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The effect of GH overexpression on GHR and IGF-I gene regulation in different genotypes of GH-transgenic zebrafish

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Abstract

Most biological actions of growth hormone (GH) are mediated by the insulin-like growth factor I (IGF-I) that is produced after the interaction of the hormone with a specific cell surface receptor, the GH receptor (GHR). Even though the GH excess on fish metabolism is poorly known, several species have been genetically engineered for this hormone in order to improve growth for aquaculture. In some GH-transgenic fish growth has been dramatically increased, while in others high levels of transgene expression have shown inhibition of the growth response. In this study, we used for the first time different genotypes (hemizygous and homozygous) of a GH-transgenic zebrafish (*Danio rerio*) lineage as a model for studying the GH resistance induced by different GH transgene expression levels. The results obtained here demonstrated that homozygous fish did not grow as expected and have a lower condition factor, which implies a catabolic state. These findings are explained by a decreased IGF-I and GHR gene expression as a consequence of GH resistance. Together, our results demonstrated that homozygous GH-transgenic fish showed similar characteristics to the starvation-induced fish and could be an interesting model for studying the regulation of the GH/GHR/IGF-I axis in fish.

Keywords: Homozygous GH-transgenic fish; Growth hormone receptor; Insulin-like growth factor-I; GH resistance

1. Introduction

Growth is a multifactorial characteristic resulting from complex genetic and molecular interactions in which the growth hormone (GH) plays a major role in all vertebrates. Besides its essential function as a growth regulator, GH is also involved in several other physiological processes which affect lipid, carbohydrate, and protein metabolism (Davidson, 1987; Moller and Norrelund, 2003), immune system maintenance (Jeay et al., 2002), as well as stress response and behavior (Yoshizato et al., 1998). These pleiotropic effects of GH are usually mediated in an indirect manner by the insulin-like growth factor I (IGF-I), which is produced in the liver and other tissues in response to GH stimulation.

The biological actions of GH are regulated mainly at the somatotrophs in the anterior pituitary gland (synthesis and secretion), and on the target organs through its interaction with a specific cell surface receptor, the GH receptor (GHR). Thus, the GHR synthesis regulates the GH sensibility which is dependent on biological and environmental factors (Flores-Morales et al., 2006). One of these biological factors that can affect GHR expression is the GH itself. However, controversial results have been reported concerning the regulation of GHR by GH. According to Schwartzbauer and Menon (1998), these discrepancies may be because the effects of GH on GHR expression seem to be dependent on the time of exposure, whether the experiments are conducted *in vivo* or *in vitro*, and the cell type under investigation. *In vivo* studies carried out in mice and rat models have showed down-regulation of GHR induced by acute GH treatment (Maiter et al., 1988; Iida et al., 2004).

In teleost fish, as in other vertebrates, growth is regulated by the GH/IGF axis. Even though the effect of GH excess on fish metabolism is poorly known, several species have been genetically engineered for this hormone in order to improve

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growth for aquaculture (Zbikowska, 2003). Indeed, a few species of GH-transgenic fish have demonstrated extraordinary growth (Devlin et al., 1994; Nam et al., 2001). However, other GH-transgenic fish lineages with high levels of transgene expression have showed inhibition of the growth response (Zhang et al., 1990; Hernandez et al., 1997). In addition, De la Fuente et al. (1999) reported that transgenic tilapia with low ectopic GH expression levels resulted in improved growth performance. Additionally, comparisons of hemizygous and homozygous transgenic tilapia have demonstrated that there is not a direct correlation between GH circulating levels and growth performance (Rahman et al., 1998; Martínez et al., 1999). These observations indicate that there seems to be an optimal GH dose for maximum growth stimulation. In the present study, we have used for the first time different genotypes (hemizygous and homozygous) of a GH-transgenic zebrafish (*Danio rerio*) lineage as a model for studying the GH resistance induced by different GH transgene expression levels.

2. Materials and methods

2.1. Generation of hemizygous and homozygous GH-transgenic zebrafish

The GH-transgenic zebrafish (*Danio rerio*, Cypriniformes, Cyprinidae) used in this study were from the F0104 lineage (Figueiredo et al., 2007), which harbour a transgene comprised by the GH cDNA from marine silverside fish (*Odontesthes argentinensis*) under the transcriptional control of carp β-actin promoter (Marins et al., 2002). Additionally, this transgenic lineage carries a reporter transgene, which is constituted by the same carp β-actin promoter driving the green fluorescent protein (GFP) gene expression as a transgenesis label. Hemizygous transgenic and non-transgenic sibling fish were produced by crossing hemizygous males from the F0104 lineage with wild type females. The offspring were classified in groups using an epifluorescence microscope (excitation 485 nm, emission 520 nm). Homozygous transgenic fish were produced by crossing homozygous animals of the F0104 lineage.

2.2. Rearing and growth analysis

Transgenic and non-transgenic fish were reared until 6 months of age in a closed circulation water system composed of 25-L tanks. Each genetic group (homozygous, hemizygous and non-transgenic siblings) was replicated three times, with twenty fish each. Water quality was monitored at least once a day, and temperature, pH, nitrogenous compounds and photoperiod were maintained according to zebrafish requirements (Westerfield, 1995). Fish were fed with high-protein commercial food (47.5%), 5% of body weight/day. In all situations food was completely consumed within 10 min. After the first month, fish were anesthetized (Tricaine, 0.1 mg/mL) and weighed every 2 weeks in order to maintain the percentage of food by biomass in each tank. At the end of the experiment, the mean weight was compared among the three test groups. In addition, the total length of individual fish was also measured and the condition factor (K) was calculated using the formula $K = (W \cdot L^{-3}) \times 10^3$, where W is mass in milligrams and L is total length in millimetres.

2.3. RNA isolation and RT–PCR

At the end of the growth experiment, nine animals of each class were randomly sampled for confirmation of transgene cβA/msGH presence and evaluation of IGF-I, GHR and endogenous GH gene expression. These animals were sacrificed for the removal of the pituitary and liver, for posterior extraction of total RNA using Trizol reagent (Invitrogen, Brazil), according to manufacturer's instructions. For the liver, approximately 4 μg RNA of each animal was used as a template for the RT–PCR, with primer AP (5′-GGCCACGCGTCGACTAGTAC (T)17-3′, Invitrogen). Synthesis of complementary DNA (cDNA) was accomplished with RT SuperScript III enzyme (Invitrogen), according to the protocol suggested by the manufacturer.

The obtained cDNA was used as a template for the msGH gene amplification using specific primers EXO 293 (5′-GAAAGCTCTCTGCAGACGGAG-3′) and GHEX6-RIG (5′-AGAGTGCAGTTTGCCTCTGG-3′), which produce a 467-bp msGH fragment but do not amplify the endogenous zebrafish growth hormone gene. PCR was carried out in a 25 μL reaction volume containing 2.5 μL of 10× PCR buffer, 0.2 μM of each primer, 0.2 mM of each dNTP, 0.75 mM of $MgCl_2$, 0.5 unit of Platinum *Taq* DNA polymerase (Invitrogen) and 1 μL of cDNA solution. The reaction was incubated at 94 °C for 2 min followed by 25 cycles of 15 s at 94 °C, 30 s at 65 °C and 30 s at 72 °C, with a final elongation step of 5 min at 72 °C.

A nested PCR strategy was necessary for the amplification of the IGF-I gene. The first-step used a more external reverse primer, while the second one used a more internal reverse primer. The primers were designed using zebrafish IGF-I cDNA sequence, available in GenBank (AF268051). For the first PCR, primers IGF-IF (5′-GGCATTGGTGTGATGTCTT-3′) and AUAP (5′-GGCCACGCGTCGACTAGTAC-3′, Invitrogen, Brazil) were used. PCR was carried out in a 25 μL reaction volume containing 2.5 μL of 10× PCR buffer, 0.2 μM of each primer, 0.2 mM of each dNTP, 0.75 mM of $MgCl_2$, 0.5 unit of Platinum *Taq* DNA polymerase (Invitrogen) and 1 μL of cDNA solution. The reaction was incubated at 94 °C for 1 min followed by 28 cycles of 30 s at 94 °C, 1 min and 30 s at 50 °C and 30 s at 72 °C, with a final elongation step of 5 min at 72 °C. In the second PCR (nested), the primers IGF-IF and IGF-IR (5′-GTGTGTCGTTGTGCTCGTA-3′) were used, generating a fragment of 345 bp. PCR was carried out in a 25 μL reaction volume containing 2.5 μL of 10× PCR buffer, 0.2 μM of each primer, 0.2 mM of each dNTP, 0.75 mM of $MgCl_2$, 0.5 unit of Platinum *Taq* DNA polymerase and 1 μL of the first PCR reaction. The reaction was incubated at 94 °C for 1 min followed by 30 cycles of 30 s at 94 °C, 1 min and 30 s at 50 °C and 30 s at 72 °C, with a final elongation step of 5 min at 72 °C.

The GHR gene was amplified using the specific primers ZFGHR-FOR (5′-CTGGTTTCCAGAAGCAGCTC-3′) and ZFGHR-REV (5′-ACACCTTGAGTTGGGAATGC-3′),

which were designed using a zebrafish GHR cDNA clone sequence, available in GenBank (CR352327). PCR was carried out in a 25 μL reaction volume containing 2.5 μL of 10× PCR buffer, 0.2 μM of each primer, 0.2 mM of each dNTP, 0.75 mM of $MgCl_2$, 0.5 unit of Platinum *Taq* DNA polymerase and 1 μL of cDNA solution. The reaction was incubated at 94 °C for 2 min followed by 31 cycles of 15 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, with a final elongation step of 5 min at 72 °C. A fragment of 335 bp was amplified.

The endogenous GH gene expression was evaluated by using pituitary cDNAs. The pituitaries of three fish were used for total RNA extraction due to the low product recovery, which was noticed previously by using 0.5 μg of RNA as a template for the RT–PCR. The obtained cDNA was used as template for the endogenous GH gene amplification using the specific primers ZFGH-F (5′-CTGAGGAACGCAGACAGTTG-3′) and ZFGH-R (5′-TGAGACTGGTCTCCCCTACG-3′), designed using zebrafish GH cDNA sequence available in GenBank (AJ937858). PCR was carried out in a 25 μL reaction volume containing 2.5 μL of 10× PCR buffer, 0.2 μM of each primer, 0.2 mM of each dNTP, 0.75 mM of $MgCl_2$, 0.5 unit of Platinum *Taq* DNA polymerase and 1 μL of cDNA solution. The reaction was incubated at 94 °C for 2 min followed by 35 cycles of 15 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C, with a final elongation step of 5 min at 72 °C. A fragment of 357 bp was obtained.

The zebrafish β-actin gene was used for normalizing gene expression (endogenous GH, GHR and IGF-I) and transgene msGH. A 380-bp fragment of the β-actin gene was amplified with primers ZFBAC-FOR (5′-CCCTTGTTCACAATAACCT-3′) and ZFBACREV (5′-TCTGTTGGCTTTGGGATTCA-3′), having been designed by Pang and Ge (2002). PCR was carried out in a 25 μL reaction volume containing 2.5 μL of 10× PCR buffer, 0.2 μM of each primer, 0.2 mM of each dNTP, 0.75 mM of $MgCl_2$, 0.5 unit of Platinum *Taq* DNA polymerase and 1 μL of cDNA solution. The reaction was incubated at 94 °C for 1 min followed by 25 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C, with a final elongation step of 5 min at 72 °C.

PCR products were then analysed on 1% agarose gels stained with ethidium bromide (0.5 μg/mL), and amplified bands were visualized by ultraviolet transillumination. The gels were photographed for posterior analysis of band densitometry.

2.4. Statistical analysis

Data were subjected to an analysis of variance (ANOVA). Post-hoc comparisons were made by Tukey's test. Normality and variance homogeneity were previously checked and a significance level of 5% was adopted in every case. All statistical tests used fixed type I error of 5% ($\alpha = 0.05$).

3. Results and discussion

The present work evaluated the growth of transgenic (homozygous and hemizygous) and non-transgenic individuals and, furthermore, it evaluated the expression of main genes related to growth. The results found in this study showed that homozygous GH-transgenic zebrafish did not grow as expected. At the end of the growth experiment, transgenic hemizygous fishes reached a final average mass (414.8±22.8 mg) higher than the transgenic homozygous (266.4±15.3 mg) and the non-transgenic siblings (272.6±11.7 mg) (Fig. 1; $P<0.01$). At first glance, these findings might seem quite unexpected; however similar results have been observed by Martínez et al. (1999) working with the transgenic tilapia *Oreochromis hornorum* overexpressing an exogenous GH. In the same sense, Rahman et al. (1998) verified that homozygous and hemizygous GH-transgenic tilapia *Oreochromis niloticus* grow at the same rate.

Even though, in theory, homozygous GH-transgenic fish have twice as many transgenes as hemizygous fish, why then do these fish not grow proportionally? In order to be sure that homozygous fish used in the present study were really carrying more transgenes than hemizygous fish, we have analysed the exogenous GH gene expression through RT–PCR. Homozygous fish showed a gene expression 73.5% higher than the hemizygous fish, after normalization of data by β-actin gene expression (Fig. 2; $P<0.01$). However, this higher level of exogenous GH gene expression may not represent the production of a biologically active hormone. Since we were not able to measure the circulating GH in zebrafish, we have chosen to analyse the endogenous GH gene expression because the negative feedback system should down-regulate the endogenous GH gene expression in the case of high levels of biologically active circulating GH. Fig. 3 shows the results of the pituitary endogenous GH gene expression. Both transgenic fish were expressing only approximately one third of the expression observed for non-transgenic siblings ($P<0.01$). This result indicated that both transgenic fish genotypes are producing a high level of biologically active GH and that the endogenous GH in the transgenic individuals is regulated by the negative feedback probably due to the excess of exogenous GH. The same has been observed for GH-transgenic tilapia *O. niloticus* (Caelers et al., 2005).

Once we have demonstrated that homozygous and hemizygous GH-transgenic zebrafish are expressing different levels of the exogenous GH and the hormone excess is inducing a

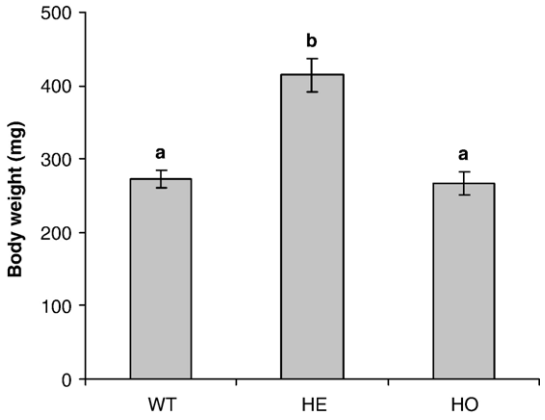

Fig. 1. Body mass average of 6-month-old zebrafish (*Danio rerio*). WT: wild type ($N=71$), HE: hemizygous ($N=57$), and HO: homozygous ($N=61$) GH-transgenic zebrafish. Different letters indicate significant differences ($P<0.01$) among fish groups.

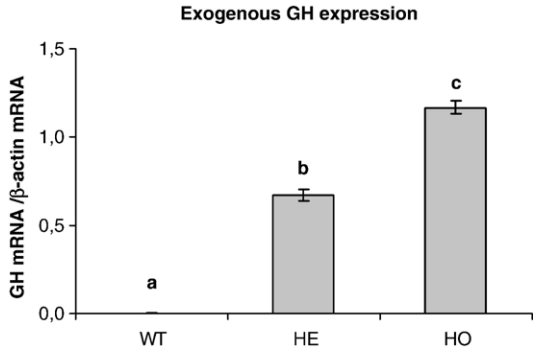

Fig. 2. Exogenous growth hormone (GH) gene expression in wild type (WT) and different genotypes of GH-transgenic zebrafish (HE: hemizygous; HO: homozygous). For all genotypes $N=7$. Different letters indicate significant differences ($P<0.01$) among fish groups.

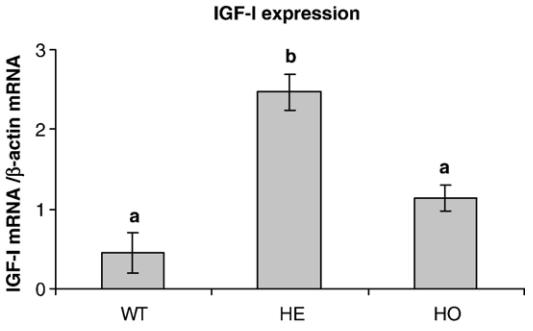

Fig. 4. Liver insulin-like growth factor-I (IGF-I) gene expression in wild type (WT) and different genotypes of GH-transgenic zebrafish (HE: hemizygous; HO: homozygous). For all genotypes $N=7$. Different letters indicate significant differences ($P<0.01$) among fish groups.

negative feedback over the endogenous GH expression, the question that arises is whether the GH level produced by homozygous fish is causing GH resistance and down-regulating the IGF-I expression since these fish did not grow at the expected rate. In order to address this issue, we have analysed the liver IGF-I gene expression in both genotypes of the transgenic fish comparing it with non-transgenic siblings. The results for IGF-I expression analysis showed that hemizygous fish are expressing this gene approximately five times higher than the controls, while homozygous fish are not statistically different from non-transgenic fish (Fig. 4; $P<0.01$). This finding provides an explanation for the result observed in the growth experiment when homozygous and non-transgenic fish grew at the same rate. Despite the fact that homozygous and non-transgenic probably have very different levels of circulating GH, both are expressing the same level of IGF-I. The unexpected low level of IGF-I expression in homozygous fish is corroborated by the GHR gene expression analysis. The results obtained for the GHR gene show a similar pattern between the expressions of both genes. Hemizygous fish demonstrated a significant increase in GHR transcripts compared to non-transgenic and homozygous fish (Fig. 5; $P<0.01$), while homozygous and non-transgenic fish showed the same expression level.

The results obtained here indicate that the GH excess does not represent a concomitant increase in the hormone biological actions. Even though the homozygous GH-transgenic zebrafish are probably producing high levels of circulating GH, the decreased level of GHR and IGF-I expression characterises the GH resistance effect. The nutritional status of the fish is probably the major environmental factor causing GH resistance (Duan, 1998; Moriyama et al., 2000; Thissen et al., 1999). Growth adjusts to nutritional conditions and during fasting plasma IGF-I decreases, whereas plasma GH generally increases. These seemingly paradoxical changes are explained by the development of liver GH resistance (Pierce et al., 2005). Indeed, studies on fasted fish such as black seabream (Deng et al., 2004) and catfish (Small et al., 2006) as well as in fasted rats (Thissen et al., 1999) have suggested that a diminished hepatic GHR gene expression could reduce plasma IGF-I and growth during the period of food deprivation (Fox et al., 2006). In fact, our homozygous GH-transgenic model resembles a starvation-induced non-transgenic fish. Plasma IGF-I levels of fasted tilapia (*Oreochromis mossambicus*) were correlated significantly with specific growth rate and condition factor, indicating that plasma IGF-I is a good indicator of growth (Uchida et al., 2003). The condition factor calculated for all zebrafish geno-

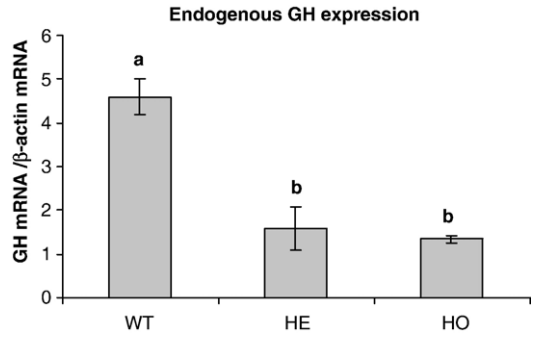

Fig. 3. Endogenous pituitary growth hormone (GH) gene expression in wild type (WT) and different genotypes of GH-transgenic zebrafish (HE: hemizygous; HO: homozygous). For all genotypes $N=3$ (each sample represents three pituitaries pooled). Different letters indicate significant differences ($P<0.01$) among fish groups.

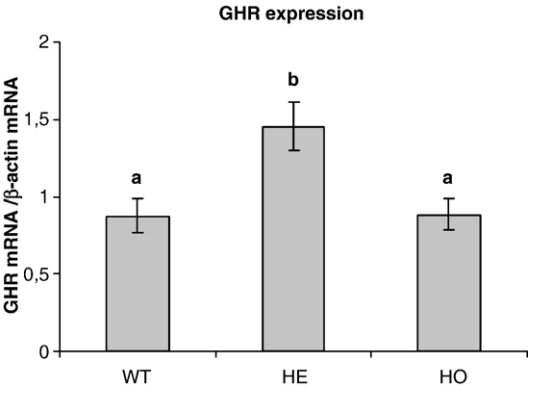

Fig. 5. Liver growth hormone receptor (GHR) gene expression in wild type (WT) and different genotypes of GH-transgenic zebrafish (HE: hemizygous; HO: homozygous). For all genotypes $N=7$. Different letters indicate significant differences ($P<0.01$) among fish groups.

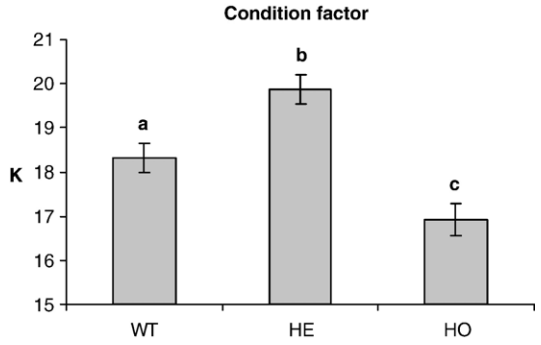

Fig. 6. Condition factor (K) in wild type (WT) and different genotypes of GH-transgenic zebrafish (HE: hemizygous; HO: homozygous) with 6 months of age. The number of fish used was WT=71, HE=57, and HO=61. Different letters indicate significant differences ($P<0.05$) among fish groups.

types used in the present study are shown in Fig. 6. The results demonstrate that homozygous fish have a decreased condition factor in comparison with the other two groups ($P<0.05$), which implies a catabolic state. It is probable that the homozygous GH-transgenic zebrafish have a higher metabolic demand than the other genotypes studied here and the quantity of ration offered (5% of body weight/day) was not sufficient to supply the energetic cost imposed by the GH excess.

In the present study, genetic analysis indicated that homozygous GH-transgenic zebrafish show a lower GHR and IGF-I gene expression when compared to hemizygous fish, although exhibiting a higher exogenous GH gene expression. These findings are corroborated by the results obtained from the growth experiments. Overall, these results demonstrate that homozygous fish have similar characteristics to the starvation-induced fish, and therefore could be used as a model for studying the regulation of the GH/GHR/IGF-I axis in fish.

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