Metabolic hormones modulate the effect of growth hormone (GH) on insulin-like growth factor-I (IGF-I) mRNA level in primary culture of salmon hepatocytes

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Abstract

Liver production of insulin-like growth factor-I (IGF-I) is a major point of control in the growth hormone (GH)/IGF axis, the endocrine system regulating body growth in fishes and other vertebrates. Pituitary GH stimulates hepatocyte production of IGF-I; however, in catabolic states, hepatocyte GH resistance results in decreases in liver IGF-I production. To investigate endocrine mechanisms leading to the development of hepatocyte GH resistance, we examined the regulation of IGF-I mRNA level by GH and metabolic hormones in primary culture of salmon hepatocytes. Cells were cultured in RPMI medium, and exposed to insulin (Ins, 10^{-6} M), glucagon (Glu, 10^{-6} M), triiodothyronine (T3, 10^{-7} M), dexamethasone (Dex, 10^{-6} M) and glucagon-like peptide (GLP, 10^{-6} M), in the presence and absence of GH (5 \times 10^{-9} M). GH always increased IGF-I mRNA. None of the other hormones tested alone affected IGF-I mRNA. However, Dex, Ins and Glu reduced the response to GH. The response to GH was inhibited by Dex at concentrations of 10^{-12} M and above, by Ins at 10^{-9} M and above, and by Glu only at 10^{-6} M. Inhibition of GH response by glucocorticoids is found in other vertebrates. Salmon hepatocytes were very sensitive to Dex, suggesting that glucocorticoids may play an important role in salmon growth regulation even in unstressed conditions. Inhibition of GH response by Ins is the opposite of what is found in mammals and chickens, suggesting that the role of Ins in growth regulation may differ between fishes and tetrapods.

To examine mechanisms for modulation of GH sensitivity, we measured hepatocyte GH receptor (GHR) mRNA, suggesting that different mechanisms mediate the inhibition of GH response by these hormones. This study shows that glucocorticoids, Ins, and Glu induce GH resistance in cultured salmon hepatocytes.

Introduction

The growth hormone (GH)/insulin-like growth factor (IGF) axis is the primary endocrine system that regulates body growth in vertebrates (reviewed in Rosenfeld & Roberts 1999). The principal hormones in the GH/IGF axis are pituitary GH and liver-derived endocrine insulin-like growth factor-I (IGF-I). IGF-I circulates bound to a number of IGF-binding proteins (IGFBPs), which modulate its growth-stimulatory effects. GH stimulates the production of IGF-I by hepatocytes. During fasting, liver IGF-I mRNA and circulating IGF-I levels decline in all vertebrate species studied, whereas plasma GH increases in most species. This apparent paradox is explained by the development of hepatocyte GH resistance (Thissen et al. 1994, 1999). Hepatocyte IGF-I production becomes less sensitive to stimulation by GH, an effect which more than counteracts the increase in GH during fasting. Hepatocyte GH resistance appears to be a common outcome of catabolic states brought about by a variety of causes, and has been found in diverse species (Thissen et al. 1999). IGF-I mRNA expression in nonhepatic tissues may also be regulated by GH; however, most circulating IGF-I is of hepatic origin.

The endocrine and cellular mechanisms that lead to hepatocyte GH resistance are not completely understood. Primary hepatocyte culture studies in mammals and birds have shown that pancreatic, stress and thyroid hormones can both regulate IGF-I production independently of GH, and modulate the effect of GH on IGF-I production. Insulin, thyroid hormones and low concentrations of glucocorticoids potentiate GH-stimulated IGF-I mRNA expression, whereas glucagon and high concentrations of glucocorticoids inhibit basal or GH-stimulated IGF-I

The components and operation of the GH/IGF axis in teleost fishes are similar to what is found in other vertebrates (Nicoll et al. 1999, Perez-Sanchez & Le Bail 1999, Mommsen 2001). GH injection increases liver IGF-I mRNA and plasma IGF-I levels in numerous fish species. As in mammals, hepatic GH resistance occurs in a variety of catabolic states in fishes (Perez-Sanchez & Le Bail 1999). Liver IGF-I mRNA and circulating IGF-I levels were reduced in spite of increased GH in coho salmon (Oncorhynchus kisutch) suffering from growth arrest (‘stunting’) due to premature transfer to saltwater (Duan et al. 1995). Liver membrane GH binding was reduced in stunted salmon (Gray et al. 1992), hypophysectomized gobies (Gillychthys mitabilis) (Gray & Kelley 1991) and nutritionally restricted gilthead sea bream (Sparus aurata) (Perez-Sanchez et al. 1995). However, the endocrine mechanisms leading to GH resistance in fishes are relatively unexplored. Insulin injection increased plasma IGF-I in brown trout (Salmo trutta) (Banos et al. 1999), and cortisol injection decreased plasma IGF-I in tilapia (Kajimura et al. 2003), suggesting that mechanisms similar to those in mammalian hepatocytes may be operating.

To investigate endocrine mechanisms modulating hepatocyte GH sensitivity in salmon, we performed a series of hepatocyte culture experiments. Primary hepatocytes were isolated and cultured by the procedure of Mommsen and co-workers, as previously described (Pierce et al. 2004a). Fish were fasted for 1 day before isolation of hepatocytes. Cells were cultured on Falcon Primaria 24-well plates at 15°C in modified RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) (buffer changed to 20 mM HEPES, 5 mM NaHCO₃). Hepatocytes were plated at a density of ~4 × 10⁶ cells/well in plain medium and allowed to adhere for 24 h, and then the medium was changed to test medium containing hormones. Cultures were stopped 18 h later by adding Tri-Reagenent (MRC, Cincinnati, OH, USA) to wells. The 18-h time point was selected on the basis of a previous time-course experiment (Pierce et al. 2004a). RNA was harvested according to the MRC protocol, with bromochloroapane as the phase separation reagent and two 70% ethanol washes. RNA was quantitated by spectrophotometry (260:280 ratios 1.8–2.0), and diluted to 100 ng/µl.

Hormones
Native coho salmon GH (sGH) was purified by HPLC. Native coho salmon insulin (sIns), glucagon (sGlu) and glucagon-like peptide (sGLP) were a generous gift from Dr Erika Plisetskaya (University of Washington, Seattle). Triiodothyronine (T₃), water-soluble dexamethasone (Dex), native bovine insulin (bIns) and native bovine glucagon (bGlu) were purchased from Sigma.

Experiments
The standard stimulatory concentration of 5 × 10⁻⁹ M sGH was based on previous sGH concentration–response studies (Pierce et al. 2004a). The survey of hormonal effects (Figs 1 and 5) was performed on cells cultured separately from three female fish, 149.7 ± 4.1 g (mean ± s.e.m.) body weight; one 24-well culture plate was used per experiment per fish. Confirmation of the insulin and glucagon effects (Fig. 2) and the Dex concentration–response (Fig. 3) experiments was performed on pooled cells from two male fish, 162 ± 16.5 g body weight, using one and two culture plates respectively. The sIns and bGlu concentration–responses (Fig. 4) were performed on pooled cells from four male fish, 182.5 ± 5.1 g body weight, using two culture plates each.

Real-time quantitative RT-PCR (qPCR) assays
Hepatocyte IGF-I mRNA expression levels were quantified with a qPCR amplicon in the leader peptide region of the gene, as previously described (Pierce et al. 2004a). Growth hormone receptor (GHR) mRNA expression levels were quantified with a qPCR amplicon in the extracellular domain, as previously described (Fukada et al. 2004). The primers and probe for the GHR qPCR assay.
fall in a region where the two known isoforms of the coho GHR are identical. Thus, the assay measures all known GHR transcripts. Gene expression data were normalized to the expression level of acidic ribosomal phosphoprotein P0 (ARP), using an efficiency corrected relative expression technique. Assays were run in 96-well format using TaqMan probes. All samples to be compared were run on the same PCR plate.

Data analysis

Gene expression levels were expressed relative to the control treatment for each fish or hepatocyte pool. In the hormone survey experiment, for each fish, the magnitude of responses was normalized to the average stimulation by GH alone found in all the fish. In the concentration–response experiments, gene expression levels were expressed relative to wells containing the control treatment for each culture plate. One- or two-way ANOVA followed by the Student–Newman–Keuls test was used to examine treatment and fish effects. Results were considered statistically significant at $P < 0.05$.

Results

As expected, sGH ($5 \times 10^{-9}$ M) significantly increased IGF-I mRNA level in all experiments; IGF-I mRNA increased by $3.62 \pm 0.27$-fold (mean $\pm$ S.E.M.; $n=12$). The threshold cycle for ARP did not vary between treatments in any experiment, indicating that treatments did not affect our reference gene.

Hormonal treatment significantly affected IGF-I mRNA levels in the hormone survey experiments (Fig. 1). The effect of fish was not significant after normalization in the $T_3$ and Dex experiment, but was significant in the bIns and sGlu experiment. Besides sGH, none of the hormones tested changed IGF-I from control levels by itself. $T_3$ ($10^{-7}$ M) did not modify the stimulation of IGF-I mRNA levels by sGH. RNA was degraded in some...
wells from the sGLP experiment, resulting in a decreased number of replicates; however, sGLP (10^{-6} M) did not modify GH stimulated IGF-I mRNA expression (data not shown). Dex (10^{-6} M) significantly inhibited the response to GH to 27% of the level without Dex, bIns (10^{-6} M) inhibited the response significantly in all fish to 43 ± 7%, and sGlu (10^{-6} M) inhibited the response significantly in two of three fish to 75 ± 11%. Inhibition of the GH response by bIns was unexpected. Therefore, the effects of bovine Ins (bIns) and salmon Glu (sGlu) were tested by alternate hormone sources (Fig. 2). However, similar results were obtained. Salmon Ins (sIns, 10^{-6} M) inhibited the IGF-I mRNA response to 33% of the level with sGH alone, and bovine Glu (bGlu, 10^{-6} M) inhibited the response to 71% of the level with sGH alone.

The inhibition of the IGF-I mRNA response to sGH by Dex was concentration-dependent over the range of 10^{-12} to 10^{-8} M (Fig. 3). Dex concentrations from 10^{-8} to 10^{-9} M inhibited the response to 14% of the level without Dex, not significantly different from the no-GH treatment.

The inhibition of the IGF-I mRNA response to sGH by sIns was concentration-dependent (Fig. 4A). Concentrations of 10^{-11} and 10^{-10} M sIns were ineffective, but sIns concentrations from 10^{-9} to 10^{-6} M progressively inhibited the sGH response. Maximal inhibition was observed at 10^{-8} M sIns, which suppressed the sGH response to 36% of the level with sGH alone. In contrast, inhibition of the sGH response by bGlu was observed only at 10^{-6} M (Fig 4b), which suppressed the response to 62% of the level with sGH alone. bGlu concentrations from 10^{-11} to 10^{-7} M did not significantly attenuate the response to sGH.

GHR mRNA levels were significantly affected by hormonal treatment (Fig. 5). bIns inhibited GHR mRNA significantly in 2/3 fish to 55 ± 11% of control levels. In contrast, Dex increased GHR significantly in 3/3 fish to 309 ± 40% of control levels. T3 and sGlu did not significantly affect GHR mRNA.

**Discussion**

GH consistently stimulated IGF-I mRNA expression in salmon hepatocytes, as in previous studies using salmonid hepatocyte culture (Duan et al. 1993, Shamblott et al. 1995, Pierce et al. 2004a) and tilapia hepatocyte culture (Schmid et al. 2000). Consistent stimulation of IGF-I mRNA expression by GH in fish hepatocyte culture confirms that this functional relationship in the GH/IGF axis is conserved in fishes (Nicoll et al. 1999, Perez-Sanchez & Le Bail 1999, Mommsen 2001). All fish hepatocyte culture studies have measured IGF-I mRNA levels rather than IGF-I protein secreted into the medium. It is not known how closely IGF-I mRNA expression reflects IGF-I protein secretion in fish hepatocyte culture. However, cellular IGF-I mRNA levels correlate well with medium IGF-I protein levels in rat hepatocyte culture (Phillips et al. 1991).

The glucocorticoid receptor agonist dexamethasone substantially inhibited the IGF-I mRNA response to GH,
without affecting basal IGF-I mRNA expression. To our knowledge, no previous studies have examined the effects of glucocorticoid hormones on IGF-I mRNA expression in fish hepatocyte culture. However, primary salmonid hepatocytes respond to glucocorticoids in terms of many other endpoints (Mommsen et al. 1999). In tilapia, cortisol injection decreased liver IGF-I mRNA in a dose-dependent manner and decreased plasma IGF-I, without changing plasma GH, suggesting that the cortisol effects may be mediated by GH resistance (Kajimura et al. 2003).

Our results in salmon hepatocytes support this idea. Consistent with inhibition of liver IGF-I production, cortisol injection and stress suppress growth in fishes (Barton & Iwama 1991).

Salmon hepatocytes were extremely sensitive to dexamethasone: significant inhibition of the GH response occurred at $10^{-12}$ M. Cortisol is the primary glucocorticoid hormone in fishes, and plasma cortisol levels range from approximately $10^{-8}$ M in unstressed to $10^{-6}$ M in stressed salmonids (Barton & Iwama 1991).

Figure 4 Concentration dependence of insulin (A) and glucagon (B) inhibition of the IGF-I mRNA expression response to GH. Cells were isolated and cultured as in Fig. 1 (four male fish, average body weight 182.5 ± 51 g). n=6 wells per treatment. Hormone abbreviations, error bars and significances are as shown in previous figures.
Thus, significant inhibition of the IGF-I mRNA response to GH occurred at dexamethasone concentrations 10,000-fold lower than circulating cortisol levels. This may be due in part to the greater potency of dexamethasone. Dexamethasone binds as or more strongly than cortisol to the glucocorticoid receptor in fish (Mommsen et al. 1999).

However, even allowing for higher dexamethasone potency, the results of the present study suggest that GH-dependent liver IGF-I production may be tonically inhibited by cortisol. Furthermore, dexamethasone inhibited the GH response substantially, to less than 30% of the response without dexamethasone at $10^{-6}$ M. Taken together, these results suggest that cortisol may be a major regulator of GH-dependent liver IGF-I production in salmon.

Adrenal glucocorticoids are primarily responsible for the GH resistance found in streptozotocin diabetic rats (Rodgers et al. 1994), suggesting that glucocorticoid induction of liver GH resistance may be conserved across vertebrates. However, lower levels of glucocorticoids increase GH sensitivity in mammalian hepatocyte culture. In rat hepatocytes, dexamethasone increased GHR mRNA expression and enhanced the IGF-I response to GH at low

![Figure 5](image-url)
Insulin had no response, insulin decreased GHR mRNA expression. Consistent with the inhibition of GH observed in five di- salmon hepatocyte culture. This surprising result was cellular mechanism in salmon than in mammals.

Insulin inhibited the IGF-I mRNA response to GH in salmon hepatocyte culture. This surprising result was observed in five different hepatocyte preparations, from both male and female fish, and with both bovine and salmon insulin. Consistent with the inhibition of GH response, insulin decreased GHR mRNA expression. Insulin had no effect on basal IGF-I mRNA expression. The inhibitory effect of insulin is most likely due to action through the insulin receptor. Salmon are tetraploid, and have multiple insulin receptor genes (Chan et al. 1997); however, there is no evidence for divergent function between receptor isotypes. Although insulin binds to the IGF type 1 receptor (IGF-IR) in brown trout, the affinity is ∼200-fold lower than that of IGF-I (Moon et al. 1996), and 5 × 10⁻⁹ M IGF-I did not inhibit the IGF-I mRNA response to GH in salmon hepatocytes (Pierce et al. 2004a). Salmo- nid hepatic portal insulin levels would be expected to range from less than 0.5 × 10⁻⁷ M to approximately 15 × 10⁻⁹ M (Mommsen & Plisetskaya 1991, Navarro & Gutierrez 1995). Metabolic responses to insulin occur at similar concentrations in salmonid hepatocyte culture (Mommsen & Plisetskaya 1991). Thus, inhibition of GH-dependent IGF-I mRNA expression occurred at physiologic concentrations of insulin.

Injection of insulin in brown trout increased plasma IGF-I levels 3 h later (Banos et al. 1999). This result may be reconciled with the present study if the injection effect is indirect, possibly through increases in circulating GH in response to insulin-induced hypoglycemia. Streptozotocin injection decreased plasma insulin and liver IGF-I mRNA or circulating IGF-I levels in coho salmon and brown trout (Plisetskaya & Duan 1994, Banos et al. 1999). Our cell culture results suggest that this could be due to toxic effects of streptozotocin rather than regulation of liver IGF-I mRNA expression by insulin. In the isletectomized (endocrine pancreatectomized) goby, GH injection stimulated growth and plasma cartilage sulfation activity, suggesting that GH sensitivity was maintained (Kelley et al. 1993). If gobies are similar to salmon, our results suggest that removal of insulin and glucagon would increase liver GH sensitivity, a result which might contribute (via increased IGF-I) to the delayed development of hyperglycemia in isletectomized gobies (Haigwood et al. 2000). During fasting in salmon, liver IGF-I mRNA and plasma IGF-I decrease before insulin decreases (Duan & Plisetskaya 1993, Dr Dianne Baker, University of Massachusetts, Amherst, in review; Pierce et al. 2004b), suggesting that decreased insulin may not be responsible for the development of GH resistance during fasting in salmon.

The inhibition of the response to GH by insulin in salmon hepatocytes is the inverse of what is found in tetrapods. In rat hepatocytes, insulin increased the IGF-I mRNA response to GH (Tollet et al. 1990, Boni-Schnetzler et al. 1991). Insulin potentiated the GH response at the level of IGF-I protein secretion into medium in chicken and rat hepatocytes (Houston & O’Neill 1991, Villafuerte et al. 1992). Studies in diabetic mammals support a requirement for normal portal insulin levels to maintain liver IGF-I production, plasma IGF-I levels and growth (Griffen et al. 1987). As far as we are aware, no study in any tetrapod species has shown inhibition by insulin of basal or GH-dependent hepatocyte IGF-I mRNA expression or protein secretion. This suggests that the role of insulin in growth regulation in salmon is different from that in mammals and birds. Glucose homeostasis is less important in the metabolism of fishes than in tetrapods. Consequently, the metabolic functions of insulin may be less important and the growth stimulatory functions more important in fishes than in tetrapods (Mommsen & Plisetskaya 1991, Kelley et al. 1993, Mommsen 2001).

No effect of T₃ (10⁻⁷ M) was found on basal or GH-stimulated IGF-I mRNA expression in salmon hepatocytes, and T₃ did not affect GHR mRNA expression. Schmid (2003) found a modest (1.5-fold) stimulation of basal IGF-I mRNA by T₃ (10⁻⁷ M) in tilapia hepatocytes, suggesting that species differences exist in this response. T₃ strongly potentiated the response to GH in rat hepatocyte culture (Tollet et al. 1990), and both strongly potentiated the response to GH and induced GHR in pig hepatocyte culture (Brameld et al. 1995). In hypothyroid chickens, thyroid hormone replacement normalized decreases in serum IGF-I, liver IGF-I mRNA, liver GHR mRNA and liver GH binding (Tsukada et al. 1998). Thyroid hormones may be less important in the regulation of liver IGF-I production in fishes than in tetrapods.

High concentrations (10⁻⁶ M) of glucagon inhibited the IGF-I mRNA response to GH, without significantly changing GHR mRNA expression. However, inhibition did not occur at lower concentrations of glucagon. Physiologic concentrations of glucagon experienced by the liver are ∼10⁻¹⁰ M, and primary fish hepatocytes respond metabolically to glucagon concentrations as low as 10⁻⁸ M (Plisetskaya & Mommsen 1996). Therefore, the effect of glucagon found in the present study is likely to be pharmacologic. In contrast, physiologic concentrations of glucagon inhibited both basal and GH-stimulated IGF-I mRNA expression and secretion into culture medium in rat hepatocytes (Denver & Nicoll 1994). GLP is a pancreatic hormone with metabolic effects similar

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to glucagon on the liver in fishes, and is often more potent than glucagon (Plisetskaya & Mommsen 1996). The present study provides no evidence for a role for GLP in the regulation of the GH/IGF axis.

None of the hormones tested affected IGF-I mRNA expression by themselves; however, strong modulatory effects on GH sensitivity were found. This clear result suggests that hepatocyte GH sensitivity is an important point of control in the regulation of IGF-I production in salmon. Basal effects of insulin, glucagon, T3, and dexamethasone are found in mammalian hepatocyte culture, and insulin and dexamethasone may be required to maintain cell viability and responsiveness in cultured mammal hepatocytes. Thus, primary cultured salmon hepatocytes provide a simpler model than mammal hepatocytes for studying the regulation of GH sensitivity. Insulin, dexamethasone and glucagon decreased, increased and did not change hepatocyte GHR mRNA expression respectively. However, all three hormones induced GH resistance in terms of IGF-I mRNA expression, suggesting that multiple mechanisms within salmon hepatocytes regulate responsiveness to GH.

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