Gene 534 (2014) 1–9

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Conservation of lipid metabolic gene transcriptional regulatory networks in fish and mammals

Greta Carmona-Antoñanzas *, Douglas R. Tocher, Laura Martinez-Rubio, Michael J. Leaver

School of Natural Sciences, University of Stirling, Institute of Aquaculture, Stirling FK9 4LA, UK

ARTICLE INFO

Article history:
Accepted 21 October 2013
Available online 28 October 2013

Keywords:
Atlantic salmon
Fatty acid
Gene expression
Lipid
Pyloric caeca
Transcription factor

ABSTRACT

Lipid content and composition in aquafeeds have changed rapidly as a result of the recent drive to replace ecologically limited marine ingredients, fishmeal and fish oil (FO). Terrestrial plant products are the most economic and sustainable alternative; however, plant meals and oils are devoid of physiologically important cholesterol and long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic (EPA), docosahexaenoic (DHA) and arachidonic (ARA) acids. Although replacement of dietary FO with vegetable oil (VO) has little effect on growth in Atlantic salmon (*Salmo salar*), several studies have shown major effects on the activity and expression of genes involved in lipid homeostasis. In vertebrates, sterols and LC-PUFA play crucial roles in lipid metabolism by direct interaction with lipid-sensing transcription factors (TFs) and consequent regulation of target genes. The primary aim of the present study was to elucidate the role of key TFs in the transcriptional regulation of lipid metabolism in fish by transfection and overexpression of TFs. The results show that the expression of genes of LC-PUFA biosynthesis (*elovl* and *fads2*) and cholesterol metabolism (*abca1*) are regulated by Lxr and Srebp TFs in salmon, indicating highly conserved regulatory mechanism across vertebrates. In addition, *srebp1* and *srebp2* mRNA respond to replacement of dietary FO with VO. Thus, Atlantic salmon adjust lipid metabolism in response to dietary lipid composition through the transcriptional regulation of gene expression. It may be possible to further increase efficient and effective use of sustainable alternatives to marine products in aquaculture by considering these important molecular interactions when formulating diets.

1. Introduction

Lipid content and composition in feeds for farmed fish have experienced a recent and rapid change, because, in order to sustain growth of the aquaculture industry, ecologically limited marine fish meal and fish oil (FO) ingredients have been replaced by terrestrial plant-derived meals and oils. Although replacement of up to 100% dietary FO with vegetable oil (VO) has little effect on growth in Atlantic salmon (*Salmo salar*) (Torstensen et al., 2005), some studies have shown major effects on the expression and regulation of genes involved in fatty acid and cholesterol metabolism (Leaver et al., 2008; Morais et al., 2009). This impact was consistent with the major compositional changes caused by feeding VO to fish including decreased levels of dietary cholesterol and long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoate (EPA; 20:5n-3), docosahexaenoate (DHA; 22:6n-3) and arachidonate (ARA, 20:4n-6), which are absent in terrestrial plants (Leaver et al., 2008). Cholesterol and LC-PUFA are critical functional components of cellular membranes and are important precursors of bioactive lipids required for homeostasis, cell signaling, immune and inflammatory responses (Simopoulos, 2008), and the long-term health and welfare effects of reductions in these essential dietary nutrients in fish, including salmon, are unknown.

Much effort has been directed toward the understanding of effects of dietary imbalances in LC-PUFA and cholesterol in humans and mammalian models because of the links between dyslipidemia and a range of highly prevalent cardiovascular, metabolic and inflammatory diseases (Wilson et al., 2005). These studies have shown that cholesterol and fatty acids and their metabolic derivatives can exert major effects on physiology by interactions with a range of transcription factors (TFs) (Desvergne et al., 2006). Particular attention has focused on liver

Abbreviations: aa, amino acid(s); ABCA1, ATP-binding cassette transporter 1; ACOX, acyl-CoA oxidase; ARA, arachidonic acid; bp, base pair(s); cDNA, DNA complementary to RNA; CYP7α1, cholesterol 7alpha-hydroxylase; DHA, docosahexaenoic acid; DMEM, Dulbecco's modified eagle medium; EDTA, ethylenediaminetetraacetic acid; ELF-1α, elongation factor 1 alpha; EPA, eicosapentaenoic acid; ELOVL, elongase of very long-chain fatty acids; ER, endoplasmic reticulum; FA, fatty acid; FADS, fatty acid desaturase; FAS, fatty acid synthase; FBS, fetal bovine serum; FHM, fathead minnow epithelial cells; FO, fish oil; HMG-CoAR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; LBD, ligand binding domain; LC-PUFA, long-chain polyunsaturated fatty acids; LO, linseed oil; LXR, liver X receptor; LXRE, LXR response element; mRNA, messenger RNA; ORF, open reading frame; PBS, phosphate buffer saline; PPAR, peroxisome proliferator-activated receptors; PPRE, PPAR response element; PUFA, polyunsaturated fatty acids; qPCR, quantitative RT-PCR; RE, response element; RO, rapeseed oil; RXR, retinoid X receptor; SCD, stearoyl CoA desaturase; SHK-1, salmon head kidney cells; SO, soybean oil; SRE, serum response element; SREBP, sterol regulatory element binding proteins; TF, transcription factor; UAS, upstream activation sequence; VO, vegetable oil.

* Corresponding author. Tel.: +44 178644 6593.
E-mail addresses: g.e.carmonaantonanzas@stir.ac.uk (G. Carmona-Antoñanzas), d.r.tocher@stir.ac.uk (D.R. Tocher), laura.martinez@stir.ac.uk (L. Martinez-Rubio), m.j.leaver@stir.ac.uk (M.J. Leaver).

0378-1119/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2013.10.040

X receptor (LXR), peroxisome proliferator-activated receptors (PPARs) and sterol regulatory element binding proteins (SREBPs) and their activities in liver and monocyte cells. LXR has a pivotal role in the control of intermediary metabolism mediating cross-regulation between fatty acid and sterol metabolism (Jakobsson et al., 2012). LXR activity is activated by binding oxysterol ligands, catabolic products of cholesterol (Reschly et al., 2008). In response to cholesterol overloading, and consequent oxysterol production, LXR modulates intracellular cholesterol levels by transactivating the expression of cholesterol ester transfer protein, apolipoproteins, cholesterol 7alpha-hydroxylase (CYP7α1) and the ATP-binding cassette transporter 1 (ABCA1), which regulate cholesterol efflux from cells (Jakobsson et al., 2012).

In response to cholesterol depletion, SREBPs, a family of membrane-bound transcription factors, are activated. SREBP1 plays a crucial role in the regulation of many lipogenic genes and SREBP2 primarily regulates the transcription of cholesterogenic enzymes (Jeon and Osborne, 2012). Interactions between these pathways are to some extent mediated through LXR activating SREBP1 transcription, inducing the expression of enzymes involved in the synthesis of fatty acids, triacylglycerols and phospholipids. In addition, some important lipid metabolizing genes, such as fatty acid synthase (FAS), are both LXR and SREBP1 targets (Joseph et al., 2002). PPARs, encoded by three genes in mammals, are activated by binding fatty acids or their oxidized derivatives and act to regulate expression of genes of lipid degradation and biosynthesis. PPARα and PPARβ, regulate the expression of genes encoding mitochondrial and peroxisomal fatty acid-catabolizing enzymes, while PPARγ has a central role in fat storage by promoting and maintaining the adipocyte phenotype (Desvergne et al., 2006). Thus, LXR, SREBP and PPAR transcription factors act as lipid sensors that translate changes in cellular sterol and fatty acid content and composition into metabolic activity.

Compared to mammals, few studies have addressed the existence or roles of these transcriptional regulators of lipid metabolism in fish. Our contention is that a greater understanding of lipid-mediated gene regulatory networks in Atlantic salmon will facilitate the efficient, effective and safe use of sustainable alternatives to marine products in aquaculture feeds. Recently the genes for Atlantic salmon Lxr, Srebp1 and Srebp2 and Ppars have been characterized (Cruz-Garcia et al., 2009; Leaver et al., 2007; Minghetti et al., 2011). In addition, studies on an Atlantic salmon cell line (SHK-1) have shown that several lipid metabolic genes are transcriptionally regulated in response to changes in lipid composition of the medium (Minghetti et al., 2011). The primary aim of the present study was to elucidate Lxr, Srebp and Ppar gene regulatory mechanisms and key lipid metabolic target genes in Atlantic salmon and to determine the extent to which dietary modulation of lipid and fatty acid metabolism in salmon reflects or varies from the patterns of gene regulation described for mammals.

2. Materials & methods

2.1. Cell lines and cell culture

The established Atlantic salmon cell line derived from head kidney (SHK-1) was grown at 22 °C in an atmosphere of 4% carbon dioxide in Dulbecco's modified eagle medium (DMEM) containing 3 g L^{-1} D-glucose and 55 mg L^{-1} sodium pyruvate, and supplemented with 10% fetal bovine serum (FBS), 50 U mL^{-1} penicillin, 50 μg mL^{-1} streptomycin, 40 μM 2-mercaptoethanol and 4 mM L-glutamine. For gene promoter transactivation assays, fathead minnow (*Pimephales promelas*; FHM) epithelial cells were maintained at 22 °C in Leibovitz's L-15 with GlutaMAX™-1 medium containing 900 mg L^{-1} D + galactose and 550 mg L^{-1} sodium pyruvate and 10% FBS. All media and supplements were obtained from Life Technologies (Glasgow, UK).

For subculturing, the cell monolayer was washed twice with phosphate buffer saline (PBS) without $CaCl_2$ or $MgCl_2$ (Invitrogen, UK), cells detached by incubation with 0.05% trypsin/0.02% EDTA and re-suspended in medium. Viable cells were counted after harvesting using a Neubauer hemocytometer, 0.4% Trypan blue (Sigma, Dorset, UK) and an inverted microscope (IMT-2, Olympus).

For TF ligand treatments, SHK-1 cells were seeded in 6-well clear plates (Nunc, Denmark) at a density of 4×10^5 cells per well in a volume of 3 mL Leibovitz's L-15 medium. Cells were approximately 70% confluent after 48 h growth, when medium was aspirated, cells washed twice with PBS and fresh medium containing the various treatments as ethanol solutions were added. Final concentrations were WY14643 (25 μM), 2-bromopalmitate (25 μM), LXR agonists (GW3965 and T0901317, 10 μM), or ethanol carrier alone (100% ethanol). After 24 h, the medium was aspirated, the cell monolayer washed twice (PBS) and cells scraped from each well in 0.5 mL of PBS. Cells were centrifuged for 5 min at 3000 xg, PBS discarded and replaced by 0.5 mL of TriReagent (Ambion, UK), followed by vigorous mixing to lyse and digest cells. Cells from two wells were pooled to produce three replicates per treatment.

2.2. Fish, diets and sampling protocols

Four diets (4 mm pellets) with the same basal protein composition, but coated with four different oils were formulated at Skretting Technology Centre (Stavanger, Norway) to satisfy the nutritional requirements of salmonid fish (National Research Council (NRC), 2011). The oils used were FO (anchovy oil), or 100% replacement with rapeseed oil (RO), linseed oil (LO) or soybean oil (SO). Atlantic salmon post-smolts (130 g) were randomly distributed into 16 tanks at the Skretting Aquaculture Research Centre (Stavanger, Norway). After a conditioning period of 3 weeks during which the fish received a commercial diet containing FO, the fish were fed the experimental diets to satiation for a period of 16 weeks. Full descriptions of the diet compositions and experimental conditions were reported previously (Leaver et al., 2008). At the end of the trial fish were anesthetized with metacain (50 mg/L) and pyloric caeca (intestine), a major organ involved in uptake and transport of lipids, were dissected from five randomly selected fish from each dietary treatment. Samples of 0.5 g of caeca were immediately and rapidly disrupted in 5 mL of TriReagent (Ambion, UK) using an Ultra-Turrax homogenizer (Fisher Scientific, UK), and stored at −80 °C prior to RNA extraction. The dietary trial and all procedures on Atlantic salmon conformed to European ethical regulations regarding the care and use of farmed animals in research.

2.3. Atlantic salmon LXR activation assay

The ligand binding domain (LBD) (amino acid residues 191–462) [GenBank: FJ470290] of Lxr was amplified by PCR from salmon pyloric caeca cDNA using primers (Supplementary Table 1) with 5′ restriction sites to allow in-frame subcloning between the *Bam*HI and *Xba*I sites of the pBIND vector (Promega, Southampton, UK), which contains the yeast Gal4 DNA-binding domain (Reschly et al., 2008). The resulting Gal4-LxrLBD chimeras were co-transfected with a reporter gene plasmid in which the *Firefly* luciferase gene is under the control of a promoter containing UAS (upstream activation sequence), which is recognized by Gal4. To control differences in transfection efficiency, a constitutively expressed control reporter construct encoding for *Renilla* luciferase was included. Ligand activation of Lxr was determined by a luciferase-based functional assay using the FHM cell line as described previously (Colliar et al., 2011). Twenty-four hours prior to transfection, 2×10^4 cells per well were seeded in a 96-well black-sided, clear-bottom microtitre plate (Corning, NY, USA). Transfection mixtures contained, per well; 100 ng pBIND-Lxr construct, 60 ng of luc2P/GAL4UAS reporter plasmid (pGL4.31, Promega), 20 ng of hRluc/CMV internal control reporter plasmid (pGL4.75, Promega) and 1.5 μL of Polyfect transfection reagent (Qiagen) in 100 μL of L-15 medium. Within each experiment, treatments were performed in triplicate. Experimental controls included treatments in which the pBIND-Lxr construct was replaced by empty pBIND vectors during transfection, as well as cells transfected with