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Roles of leptin and ghrelin in adipogenesis and lipid metabolism of rainbow trout adipocytes *in vitro*

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ABSTRACT

Leptin and ghrelin are important regulators of energy homeostasis in mammals, whereas their physiological roles in fish have not been fully elucidated. In the present study, the effects of leptin and ghrelin on adipogenesis, lipolysis and on expression of lipid metabolism-related genes were examined in rainbow trout adipocytes *in vitro*. Leptin expression and release increased from preadipocytes to mature adipocytes in culture, but did not affect the process of adipogenesis. While ghrelin and its receptor were identified in cultured differentiated adipocytes, ghrelin did not influence either preadipocyte proliferation or differentiation, indicating that it may have other adipose-related roles. Leptin and ghrelin increased lipolysis in mature freshly isolated adipocytes, but mRNA expression of lipolysis markers was not significantly modified. Leptin significantly suppressed the fatty acid transporter-1 expression, suggesting a decrease in fatty acid uptake and storage, but did not affect expression of any of the lipogenesis or β-oxidation genes studied. Ghrelin significantly increased the mRNA levels of lipoprotein lipase, fatty acid synthase and peroxisome proliferator-activated receptor-β, and thus appears to stimulate synthesis of triglycerides as well as their mobilization. Overall, the study indicates that ghrelin, but not leptin seems to be an enhancer of lipid turn-over in adipose tissue of rainbow trout, and this regulation may at least partly be mediated through autocrine/paracrine mechanisms. The mode of action of both hormones needs to be further explored to better understand their roles in regulating adiposity in fish.

1. Introduction

In addition to the classical functions exerted on lipid metabolism, the adipose tissue has more recently been recognized as an important endocrine organ through the secretion of a wide range of factors (Won and Borski, 2013; Londraville et al., 2014). Among these, leptin and ghrelin together with their corresponding functional receptors, the long isoform leptin receptor (LepRL) and the growth hormone secretagogue receptor 1a (GHS-R1a), respectively have been identified in a number of fish species including salmonids (Kaiya et al., 2009a; Rønnestad et al., 2010; Angotzi et al., 2013; Gong et al., 2013; Jönsson, 2013).

Most *in vivo* studies on the role of leptin in lipid metabolism of fish have used heterologous mammalian leptin, although the low degree of leptin sequence conservation among vertebrates (Londraville et al., 2014) makes difficult to gauge the relevance of the obtained data. In goldfish (*Carassius auratus*), for example, treatment with human leptin decreases hepatic lipid content (de Pedro et al., 2006), but it is not known if homologous leptin will have a similar effect. In terms of the modulatory effect of leptin on the expression of lipid-related genes, homologous leptin treatment decreases hepatic stearoyl CoA desaturase and lipoprotein lipase (LPL), as well as increases hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL) and carnitine palmitoyltransferase-1 (CPT1) in grass carp (*Ctenopharyngodon idellus*) (Li et al., 2010a; Lu et al., 2012), supporting an anti-adipogenic role for leptin in fish. Moreover, the LepRL$^{-/-}$ mutant medaka (*Oryzias latipes*) show large deposits of visceral fat not found in the wild type indicating that leptin signaling is involved in lipid allocation in teleosts (Chisada et al., 2014). In mammals, leptin treatment of primary preadipocytes and 3T3-L1 cells has inconsistent effects on proliferation and differentiation, in some cases depending on the

Abbreviations: ATGL, adipose triglyceride lipase; CPT1, carnitine palmitoyltransferase-1; EF1α, elongation factor 1α; FAS, fatty acid synthase; FATP1, fatty acid transporter-1; GHS-R1a, growth hormone secretagogue receptor 1a, ghrelin receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSL, hormone sensitive lipase; LepA1, leptin paralogue A1; LepA2, leptin paralogue A2; LepRL, leptin receptor long isoform; LPL, lipoprotein lipase; PPARs, peroxisome proliferator-activated receptors; qPCR, quantitative real-time polymerase chain reaction.

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hormone concentrations used (Wagoner et al., 2006; Kim et al., 2008). However, leptin consistently increases energy expenditure *in vivo* enhancing lipid oxidation while it inhibits insulin-induced lipogenesis and promotes lipolysis in adipocytes, hepatocytes and other cell types (Hwa et al., 1997; Reidy and Weber, 2000). Importantly, some of these effects appear to be modulated through autocrine or paracrine signaling (Frühbeck et al., 1997; Kim et al., 2008).

In fish, *in vivo* ghrelin treatment increases lipid deposition in the liver and muscles of Mozambique tilapia (*Oreochromis mossambicus*) and goldfish (Riley et al., 2005; Kang et al., 2011), but not in rainbow trout (*Oncorhynchus mykiss*) (Jönsson et al., 2010). No data are available about ghrelin effects on adipogenesis or adipocyte lipid metabolism in piscine models. Ghrelin has been shown to promote growth and adiposity in rat (Tschöp et al., 2000; Choi et al., 2003; Castañeda et al., 2010), and stimulatory effects of ghrelin on preadipocyte proliferation have been well described in mammalian models such as the 3T3-L1 cells, with the proliferation-promoting effect showing time-dependence (Kim et al., 2004; Zhang et al., 2004; Zwirska-Korczala et al., 2007; Liu et al., 2009). In addition, ghrelin enhances adipocyte differentiation *in vitro*, with a concomitant increase in the mRNA expression of two key adipogenic transcription factors: the peroxisome proliferator-activated receptor-γ (PPARγ) and the CCAAT/enhancer binding protein-α (Liu et al., 2009; Pulkkinen et al., 2010). Further, ghrelin induces abdominal obesity and hepatic steatosis in rodents by increasing the number of lipid droplets and their lipid content as well as decreasing fat utilization (Tschöp et al., 2000; Davies et al., 2009). In visceral adipose tissue, ghrelin also stimulates lipid accumulation by enhancing the expression of several genes including fatty acid synthase (FAS), LPL and perilipin among others (Pulkkinen et al., 2010).

The aim of this study was to elucidate the role of leptin and ghrelin in the regulation of adipogenesis and lipid metabolism in rainbow trout by using well-established *in vitro* systems for cultured preadipocytes and freshly isolated mature adipocytes (Albalat et al., 2005a; Bouraoui et al., 2008; Salmerón et al., 2013, 2015; Cruz-Garcia et al., 2015).

2. Materials and methods

2.1. Fish, holding conditions and sampling procedures

Adult rainbow trout from the fish farm Viveros de los Pirineos S.A. (El Grado, Huesca, Spain) were held in a recirculation system at the University of Barcelona in round fiberglass tanks (80 cm ø) of 400 L (8–9 trout/tank), at 15 ± 1 °C, with 12 h light:12 h dark photoperiod, and fed to satiation twice daily with a commercial feed (Trout evolution, Dibaq Diproteg S.A., Segovia, Spain). Prior to sampling, the fish were fasted for 24 h to avoid regurgitation of food and to ensure clean intestines. The fish were anesthetized with MS-222 (0.1 g/L) and sacrificed by a blow to the head and medullar section, after which they were weighed, externally sterilized with 70% ethanol and visceral adipose tissue was taken with sterile dissection material.

The study was divided in two parts. The first part concerned the effects of leptin and ghrelin on the development of preadipocytes, in which the number and mean body weight of the fish used was: 45 fish of 430.5 ± 18 g for the leptin expression and secretion experiment; and 50 fish of 181.1 ± 10 g for the immunofluorescence, proliferation and differentiation experiments. The second part of the study was with mature isolated adipocytes and encompassed 49 fish of 264.5 ± 25 g. All animal handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the established legislation of the European Union, Spanish and Catalan Governments (reference numbers CEEA 237/12 and DAAM 6755).

2.2. Peptides

Recombinant rainbow trout leptin was produced at the Department of Biology, University of Bergen (Norway) following the procedure described in Murashita et al. (2008). Synthetic 20 amino-acid octanoylated rainbow trout ghrelin was obtained from the Peptide Institute Inc., Osaka (Japan). Porcine insulin was obtained from Sigma (Tres Cantos, Spain) and used instead of piscine insulin as its bioactivity in fish cells has been shown previously (Albalat et al., 2005a, 2005b; Bouraoui et al., 2012; Salmerón et al., 2015).

2.3. Roles of leptin and ghrelin in adipogenesis

Preadipocytes were obtained following the protocol of Bouraoui et al. (2008) with minor modifications. For each experiment, a pool of adipose tissue of approximately 28 g obtained from 4 to 9 fish was kept in Krebs–Hepes buffer pre-gassed with a mixture of O_2–CO_2 to stabilize the pH (pH 7.3–7.4) and supplemented with 1% (*v*/*v*) antibiotic/antimycotic solution. The adipose tissue was minced into small pieces and incubated for 60 min in Krebs–Hepes buffer containing collagenase type II (130 U/mL) and 1% bovine serum albumin at 18 °C with gentle agitation. Cell suspension was filtered through a 100 μm cell strainer and centrifuged at 2.000 ×*g* for 10 min. The pellet obtained was treated with erythrocyte lysing buffer for 10 min at room temperature. Cells were centrifuged again and resuspended in growth medium containing Leibovitz's L-15, 10% fetal bovine serum and 1% antibiotic/antimycotic solution. Finally, cells were counted, diluted, and plated into 6- or 12-well plates treated with 1% gelatin at a density of 3.0 and 1.2 × 10^5 cells per well, respectively and incubated at 18 °C in growth medium.

2.3.1. Immunofluorescence

To detect the presence of leptin and ghrelin and their receptors in mature adipocytes, cells from four independent experiments were plated on glass cover slips and incubated in growth medium up to day 7. When confluence was reached, differentiation into adipocytes was induced by using a differentiation medium composed of growth medium plus 10 μg/mL insulin, 0.5 mM 1-methyl-3-isobutylxanthine and 0.25 μM dexamethasone; supplemented also with lipid mixture (5 μL/mL), as lipids are required to induce complete maturation of fish adipocytes (Vegusdal et al., 2003; Bouraoui et al., 2008; Salmerón et al., 2013). After 3 days, the culture conditions were changed to an adipocyte medium consisting of growth medium plus lipid mixture (5 μL/mL) to keep the cells already differentiating until day 13, when the cells were fixed.

Immunostaining was performed as described by Capilla et al. (2007) using 3T3-L1 adipocytes. The primary antibodies were tested at the following dilutions: leptin (1:100 and 1:500), LepRL (1:10000), ghrelin (1:10,000) and GHS-R1a (1:20,000). The anti-rat ghrelin antibody was kindly provided by Dr. Hiroshi Hosoda (Japan) and all other antibodies were developed against rainbow trout in rabbits by Agrisera (Vännäs, Sweden) as previously described (Jönsson et al., 2010; Einarsdóttir et al., 2011). The secondary Alexa Fluor 568-conjugated goat anti-rabbit antibody (A21069 LifeTechnologies, Alcobendas, Spain; 1:1000) was used in combination with a Hoechst 342 stain (LifeTechnologies, Alcobendas, Spain; 1:2000). The cells were mounted with Prolong (Invitrogen, Alcobendas, Spain) and immunofluorescence was captured with a confocal microscope (Leica TCS SP2). The images were analyzed with the image processing software ImageJ version 1.47 (National Institutes of Health, USA).

2.3.2. Expression and secretion of leptin during adipogenesis

To study leptin mRNA expression and secretion during adipogenesis, plated cells were incubated up to day 7 in growth medium and then, it was changed to differentiation medium including 5 μL/mL of lipid mixture to induce differentiation. At day 10, and then every 2 days, the medium was changed again to adipogenic medium to maintain differentiation. Media and cell samples were obtained from 6 to 8 independent experiments at days 7 (preadipocytes) and 16 (differentiated cells) of culture. Cell culture medium (0.5 mL) from individual wells of a 6-well plate was collected to analyze leptin secretion by radioimmunoassay using the established homologous protocol (Kling et al., 2009)