

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

A L Pierce^{1,2}, H Fukada³ and W W Dickhoff^{1,2}

¹Integrative Fish Biology Program, Northwest Fisheries Science Center, National Marine Fisheries Service, 2725 Montlake Boulevard East, Seattle, Washington State 98112, USA

²School of Aquatic and Fishery Sciences, University of Washington, Seattle, Washington State 98195, USA

³Faculty of Agriculture, Kochi University, 2-5-1 Akebono-cho, Kochi, 780-8520 Japan

(Requests for offprints should be addressed to A L Pierce; Email: piercea@u.washington.edu)

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 (T_3 , 10^{-7} M), dexamethasone (Dex, 10^{-6} M) and glucagon-like peptide (GLP, 10^{-6} M), in the presence and absence of GH (5×10^{-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 levels. Ins inhibited and Dex stimulated 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.

Journal of Endocrinology (2005) **184**, 341–349

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

expression (Tollet *et al.* 1990, Boni-Schnetzler *et al.* 1991, Houston & O'Neill 1991, Denver & Nicoll 1994, Brameld *et al.* 1995, Beauloye *et al.* 1999). *In vivo* data support a physiologically significant positive role for insulin in the regulation of GH sensitivity (Griffen *et al.* 1987). Within the hepatocyte, changes in GH sensitivity occur at both the receptor and post-receptor level (Thissen *et al.* 1994, 1999).

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 (*Gillichthys mirabilis*) (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, cultured and exposed to pancreatic, thyroid and glucocorticoid hormones in the presence and absence of GH. Significant modulatory effects on GH sensitivity were found for dexamethasone, insulin and glucagon. To begin to investigate cellular mechanisms underlying changes in GH sensitivity, GH receptor (GHR) mRNA expression levels were measured in selected samples.

Materials and Methods

Animals

Coho salmon were raised in 1.3-m diameter cylindrical tanks with recirculated fresh water at 11–12 °C under simulated natural photoperiod at the Northwest Fisheries Science Center (Seattle, WA, USA). Fish were fed BioOregon-grower pellets at a ration of 0.6% body weight per day. Two-year-old coho salmon were used. Experiments took place from September to January 2002–3.

Hepatocyte culture and RNA isolation

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 $\sim 4 \times 10^6$ 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-Reagent (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 bromochloropropane 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/ μ l.

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 \pm 4.1 g (mean \pm 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 \pm 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 \pm 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

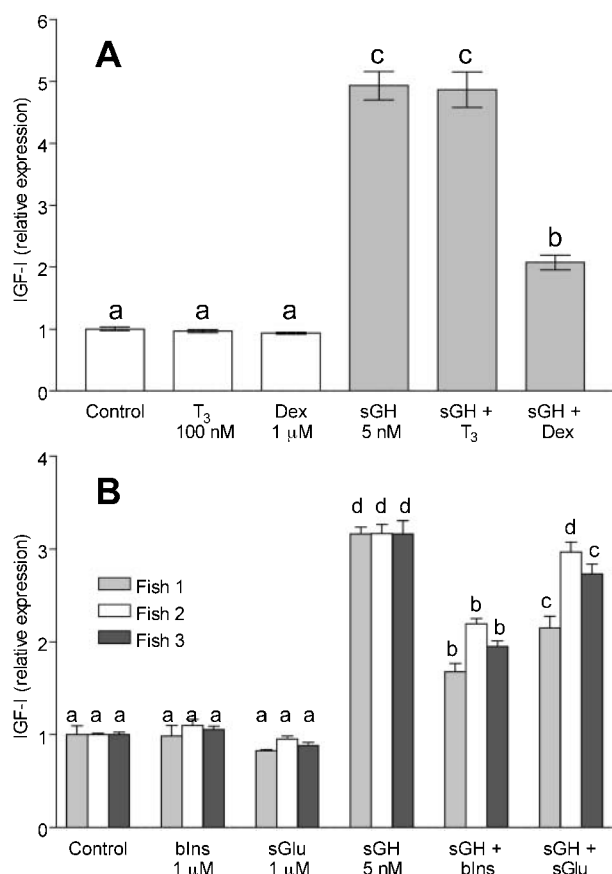


Figure 1 Effects of metabolic hormones on basal and GH-stimulated IGF-I mRNA expression in primary salmon hepatocyte culture. Hepatocytes were isolated, plated in plain RPMI medium and incubated for 24 h at 15 °C. The medium was then changed to test medium containing the indicated hormones (sGH: salmon growth hormone; T₃: triiodothyronine; Dex: dexamethasone; bIns: bovine insulin; sGlu: salmon glucagon), and cells were incubated for an additional 18 h. Cells from three fish (female, average body weight 149.7 ± 4.1 g) were kept separately and used in independent experiments. Data were normalized so that the response of each fish to GH alone was set equal to the average response of all the fish. After normalization, the effects of fish and fish times treatment were not significant in panel A; however, significant effects remained in panel B. Therefore, fish are presented separately in panel B. (A) GH responses 5.18-, 5.48- and 4.14-fold control levels; *n* = 12 wells per treatment. (B) GH responses 3.19-, 3.25- and 3.06-fold control levels; *n* = 4 wells per treatment for each fish. Error bars represent standard error of the mean. Bars with different letters are significantly different (*P* < 0.05). In panel B, letters refer to significant differences between treatments within each fish.

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

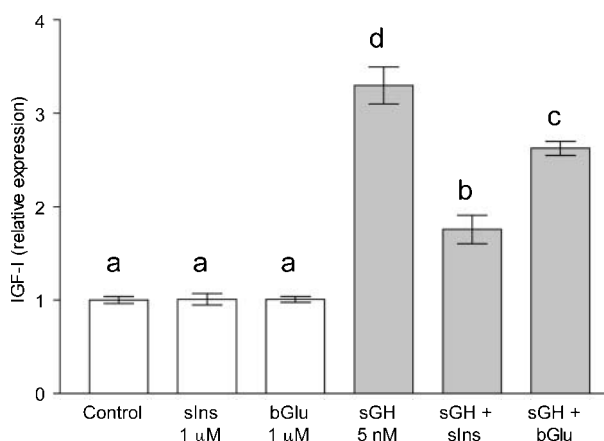


Figure 2 Confirmation of insulin and glucagon effects by alternative hormone sources. Cells were isolated and cultured as in Fig. 1 (two male fish, 162.5 ± 16.5 g). The experiment was performed as in Fig. 1, except salmon insulin (sIns) and bovine glucagon (bGlu) were used. *n* = 4 wells per treatment. Error bars and significances are as shown in previous figure.

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 (5×10^{-9} M) significantly increased IGF-I mRNA level in all experiments; IGF-I mRNA increased by 3.62 ± 0.27 -fold (mean ± 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 T₃ and Dex experiment, but it was significant in the bIns and sGlu experiment. Besides sGH, none of the hormones tested changed IGF-I from control levels by itself. T₃ (10^{-7} M) did not modify the stimulation of IGF-I mRNA levels by sGH. RNA was degraded in some

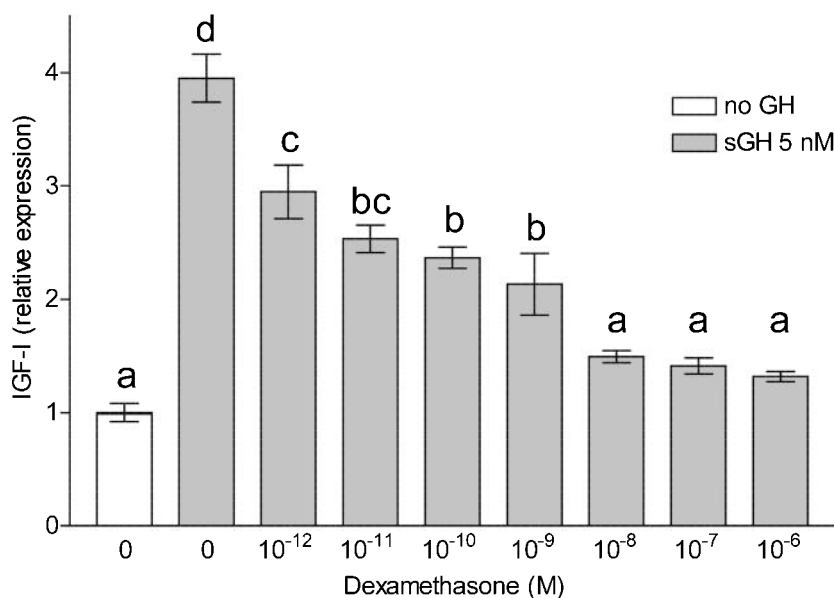


Figure 3 Concentration dependence of dexamethasone inhibition of the IGF-I mRNA expression response to GH. Cells were isolated and cultured as in Fig. 1 (two male fish, average body weight 162.5 ± 16.5 g). $n=4-5$ wells per treatment. Hormone abbreviations, error bars and significances are as shown in previous figures.

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 $43 \pm 7\%$, and sGlu (10^{-6} M) inhibited the response significantly in two of three fish to $75 \pm 11\%$. 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} – 10^{-8} M (Fig. 3). Dex concentrations from 10^{-8} to 10^{-6} 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^{-6} 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 $55 \pm 11\%$ of control levels. In contrast, Dex increased GHR significantly in 3/3 fish to $309 \pm 40\%$ of control levels. T_3 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, Shamblo *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,

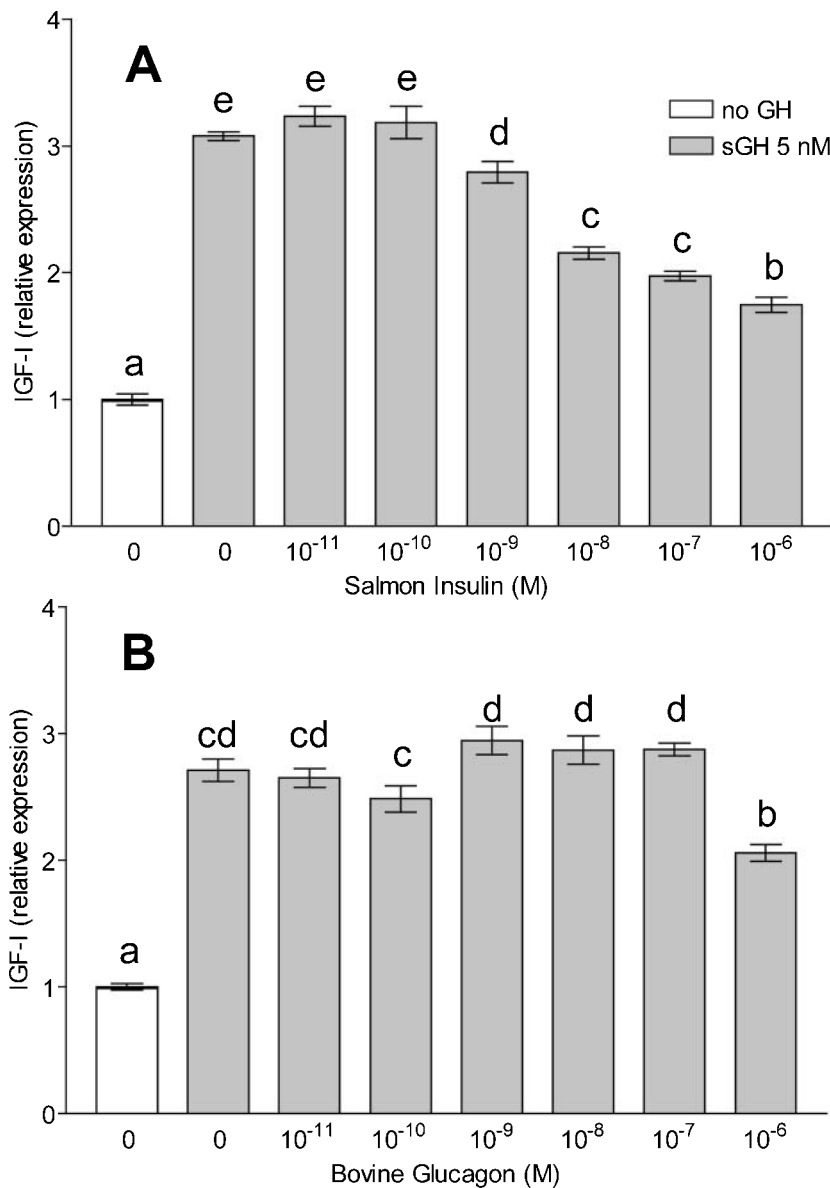


Figure 4 Concentration dependence of insulin (A) and glucagon (B) inhibition of the IGF-I mRNA expression response to GH. Cells were isolated and cultured as in Fig. 1 (four male fish, average body weight 182.5 ± 5.1 g). $n=6$ wells per treatment. Hormone abbreviations, error bars and significances are as shown in previous figures.

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 (Mommensen *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).

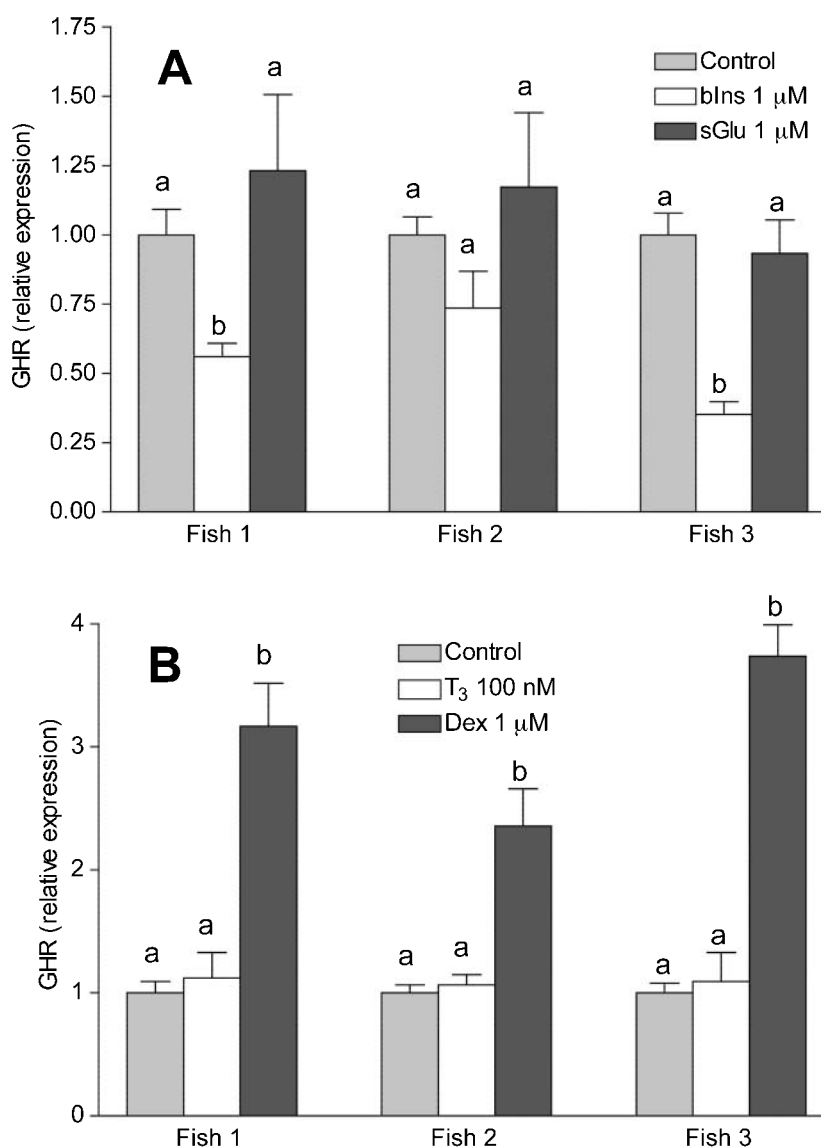


Figure 5 Effects of insulin and glucagon (A), and triiodothyronine and dexamethasone (B) on growth hormone receptor (GHR) mRNA expression in primary salmon hepatocyte culture. GHR mRNA was measured in selected samples from the experiment described in Fig. 1. Hormone abbreviations, error bars and significances are as shown in previous figures. Letters refer to significant differences between treatments within each fish.

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 (Mommensen *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

concentrations (10^{-10} M), and decreased GHR and the response to GH at higher concentrations (Beauloye *et al.* 1999). In pig hepatocytes, 10^{-7} M dexamethasone increased GHR mRNA levels and enhanced the IGF-I mRNA response to GH (Brameld *et al.* 1995). Dexamethasone also increased basal IGF-I mRNA in these studies. In the present study, dexamethasone increased GHR mRNA expression but diminished the response to GH, suggesting that glucocorticoid regulation of hepatocyte GH sensitivity may operate through a different 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 isoforms. 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). Salmonid hepatic portal insulin levels would be expected to range from less than 0.5×10^{-9} 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 T_3 (10^{-7} M) was found on basal or GH-stimulated IGF-I mRNA expression in salmon hepatocytes, and T_3 did not affect GHR mRNA expression. Schmid (2003) found a modest (1.5-fold) stimulation of basal IGF-I mRNA by T_3 (10^{-7} M) in tilapia hepatocytes, suggesting that species differences exist in this response. T_3 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 $\sim 10^{-10}$ 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

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.

Acknowledgements

This work was supported by fellowships from the University of Washington School of Aquatic and Fishery Sciences, and grants from the US Department of Agriculture (no. 2001-03320) and the Bonneville Power Administration (projects 92-022 and 93-056). We thank Dr Erika Plisetskaya for the generous gift of salmon insulin, glucagon and GLP, and Dr Penny Swanson for purification of salmon GH. We thank Brad Gadberry and Paul Parkins for fish care, and Dr Brian Beckman for hatchery management. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Banos N, Planas JV, Gutierrez J & Navarro I 1999 Regulation of plasma insulin-like growth factor-I levels in brown trout (*Salmo trutta*). *Comparative Biochemistry and Physiology* **124C** 33–40.
- Barton BA & Iwama GK 1991 Physiological change in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases* **1** 3–26.
- Beaulieu V, Ketelslegers JM, Moreau B & Thissen JP 1999 Dexamethasone inhibits both growth hormone (GH)-induction of insulin-like growth factor-I (IGF-I) mRNA and GH receptor (GHR) mRNA levels in rat primary cultured hepatocytes. *Growth Hormone and IGF Research* **9** 205–211.
- Boni-Schnetzler M, Schmid C, Meier PJ & Froesch ER 1991 Insulin regulates insulin-like growth factor I mRNA in rat hepatocytes. *American Journal of Physiology – Endocrinology and Metabolism* **260** E846–E851.
- Brameld JM, Weller PA, Saunders JC, Buttery PJ & Gilmour RS 1995 Hormonal control of insulin-like growth factor-I and growth hormone receptor mRNA expression by porcine hepatocytes in culture. *Journal of Endocrinology* **146** 239–245.
- Chan SJ, Plisetskaya EM, Urbinati E, Jin Y & Steiner DF 1997 Expression of multiple insulin and insulin-like growth factor receptor genes in salmon gill cartilage. *PNAS* **94** 12446–12451.
- Denver RJ & Nicoll CS 1994 Pancreatic hormones differentially regulate insulin-like growth factor (IGF)-I and IGF-binding protein production by primary rat hepatocytes. *Journal of Endocrinology* **142** 299–310.
- Duan C & Plisetskaya EM 1993 Nutritional regulation of insulin-like growth factor-I mRNA expression in salmon tissues. *Journal of Endocrinology* **139** 243–252.
- Duan C, Duguay SJ & Plisetskaya EM 1993 Insulin-like growth factor I (IGF-I) mRNA expression in coho salmon, *Oncorhynchus kisutch*: tissue distribution and effects of growth hormone/prolactin family proteins. *Fish Physiology and Biochemistry* **11** 371–379.
- Duan C, Plisetskaya EM & Dickhoff WW 1995 Expression of insulin-like growth factor I in normally and abnormally developing coho salmon (*Oncorhynchus kisutch*). *Endocrinology* **136** 446–452.
- Fukada H, Ozaki Y, Pierce AL, Adachi S, Yamauchi K, Hara A, Swanson P & Dickhoff WW 2004 Salmon growth hormone receptor: molecular cloning, protein expression, binding assay and gene expression. *General and Comparative Endocrinology* **139** 61–71.
- Gray ES & Kelley KM 1991 Growth regulation in the gobiid teleost, *Gillichthys mirabilis*: roles of growth hormone, hepatic growth hormone receptors and insulin-like growth factor-I. *Journal of Endocrinology* **131** 57–66.
- Gray ES, Kelley KM, Law S, Tsai R, Young G & Bern HA 1992 Regulation of hepatic growth hormone receptors in coho salmon (*Oncorhynchus kisutch*). *General and Comparative Endocrinology* **88** 243–252.
- Griffen SC, Russell SM, Katz LS & Nicoll CS 1987 Insulin exerts metabolic and growth-promoting effects by a direct action on the liver in vivo: clarification of the functional significance of the portal vascular link between the beta cells of the pancreatic islets and the liver. *PNAS* **84** 7300–7304.
- Haigwood JT, Flores RM, Mazloumi R, Ngan G & Kelley KM 2000 Diabetogenic role of insulin's counterregulatory hormones in the isletectomized, diabetic goby. *Endocrine* **13** 273–280.
- Houston B & O'Neill IE 1991 Insulin and growth hormone act synergistically to stimulate insulin-like growth factor-I production by cultured chicken hepatocytes. *Journal of Endocrinology* **128** 389–393.
- Kajimura S, Hirano T, Visitacion N, Moriyama S, Aida K & Grau EG 2003 Dual mode of cortisol action on GH/IGF-I/IGF binding proteins in the tilapia, *Oreochromis mossambicus*. *Journal of Endocrinology* **178** 91–99.
- Kelley KM, Gray ES, Siharath K, Nicoll CS & Bern HA 1993 Experimental diabetes mellitus in a teleost fish. II. Roles of insulin, growth hormone (GH), insulin-like growth factor-I, and hepatic GH receptors in diabetic growth inhibition in the goby, *Gillichthys mirabilis*. *Endocrinology* **132** 2696–2702.
- Mommsen TP 2001 Hormonal regulation of muscle growth. In *Fish Physiology*, vol 18, pp 251–308. Ed IA Johnston. San Diego, CA: Academic Press.
- Mommsen TP & Plisetskaya EM 1991 Insulin in fishes and agnathans: history, structure, and metabolic regulation. *Reviews in Aquatic Sciences* **4** 225–259.
- Mommsen TP, Vijayan MM & Moon TW 1999 Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries* **9** 211–268.
- Moon TW, Castejon C, Banos N, Maestro MA, Plisetskaya EM, Gutierrez J & Navarro I 1996 Insulin and IGF-I binding in isolated trout cardiomyocytes. *General and Comparative Endocrinology* **103** 264–272.
- Navarro I & Gutierrez J 1995 Fasting and starvation. In *Biochemistry and Molecular Biology of Fishes*, vol 4, pp 393–434. Eds PW Hochachka & TP Mommsen. Amsterdam: Elsevier Science.

- Nicoll CS, Rodgers BD & Kelley KM 1999 Hormonal regulation of growth and development of nonmammalian vertebrates. In *Handbook of Physiology, Endocrinology*, vol 5, pp 73–98. Ed JL Kostyo. London: Oxford University Press.
- Perez-Sanchez J & Le Bail P-Y 1999 Growth hormone axis as marker of nutritional status and growth performance in fish. *Aquaculture* **177** 117–128.
- Perez-Sanchez J, Marti-Palanca H & Kaushik SJ 1995 Ration size and protein intake affect circulating growth hormone concentration, hepatic growth hormone binding and plasma insulin-like growth factor-I immunoreactivity in a marine teleost, the gilthead sea bream (*Sparus aurata*). *Journal of Nutrition* **125** 546–552.
- Phillips LS, Goldstein S & Pao CI 1991 Nutrition and somatomedin. XXVI. Molecular regulation of IGF-I by insulin in cultured rat hepatocytes. *Diabetes* **40** 1525–1530.
- Pierce AL, Dickey JT, Larsen DA, Fukada H, Swanson P & Dickhoff WW 2004a A quantitative real-time RT-PCR assay for salmon IGF-I mRNA, and its application in the study of GH regulation of IGF-I gene expression in primary culture of salmon hepatocytes. *General and Comparative Endocrinology* **135** 401–411.
- Pierce AL, Shimizu M, Beckman BR, Baker DM & Dickhoff WW 2004b Time course of the GH/IGF axis response to fasting and increased ration in chinook salmon (*Oncorhynchus tshawytscha*). *General and Comparative Endocrinology* (In press).
- Plisetskaya EM & Duan C 1994 Insulin and insulin-like growth factor I in coho salmon *Oncorhynchus kisutch* injected with streptozotocin. *American Journal of Physiology – Regulatory, Integrative and Comparative Physiology* **267** 1408–1412.
- Plisetskaya EM & Mommsen TP 1996 Glucagon and glucagon-like peptides in fishes. *International Review of Cytology* **168** 187–257.
- Rodgers BD, Lau AO & Nicoll CS 1994 Hypophysectomy or adrenalectomy of rats with insulin-dependent diabetes mellitus partially restores their responsiveness to growth hormone. *Proceedings of the Society for Experimental Biology and Medicine* **207** 220–226.
- Rosenfeld RG & Roberts CTJ 1999 The IGF system: molecular biology, physiology, and clinical applications. In: *Contemporary Endocrinology*, vol. 17, pp 1–575. Ed PM Conn. Totowa, NJ: Humana Press.
- Schmid AC, Reinecke M & Kloas W 2000 Primary cultured hepatocytes of the bony fish, *Oreochromis mossambicus*, the tilapia: a valid tool for physiological studies on IGF-I expression in liver. *Journal of Endocrinology* **166** 265–273.
- Schmid AC, Lutz I, Kloas W & Reinecke M 2003 Thyroid hormone stimulates hepatic IGF-I mRNA expression in a bony fish, the tilapia *Oreochromis mossambicus*, *in vitro* and *in vivo*. *General and Comparative Endocrinology* **130** 129–134.
- Shamblott MJ, Cheng CM, Bolt D & Chen TT 1995 Appearance of insulin-like growth factor mRNA in the liver and pyloric ceca of a teleost in response to exogenous growth hormone. *PNAS* **92** 6943–6946.
- Thissen JP, Ketelslegers JM & Underwood LE 1994 Nutritional regulation of the insulin-like growth factors. *Endocrine Reviews* **15** 80–101.
- Thissen JP, Underwood LE & Ketelslegers JM 1999 Regulation of insulin-like growth factor-I in starvation and injury. *Nutrition Reviews* **57** 167–176.
- Tollet P, Enberg B & Mode A 1990 Growth hormone (GH) regulation of cytochrome P-450 IIC12, insulin-like growth factor-I (IGF-I), and GH receptor messenger RNA expression in primary rat hepatocytes: a hormonal interplay with insulin, IGF-I, and thyroid hormone. *Molecular Endocrinology* **4** 1934–1942.
- Tsukada A, Ohkubo T, Sakaguchi K, Tanaka M, Nakashima K, Hayashida Y, Wakita M & Hoshino S 1998 Thyroid hormones are involved in insulin-like growth factor-I production by stimulating hepatic growth hormone receptor (GHR) gene expression in the chicken. *Growth Hormone and IGF Research* **8** 235–242.
- Villafuerte BC, Goldstein S, Robertson DG, Pao CI, Murphy LJ & Phillips LS 1992 Nutrition and somatomedin. XXIX. Molecular regulation of IGFBP-1 in hepatocyte primary culture. *Diabetes* **41** 835–842.

Received in final form 1 November 2004

Accepted 11 November 2004