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Sex steroids stimulate leptin gene expression in Atlantic salmon parr hepatocytes *in vitro*

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ABSTRACT

In mammals, leptin plays an important role in puberty and reproduction and leptin is regulated by sex steroids. Elevated leptin levels have been associated with sexual maturation in some teleosts such as Atlantic salmon. In the present study, primary cultures of Atlantic salmon hepatocytes were used to investigate the direct effects of different sex steroids on expression of the two salmon leptin-a genes, *lepa1* and *lepa2*. Testosterone (T) stimulated both *lepa1* and *lepa2* in a dose dependent manner after four days of incubation. The stimulatory effect of T on leptin expression was not prevented by co-incubation with the aromatase inhibitor fadrozole, indicating a direct androgen effect on transcription. The non-aromatizable androgen 11-ketotestosterone (11-KT), which is the main androgen in fish, was generally slightly less potent than T in stimulating *lepa1* and *lepa2*. The strongest stimulatory response was seen for 17β-estradiol (E2). E2 treatment significantly up-regulated *lepa1* and *lepa2* gene expression at doses of 10 nM and 1 nM for each gene, respectively. *Lepa1*, but not *lepa2*, was stimulated by T and 11-KT in immature male and immature female parr, while E2 stimulated expression of both genes. The sensitivity to sex steroid stimulation differed in maturing males compared to immature. In maturing males, the androgens and E2 stimulated *lepa2* but not *lepa1*, while in immature males, the androgens and E2 stimulated *lepa1*, but only E2 stimulated *lepa2*. The differential response of the two leptin paralogues to the sex steroids suggests differences in regulation of the two leptin genes during maturation. Altogether, these results indicate that leptin expression in Atlantic salmon hepatocytes is directly regulated at the transcriptional level by the main teleost androgens and an estrogen, and that the response might depend on the developmental stage of the fish.

1. Introduction

Leptin is a key hormone in the regulation of body weight, appetite and metabolism (Ahima and Osei, 2004; Friedman, 2009) that also influences a large number of other physiological processes including puberty onset, reproductive function and fertility (Tena-Sempere, 2007). Leptin modulates all levels of the reproductive axis and is in turn regulated by sex steroids i.e. there is a cross talk between the leptin system and the reproductive tissues (Casabiell et al., 2001; Tena-Sempere, 2007). Leptin is predominantly produced by adipocytes and the main determinant of circulating leptin levels in mammals is the amount of body fat (Maffei et al., 1995), but gene expression as well as production and release of leptin is also influenced by a number of other factors, including sex steroids (Margetic et al., 2002).

In humans, circulating leptin levels are higher in women than in men and this sexual dimorphism is proposed to be due to higher estrogen levels in females (Casabiell et al., 2001). The estrogen 17β-estradiol (E2) has been shown to be a potent stimulator of leptin transcription and release in cultured rat adipocytes (Machinal et al., 1999) and E2 stimulates both leptin mRNA levels and release also in women (Machinal-Quelin et al., 2002). Androgens, on the other hand, generally have no or an inhibitory effect on leptin secretion (Wabitsch et al., 1997; Pineiro et al., 1999; Casabiell et al., 2001; Apostolova et al., 2005), although stimulatory effects have also been reported (Machinal-Quelin et al., 2002).

In fish, leptin is primarily expressed in the liver and not in adipose tissue as in mammals (Kurokawa et al., 2005; Kurokawa and Murashita, 2009; Trombley et al., 2012). Leptin is poorly conserved between mammals and fish, and many fish species have duplicate leptin genes (Huising et al., 2006; Gorissen et al., 2009; Won et al., 2012) among them Atlantic salmon, *Salmo salar* L. (Rønnestad et al., 2010; Angotzi et al., 2013). While sex steroids modulate leptin expression and release in mammals, only indirect evidence exists

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for this regulatory mechanism also being present in fish. It has previously been demonstrated that hepatic *lepa1* and *lepa2* gene expression levels are elevated during spermatogenesis in early sexually maturing one-year old Atlantic salmon males (Trombley and Schmitz, 2013; Trombley et al., 2014) and a similar increase in leptin expression associated with sexual maturation has been found also in Arctic charr, *Salvelinus alpinus* (Frøiland et al., 2010). Moreover, high plasma leptin levels were measured in the salmonid species Ayu (*Plecoglossus altivelis*) during and after spawning and this elevation coincided with high plasma levels of E2 and prolactin, suggesting the possibility of a regulatory link between sex hormones and leptin (Nagasaka et al., 2006). The association found between high leptin levels and increased sex steroid levels in some fish species, indicates that sex steroids might regulate leptin expression also in fish as has been shown in mammals.

Atlantic salmon is a seasonal spawner where males can mature already as one-year olds while still in freshwater. Early sexual maturation is strongly influenced by the individual's metabolic status in this species (Rowe et al., 1991; Silverstein et al., 1998) and salmon therefore provides an interesting model to study the link between leptin and reproduction in teleosts. The present study was aimed at investigating the regulation of the two salmon leptin-a genes, *lepa1* and *lepa2*, at the transcriptional level *in vitro* by sex steroids using primary hepatocyte cultures from Atlantic salmon parr. The two most important androgens in teleosts testosterone (T) and 11-ketotestosterone (11-KT), 11-KT being considered the main androgen (Borg, 1994), were tested as well as the estrogen E2. E2 is important in female salmon reproduction but is also present in male salmon blood plasma (Mayer et al., 1990) and is likely to play a role also in male maturation (reviewed in Schulz et al., 2010). T, but not 11-KT, can be aromatized to estrogens by the enzyme aromatase. We therefore tested whether any effects seen on leptin gene expression by T treatment was a direct androgen effect or caused by the aromatization of T into estrogen by co-incubation of T with an aromatase inhibitor.

2. Materials and methods

2.1. Experimental animals

Fish used for the experiments were 1 and 2-year old Atlantic salmon parr reared at the SLU Fishery research station in Älvkarleby, Sweden. The fish were transported to the Uppsala University fish holding facilities, kept under simulated natural photoperiod in 10° C municipal water and left to acclimatize for at least 10 days before experiments were carried out. Fish were fed a commercial diet (Aller Performa, AllerAqua, Christiansfeld, Denmark) *ad libitum* using automatic feeders. Food was withheld for 24 h prior to sampling. Livers from three fish were used for each primary hepatocyte culture. Fish were killed by decapitation and subsequently weighed, measured, sexed and livers were dissected out. Males were categorized as sexually maturing based on visual inspection of the gonads. Gonads of maturing males were removed and weighed and gonadosomatic indices (GSI) were calculated according to: GSI = 100 × gonad weight (g)/body weight (g). Body parameter data, age, sex, sexual stage, and gonadosomatic index of the fish for the different experiments are shown in Table 1. Maturing males were categorized as being at the mid-spermatogenesis stage based on their GSI values (Maugars and Schmitz, 2008). All animal experimentation was approved by the local committee of the Swedish Board for Laboratory Animals (no. C53/11).

2.2. Primary hepatocyte culture

2.2.1. Isolation

Hepatocytes were isolated following the procedure of Mommsen et al. (1994) with slight modifications. Livers from three fish were used for each primary culture. Livers were dissected out and placed into a sterile petri dish filled with Dulbecco's phosphate buffered saline without $CaCl_2$ and $MgCl_2$ (DPBS) (Gibco® Life Technologies, Carlsbad, CA, USA) supplemented with 1% antibiotic/antimycotic solution (Gibco® Life Technologies, Carlsbad, CA, USA). The livers were cut into smaller pieces and washed repeatedly with DPBS until blood was cleared. Liver digestion was performed by incubation at 25 °C for 30 min using collagenase solution (type IV, 0.5 ml collagenase/ml DPBS, Sigma, Saint Louis, MO, USA). Enzyme reaction was stopped by removing the collagenase solution and washing the digested tissue with DPBS three times. Cells were dispersed by repeated passages through a plastic transfer pipette. Cells were then filtered through a fine 70 μm mesh filter and subsequently collected by low speed centrifugation at 60*g* for 5 min. DPBS was removed and cells were re-suspended in fresh DPBS, counted and viability was assessed by the Trypan blue exclusion test. Cell viability was >90% and yields were typically in the order of 1×10^8 cells/g liver. Cells were then collected by low speed centrifugation at 60*g* for 5 min and left to sediment on ice for a minimum of 30 min. DPBS was removed and cells were re-suspended in L-15 medium (Leibowitz) (Sigma, Saint Louis, MO, USA) supplemented with 1% antibiotic/antimycotic solution.

2.2.2. Cell cultures and RNA extraction

Cells were cultured on 96-well plates (Costar) coated with poly-L-lysine (Sigma), for increased cell attachment, at 16 °C under plain air. A total volume of 125 μl cell suspension was added to each well and cells density was 1×10^5 cells/well. Cells were allowed to adhere for approximately 36 h prior to start of treatment. At start of treatment medium was changed to fresh medium (final volume was 250 μl/well) with or without hormone. Medium was changed every three days for up to six days of treatment. Cultures were terminated by placing the plates on ice and carefully removing the medium followed by an addition of 200 μl of Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Trizol was pipetted up and down repeatedly to ensure complete cell lysis and samples were then frozen in −80 °C until further analysis.

2.3. In vitro treatments

Experiments were performed on hepatocytes from samples of immature male parr, immature female parr, mature male parr

Table 1
Length (cm), weight (g) and gonadosomatic index (GSI) (%) of male and female Atlantic salmon parr used for hepatocyte isolation at different times of the year and developmental stages.

Sex	Developmental stage	Age	Month	Length (cm)	Weight (g)	GSI (%)
Male	Immature	1+	June	10.5 (±0.5)	10.3 (±1.1)	<0.01
Female	Immature	1+	June	10.1 (±0.2)	8.9 (±0.6)	<0.01
Male	Immature	1+	August	13.0 (±1.1)	31.8 (±8.2)	<0.01
Male	Mid-spermatogenesis	1+	July	11.2 (±0.5)	18.1 (±2.8)	2.2 (±0.5)
Male	Previously mature	2+	March	17.3 (±0.3)	52.5 (±2.0)	3.7 (±0.6)

Values are means ± SE ($n = 3$).