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Expression of leptin receptor gene in developing and adult zebrafish

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ABSTRACT

Interactions of leptin and leptin receptors play crucial roles during animal development and regulation of appetite and energy balance. In this study we analyzed expression pattern of a zebrafish leptin receptor gene in both developing and adult zebrafish using in situ hybridization and Q-PCR methods. Zebrafish leptin receptor message (*lepr*) was detected in all embryonic and larval stages examined, and in adult zebrafish. In embryonic zebrafish, *lepr* was mainly expressed in the notochord. As development proceeded, *lepr* expression in the notochord decreased, while its expression in several other tissues, including the trunk muscles and gut, became evident. In both larval and adult brains, large *lepr* expressing cells were detected in similar regions of the hindbrain. In adult zebrafish, *lepr* expression was also observed in several other brain regions including the hypothalamic lateral tuberal nucleus, the fish homolog of the arcuate nucleus. Q-PCR experiments confirmed *lepr* expression in the adult fish brain, and also showed *lepr* expression in several adult tissues including liver, muscle and gonads. Our results showed that *lepr* expression was both spatially and temporally regulated.

1. Introduction

Leptin is a small (16 kDa) protein hormone, whose discovery in 1994 by Jeff Friedman's laboratory (Zhang et al., 1994) led to intense research into its properties and physiological action (>25,000 reports to date; Friedman, 2009). Although initially characterized exclusively as a product of adipose tissue, more recent investigations document leptin expression in stomach (Bado et al., 1998), placenta (Sagawa et al., 2002), brain, and pituitary (Wilkinson et al., 2007). Similarly, the initial emphasis on leptin's physiological effects focused upon its influence on metabolic rate and mobilization of fat stores. We now know that leptin (in mammals) is pleiotropic, exerting effects on reproduction, immune function, capillary growth, and bone remodeling (see Friedman, 2009 for review). Ahima and Flier recognized this pleiotropy relatively early in leptin's history, and called for an evolutionary approach to unraveling leptin's many functions/effects (Ahima and Flier, 2000). They advanced the hypothesis that leptin signaling evolved as a sensitive indicator of starvation; leptin signaling mediates decreased activity of energetically demanding pathways (e.g., reproduction and immunity) and thus increases the chance of surviving the starvation event.

This compelling idea was extended by comparative endocrinologists investigating leptin signaling in lower vertebrates; such a signaling system would have obvious selective benefits. The search for non-mammalian leptin orthologs was difficult, however, with the first accepted non-mammal leptin sequence published 11 years after leptin was cloned in mice (Kurokawa et al., 2005). Kurokawa's group identified pufferfish leptin via gene synteny, which revealed that leptin sequence is poorly conserved among vertebrates (11–30% amino acid conservation between non-mammals and mammals; Londraville and Niewiarowski, 2010) although with apparently strong conservation of tertiary structure. In contrast, the leptin receptor was more tractable, with the first non-mammalian receptor (chicken) published in 2000 (Dunn et al., 2000; Horev et al., 2000), although no chicken leptin sequence has been identified in the (largely solved) chicken genome (Sharp et al., 2008). To date there are several verified leptin sequences from non-mammals, including amphibians *Xenopus* (Crespi and Denver, 2006), and several species of fish (Fugu; Kurokawa et al., 2005, carp; Huising et al., 2006, Medaka; Kurokawa and Murashita, 2009, and rainbow trout; Murashita et al., 2008). For the leptin receptor, several sequences have been cloned in birds (Horev et al., 2000), amphibians (Crespi and Denver, 2006), and fishes (Kurokawa et al., 2008; Kurokawa and Murashita, 2009). A notable gap in our knowledge of leptin evolution is that there are no reptilian sequences for either leptin or its receptor, although reptiles do respond to injections of mammalian leptin (Niewiarowski et al., 2000) and anti-mammal leptin antibodies recognize 'leptins' in reptiles (Paolucci et al., 2001; Spanovich et al., 2006).

Although there has been considerable progress on the molecular evolution of leptin and the leptin receptor, little physiological

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data exist on the expression or function of native leptins in non-mammals. Wong et al. (2007) characterized expression of leptin receptor in adult marine medaka (*Oryzias melastigma*) and its response to hypoxia, but their study did not include developmental studies. Most physiologically oriented leptin studies in fish document response to a mammalian leptin (Londraville and Duvall, 2002; Volkoff et al., 2003), with only one study (to date) that investigates the effects of native leptin in fish (Murashita et al., 2008). The only study that addresses leptin's effects on developing and adult lower vertebrates is Crespi and Denver's seminal work on *Xenopus* (2006). It is our long-term goal to characterize the physiology of both leptin and its receptor in zebrafish (*Danio rerio*), to elucidate its roles in both development and adults. In this study, we used Q-PCR and in situ hybridization to describe tissue level-expression of zebrafish leptin receptor. The expression pattern is strikingly similar to that described for mammalian leptin receptors and for medaka, which both supports our contention that this is a long-form leptin receptor ortholog, and that the zebrafish model system is ideally suited for the study of how leptin function evolved.

2. Materials and methods

2.1. Animals

Zebrafish embryos were obtained from in house breeding, and maintained as described in the Zebrafish Book (Westerfield, 2005). Adult zebrafish were raised from embryos obtained from in house breeding. Embryos for whole mount in situ hybridization were raised in PTU (1-phenyl-2-thiourea, 0.003%) treated fish tank water, while embryos for in situ hybridization on tissue sections were raised in regular fish tank water, both at 28.5 °C. Ages of the embryos or larvae are given as hours post-fertilization (hpf) or days post-fertilization. All animal-related procedures were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Akron Committee on Use and Care of Animals.

2.2. Tissue preparation

Animals were anesthetized in 0.02% MS-222. Adult fish were killed by cervical transection. The brain of adult fish or whole embryos and larvae were fixed for 2 h at room temperature or overnight at 4 °C, in phosphate-buffered 4% paraformaldehyde (pH 7.4). Embryos for whole mount in situ hybridization were washed in 0.1 M phosphate-buffered saline (PBS, pH 7.4), placed in increasing concentrations of methanol and stored in 100% methanol at −20 °C till use. Whole larval fish or adult brains were rinsed in PBS and prepared for cryosections (15 μm) as described previously (Barthel and Raymond, 1990).

2.3. Probe synthesis and in situ hybridization

To obtain a zebrafish *lepr* cDNA fragment as a template for synthesizing in situ hybridization cRNA probes, RT-PCR was performed using zebrafish *lepr* specific primers (forward primer 1, 5′-GGTCTCACTGCCTGTCCATT-3′; reverse primer 1, 5′-AGATGGTGCTGCTCCACT-3′) and total RNA from zebrafish 20–50 hpf embryos. The resulting DNA fragment, corresponding to nucleotides 2565–3303 of the published zebrafish *lepr* sequence (GenBank Accession Number: NM_001113376), was cloned into the pCRII-TOPO vector (Invitrogen), and was verified by restriction enzymes digestion, a PCR experiment using a pair of zebrafish *lepr* specific primers that were internal to the last set of primers (forward primer 2, 5′-GACGAAGGCAACTTCTCTGC-3′; reverse primer 2, 5′-TTCTTTCTCCTCTCCGGTCA-3′), and sequencing.

Detailed procedures for digoxigenin-labeled cRNA probe synthesis, whole mount in situ hybridization, and in situ hybridization on tissue sections were described previously (Liu et al., 1999). To verify the specificity of the above *lepr* cRNA probe, we also performed in situ hybridization using a shorter cRNA probe (transcribed from a *lepr* cDNA fragment corresponding to nucleotides 2565–3168 of the published zebrafish *lepr* sequence). Staining patterns in both developing and adult tissues were identical. Moreover, there was no staining in zebrafish embryos from 21 to 72 hpf using sense *lepr* probes (data not shown).

2.4. Q-PCR experiments

Total RNA was isolated from adult tissues or developing embryos at 0, 5–6, 12, 24, 36, 48, 72 hpf intervals using a column based extraction (EZRNA, Omega Biotech). Approximately 20–50 embryos were pooled and frozen at −80 °C for each stage; adult tissues were pooled from two individuals (fresh tissue, immediately extracted). Samples were homogenized in a bead mill to avoid cross-contamination. RNA samples were digested with DNAse during extraction to reduce possible genomic DNA contamination. cDNA was synthesized from total RNA using a high efficiency reverse transcriptase (Applied Biosystems) primed with random hexamers. cDNA was quantified with a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies). Duplicate reactions without reverse transcriptase were performed for negative control-templates for quantitative PCR. Q-PCR analysis of *lepr* temporal and tissue expression profiles was performed using *lepr* primers (forward primer 5′-CTCCAGTGACGAAGGCAACTT-3′; reverse primer 5′-GGGAAGGAGCCGGAAATGT-3′), and primers for zebrafish ribosomal protein L13A (60s) as a reference gene (forward primer 5′-TCTGGAGGACTGTAAGAGGTATGC-3′; reverse primer 5′-AGACGCACAATCTTGAGAGCAG-3′) as in Tang et al. (2007). L13A is a validated control gene for zebrafish, showing no significant change in expression among tissues or during early development (Tang et al., 2007). cDNAs (100 ng/reaction) were amplified and quantified with SYBR green master mix (Sigma) on an Applied Biosystems 7300 (ABI).

3. Results

Alignment of several leptin receptor sequences indicate that the zebrafish receptor is relatively divergent from other vertebrate receptors (~20% primary sequence identity), and is most similar to other fish (32%; Fig. 1 alignment and identity table). Regions of the sequence contain blocks of sequence that are highly conserved, including several blocks within the putative leptin binding region identified by Kurokawa et al. (2008) and Kurokawa and Murashita (2009) (residues 387–592 on the *Takifugu* sequence).

3.1. Q-PCR analysis of lepr expression in embryonic and adult zebrafish

We measured relative *lepr* expression in embryonic and adult zebrafish using quantitative PCR. *lepr* transcripts were detected in all the stages examined, but their expression levels varied during development and among adult tissues. *lepr* transcripts were weakly expressed by young embryos (0–12 hpf), increased expression in 24–36 hpf embryos, followed by a decrease in expression at 48 hpf and an increase at 72 hpf (Fig. 2A. In adult zebrafish, *lepr* transcripts were detected in all the tissues examined, with strongest expression in liver, muscle, gill, and testes (Fig. 2B).

3.2. In situ hybridization analysis of lepr expression

To determine spatial and temporal expression pattern of *lepr* in embryonic and larval zebrafish, and distribution of *lepr* in adult