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Effects of nutritional status on plasma leptin levels and *in vitro* regulation of adipocyte leptin expression and secretion in rainbow trout

Cristina Salmerón [a], Marcus Johansson [b], Anna R. Angotzi [c], Ivar Rønnestad [c], Elisabeth Jönsson [b], Björn Thrandur Björnsson [b], Joaquim Gutiérrez [a], Isabel Navarro [a], Encarnación Capilla [a,*]

[a] Department of Physiology and Immunology, Faculty of Biology, University of Barcelona, Barcelona 08028, Spain
[b] Fish Endocrinology Laboratory, Department of Biological and Environmental Sciences, University of Gothenburg, 40590 Gothenburg, Sweden
[c] Department of Biology, University of Bergen, Bergen 5020, Norway

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ABSTRACT

As leptin has a key role on appetite, knowledge about leptin regulation is important in order to understand the control of energy balance. We aimed to explore the modulatory effects of adiposity on plasma leptin levels *in vivo* and the role of potential regulators on leptin expression and secretion in rainbow trout adipocytes *in vitro*. Fish were fed a regular diet twice daily *ad libitum* or a high-energy diet once daily at two ration levels; satiation (SA group) or restricted (RE group) to 25% of satiation, for 8 weeks. RE fish had significantly reduced growth ($p < 0.001$) and adipose tissue weight ($p < 0.001$), and higher plasma leptin levels ($p = 0.022$) compared with SA fish. Moreover, plasma leptin levels negatively correlated with mesenteric fat index ($p = 0.009$). Adipocytes isolated from the different fish were treated with insulin, ghrelin, leucine, eicosapentaenoic acid or left untreated (control). In adipocytes from fish fed regular diet, insulin and ghrelin increased leptin secretion dose-dependently ($p = 0.002$; $p = 0.033$, respectively). Leptin secretion in control adipocytes was significantly higher in RE than in SA fish ($p = 0.022$) in agreement with the *in vivo* findings, indicating that adipose tissue may contribute to the circulating leptin levels. No treatment effects were observed in adipocytes from the high-energy diet groups, neither in leptin expression nor secretion, except that leptin secretion was significantly reduced by leucine in RE fish adipocytes ($p = 0.025$). Overall, these data show that the regulation of leptin in rainbow trout adipocytes by hormones and nutrients seems to be on secretion, rather than at the transcriptional level.

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1. Introduction

Leptin is a pleiotropic 16-kDa peptide hormone implicated in the regulation of energy homeostasis, obesity, reproduction, bone formation, wound healing and immunity among other biological functions (Peelman et al., 2006). Leptin in mammals is produced and secreted primarily by mature white adipocytes (Matson et al., 1996; Zhang et al., 1994) and is considered to be a lipostatic and satiety signal, acting on hypothalamic orexigenic and anorexigenic neurons via transmembrane receptors to regulate food intake and energy balance (Coll et al., 2007; Harris, 2014). When fat mass decreases, circulating leptin is reduced, leading to stimulated appetite and suppressed energy expenditure (Ahima et al., 1996; Coppari and Bjørbæk, 2012).

In fish, the leptin gene was first discovered in pufferfish, *Takifugu rubripes* (Kurokawa et al., 2005), and since then, leptin A and B isoforms (LepA and LepB) resulting from an ancient fish whole genome duplication event (WGD; 3R) (Taylor et al., 2003; Volff, 2004) have been identified in zebrafish, *Danio rerio* (Gorissen et al., 2009), Japanese medaka, *Oryzias latipes* (Kurokawa and Murashita, 2009) and orange-spotted grouper, *Epinephelus coioides* (Zhang et al., 2012). Salmonids possess duplicates for both the LepA and LepB genes (Angotzi et al., 2013; Rønnestad

Abbreviations: AgRP, agouti-related protein; BL, body length; BSA, bovine serum albumin; BW, body weight; CART, cocaine and amphetamine-regulated transcript; CF, condition factor; Ef1α, elongation factor-1α; EPA, eicosapentaenoic fatty acid; FFA, free fatty acids; GH, growth hormone; HSI, hepatosomatic index; LepA1, leptin A1 gene isoform; LepA2, leptin A2 gene isoform; MFI, mesenteric fat index; NPY, neuropeptide Y; PC, Pearson correlation; POMC, pro-opiomelanocortin; PPARγ, peroxisome proliferator-activated receptor-γ; qPCR, quantitative real-time PCR; RE, restricted fish; RIA, radioimmunoassay; SA, satiated fish; SGR_L, standard growth rate for body length; SGR_W, standard growth rate for body weight; TG, triglycerides; WGD, whole genome duplication.

* Corresponding author at: Department of Physiology and Immunology, Faculty of Biology, University of Barcelona, Av. Diagonal 643, Barcelona 08028, Spain. Fax: +34 934110358.
E-mail address: ecapilla@ub.edu (E. Capilla).

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et al., 2010), most probably generated by the lineage specific WGD (4R) resulting in its tetraploidization about 25–100 million years ago (Allendorf and Thorgaard, 1984; Ohno, 1970). Nevertheless, some species such as the rainbow trout (*Oncorhynchus mykiss*) have later preserved only the LepB1 paralog (Angotzi et al., 2013). Leptin teleost primary structure is only 13–25% identical to human leptin. However, predicted tertiary structures of fish leptin are similar to that of their mammalian counterparts (Gorissen et al., 2009; Huising et al., 2006; Kurokawa et al., 2005; Li et al., 2010; Murashita et al., 2008; Rønnestad et al., 2010), thus supporting functional homology. In salmonids as in other teleost species studied, the liver appears to be the main production site of leptin, as evidenced by high hepatic Lep gene expression (Gong et al. 2013; Gorissen et al., 2009; Huising et al., 2006; Kling et al., 2012; Kurokawa et al., 2005; Kurokawa and Murashita, 2009; Murashita et al., 2008; Pfundt et al., 2009; Rønnestad et al., 2010). Although leptin is expressed at relatively low levels in adipose tissue of rainbow trout (Gong et al., 2013; Pfundt et al., 2009) and Atlantic salmon, *Salmo salar* (Rønnestad et al., 2010), it has been immunohistochemically detected in primary cultured mature adipocytes of Atlantic salmon (Vegusdal et al., 2003) and in rainbow trout adipose tissue (Pfundt et al., 2009) using mammalian antibodies.

The role of leptin as a satiety signal has been reported in mammalian as well as non-mammalian species either administering homologous or heterologous leptin by different means (reviewed by Londraville et al. (2014)). In rainbow trout, short-term injections with human or homologous leptin decreases food intake, and causes a reduction on the hypothalamic mRNA levels of the orexigenic neuropeptide Y (NPY), while it elevates the expression of the anorexigenic neuropeptides pro-opiomelanocortin (POMC) A1 and A2 (Aguilar et al., 2010; Murashita et al., 2008). Moreover, in goldfish (*Carassius auratus*) an acute or chronic treatment with human leptin also decreases food intake modulating partly the orexigenic effects of NPY and orexin A (De Pedro et al., 2006; Vivas et al., 2011; Volkoff et al., 2003). Similar results with leptin reducing feeding have been reported in grass carp, *Ctenopharyngodon idellus* (Li et al., 2010), Atlantic salmon (Murashita et al., 2011) and striped bass, *Morone saxatilis* (Won et al., 2012). Supporting the role of leptin suppressing appetite, increased food intake and up-regulated mRNA levels of the orexigenic neuropeptides NPY and agouti-related protein (AgRP), together with reduced expression of POMC has been described in a leptin receptor-deficient Japanese medaka (Chisada et al., 2014). More recently also, in rainbow trout infected with a pathogenic hemoflagellate, *Cryptobia salmositica*, which causes anorexia, a reduction in food intake has been observed associated with increased hepatic leptin expression (LepA1) and plasma leptin levels, as well as decreased mRNA levels of NPY and cocaine and amphetamine-regulated transcript (CART), and up-regulated AgRP and POMC-A2 in the hypothalamus (MacDonald et al., 2014). Furthermore, although certain controversy exists (reviewed by Londraville et al. (2014) and Won and Borski (2013)), a lipostatic model for food intake regulation has been proposed also for fish, suggesting that the central nervous system senses the amount of lipid stores and modulates/adjusts feeding behavior accordingly (Johansen et al., 2002). However, in this regard, the regulation of leptin seems to be fundamentally different between fish and mammals although being in both an anorexigenic peptide, as long-term fasting or restricted feeding increases plasma leptin levels in many fish species, such as rainbow trout, Atlantic salmon, Arctic charr (*Salvelinus alpinus*) and fine flounder (*Paralichthys adspersus*) (Frøiland et al., 2012; Fuentes et al., 2012; Johnsen et al., 2011; Kling et al., 2009, 2012; Rønnestad et al., 2010; Trombley et al., 2012).

In mammals, leptin production by adipocytes appears to be regulated at the transcriptional and translational levels, but also through storage, turnover and secretion (Lee and Fried, 2006). Several nutritional and hormonal signals have been found to modulate leptin release from isolated adipocytes, including food consumption (Lynch et al., 2006), leucine (Lynch et al., 2006; Roh et al., 2003), eicosapentaenoic fatty acid (EPA) (Pérez-Matute et al., 2005), insulin (Moreno-Aliaga et al., 2003; Ricci et al., 2005) and ghrelin (Giovambattista et al., 2008), all of which stimulate leptin secretion, whereas fasting (Szkudelski et al., 2004), fatty acids (e.g., oleic acid) (Margetic et al., 2002) and growth hormone (GH) (Margetic et al., 2002) appear to be leptin inhibitors. To date, nothing is known about the regulation of leptin expression and secretion by fish adipocytes.

The overall aim of this study was to further elucidate leptin endocrinology in teleost fish focusing on the role of adipose tissue, the contribution of which is uncertain due to its low expression of leptin in comparison to the liver, by addressing two key questions and using rainbow trout as an experimental model. Firstly, to evaluate leptin as a potential endocrine signal of adiposity at the organism level, two different states of adiposity were established in rainbow trout by means of feed restriction. Secondly, to shed light on the hormonal and/or nutritional regulation of leptin transcription and secretion by the adipose tissue at the cellular level, isolated adipocytes from rainbow trout under different feeding regimes were used as an *in vitro* model (Albalat et al., 2005; Cruz-Garcia et al., 2009).

2. Materials and methods

2.1. Fish and in vivo experimental trial

For the *in vivo* studies two groups of rainbow trout were generated with different levels of visceral adiposity by feeding them a high-energy diet once a day at two ration levels, satiation (SA group) or restricted (RE group) to 25% of satiation for 8 weeks. For the *in vitro* studies, adipocytes were isolated first from a group of fish fed *ad libitum* twice daily a regular diet and secondly, from the two groups of fish fed the high-energy diet (SA and RE).

All animal handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the European Union, Spanish and Catalan Governments established legislation (reference numbers CEEA 237/12 and DAAM 6755).

2.1.1. Regular diet fish

Rainbow trout (*O. mykiss*, Walbaum 1792) used for the regular diet experiment were obtained from Truites del Segre (Oliana, Lleida, Spain) and were held at the facilities of the University of Barcelona (Barcelona, Spain) until sampling. A total of 48 adult fish were equally distributed in 6 fiberglass tanks of 400 L each and, maintained in a recirculation system at 15 ± 1 °C with 12 h light: 12 h dark photoperiod. The fish were acclimated during 2 weeks fed twice daily *ad libitum* with a commercial diet (38% protein, 24% fat and 19.8 MJ/kg digestible energy; Trout evolution, Dibaq Diproteg S.A., Segovia, Spain), after which the fish were anesthetized with MS-222 (0.1 g/L) and sacrificed by a blow to the head and medullar section. The adipose tissue was excised and the adipocytes isolated for the *in vitro* experiments, as described below in Section 2.2.

2.1.2. High-energy diet experiment: satiated and feed restricted fish

Rainbow trout from the fish farm Viveros de los Pirineos S.A. (El Grado, Huesca, Spain) were held at the facilities of the University of Barcelona (Barcelona, Spain) where the experimental trial was carried out. A total of 98 adult fish were distributed among 6 fiberglass tanks of 400 L each at equal densities (16–17 trout/tank),

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