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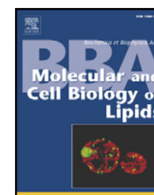

Changes in fatty acids metabolism during differentiation of Atlantic salmon preadipocytes; Effects of n-3 and n-9 fatty acids

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ABSTRACT

Atlantic salmon (*Salmo salar*) preadipocytes, isolated from visceral adipose tissue, differentiate from an unspecialized fibroblast like cell type to mature adipocytes filled with lipid droplets in culture. The expression of the adipogenic gene markers peroxisome proliferated activated receptor (PPAR) α, lipoprotein lipase (LPL), microsomal triglyceride transfer protein (MTP), fatty acid transport protein (FATP) 1 and fatty acid binding protein (FABP) 3 increased during differentiation. In addition, we describe a novel alternatively spliced form of PPARγ (PPARγ short), the expression of which increased during differentiation. Eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) lowered the triacylglycerol (TAG) accumulation in mature salmon adipocytes compared to oleic acid (18:1n-9, OA). This finding indicates that a reduced level of highly unsaturated n-3 fatty acids (HUFAs) in fish diets, when the traditional marine oil is exchanged for n-9 fatty acids (FAs) rich vegetable oils (VOs), may influence visceral fat deposition in salmonids. Moreover, major differences in the metabolism of EPA, DHA and OA at different stages during differentiation of adipocytes occur. Most of the EPA and DHA were oxidized in preadipocytes, while they were mainly stored in TAGs in mature adipocytes in contrast to OA which was primarily stored in TAGs at all stages of differentiation.

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1. Introduction

The use of high-energy feeds has been of major importance for the development of cost-effective fish farming. By increasing the energy levels in salmonid diets, growth and feed utilisation are improved [1]. However, increased dietary energy also increases fat deposition in the fish's fat storage organs and thereby reduces harvest yields [2]. It is of major importance to develop strategies to prevent excessive fat deposition in cultivated fish in order to strengthen the sustainability of the aquaculture industry. An improved knowledge of the underlying molecular events that regulate the differentiation process of preadipocytes to adipocytes in Atlantic salmon (*Salmo salar*) may open new avenues for the prevention of excessive storage of lipids in this important aquaculture species.

The primary sites for triacylglycerol (TAG) deposition in Atlantic salmon are the visceral adipose tissue [3–5] and myosepta surrounding the muscle [6,7]. Adiposity may arise from both an increased size of individual adipose cells due to lipid accumulation, and from an increased number of adipocytes arising from the proliferation of precursor cells. It has been suggested that in fish, as in mammals, this process occurs not only during early life stages [8] but also throughout life [9]. When energy intake is excessive, both the number and size of fish adipocytes increase [9,10]. The underlying molecular processes that control adipocyte differentiation in fish are poorly known. We have, however, previously shown that primary preadipocytes from Atlantic salmon differentiate to mature adipocytes in vitro and that these cells may be used as a model system for studies of adipose tissue development in fish [11]. In contrast to fish, the differentiation process in several mammalian species has been relatively well described: it is regulated by a complex network of molecular events controlled by signalling from hormones, growth factors and components of the extracellular matrix. The transcription factors peroxisome proliferator activated receptor gamma (PPAR) γ and CCAAT binding proteins (C/EBPs), are key regulators involved in initiating differentiation and inducing the expression of adipose-associated genes during differentiation [12,13].

Nutritional studies in humans and rats have demonstrated that energy balance and body fat content can be affected by changing the dietary long-chain polyunsaturated fatty acid (PUFA) level [14–16]. Diets enriched in n-3 PUFAs decrease adipose tissue mass and

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suppress the development of obesity in rats [17]. De Vos et al. [18] demonstrated that n-3 PUFAs limit the development of visceral adipose tissue by suppressing the late phase of adipocyte differentiation through modifications of PPARγ. Fish oil (FO), being a very rich source of n-3 highly unsaturated fatty acids (HUFAs), has been traditionally used as the dominating lipid component in feed for salmonids. Due to a general shortage of marine feed sources, vegetable oils (VOs) are being included to an ever-increasing degree in Atlantic salmon diets. However, to date it is more or less unknown how changing from dietary FOs to VOs affects visceral fat deposition in Atlantic salmon. In order to avoid an increase in the amount of visceral adipose tissue when replacing a diet rich in n-3 HUFAs with one rich in 18-carbon fatty acids (FAs) from VOs, the different dietary FAs from VOs should preferably be easily β-oxidized rather than being primarily deposited. The role of n-3 HUFAs in promoting FA oxidation and repressing lipid deposition has not been reported in fish so far. This study was therefore conducted in order to investigate firstly, the molecular events regulating adipocyte differentiation in Atlantic salmon; secondly, how oleic acid (18:1n-9, OA), a FA highly present in VOs, affects preadipocyte differentiation, cell morphology, FA deposition and utilisation in comparison to two typical marine FAs, namely eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA); and thirdly, how different FAs are deposited and utilised at different stages of adipocyte differentiation.

2. Materials

Atlantic salmon were obtained from AKVAFORSK (Averøy, Norway). Fetal bovine serum (FBS), essential FA free bovine serum albumin (BSA), antibiotics (mixture of pencillin, streptomycin and amphotericin B), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), L-glutamin, lipid mixture, laminin, Thermanox cover slips, Hank's balanced salt solution (HBSS), oil red O, phosphate buffered saline solution (PBS), Leibowitz-15 (L-15), albumin-solution, diethylether, 2′,7′dichlorofluorescein, petroleum ether, formalin, dexamethasone, biotin, triiodothyronine, panthothenate, isobutylmethylxanthine, non radiolabelled FAs and butylated hydroxytoluene (BHT) were all supplied from Sigma-Aldrich (St. Louis, MO, USA). Insulin (recombinat human Z) was obtained from Invitrogen (Carlsbad, CA, USA). Metacain (MS-222) was obtained from Norsk Medisinaldepot (Oslo, Norway). Collagenase (type I, 220 U/mg) was obtained from Worthington (Lakewood, NJ, USA). Chloroform and phenylethylamine were from Prolabo (Paris, France). Sodium chloride, sodium hydroxide, methanol, ammonium dihydrogenphosphate, perchloric acid, acetonitrile, hexane, acetic acid and thin layer chromatography (TLC) plates (silica gel 60) were all purchased from Merck (Darmstadt, Germany). Whatman filter paper was obtained from Schleicher & Schuell (Dassel, Germany). Nylon filters 250/100 were obtained from Sefar AG (Heiden, Switzerland). RNeasy Mini Kit, QIAshredder columns and RNase-free DNase I were purchased from Qiagen (Valencia, CA, USA). TaqMan® Gold RT-PCR Kit was bought from Applied Biosystems (Foster City, CA, USA).The radiollabeled FAs [1-^{14}C] 18:1n-9, [1-^{14}C] 20:5n-3 and [1-^{14}C] 22:6n-3 (50 mCi/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St.Louis, MO, USA). Cells in culture were observed using a Diaphot inverted light microscope Nikon (Tokyo, Japan). Scinitilant InstaGel II Plus and the dual channel liquid scintillation counter Model 1900 TR TRI-CARB Liquid Scintillation Analyzer were obtained from Packard Instrument (Downers Grove, IL, USA).

Radioactive detector A-100 was obtained from Radiomatic Instrument & Chemicals (Tampa, FL, USA). Tissue culture plastic ware was obtained from NalgeNunc International (Naperville, IL, USA). Glutaraldehyde, osmium tetraoxide, epon resin, copper grids and lead citrate were supplied by Electron Microscopy Sciences (Fort Washington, PA, USA).

3. Methods

3.1. Preadipocyte isolation and culture conditions

Atlantic salmon were reared in sea water (average temperature 12–13 °C) on a commercial diet to an average weight of 2–3 kg. Random fish were sampled and anaesthetized in metacain. After the anaesthesia, arch bows of the gills were cut. After bleeding for a couple of minutes, the fish were killed by a blow to the head and the abdomen was cut open to expose the visceral adipose depot. Visceral fat was carefully excised in order to avoid contamination with intestinal contents. Salmon preadipocytes were mainly isolated as described by Vegusdal et al. [11]. Briefly, the dissected fat tissue was washed with PBS (pH 7.4) to carefully remove blood cells, then minced, and digested in 0.1% collagenase in HBSS (1 g tissue/5 ml HBSS) at 13 °C for 1 h under shaking. Subsequently, the digested tissue suspension was filtered through 250 and 100 µm nylon filters to remove large particulate material. The resulting cell suspension was then centrifuged at 700 ×g for 10 min at 10 °C. The buoyant fat layer with mature adipocytes on the top of the centrifuge tube and the digestion medium was removed by aspiration, while the preadipocytes were pelleted on the bottom. After washing twice, the cells obtained were resuspended in a growth medium containing L-15, 10% FBS, 2 mM L-glutamine, 10 mM HEPES, and antibiotics (mixture of pencillin, streptomycin and amphotericin B) and seeded on laminin coated cell-culture flasks. The adipose tissue was weighed after excision and cells were plated at a density of approximately 10 g tissue/25 cm^2. The cells were kept at 13 °C, a temperature close to the average sea-water temperature where the fish was kept prior to isolation of cells. The medium was changed every three days. The cells reached confluence after approximately 1 week. Confluent preadipocytes were firstly differentiated in an initial differentiation-inducing medium containing growth medium supplemented with 1 µM dexamethasone, 33 µM biotin, 10 nM triiodothyronine, 17 µM panthothenate and 25 µM isobutylmethylxanthine, 20 µg/ml insulin and a lipid mixture (1 µl/ml; corresponding to 45 mg/ml cholesterol, 100 mg/ml cod liver oil FA (methyl esters)). After 48 h the cells were transferred to a maintenance differentiation medium containing growth medium only supplemented with 2 µl/ml of lipid mixture.

The cultured medium was changed every three days until the cells reached the final differentiation step, with morphology of mature adipocytes (day 21). During the cultivating period the differentiation and accumulation of lipids in the mature adipocytes was evaluated by morphological observations. Cells for electron microscopy studies were taken from cultures at three different differentiation stages: at confluence (day 7) and after induction of differentiation (day 9 and day 21). RNA was isolated from the cells at confluence (day 7) and after induction of differentiation (day 9 and day 21).

3.2. Preparation of adipocytes for electron microscopy

Cells were washed in 0.1 M PBS (pH 7.4), then fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4 °C for 24 h. The cells were then harvested, rinsed in 0.1 M cacodylate buffer and post-fixed for 60 min in 2% osmium tetroxide containing 1.5% potassium ferrocyanide, followed by en bloc staining with 1.5% uranyl acetate. Cells were dehydrated in a series of ethanol solutions (70%, 90%, 96%, and 100%) and propylene oxide, and then embedded in epon resin, which was polymerized at 60 °C for 24 h. Ultrathin sections (approximately 50 nm) were cut on a Reichert Ultracut E ultramicrotome using a diamond knife. The sections were placed onto formvar/carbon-coated 75-mesh copper grids, post-stained for 2 min with 0.2% lead citrate solution in 0.1 M sodium hydroxide, and examined in a Philips CM 100 transmission electron microscope at an accelerating voltage of 80 kV.

3.3. RNA extraction and cDNA synthesis from salmon adipocytes

Adipocytes for gene expression studies were thoroughly washed in PBS. Adipocytes from six cell flasks were pooled prior to RNA purification. Total RNA was extracted by using RNeasy® Mini Kit, according to the manufacturer's instruction. RNA was treated with RNase-free DNase I to remove any contaminating DNA. All RNA samples used in our experiments had A260/280 ratios between 1.80 and 2.30. The total RNA concentration was determined at 260 nm using spectrophotometry.

Approximately 2 µg of total RNA was reverse-transcribed into cDNA, using TaqMan® Gold real time quantitative polymerase chain reaction (qPCR) Kit, a 100 µl reaction system. All experiments were done in accordance with the protocol: 1 µg of total RNA was used in a 50 µl reaction with a final concentration of 1× TaqMan RT buffer, 5.5 mM magnesium chloride, 500 µM of each dNTP, 2.5 µM oligo d(t)$_{16}$, 0.4 U/µl RNase inhibitor, and 1.25 U/µl Reverse Transcriptase. Then the cDNA synthesis was performed with 10 min primer incubation at 25 °C, 60 min RT step at 48 °C,

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