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Leptin stimulates hepatic growth hormone receptor and insulin-like growth factor gene expression in a teleost fish, the hybrid striped bass

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ABSTRACT

Leptin is an anorexigenic peptide hormone that circulates as an indicator of adiposity in mammals, and functions to maintain energy homeostasis by balancing feeding and energy expenditure. In fish, leptin tends to be predominantly expressed in the liver, another important energy storing tissue, rather than in fat depots as it is in mammals. The liver also produces the majority of circulating insulin-like growth factors (IGFs), which comprise the mitogenic component of the growth hormone (GH)-IGF endocrine growth axis. Based on similar regulatory patterns of leptin and IGFs that we have documented in previous studies on hybrid striped bass (HSB: *Morone saxatilis* × *Morone chrysops*), and considering the co-localization of these peptides in the liver, we hypothesized that leptin might regulate the endocrine growth axis in a manner that helps coordinate somatic growth with energy availability. Using a HSB hepatocyte culture system to simulate autocrine or paracrine exposure that might occur within the liver, this study examines the potential for leptin to modulate metabolism and growth through regulation of IGF gene expression directly, or indirectly through the regulation of GH receptors (GHR), which mediate GH-induced IGF expression. First, we verified that GH (50 nM) has a classical stimulatory effect on IGF-1 and additionally show it stimulates IGF-2 transcription in hepatocytes. Leptin (5 and/or 50 nM) directly stimulated *in vitro* GHR2 gene expression within 8 h of exposure, and both GHR1 and GHR2 as well as IGF-1 and IGF-2 gene expression after 24 h. Cells were then co-incubated with submaximal concentrations of leptin and GH (25 nM each) to test if they had a synergistic effect on IGF gene expression, possibly through increased GH sensitivity following GHR upregulation by leptin. In combination, however, the treatments only had an additive effect on stimulating IGF-1 mRNA despite their capacity to increase GHR mRNA abundance. This suggests that leptin's stimulatory effect on GHRs may be limited to enhancing transcription or mRNA stability rather than inducing full translation of functional receptors, at least within a 24-h time frame. Finally, leptin was injected IP (100 ng/g and 1 μg/g BW) to test the *in vivo* regulation of hepatic IGF-1 and GHR1 gene expression. The 100 ng/g BW leptin dose significantly upregulated *in vivo* IGF-1 mRNA levels relative to controls after 24 h of fasting, but neither dosage was effective at regulating GHR1 gene expression. These studies suggest that stimulation of growth axis component transcripts by leptin may be an important mechanism for coordinating somatic growth with nutritional state in these and perhaps other fish or vertebrates, and represent the first evidence of leptin regulating GHRs in vertebrates.

1. Introduction

The growth hormone (GH)/insulin-like growth factor (IGF) axis is central to regulating somatic growth in vertebrates (Duan, 1998; Perez-Sanchez and LeBail, 1999; Reinecke, 2006). In this system, circulating pituitary GH stimulates production of IGF-1, a potent mitogen that is largely responsible for somatic growth, and IGF-2, which is less studied but appears to elicit similar actions in teleosts (Chen et al., 2000; Gabillard et al., 2006; Peterson et al., 2004; Terova et al., 2007). The systemic or endocrine source of IGFs is thought to be primarily derived from the liver (Duan, 1998; Reinecke and Collet, 1998; Terova et al., 2007). Stimulation of IGF production by GH is mediated by GH receptors (GHR), two distinct forms of which have been identified in fish (Jiao et al., 2006), although it is not clear to what extent the individual receptor types mediate the growth promoting versus metabolic effects of GH (Kittilson et al., 2011; Pierce et al., 2007). Increased expression of hepatic GHRs elevates ligand sensitivity to GH-induced IGF production during anabolic states, whereas the liver is desensitized

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to GH during catabolic states due to downregulation of GHRs (Picha et al., 2008; Saera-Vila et al., 2005). Hence, declines in hepatic GHR expression lead to reduced IGF-1 production and somatic growth, this despite an elevation in systemic GH that occurs during fasting-induced catabolism (Norbeck et al., 2007; Picha et al., 2009, 2008; Won and Borski, 2013). The endocrine mechanisms that mediate changes in the GH–IGF growth axis under differential metabolic or nutritional states are not fully understood at present.

Leptin is an anorexigenic peptide hormone that circulates as an endocrine indicator of adiposity in mammals (Ahima and Flier, 2000). Extensive mammalian and clinical research shows that circulating leptin levels communicate the status of endogenous energy reserves to the brain and periphery, and function to balance appetite and energy expenditure based on fat deposition. Positive energy states are accordingly characterized by elevated plasma leptin levels, decreased energy intake and increased energy utilization (Arora and Arora, 2008). Aside from having a similar function to mammals in suppressing appetite (de Pedro et al., 2006; Douros et al., 2014; Murashita et al., 2008; Won et al., 2012), relatively little is known about leptin actions in lower vertebrates, including fish (Londraville et al., 2014).

In many fish, leptin is produced predominantly by the liver (Douros et al., 2014; Gorissen et al., 2009; Huising et al., 2006; Kurokawa and Murashita, 2009; Rønnestad et al., 2010), which serves as an energy storage site for lipids and glycogen, although it can be found in other tissues as well. Its regulation by nutritional state varies depending on species (Ahima, 2008; Copeland et al., 2011; Douros et al., 2014). Impeding a consensus on the functional role of leptin in fish, genome duplication events specific to certain lineages have resulted in multiple leptin paralogs that may be in various stages of neofunctionalization, while some species appear to have only a single leptin gene (Angotzi et al., 2013; Londraville et al., 2014). Regulation of leptin in response to nutritional state can vary between species, tissue and leptin type, albeit evidence suggests it tends to increase with fasting in most teleost studied to date. Among several salmonids, gene expression of one or both A-type leptins (A1 and A2) rise in the liver during feed-restriction (Frøiland et al., 2012; Fuentes et al., 2012; Jorgensen et al., 2013; Rønnestad et al., 2010; Trombley et al., 2012), possibly contributing to a rise in circulating leptin during fasting (Kling et al., 2009; Trombley et al., 2012). Hepatic leptin-like immunoreactivity in European sea bass (Gambardella et al., 2012) and circulating hormone in flounder (Fuentes et al., 2012) also increased during fasting. Conversely, A1-type leptin gene expression in Atlantic salmon was upregulated in the visceral fat of fish fed to satiety compared to feed-restricted fish (Rønnestad et al., 2010). Fasting also decreased hepatic gene expression of leptin-B relative to fed controls in *danio* (Gorissen et al., 2009). The endocrine growth axis of the hybrid striped bass (HSB: *Morone saxatilis* × *Morone chrysops*), a commercially valuable finfish cultivar that was used in this study, has been extensively characterized under different metabolic states, making it a practical subject for inquiries into regulation by leptin. In previous studies, we show that hepatic mRNA levels of leptin A (*lepa*) declined with fasting and increased during refeeding (Won et al., 2012), similar to the expression patterns of IGF-1, IGF-2, GHR1 and GHR2 in the liver (Picha et al., 2014; Picha et al., 2009, 2008).

The concordant regulation of leptin, GHR and IGF expression by nutritional state in HSB, along with their colocation in the liver, suggests a possible regulatory relationship between leptin and the endocrine growth axis. In cases where leptin levels are reflective of energy state in an adipostatic manner, as in mammals, leptin might reasonably serve as an endocrine signal for opportunistic physiological processes that require surplus energy. For example, leptin levels function as a permissive factor for reproduction (Casanueva and Dieguez, 1999; Roa et al., 2010), and could ostensibly serve a similar role in regulating somatic growth. Chronic leptin treatment increased circulating IGF-1 in leptin-deficient women and in mice *in vivo*, the latter accompanied by increased muscle mass and bone formation (Bartell et al., 2011a; Chan et al., 2008) suggesting that leptin may act permissively on tissue growth by mediating IGF-1 production. Leptin's actions may occur, at least in part, in the liver, insofar as leptin was shown to increase IGF-1 mRNA levels in isolated porcine hepatocytes (Ajuwon et al., 2003). Whether leptin regulates IGF-1 production directly, or through alterations in GH sensitivity or GHR expression in these cases is unknown. A potential role for leptin in modulating GHRs or IGFs in teleost is yet to be established. The aim of this study was to examine if leptin might act alone or in synergy with GH to regulate hepatic GHR1, GHR2, IGF-1 and IGF-2 gene expression in HSB, a perciform fish representative of advanced teleosts.

2. Materials and methods

2.1. Animals

Phase I HSB were obtained from Pungo Fisheries (Pinetown, NC) and housed in fresh water recirculating aquaculture systems (hardness = 200 mg/L; alkalinity = 250 mg/L; temperature = 22 °C; photoperiod = 12L:12D) at North Carolina State University (NCSU; Raleigh, NC). For 2 weeks prior to experiments, fish were fed a maintenance diet of 1.5% body weight/day (Ziegler Silver 5 mm pellets, Gardners, PA; 40% protein, 10% lipid), which is half of the typical commercial feeding rate. This regimen was chosen in order to maintain a mild anabolic state and intermediate GHR and IGF gene expression levels compared to those elicited under higher ration levels or fasting (Picha et al., 2008). Protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee.

2.2. Hepatocyte cultures

In vitro experiments on HSB hepatocytes were conducted in three separate trials to test the regulatory effects of GH and leptin on GHR and IGF gene expression. Experiments were repeated with similar results. In the first experiment, cells were incubated in growth medium containing 5 or 50 nM bovine GH (National Hormone and Peptide Program, Torrance, CA) to validate that cells were responsive to IGF-1 stimulation and were hence functioning appropriately. This study also evaluated if GH might regulate IGF-2 synthesis in *Morone*. In the second experiment, hepatocytes were incubated with 5 and 50 nM human recombinant leptin (National Hormone and Peptide Program, Torrance, CA) for 8 or 24 h to test for effects on hepatic GHR1, GHR2, IGF-1 and IGF-2 gene expression. A third culture experiment was conducted to evaluate if leptin and GH might have a synergistic effect in regulating IGF gene expression. Leptin and GH were administered at half the highest concentration used in previous experiments (25 nM each) to avoid inducing a maximal threshold of IGF gene expression by the combined hormonal treatments, which could mask potential synergistic or additive effects.

Livers from fish (170–200 g) were used for each *in vitro* experiment in order to yield enough cells for up to six replicates per group (N = 4–6 wells). Hepatocytes were harvested as described by Mommsen et al. (1994). In short, after fish were lethally dosed with buffered MS222, the posterior intestinal vein was cannulated and the liver flushed of blood with calcium-free Hank's buffered salt solution (HBSS) for 5 min using a peristaltic pump (2 mL/min), then digested *in situ* with HBSS containing type IV collagenase (Sigma, St. Louis, MO) for 10–15 min. The bulbus of the heart was nicked to alleviate fluid backpressure as solutions were