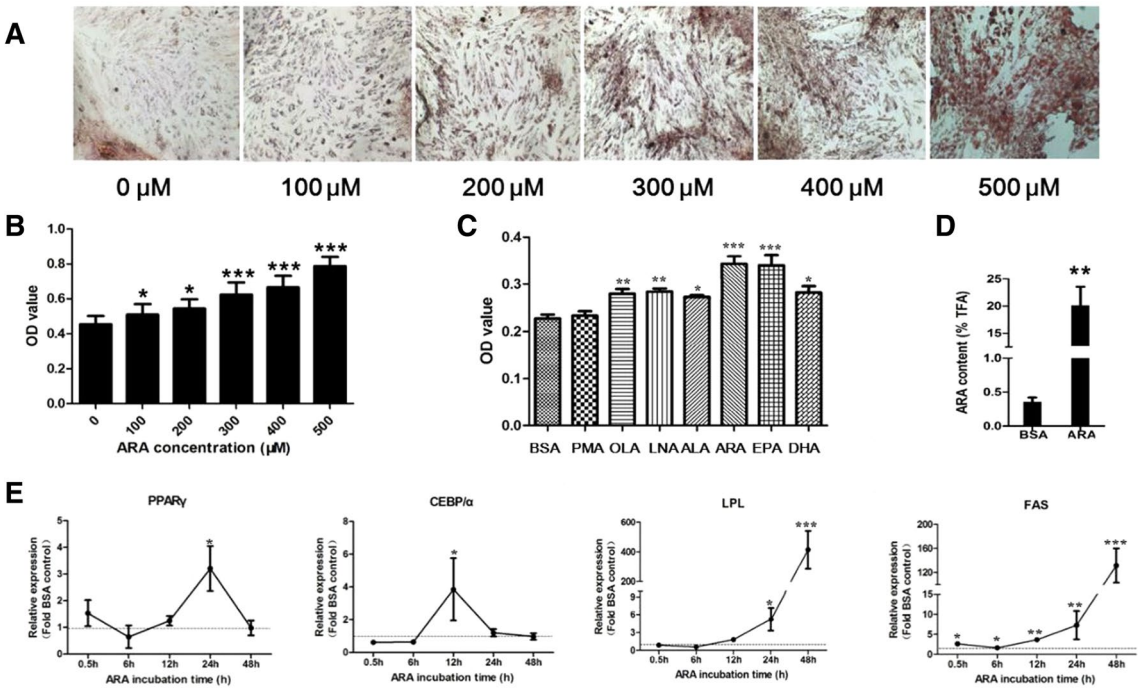


**Fig. 4** Effects of arachidonic acid (ARA) on pre-adipocyte differentiation in grass carp. **a** Oil red O staining of confluent pre-adipocyte cells (day 0) upon ARA treatment (0, 100, 200, 300, 400, and 500 μM) for 3 days. Cells were observed under a light microscope using ×10 magnification. **b** Lipid content quantified by OD 490 value after being dissolved in isopropanol ($n = 8$). **c** Lipid content of confluent pre-adipocytes (day 0) upon treatment with various fatty acids [palmitic acid (PMA), oleic acid (OLA), linoleic acid (LNA), alpha-linolenic acid (ALA), ARA, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA); 300 μM] or fatty acid-free bovine serum albumin (BSA) (vehicle) for 3 days. **d** ARA proportion of confluent pre-adipocyte cells (day 0) upon BSA or ARA treatment (300 μM) for 3 days ($n = 3$). **e** Pre-adipocyte cells (day 0) were treated with BSA or ARA (300 μM) at the indicated time and subjected to RT-PCR ($n = 3$). All results are presented as the mean ± SE (*error bars*). Statistical significances are denoted with *asterisks* as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

successfully blocked the COX pathway, because the main COX derivative $PGE_2$ had decreased in the fish after feeding for 8 weeks.

In mammals, most PGs are reported to block adipogenesis [19]. Knockdown and sustained expression of COX-2 augment and repress adipogenesis, respectively [15, 35]. However, in contrast to the negative effect, several PGs, such as 15-deoxy-$\Delta^{12,14}$-$PGJ_2$ and 15-keto-$PGE_2$, are reported to be the endogenous ligands of PPARγ and have a positive effect on adipose conversion [12, 36]. Nevertheless, many other studies have shown no effect of PG on adipogenesis [19]. In the present study, grass carp fed with 0.3 % ARA for 8 weeks showed reductions in adipose tissue content and adipocyte size, and the inhibition of COX by ASA suppressed these reductions, suggesting a potentially negative effect of PG on adipogenesis. We further demonstrated that ARA decreased expression of the adipogenic genes PPARγ, LPL, and SCD, similar to that which was observed in our earlier study of grass carp [18]. Unexpectedly, we found that unlike PPARγ, which exhibited a slight recovery, neither LPL nor SCD showed an upregulated trend in fish fed ARA + ASA, suggesting that there is no relationship between PG and adipogenic genes. However, the phenotype of the adipose tissue was not consistent with these genes in the ARA + ASA group. Consequently, we examined genes related to lipid catabolism and found that ATGL and CPT-1 increased in the ARA group but decreased in the ASA group after 8 weeks of feeding. This, at least in part, explains the increase in adipose tissue content in the ARA + ASA group and indicates that PG might play an important role in the regulation of lipid catabolism. In mammals, $PGE_2$ has been shown to reduce lipolysis, whereas $PGI_2$ has been shown to antagonize the anti-lipolytic effect of $PGE_2$ [33, 37, 38]. In the present study, ASA may have changed the balance between lipolysis-promoting and -antagonizing PGs. However, the observed $PGE_2$ content of adipose tissue was not in agreement with this speculation. Nevertheless, we did not measure the levels of $PGI_2$, and consequently our conclusions remain tentative. In the present study, fish fed ARA + ASA underwent a downregulation of lipogenic genes and an upregulation of lipid catabolic genes, while still maintaining a high level of lipid content in the adipose tissue, implying that lipid catabolism was more important than lipogenesis in controlling lipid

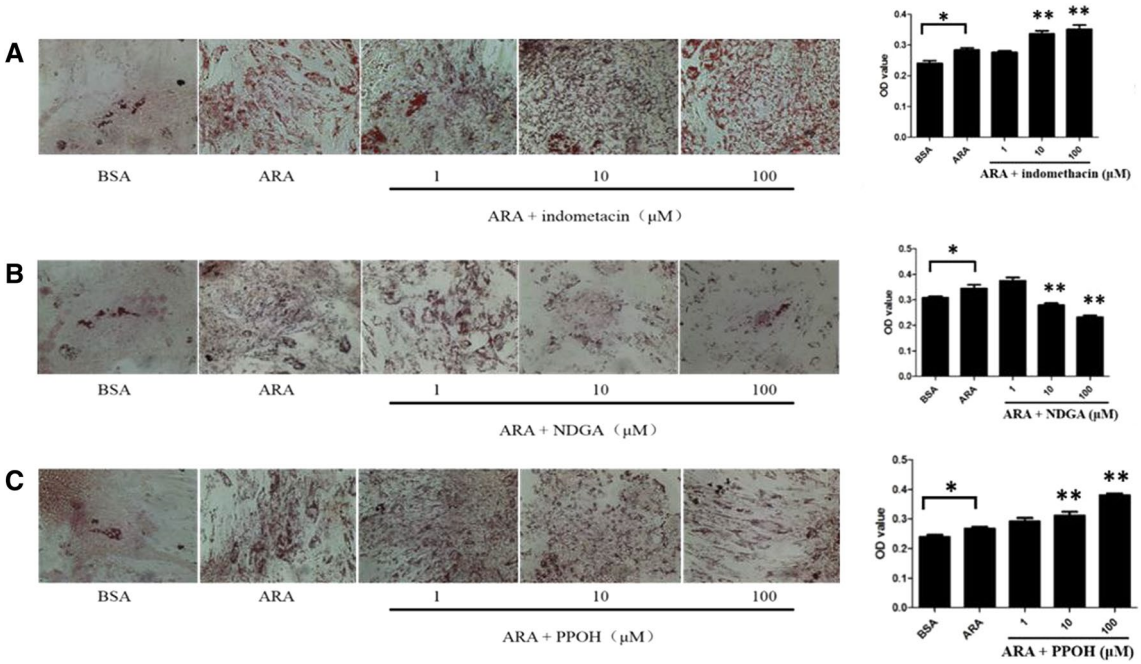


**Fig. 5** Effects of arachidonic acid (ARA) on differentiation of the pre-adipocytes of grass carp, administered with or without indomethacin (**a**), nordihydroguaiaretic acid (NDGA) (**b**), and 6-(2-propargyloxyphenyl) hexanoic acid (PPOH) (**c**) for 3 days. All results are presented as the mean ± SE (*error bars*), $n = 8$. Statistical significances are denoted with *asterisks* as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

accumulation in this group. This may be attributable to the poor ability of the fish to use carbohydrates as a source of acetyl CoA to form TAG [17].

In the present study, we observed a positive effect of ARA on adipogenesis in grass carp after feeding for 4 weeks, even though ARA was incorporated into the adipose tissue. During the feeding process, adipose tissue was in a state of development, e.g., IPFI increased from ~0.05 % (week 0) to ~0.4 % (week 4) and then to ~0.8 % (week 8), and adipocytes increased in size from ~100 $\mu m^2$ (week 4) to ~150 $\mu m^2$ (week 8). These data suggest that ARA accelerated adipocyte development at the early stage. Importantly, ARA did not apparently increase the concentration of $PGE_2$ in IPF, and ASA had no influence on the adipogenesis that was caused by ARA at week 4, suggesting that PG might not participate in the promotion of adipocyte development in grass carp.

The promotion of primary adipocyte differentiation by ARA in a dose-dependent manner once again confirms our speculation that ARA accelerates adipogenesis in grass carp. C/EBPα and PPARγ are critical transcription factors in adipogenesis [39], and in the present study, expression of C/EBPα and PPARγ genes exhibited instantaneous upregulation at 12 and 24 h after ARA treatment, respectively. This sequence is consistent with the molecular events during adipocyte differentiation in grass carp, which show that C/EBPα and PPARγ are transiently upregulated at day 1 and day 3, respectively [30]. The expression of LPL, one of the downstream genes of PPARγ [40], showed a rapid rise at 24 h after treatment with ARA, suggesting that ARA might trigger a PPARγ pathway that results in adipogenesis. Interestingly, ARA also upregulated the mRNA expression of FAS, suggesting that ARA also triggers lipogenesis, possibly through SREBP-1c, during the initial differentiation of pre-adipocytes. It has been shown that fatty acids and eicosanoids are the natural ligands of PPARγ and thus influence adipose conversion [19, 40]. However, and importantly, we have shown that ARA and EPA play a unique role in controlling adipogenesis compared with other fatty acids such as PMA, OLA, LNA, ALA, and DHA, and this suggests a possible way by which eicosanoids may participate in this process. Generally, EPA also acts as the substrate for eicosanoids, in particular the 3-series prostanoids and 5-series leukotrienes, and these eicosanoids are recognized as being less biologically active than the corresponding 2-series prostanoids and 4-series leukotrienes produced from ARA [5]. However, our data showed that EPA had a similar effect to ARA in promoting adipogenesis, and the mechanism underlying this process requires further study.

Three classes of eicosanoids can be synthesized in adipocytes, and they have been suggested to have positive effects on adipocyte differentiation: $PGI_2$ and 15-deoxy-$\Delta^{12,14}$ $PGJ_2$ in the COX pathway [11, 12, 41], hepoxilins and 15-(S)-HETE in the LOX pathway [14, 42], and 20-HETE

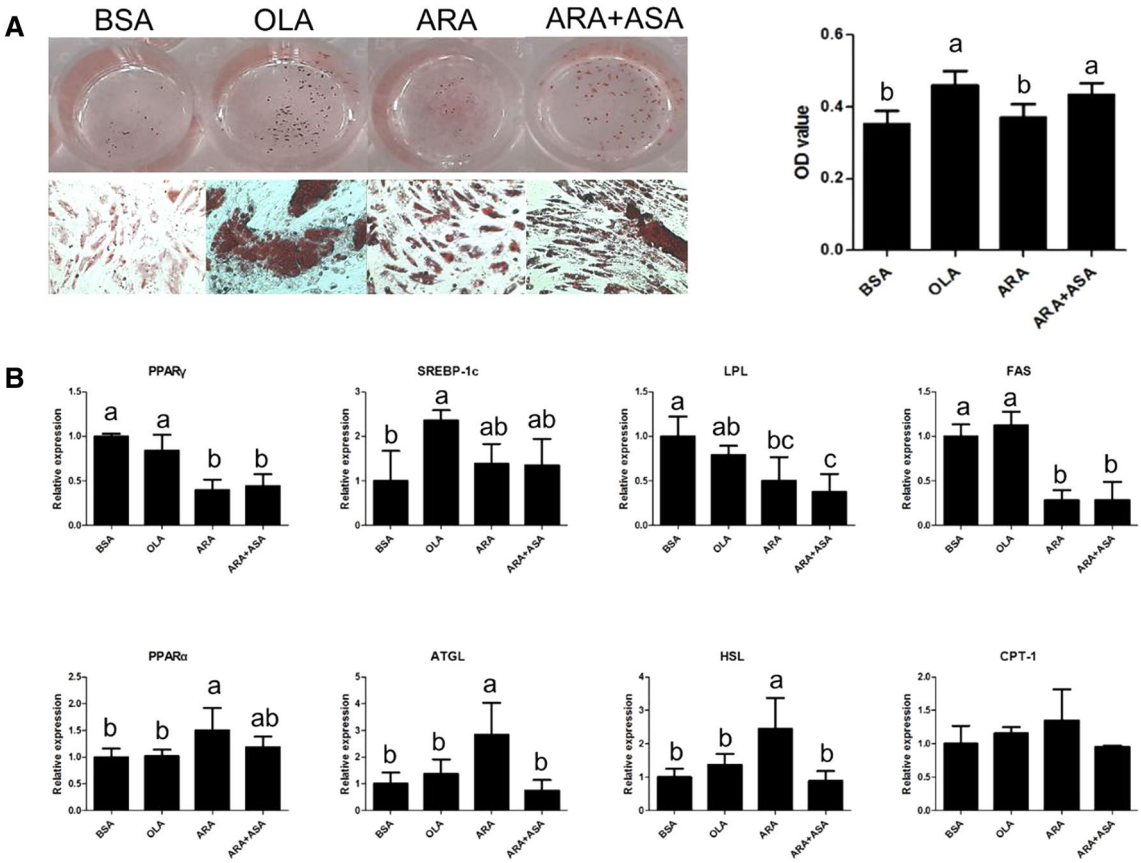


**Fig. 6** Effects of arachidonic acid (ARA) on pre-adipocyte differentiation in grass carp, administered with or without acetylsalicylic acid (ASA) for 8 days. **a** Oil red O staining of confluent pre-adipocyte cells (day 0) upon BSA, oleic acid (OLA; 300 μM), ARA (300 μM), and ARA (300 μM) + ASA (100 μM) treatment for 8 days. Cells were observed under a light microscope using ×10 magnification. The lipid content was quantified by OD 490 value after being dissolved in isopropanol ($n = 8$). **b** Relative expression of adipogenic and lipid catabolic genes ($n = 3$). *Different symbols* denote significant differences ($P < 0.05$)

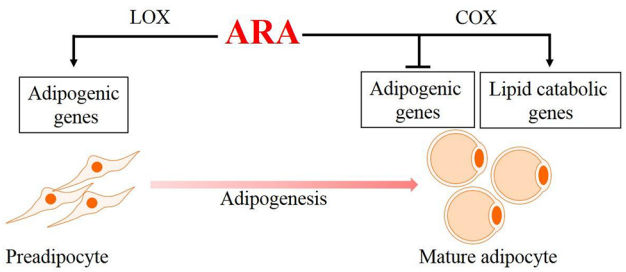


**Fig. 7** Schematic overview of the proposed role of arachidonic acid (ARA) in the adipogenesis of grass carp. ARA increases the adipogenic genes through the lipoxygenase (LOX) pathway at the initial stage of differentiation, which stimulates adipogenesis. However, at the mature adipocyte stage, ARA decreases the adipogenic genes and also increases the lipid catabolic genes through the cyclooxygenase (COX) pathway, resulting in reduced lipid accumulation in adipose tissue

in the CYP450 pathway [43]. In this study, we have shown that NDGA can attenuate the adipogenesis of pre-adipocytes affected by ARA, suggesting an important role for LOX in regulating lipid accumulation in grass carp adipocytes. This is in agreement with the study of 3T3-L1 pre-adipocytes by Lise et al. in 2003 [15]. However, which LOX-derived eicosanoid mediates this process has yet to be determined. In 3T3-L1 pre-adipocytes, only one LOX, the hydroperoxide isomerase eLOX3, is expressed. Additionally, the products of eLOX3, namely, hepoxilins, are able to bind to and activate the PPARγ-LBD in fibroblasts [14, 19]. More extensive study of the metabolites in the LOX pathway is required to explore this in detail; however, these data do suggest that the LOX pathway is an important regulator of ARA-induced adipogenesis in its initial stages.

Results from long-term treatment of adipocytes with OLA, ARA, and ARA + ASA are consistent with the *in vivo* study. In fact, in the feeding experiment, ARA was used to replace OLA, and thus the lipid content of the diets was equal. It is known that OLA is a fatty acid that acts as the main component of triglyceride and has the function of inducing adipogenesis [5, 44]. Although it is a fatty acid, ARA has no obvious effect on promoting adipogenesis

after long-term treatment; however, ASA administration can lead to lipid accumulation in adipocytes, suggesting a particular role for ARA, particularly COX-mediated PG, in adipocyte differentiation. Moreover, these PGs might play important roles in regulating lipolytic genes rather than adipogenic genes, as seen in the feeding experiment.

Although ARA has a dual role in inducing adipogenesis, the conversion mechanism from pro- and anti-adipogenesis has not been thoroughly investigated. In mammals, the role of ARA in obesity remains controversial, despite studies using both *in vivo* and *in vitro* models [44–47]. It has been proposed that cAMP levels govern the action of ARA in adipogenesis [44]. In addition, Nikolopoulou et al. demonstrated in 2014 that Fos-related antigen 1 (Fra-1) upregulation at the early stages causes the inhibition of adipocyte differentiation by ARA [47]. Nevertheless, further experiments should be performed to address this issue in fish.

In conclusion, the present study provides novel insights into the behavior of grass carp adipocytes following exposure to exogenous ARA. We show that ARA plays a dual role in adipogenesis in grass carp. On the one hand, ARA accelerates the early stages of pre-adipocyte differentiation, during which LOX might be the key regulator. On the other hand, after long-term treatment, ARA inhibits adipogenesis, during which COX might be the important modulator in controlling the lipid catabolic genes. Overall, we believe these data provide a basis for the use of this fatty acid in aquaculture.

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