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The Wnt/β-catenin pathway contributes to the regulation of adipocyte development induced by docosahexaenoic acid in grass carp, *Ctenopharyngodon idellus*

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ABSTRACT

Docosahexaenoic acid (DHA) has been suggested to inhibit adipogenesis in fish; the mechanism behind this observation remains largely unknown. The present study was to investigate the effects of DHA on the adipocyte differentiation of grass carp by using transcriptomic technology. Confluent primary cultured differentiating adipocytes were co-incubated with either 0 μM or 100 μM DHA for 2 days (early phase of differentiation) and 7 days (late phase). The results showed that 100 μM DHA promoted adipogenesis at 2 days but decreased lipid accumulation at 7 days of treatment compared to that of the control. Cells treated with DHA for 2 days showed up-regulation of adipogenic genes, such as PPARγ, C/EBPs, and FAS; 7 day treatment showed down-regulation of these genes. Interestingly, β-catenin, which plays important roles in suppressing adipogenesis in mammals, was also up-regulated after 7 days of treatment. To address this, administration of β-catenin inhibitor FH535 was used and abolished the inhibition of the adipogenesis induced by DHA for 7 days. However, FH535 did not rescue the inhibition of adipogenic gene expression (including that of PPARγ, C/EBPβ, and C/EBPγ) in the presence of 100 μM DHA, but it attenuated the up-regulation of β-catenin downstream target genes, e.g. c-myc and cyc D1. This suggests that DHA might regulate the adipogenic junction through the β-catenin pathway. Overall, our results demonstrate that continuous DHA treatment could promote the early step of adipocyte differentiation and suppress late phase adipogenesis in grass carp. Moreover, Wnt/β-catenin might be a new potential signaling pathway by which DHA suppresses adipocyte development.

1. Introduction

Adipose tissue plays important roles in energy storage in the form of triacylglycerol and the regulation of energy homeostasis in the body. An energy shortage situation will induce the lipolysis of triacylglycerol stored in adipocytes and the transportation of free fatty acid to organs to fulfill their energy requirements (Frühbeck et al., 2014; Rangwala and Lazar, 2000). However, excessive lipid accumulation in adipose tissue is strongly associated with conditions such as chronic low grade inflammation, insulin resistance, type II diabetes, and cardiovascular disease (Gregor and Hotamisligil, 2011). Adipocytes are a main component of adipose tissue (Rosen and Spiegelman, 2014). The development of adipocytes includes adipocyte proliferation and differentiation, which determine the number and size of the adipocytes, respectively. Adipocyte differentiation is regulated by a complex network of molecular events (Farmer, 2006; Oku and Umino, 2008). Peroxisome proliferator-activated receptor γ (PPAR γ), CCAAT/enhancer-binding proteins α (C/EBP α) and sterol regulatory element-binding protein 1c (SREBP-1c) are believed to be requisites for adipocyte differentiation (Rangwala and Lazar, 2000). These transcription factors regulate a series of genes, such as lipoprotein lipase (LPL), acetyl-CoA carboxylase 1 (ACC1), and fatty acid synthetase (FAS) (Rangwala and Lazar, 2000). Moreover, many signal transduction pathways participate in the adipocyte differentiation process, including the MAPK/ERK,

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insulin and insulin like growth factor, TGFs/BMPs, and Wnt/β-catenin signaling pathways (Rosen and MacDougald, 2006). Among these signaling pathways, the Wnt/β-catenin signaling pathway mainly acts as a negative regulator in the process of adipocyte differentiation (Christodoulides et al., 2009; Prestwich and MacDougald, 2007). For the past few several years, information regarding adipocyte development has been studied in vitro in many fish species, such as Atlantic salmon (Vegusdal et al., 2003), red sea bream (Oku et al., 2006), rainbow trout (Bouraoui et al., 2008; Lutfi et al., 2017; Salmerón et al., 2015), large yellow croaker (Wang et al., 2012), gilthead sea bream (Salmerón et al., 2013; Salmerón et al., 2016), cobia (Cheng and Chen, 2015), and grass carp (Li, 2012; Liu et al., 2015). Moreover, some key adipogenic genes, such as PPARγ, FATP, LPL, FAS, and ACC, have also been characterized in fish (Cheng et al., 2011; Leng et al., 2012; Saera-Vila et al., 2005; Wafer et al., 2017). These studies demonstrate that the adipocyte differentiation process of fish is mostly similar to that of mammals (Wafer et al., 2017).

The development of adipocytes is regulated by various external factors, including hormones, cytokines, and nutrients. Docosahexaenoic acid (22:6n-3; DHA) is one of the main n-3 highly unsaturated fatty acids (HUFAs) and has been suggested to have anti-obesity effects in animals (Todorčević and Hodson, 2015). Studies in mammals have largely uncovered the mechanism of adipogenic regulation by DHA, involved in inhibiting lipid accumulation and enhancing mitochondrial biogenesis through SIRT1 and Hh signaling in humans (Chen et al., 2015), and inducing mitochondria-mediated adipocyte apoptosis by down-regulation of Akt and ERK in 3 T3-L1 (Wang et al., 2016). However, though several studies have shown a negative effect of DHA on lipid accumulation in the adipocytes of fish, the mechanism elucidated was limited to several gene expression analyses such as adipose triglycerol lipase (ATGL), FAS, and LPL (Cheng and Chen, 2015; Wang et al., 2012). Over the past several years, the development of next-generation sequencing methods has provided a cost- and time-saving tool for both genomic and transcriptomic resources, as well as finding new key pathways in the physiological process (Metzker, 2010; Wang et al., 2009). However, the use of RNA−Seq analyses in the adipocytes of fish remains limited.

Grass carp, *Ctenopharyngodon idellus*, a typical herbivorous freshwater fish species that is widely cultured for food in China, accumulate excessive lipid easily in the abdominal cavity during aquaculture, which results in a negative effect on the health status of this fish species (Tian et al., 2015). We have previously shown that n-3 HUFA could depress the lipid accumulation and regulate the expression level of several key lipid metabolism related genes in the intraperitoneal adipose tissue of grass carp (Ji et al., 2011; Li et al., 2015; Liu et al., 2014). However, the function and mechanism of the sole n-3 fatty acid (such as DHA) was not determined. Therefore, the aim of the present study was to perform an in depth investigation into the influence of DHA on the adipocyte differentiation in primary cultured grass carp adipocytes; the effects of DHA and its regulatory mechanism in differentiating adipocytes were investigated by next-generation sequencing and gene expression analyses.

2. Materials and methods

2.1. Cell culture

Grass carp that had a body weight of approximately 1–2 kg were obtained from a commercial fish farm in Yangling (Shaanxi, China). Fish were acclimated in a 220 L aquarium for 1 day. The preparation of pre-adipocytes was performed by enzymatic digestion according to the method as previously described (Liu et al., 2015; Tian et al., 2016a). Briefly, grass carp were anesthetized with MS-222 (0.1 g/l) before extraction of adipose tissue. Subsequently, the fish were knocked to be dizzy by a crabstick, the arch bows of the gills were cut and the fish was exsanguinated. In a bacteria-free environment, adipose tissue was carefully isolated from the abdominal cavity. Collected adipose tissue was washed three times with PBS (pH 7.4) in a laminar 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 large particulate matter. 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 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 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, 25% linoleic acid, 25% alpha-linolenic acid], 10 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine (IBMX). The medium was changed every 2 days. DHA (100 μM; Sigma-Aldrich, USA) and/or FH535 (10 nM; Sigma-Aldrich, USA) were not added to the incubation medium until the pre-adipocytes were confluent. The DHA was delivered to the cells as fatty acid/bovine serum albumin (BSA; Sigma-Aldrich, USA) complexes. Adipocytes were harvested after differentiation in the MDI for 2 and 7 days. The cells differentiated without DHA were defined as C2 and C7, and those treated with 100 μM DHA were defined as D2 and D7. For inhibitor treatment, cells were pre-incubated with FH535 for 2 h, followed by further incubation with or without the addition of DHA. Cells were harvested after 7 days. The cell culture experiments were performed three times independently, with three replicates each time. The procedures were carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

2.2. Lipid content measurement

The lipid contents of cells were determined using Oil Red O staining according to the previously described method (Ramirez-Zacarias et al., 1992). The stained culture dishes were then subjected to dye extraction with isopropanol. The cellular lipid content was estimated at an absorbance 510 nm with a microtiter plate spectrophotometer (Multiskan MK3, Thermo). The cellular lipid content was calculated using the OD value.

2.3. RNA extraction, transcriptome library preparation and Illumina sequencing

Total RNA was extracted from the cells with RNAiso Plus (TaKaRa, Otsu, Japan) following the manufacturer's instructions. The transcriptome library preparation and Illumina sequencing were performed as previously described (Tian et al., 2015).

2.4. Transcriptome de novo assembly and sequence annotation

The raw reads of the two samples were separately assembled using the Trinity (http://trinityrnaseq.sourceforge.net/) software (Grabherr et al., 2011). Contigs were obtained by extending based on the overlap between sequences and then the resultant contigs were joined into transcripts with the paired-end information. We selected the longest transcripts from potential alternative splicing transcripts as the unigene sequences of this sample. To annotate the transcriptome, unigenes were searched against Nr, Nt, Swissprot, and TrEMBL using BLAST with a cut-off e-value of 0.00001.

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