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The effects of 2-bromopalmitate on the fatty acid composition in differentiating adipocytes of red sea bream (*Pagrus major*)

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ABSTRACT

To determine whether external factors affect the adipogenic function of fish adipocytes, the effects of 2-bromopalmitate (a PPAR agonist) on the fatty acid composition in differentiating adipocytes of red sea bream were investigated *in vitro*. In the presence of 2-bromopalmitate, the red sea bream adipocytes were differentiated and the effects on the fatty acid composition and the adipogenic gene expression were analyzed. With the level of 2-bromopalmitate, the content of 16:1n-7, a delta-9 desaturation product, increased in association with the increase in a stearoyl CoA desaturase (SCD) gene expression level while the triglyceride accumulation was not affected. Subsequently, the effects on the bioconversion of the n-3 and n-6 fatty acids, which are main series of dietary essential fatty acids, were examined. In the presence of 300 μM of 18:3n-3 or 18:2n-6, red sea bream stromal-vascular cells accumulated the lipid in the cytoplasm within 3 days by the fatty acid uptake with the increase of corresponding fatty acid contents. Furthermore, in both the 18:3n-3 and 18:2n-6 stored cells, the products of delta-6 desaturation (18:4n-3 and 18:3n-6, respectively) and C_{18-20} elongation (20:3n-3 and 20:2n-6, respectively) were detected. However, neither the delta-6 desatutration nor C_{18-20} elongation of 18:3n-3 and 18:2n-6 were enhanced by 2-bromopalmitate treatment. In conclusion, the results indicate that the adipocyte function in fish, e.g. adipogenic gene expression and fatty acid composition, can be modified by external factors and a main effect of 2-bromopalmitate is the increase in the content of delta-9 desaturation product by stimulating the SCD gene expression.

1. Introduction

Adipocytes are major sites of body lipid deposition in fish. In adipose tissue, the stored lipids, most of which consist of triacylglycerols (TGs), are derived from dietary lipids but some of them can produced by *de novo* fatty acid synthesis. Prior to the esterification to TGs for storage, both dietary derived and *de novo* synthesized fatty acids are postulated to be subjected to the bioconversion such as introduction of a double bond (desaturation) and elongation of carbon chains (Cook, 1991; Nakamura and Nara, 2004).

Recently, the primary culture systems of fish adipocytes have been developed in several species and the lipid accumulation process has been investigated *in vitro* (Vegusdal et al., 2003; Oku et al., 2006; Bouraoui et al., 2008). In salmon adipocytes, the cellular uptake of exogenous fatty acids increases in association with adipocyte differentiation (Todorcevic et al., 2008). In red sea bream differentiating adipocytes, triglyceride accumulation by lipid synthesis in the cytoplasm was observed within 2 weeks in a serum free culture system without lipid supplementation (Oku et al., 2006). Furthermore, the expression of adipogenic genes including fatty acid synthetase (FAS), delta-9 desaturase (so-called stearoyl CoA desaturase, SCD) and delta-6 desaturase are activated during adipocyte differentiaton in fish (Oku and Umino, 2008).

It is well known that the adipogenic gene expression is regulated by transcription factor peroxisome proliferator-activated receptors (PPARs) in mammalian adipocytes (Gregoire et al., 1998; Morrison and Farmer, 1999). PPAR, which includes three subtypes termed α, β and γ, is a ligand-dependent transcriptional factor and various kinds of agonists have been reported in mammals (Schoonjans et al., 1996; Kersten and Wahli, 2000). In our previous study, it has been revealed that 2-bromopalmitate, an agonist of mammalian PPAR (Amri et al., 1994; Bastie et al., 1999), increases the gene expression levels of PPARγ and related adipogenic genes in an early stage of adipocyte differentiation of red sea bream *in vitro* (Oku and Umino, 2008). The results suggested the potential ability of PPAR agonists for functional modification in fish adipocytes.

In this study, to determine whether the modification of adipogenic gene expression by a PPAR agonist actually modulates the adipocyte function in fish, we examined the effects of 2-bromopalmitate on the fatty acid composition, as a representative of the adipogenic property. Firstly, the effects of 2-bromopalmitate on the triglyceride accumulation and fatty acid composition were analyzed in the differentiating adipocytes of red sea bream *in vitro*. Subsequently, the two main series

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of dietary essential fatty acids (18:3n-3 and 18:2n-6) were taken up by red sea bream adipocytes and the effects of 2-bromopalmitate on the bioconversion of these fatty acids were examined.

2. Materials and methods

2.1. Experimental fish

Red sea bream (*Pagrus major*) weighing 1–2 kg were purchased from a local dealer (Nansei Suisan, Minami-Ise, Mie, Japan) and maintained in our institute (National Research Institute of Aquaculture, Minami-Ise, Mie, Japan).

2.2. Culture media

The plating medium and the serum-free induction medium were prepared as described previously (Oku et al., 2006). The plating medium consisted of the DME/F12 (1:1) medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 65 mM NaCl, 10% fetal bovine serum, 100 μg/mL streptomycin and 100 U/mL penicillin (Invitrogen). The induction medium (FA(−) induction medium) was prepared without supplementation of lipids to eliminate the influence of any exogenous fatty acids (Oku et al., 2006). The FA(−) induction medium was prepared with 1:1 mixture of DME (Invitrogen) and Ham F12K nutrient (Invitrogen) supplemented with 65 mM NaCl, 1x ITS (5 μg/mL bovine insulin, 50 μg/mL transferrin, 5 ng/mL sodium selenite, Sigma-Aldrich, St. Louis, MO, US), 50 ng/mL hydrocortisone (Sigma), 100 μg/mL streptomycin and 100 U/mL penicillin (Invitrogen). Linoleic acid (18:2n-6), α-linolenic acid (18:3n-3) and 2-bromopalmitate (2-hexadecanoic acid) (all from Sigma) were prepared in ethanol and supplemented to the induction medium with fatty acid free bovine serum albumin (final concentration 1%).

2.3. Preparation of stromal-vascular cells (SV cells) from red sea bream adipose tissue

Preparation of red sea bream SV cells was carried out as described previously (Oku et al., 2006). In brief, the red sea bream SV cells were prepared from visceral adipose tissue. The visceral adipose tissue was minced in PBS containing 5% bovine serum albumin, followed by digestion with 1 mg/mL type I collagenase (Invitrogen) for 1 h at room temperature. The digested tissue suspension was filtered through 200 μm nylon mesh and centrifuged at 800× *g* for 5 min to separate SV fraction and matured adipocytes. The SV fraction was suspended in the plating medium and seeded in 35 mm culture dishes unless otherwise stated. The cell density was approximately $4.3\times10^4/cm^2$. The seeded cells were cultured in the plating medium at 25 °C in 5% CO_2 in air. After 2 days, the attached SV cells were washed with the plating medium without fetal bovine serum, followed by additional 2 days cultivation in the plating medium.

2.4. Adipocyte differentiation of red sea bream SV cells

The adipocyte differentiation was initiated by switching the medium to the induction medium. For the analyses of lipid accumulation during adipocyte differentiation, the SV cells were cultured for 1–2 weeks in the FA(−) induction medium in the presence or absence of 2-bromopalmitate. On the other hand, for the analyses of fatty acid bioconversion, the SV cells were cultured in the FA(−) induction medium supplemented with 300 μM of fatty acids (18:2n-6 or 18:3n-3) for 3 days. In this experiment, to eliminate the influence of extracellular fatty acids in the fatty acid composition analyses, the medium was replaced with the FA(−) induction medium without fatty acid supplementation and cultured for 24 h prior to harvest whereas 2-bromopalmitate (0–30 μM) was supplemented continuously throughout the cultivation period.

SV cells were maintained at 25 °C in 5% CO_2 in air and the culture medium was changed every 2 or 3 days unless otherwise stated.

2.5. Cytological observation

The cultured SV cells were fixed in 10% formaldehyde and subjected to Sudan black B and nuclear fast red staining.

2.6. Analysis of triglyceride amount

The analysis of triglyceride was carried out as described previously (Oku et al., 2006). In brief, the cultured SV cells were scraped and homogenized in 25 mM Tris–HCl pH7.5–1 mM EDTA and subjected to the analyses of trigriceride amount. Total cellular lipids were extracted with chloroform–methanol (2:1), followed by the analysis of triglyceride amounts. The triglyceride amount was determined by the enzymatic method with TG Test Wako (Wako, Osaka, Japan).

2.7. Analyses of fatty acid composition by gas chromatography/mass spectrometry (GC/MS)

The cultured cells (2–4 dishes for each analysis) were harvested into 0.3 mL/dish of 25 mM Tris pH 7.5–1 mM EDTA and the total cellular lipids were extracted with 3 vol of chloroform–methanol (2:1). The extracted cellular total lipids were methylated with 5% HCl–methanol. The fatty acid methyl esters derived from the total cellular lipids were subjected to the GC/MS analyses with gas chromatography mass spectorometry system QP-5050 (Shimadzu, Kyoto, Japan) and DB-WAX column (Shimadzu). The temperature conditions were as follows; Firstly, the temperature was held at 120 °C for 10 min, then gradually increased to 191 °C at a rate of 20 °C/min. After holding at 191 °C for 30 min, the heating temperature was gradually increased to 220 °C at a rate of 10 °C/min, followed by the final heating step at 220 °C for 80 min. Each fatty acid methyl ester was detected based on the total ionic strength of EI mass. The species of fatty acid was identified by comparing the retention time and its mass spectrometric data with those of standard fatty acids. The fatty acid composition was calculated with the intensity of total ion chromatogram.

2.8. RNA extraction, cDNA synthesis and quantification of adipogenic gene transcripts

The expression levels of the adipogenic genes identified in red sea bream were measured by real time PCR method. The oligonucleotide sequences and GenBank accession numbers of genes analyzed in this study are indicated in Table 1.

Table 1
Oligonucleotide sequences for real time PCR

Target gene	GenBank accession number	(Forward) Sequences (Reverse)	Amplified fragment length
PPAR α	AB298547	5'-GACAAGTGTGAGCGCCG-3' 5'-CATCTTTGCCACCAGGGT-3'	(362 bp)
PPARβ	AB298548	5'-CTTTGGGAATGTCCCATGA-3' 5'-GATAGAAAACATGGACCCC-3'	(339 bp)
PPARγ	AB298549	5'-CGACATGGAGCACATGCA-3' 5'-TTCTCTCACCGCTTCGGC-3'	(360 bp)
Fatty acid synthetase (FAS)	AB298550	5'-AGCTGTTCATCTGGGGAT-3' 5'-CTGGGAAGAGGGCCATC-3'	(345 bp)
Stearoyl CoA desaturase a (SCDa)	AB298551	5'-CTTCGCCCACATTGGTTG-3' 5'-CACTCAAAGCAACCATTGC-3'	(342 bp)
Stearoyl CoA desaturase b (SCDb)	AB298552	5'-CTTTGCTCACATCGGCTG-3' 5'-CGCTGAACGTGACAAACTT-3'	(342 bp)
Delta-6-desaturase (d6DES)	AB298553	5'-GCACTTCCAGCATCACGC-3' 5'-ACGAAGCTGATGAGCGC-3'	(330 bp)
β actin	AB252854	5'-GGCACTGCTGCCTCCTC-3' 5'-GCCAGGATGGAGCCTCC-3'	(309 bp)