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Short communication

Leptin: Clue to poor appetite in oxygen-starved fish

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ABSTRACT

Hypoxia is the most widespread deleterious consequence of eutrophication and has become a major cause of fishery decline. One feature of chronic exposure to hypoxia in fish is inhibition of feeding. In this study, we investigated if the gene that encodes the appetite-suppressing hormone leptin is regulated by hypoxia in zebrafish (*Danio rerio*). Exposure of adult zebrafish to hypoxic conditions (1 ± 0.2 mg O_2 L^{-1}) for 4 and 10 days significantly increased leptin-a (*zlep-a*) mRNA levels in the liver. To evaluate the role of hypoxia-inducible factor 1 (HIF-1) in regulating *zlep-a* expression, zebrafish embryos were exposed to cobalt chloride ($CoCl_2$, a HIF-1 inducer) and overexpressed with HIF-1α mRNA. Both $CoCl_2$ treatment and HIF-1α overexpression markedly increased *zlep-a* expression in developing embryos, indicating the possible involvement of HIF-1 in *zlep-a* regulation. *In vivo* promoter analysis indicated that *zlep-a* promoter activity is found in the muscle fibers of zebrafish embryos and enhanced by $CoCl_2$. This is the first report to show that leptin gene expression in fish is regulated by hypoxia possibly via the involvement of HIF-1.

Hypoxia (<2 mg O_2 L^{-1}) arising as a result of euthrophication affects vast stretches of aquatic ecosystems worldwide and often leads to population decline and changes in community structure by eliminating oxygen-sensitive species (Wu, 2002). Because of increased nutrient inputs and climate change, the severity and global distribution of aquatic hypoxia have increased in the last few decades, and this problem is likely to be exacerbated in the coming years (Wu, 2002; Diaz and Rosenberg, 2008). Periods of hypoxia also occur naturally in non-eutrophic waters (Nikinmaa and Rees, 2005). To survive regular episodes of low dissolved oxygen, fish have evolved a number of behavioral responses such as inhibition of feeding and movement to lower temperatures to conserve energy and reduce oxygen consumption. Although inhibition of feeding reduces the demand for energy and oxygen, it is a major cause of growth impairment in hypoxic fish. The appetite-suppressing effects of hypoxia in fish are well documented (Pichavant et al., 2001; Zhou et al., 2001; Chabot and Claireaux, 2008), however, little is known so far about the molecular mechanisms involved, in particular those mediated by the oxygen-sensitive transcription factor hypoxia-inducible factor 1 (HIF-1). Understanding these mechanisms and the associated gene responses may provide the basis for developing sensitive molecular biomarkers for monitoring aquatic hypoxia.

The anti-obesity hormone leptin is well known for its function in controlling food intake in mammals (Wynne et al., 2005). This peptide hormone is synthesized primarily by adipocytes (Zhang et al., 1994) and circulates in the blood at a concentration proportional to fat mass content (Maffei et al., 1995). The binding of leptin to its receptor in the hypothalamus suppresses food intake through inhibition of orexigenic neuropeptide Y (NYP) neurons and stimulation of anorexigenic proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neurons (Meister, 2007). In teleosts, there are at least two duplicate forms of leptin, namely leptin-a and leptin-b, which are differentially expressed in different organs (Gorissen et al., 2009). In the liver (a major fat reserve in some fish), leptin-a is strongly expressed (Kurokawa et al., 2005; Huising et al., 2006; Gorissen et al., 2009), whereas leptin-b is expressed at much lower levels (Gorissen et al., 2009). Recently, a possible role of leptin-a in food intake regulation has been demonstrated in rainbow trout (*Oncorhynchus mykiss*); intraperitoneal injection of recombinant trout leptin-a in the fish led to suppressed food intake, together with transient reduction and elevation of NPY and POMC mRNA levels, respectively (Murashita et al., 2008). Previously, hypoxia has been shown to increase leptin expression and secretion in mammalian systems (Snyder et al., 2008; Wang et al., 2008), and the involvement of HIF-1 in these responses has been suggested (Cascio et al., 2008). Although a similar HIF-1 pathway also exists in fish (Nikinmaa and Rees, 2005; Kajimura et al., 2006), no published information is available to date on the regulation of fish leptin gene expression by hypoxia. In this study, the effects of hypoxia and HIF-1 on leptin gene expression were examined in the livers of adult zebrafish (*Danio rerio*) and their embryos,

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respectively. Our results indicate that zebrafish leptin-a (*zlep-a*) is a hypoxia-inducible gene that may be stimulated through HIF-1 interaction.

Adult wild-type zebrafish (ca. 6-month old) were maintained on a 14:10 h light:dark cycle at 28.5 °C and fed twice daily. Exposure experiments were conducted using a normoxic (5.8 ± 0.2 mg O_2 L^{-1}) system and a hypoxic (1 ± 0.2 mg O_2 L^{-1}) system as previously described (Shang et al., 2006). To minimize ammonia buildup in fish tanks, 50% of tank water (hypoxic water was pre-mixed with nitrogen to reach 1 mg O_2 L^{-1}) was changed daily during the whole course of exposure. No significant difference in fish mortality was found between the normoxic and hypoxic groups. After 4 and 10 days of exposure, fish ($n = 10$) were sampled at random and the liver was dissected, snap-frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

To investigate the role of HIF-1 in the regulation of *zlep-a* expression, developing zebrafish embryos were exposed to cobalt chloride ($CoCl_2$, a HIF-1 inducer) as well as subjected to HIF-1 overexpression and *in vivo* analysis of the *zlep-a* promoter. Embryos were obtained by natural spawning and cultured in petri dishes containing E3 medium at 28.5 °C. At 24 h post-fertilization (hpf), embryos were transferred to 10 mM $CoCl_2$ or E3 medium alone (control) and incubated further for 24 and 48 h until RNA extraction. No $CoCl_2$-induced developmental toxicity was found at the end of the exposure periods. In the HIF-1 overexpression experiment, *in vitro* synthesis of zebrafish HIF-1α mRNA (GenBank accession no. AY326951) and microinjection were conducted according to Kajimura et al. (2006). Changes in *zlep-a* mRNA levels were measured at 12 hpf using real-time PCR. The *in vivo* promoter activity of *zlep-a* was examined under the effect of $CoCl_2$. Briefly, an EGFP construct driven by a 2-kb 5′-flanking region of *zlep-a* (GenBank accession no. BX927136) was generated and injected into embryos at the 1-cell stage. Until 48 hpf, injected embryos were transferred to 10 mM $CoCl_2$ or E3 medium alone (control) and further incubated for 24 h. At the end of the exposure, embryos (72 hpf) were observed for GFP signals using fluorescence microscopy and GFP transcripts were quantified by real-time PCR. RNA extraction and real-time PCR for measuring *zlep-a* and GFP mRNA levels were performed as previously described (Yu et al., 2008). Sequences of primer sets for real-time PCR were as follows: *zlep-a*, 5′-CAGGGAACACATTGACGGGCA-3′ and 5′-ATGGAGCCGAGCCCTTGGATG-3′; GFP, 5′-GACAACCACTACCTGAGCAC-3′ and 5′-ACTTGTACAGCTCGTCCATG-3′; *igfbp-1*, 5′-GTCAATGAAGGCAGCTCCAC-3′ and 5′-TCTTGCGTATCGCGTTGACT-3′; 18S rRNA (normalizer), 5′-GGACACGGAAAGGATTGACAG-3′ and 5′-CCGGAGTCTCGTTCGTTATCG-3′; and β-actin (normalizer), 5′-CGAGCAGGAGATGGGAACC-3′ and 5′-CAACGGAAACGCTCATTAC-3′.

The effects of hypoxia on *zlep-a* expression in the adult zebrafish liver were assessed by real-time PCR upon exposure at 1 mg O_2 L^{-1} for 4 and 10 days. The rationale for studying the liver in particular was based on the previous findings that this organ is the major site of *lep-a* expression in zebrafish (Gorissen et al., 2009) and other fish species (Kurokawa et al., 2005; Huising et al., 2006; Murashita et al., 2008) and *lep-a* expression was shown to increase in the liver after feeding in common carp (*Cyprinus carpio*) (Huising et al., 2006). In the present study, *zlep-a* mRNA levels in the adult zebrafish liver were significantly increased by ca. 7.5- and 5-fold after 4 and 10 days of exposure to hypoxia, respectively (Fig. 1). Likewise, hepatic expression of *igfbp-1* (a proven HIF-1-regulated gene; Kajimura et al., 2006) was also significantly elevated by 5.5- and 6.5-fold ($p < 0.05$; data not shown) after 4 and 10 days of exposure to hypoxia, respectively, supporting the activation of the HIF-1 pathway under our hypoxic conditions. Although *zlep-a* is also expressed in other adult tissues (moderate-to-high expression: brain, eye, gill, gut, ovary and testis; low expression: head kidney, heart, muscle and spleen; unpublished data), hypoxia-induced expression of *zlep-a* was exclusively detected in the liver but not in the rest of the tissues. In the whole embryos, treatment with $CoCl_2$ (which mimics hypoxic states by inducing HIF-1α levels) significantly increased *zlep-a* mRNA levels by ca. 8.5- and 11-fold after 24 and 48 h, respectively (Fig. 2A). Moreover, overexpression of HIF-1α mRNA in normoxic embryos increased *zlep-a* mRNA levels by ca. 2.5-fold (Fig. 2B), providing a clue that HIF-1 may have a regulatory function in *zlep-a* transcription. We failed to localize *zlep-a* mRNA in the developing embryos (24–72 hpf) using whole-mount in situ hybridization (WISH), possibly because of low expression levels of *zlep-a* during embryogenesis. Instead, we monitored the *zlep-a* promoter activity *in vivo* with an injected *zlep-a* promoter-driven EGFP construct. Fluorescence microscopy revealed that *zlep-a* promoter activity (indicated by mosaic GFP expression) was localized only in muscle fibers (Fig. 3A) and markedly increased (indicated by a 3-fold increase in GFP transcript levels; Fig. 3B) following a 24-h period of exposure to $CoCl_2$.

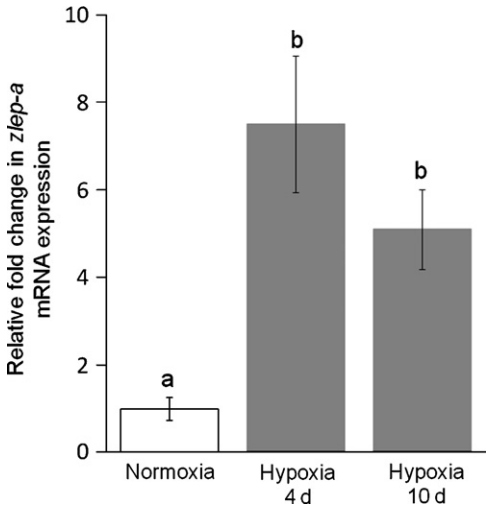

Fig. 1. Hypoxic induction of *zlep-a* expression in the adult zebrafish liver. Fish were maintained in normoxic (5.8 ± 0.2 mg O_2 L^{-1}) or hypoxic (1 ± 0.2 mg O_2 L^{-1}) conditions for 4 and 10 days. Expression of *zlep-a* mRNA was quantified using real-time PCR and normalized against 18S rRNA and β-actin. Data are the mean relative fold changes ± SE ($n = 10$) with respect to the control (normoxia) level (arbitrarily set to 1). Letters above the bars indicate significant differences between the indicated groups ($p < 0.05$; one-way ANOVA followed by Tukey's test).

To our knowledge, this is the first report to show that leptin gene expression in fish is regulated by hypoxia possibly via the involvement of HIF-1. The direct involvement of fish HIF-1 in oxygen-dependent gene expression was first described in zebrafish embryos, where HIF-1 was shown to selectively interact with a functional hypoxia-responsive element (HRE; ACGTG) in *igfbp-1* (Kajimura et al., 2006). Although this zebrafish HRE sequence is not present in the immediate 5′-flanking region (2 kb) of *zlep-a*, a consensus core HRE (A/GCGTG; Wenger et al., 2005) and a novel HRE (GATGTG) recently characterized in the killifish (*Fundulus heteroclitus*) lactate dehydrogenase gene-B gene (Rees et al., 2009), were identified 1156 and 531 bp upstream of the ATG codon, respectively. Further analyses such as cell transfection, EMSA and CHIPS are required to elucidate the functional role of these putative HREs in hypoxia-induced transactivation of the *zlep-a* promoter. Despite demonstrating the hypoxia-induced responses of *zlep-a* in the adult zebrafish livers (Fig. 1) and embryo muscle fibers (Fig. 3), whether this differential gene expression changes blood serum leptin levels and thereby affects food intake remains elusive. In mice, exposure to short-time intermittent hypoxia has been shown to increase serum leptin levels (Polotsky et al., 2003). Interestingly, obstructive sleep apnoea, a syndrome that leads to recurrent intermittent