Differential regulation of *Igf1* and *Igf2* mRNA levels in tilapia hepatocytes: effects of insulin and cortisol on GH sensitivity

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Abstract

Igf1 and Igf2 stimulate growth and development of vertebrates. In mammals, liver-derived endocrine Igf1 mediates the growth promoting effects of GH during postnatal life, whereas Igf2 stimulates placental and fetal growth and is not regulated by GH. Insulin enhances Igf1 production by the mammalian liver directly, and by increasing hepatocyte sensitivity to GH. We examined the regulation of *igf1* and *igf2* mRNA levels by GH, insulin, and cortisol, and the effects of insulin and cortisol on GH sensitivity in primary cultured hepatocytes of tilapia, a cichlid teleost. GH increased mRNA levels of both *igf1* and *igf2* in a concentration-related and biphasic manner over the physiological range, with a greater effect on *igf2* mRNA level. Insulin increased basal *igf2*

mRNA level, and strongly increased GH-stimulated *igf2* mRNA level, but slightly reduced basal *igf1* mRNA level and did not affect GH-stimulated *igf1* mRNA level. Cortisol inhibited GH stimulation of *igf1*, but increased GH stimulation of *igf2* mRNA level. The synergistic effect of insulin and GH on *igf2* mRNA level was confirmed *in vivo*. These results indicate that insulin and cortisol differentially modulate the response of *igf1* and *igf2* mRNA to GH in tilapia hepatocytes, and suggest that the regulation of liver Igf2 production differs between fish and mammals. Regulation of liver Igf2 production in fish appears to be similar to regulation of liver Igf1 production in mammals.

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Introduction

The insulin-like growth factors (Igf1 and Igf2) are mitogenic peptides that regulate vertebrate growth (Humbel 1990, Jones & Clemmons 1995, Reinecke & Collet 1998). The Igfs are members of the evolutionarily ancient insulin-like family of peptides, found throughout the metazoans. Conserved features of the insulin-like peptides include regulation by nutritional status, and roles in nutrient metabolism, growth, development, reproduction, and aging (Tatar *et al.* 2003). Igf1 is thought to have arisen from insulin during the transition from chordates to primitive vertebrates, and Igf2 to have arisen from Igf1 in the common ancestor of the bony and cartilaginous fishes (Reinecke & Collet 1998). Both Igfs act through the same membrane receptor, the type 1 Igf receptor, and the activity of both Igfs is modulated by multiple Igf-binding proteins. Both Igfs have a dual mode of action as local and as endocrine growth factors. Local Igfs are produced throughout the body; whereas endocrine Igfs are mainly produced by the liver.

Liver-derived circulating Igf1 plays an essential role in postembryonic mammals as the primary mediator of GH-dependent growth. As developed in the somatomedin hypothesis, pituitary GH stimulates liver production of Igf1,

which feeds back to inhibit GH secretion in the GH/Igf endocrine axis (Humbel 1990, Jones & Clemmons 1995, Phillips *et al.* 1998). In mice, both endocrine and local Igf1 play biologically significant roles in growth regulation (Stratikopoulos *et al.* 2008). Nutritional and metabolic status regulate the GH/Igf axis through direct effects on the liver, and by modulating liver sensitivity to GH. In catabolic states such as fasting and disease, liver production of Igf1 becomes resistant to stimulation by GH (Thissen *et al.* 1994, 1999). Metabolically responsive hormones link the anabolic/catabolic state of the animal to liver GH sensitivity. Insulin directly increases hepatocyte Igf1 production, and strongly increases hepatocyte responsiveness to GH *in vivo* (Griffen *et al.* 1987, Butler *et al.* 2003), and in mammalian and avian primary hepatocyte culture (Tollet *et al.* 1990, Boni-Schnetzler *et al.* 1991, Houston & O'Neill 1991, Phillips *et al.* 1998). Glucocorticoids induce liver GH resistance (Rodgers *et al.* 1994, Brameld *et al.* 1995, Beauloye *et al.* 1999).

In all vertebrate classes, *igf2* is highly expressed during embryogenesis, and stimulates growth in embryonic tissues (Jones & Clemmons 1995, Reinecke & Collet 1998, Wood *et al.* 2005, White *et al.* 2009). In contrast, *igf1* expression is low during embryogenesis and increases with the onset of postnatal GH-dependent growth. It is thought that liver Igf2

production is not strongly stimulated by GH or other hormones in postnatal mammals (Humbel 1990, Holly 1998). The lack of a clear picture of the regulation and function of endocrine Igf2 in postnatal mammals may be due to the central role of Igf2 in the development of the placenta. Igf2 is a principal regulator of the size and exchange capacity of the placenta. The parent–offspring evolutionary conflict theory predicts that this role as a mediator of maternal–fetal resource partitioning has resulted in selection on *igf2* and divergence of Igf2 regulation and function (Haig 1993, O'Neill *et al.* 2007). A recent study in placental guppies supports this scenario (O'Neill *et al.* 2007). This suggests that Igf2 physiology may fundamentally differ between placental and non-placental vertebrates.

Recent studies show that teleost fishes including salmonids, sea bream, carp, catfish, eels, rabbitfish, sea bass, and hybrid striped bass, as well as the dogfish, an elasmobranch, differ from mammals in that liver *igf2* gene expression and plasma Igf2 levels are stimulated by GH and respond to metabolic status (Shamblott *et al.* 1995, Tse *et al.* 2002, Vong *et al.* 2003a, Peterson *et al.* 2004, 2005, Carnevali *et al.* 2005, Gabillard *et al.* 2006, Wilkinson *et al.* 2006, Ayson *et al.* 2007, Terova *et al.* 2007, Gahr *et al.* 2008, Moriyama *et al.* 2008a, b, Picha *et al.* 2008b, Ponce *et al.* 2008, Devlin *et al.* 2009, Peterson & Waldbieser 2009, Eppler *et al.* 2010, Pierce *et al.* 2010). In primary cultured coho salmon hepatocytes, insulin directly increased *igf2* mRNA, and strongly increased the response of *igf2* mRNA to GH, but suppressed the response of *igf1* mRNA to GH (Pierce *et al.* 2005, 2010). Thus, the regulation of liver *igf2* gene expression in coho salmon is similar to the regulation of liver *igf1* gene expression in mammals. We hypothesized that this may be the case in teleost fishes in general. To test this hypothesis, we examined the regulation of *igf1* and *igf2* gene expression by GH, insulin, and cortisol, and the effects of insulin and cortisol on GH sensitivity in primary cultured hepatocytes of a cichlid teleost, the Mozambique tilapia (*Oreochromis mossambicus*).

Materials and Methods

Animals

Mozambique tilapia were maintained in 700 l outdoor freshwater flow-through tanks at the Hawaii Institute of Marine Biology. Fish were fed ~2% body weight per day in two daily feedings (Silver Cup Trout Chow, Portco Corporation, Vancouver, BC, Canada). Water temperature was maintained at 25–28 °C. Fish used in hepatocyte culture studies were 2- to 3-year-old males weighing 300–600 g, whereas fish used in the *in vivo* injection experiment were 1-year-old males weighing 60–80 g. All experiments were conducted under approved protocols in accordance with the principles and procedures of the Institutional Animal Care and Use Committee, University of Hawaii.

Hepatocyte isolation and culture

Fish were fasted for 1 day before hepatocyte isolation. Fish were first anesthetized by immersion in a nonlethal concentration of buffered tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA, USA, 0.5 g/l), and i.p. injected with lithium heparin (Sigma, 10 000 U/kg). After 5 min, fish were killed by immersion in a lethal concentration of MS-222 (2.0 g/l). Immediately after opercular movements stopped, fish were placed left side down on a sterile elevated screen. The right side of the peritoneal cavity was cut away. The gall bladder was punctured and drained, and the empty gall bladder and fat and connective tissue were dissected away, exposing the hepatic portal vein. The vessel was cut partway through and a polyethylene cannula was inserted and pushed toward the liver, until the tip was near where the vessel begins to ramify into the liver, and tied in place. The heart was removed to allow drainage. The liver was perfused with Ca⁺⁺-free Hanks' buffered saline solution (HBSS) for 20–30 min at a flow rate of 1.5 ml/min. Perfusion then continued with 6.5–7.5 mg/100 ml collagenase (type IV, Sigma C5138, lot #085K8618), made fresh in Ca⁺⁺-free HBSS and sterile filtered immediately before use. Collagenase perfusion continued until cracks appeared on the surface of the liver, typically 8–12 min. During Ca⁺⁺-free HBSS and collagenase perfusion, the liver was massaged gently every 5 min. When digestion was complete, the liver was removed from the fish and placed in a sterile Petri dish in ice cold Ca⁺⁺-free HBSS. Undigested sections of liver tissue, fat, connective tissue, and blood clots were dissected away, digested tissue was chopped fine with a razor blade, and cells were mashed through a 70 µm filter. Cells were collected in 50 µl of ice cold Ca⁺⁺-free HBSS and pelleted (60 g, 5 min). Cells were resuspended in recovery medium (Ca⁺⁺-free HBSS supplemented with 1.5 mM CaCl₂, 2% cell culture grade BSA, 3 mM glucose, and 1 × Gibco MEM essential and non-essential amino acid solutions). Cells were washed three times in recovery medium, and then settled on ice for 1–2 h. After settling, cells were resuspended in culture medium (modified RPMI 1640 medium, Gibco BRL; 20 mM HEPES, 5 mM NaHCO₃, and 0.2% BSA) and washed twice. Cells were counted and examined for viability by trypan blue exclusion, and then plated at a density of 1–2 × 10⁶ cells/ml, 0.5 ml/well on Falcon Primaria 24-well plates and incubated at 25 °C under plain air. Cells were isolated from individual fish and pooled if necessary. Cells were allowed to adhere to culture plates for an initial period as described in the Results section, and then medium was changed to test medium containing hormones. Native tilapia GH was purified by HPLC (Specker *et al.* 1985). Native bovine insulin and cortisol were purchased from Sigma (insulin Sigma I6634, lot 096K16911, activity 28 USP units/mg by HPLC). Ethanol (0.1%) was added to medium for all treatments in experiments testing the effects of cortisol. Cultures were stopped by adding Tri-Reagent (MRC, Cincinnati, OH, USA) to wells and freezing culture plates at –80 °C.

In vivo injection experiment

Freshwater-acclimated fish were fasted for 24 h and injected with hormones. Fish were not fed post-injection. During injection, fish were anesthetized (MS-222, 0.5 g/l) and injected with saline vehicle (0.9% NaCl, 1 µl/g body weight), native ovine GH (5 µg/g body weight, NIDDK oGH-15, lot AFP 9220A, activity 1.52 IU/mg in terms of Bovine GH International Standard 55/1), native bovine insulin (5 µg/g body weight, Sigma), or insulin and GH. Fish were fatally anesthetized 6 h later, and liver tissue was collected. Liver was homogenized immediately in Tri-Reagent and stored at -80°C until RNA isolation.

RNA isolation, real-time quantitative RT-PCR, and plasma glucose assays

RNA was isolated following the MRC protocol, with bromochloropropane as the phase separation reagent and two 70% ethanol washes. RNA was quantified and purity assessed by spectrophotometry (Nanodrop ND-1000, Wilmington, DE, USA; RNA yield 286–906 ng/well, A260/280 1.90–2.18), RNA was diluted to 10–50 ng/µl, and first strand cDNA was synthesized with a kit (qScript cDNA Supermix, Quanta BioSciences, Gaithersburg, MD, USA). Hepatocyte and liver *igf1* and reference gene (acidic ribosomal phosphoprotein P0 (*arp*) and elongation factor 1 α (*ef1a*)) mRNA expression levels were quantified as described previously (Pierce *et al.* 2007, Breves *et al.* 2010). Hepatocyte and liver *igf2* mRNA levels were quantified with a quantitative RT-PCR (qPCR) assay across the predicted exons 3/4 boundary (F primer: AGCCACCTCTCTACAGGTCATACC and R primer: ACTTCACGGTCACATGTTGCTT, hydrolysis probe: FAM-TGCCCGCACTAAAACAGGAAGTTCA-GA-BHQ, amplicon size: 80 bp), which was validated (PCR efficiency 91–95%, biological template serial dilutions parallel to PCR product standard curve, no bias across PCR plate, no signal from genomic DNA or RT reactions with the reverse transcriptase omitted, single peak in melting curve analysis in assay run with SYBER green and probe omitted). All qPCR assays were run in 96-well format on an Applied BioSystems (ABI, Foster City, CA, USA) StepOne Plus qPCR machine, using standard cycling conditions. Assays were run using ABI Gene Expression Master Mix in a reaction volume of 15 µl, with primers and probe at a concentration of 200 nM. Gene expression levels for individual targets were quantified using PCR product standard curves and then normalized by dividing the expression level of a gene of interest in a given sample by the expression level of an appropriate reference gene in that sample as described previously (Pierce *et al.* 2007). Gene expression data for hepatocyte culture experiments were normalized to the expression level of *arp*, whereas gene expression data for the *in vivo* injection experiment were normalized to the expression level of *ef1a*. Statistically detectable differences in the C_T for *arp* were not found between treatments in any hepatocyte culture experiment, and

statistically detectable differences in the C_T for *ef1a* were not found between treatments in the injection experiment, providing evidence that treatments did not affect our reference genes (ANOVA on replicate culture plate wells or fish). Plasma glucose was measured by the hexokinase method by a kit (GAHK-20, Sigma).

Data analysis

Gene expression data were log2 transformed before analysis. Treatment effects were examined by one- or two-way ANOVA, followed by the Student–Newman–Keuls test. Results were considered statistically detectable at $P < 0.05$. Data analysis was conducted with Prism (GraphPad Software, San Diego, CA, USA). Data are given as mean $\pm$ s.d. To establish tilapia hepatocyte culture, we used pooled cells (time course experiment: pooled cells from two fish and GH concentration–response experiment: pooled cells from two fish). In these experiments, our experimental unit is the well, and our statistical inferences are restricted to the pool of cells used. To show that effects were repeatable, we used independent cultures (insulin GH and factorial experiment: separately cultured cells from four fish and cortisol and GH factorial experiment: separately cultured cells from three fish). In these experiments, our experimental unit is the cell preparation, and our statistical inferences are to tilapia hepatocytes cultured using our methods. Owing to differences in the magnitude of response of different cultures, log2

Figure 1 Effects of incubation time and GH treatment on (A) *igf1* and (B) *igf2* mRNA level in primary cultured tilapia hepatocytes and expressed relative to the time 0 control treatment (rel. expr.). Filled circles, control; open triangles, native tilapia GH, 100 ng/ml (4.5×10^{-9} M). Hepatocytes were plated and preincubated in hormone-free medium for 4 h before addition of test medium. Letters indicate statistically detectable differences over time within each treatment (Student–Newman–Keuls test, $P < 0.05$, $n = 6$ –8 wells per point). Points connected by a vertical line to the left are not statistically detectably different between treatments. Symbols show the mean value for each treatment at each time point; error bars show s.d., and are shown only when larger than symbols.

Figure 2 Effect of native tilapia GH concentration on (A) *igf1* and (B) *igf2* mRNA level in primary cultured tilapia hepatocytes and expressed relative to the 0 GH control treatment (rel. expr.). Hepatocytes were preincubated for 4 h, and incubated with GH-containing medium for 6 h. Letters indicate statistically detectable differences among GH concentrations (Student–Newman–Keuls test, $P < 0.05$, $n = 7$ –12 wells per point). Symbols show the mean value at each GH concentration; error bars show s.d.

transformed data from these experiments were normalized by multiplying by the average response to GH in all cultures divided by the response to GH in a given culture. To characterize concentration–response relationships, we used pooled cells (insulin concentration–response experiment: pooled cells from two fish and cortisol concentration–response experiment: pooled cells from two fish). In these experiments, our experimental unit is the well, and our statistical inferences are restricted to the pool of cells used.

Results

Under our experimental conditions, freshly isolated tilapia hepatocytes adhered to culture plates after 4 h. Shorter adherence times resulted in substantial loss of cells when changing medium. Consequently, initial 4 h incubation in plain medium without hormones or serum was employed in all experiments. Gene expression levels of both *igf1* and *igf2* decreased rapidly during primary culture (Fig. 1, $n = 6$ –8 wells per point, two-way ANOVA *igf1*: time 55% of variation, $P < 0.0001$; GH 30%, $P < 0.0001$; interaction 12%, $P < 0.0001$ and *igf2*: time 38%, $P < 0.0001$; GH 30%, $P < 0.0001$; interaction 26%, $P < 0.0001$). After 24 h, control *igf1* mRNA levels had decreased to 0.091 ± 0.011 and *igf2* mRNA levels to 0.128 ± 0.009 -fold time 0 levels. The addition of GH ($100 \text{ ng/ml} = 4.5 \times 10^{-9} \text{ M}$) to the medium increased mRNA levels of both *igfs* versus time-matched controls. Basal and GH-stimulated *igf1* decreased with time in previous studies with primary tilapia and salmon hepatocytes (Schmid *et al.* 2000, Pierce *et al.* 2004). Based on the results for these and other transcripts, an incubation time of 6 h was selected, giving a total culture age of 10 h.

The response of both *igfs*–GH was concentration-dependent and biphasic (Fig. 2, $n = 7$ –12 wells per point, one-way ANOVA *igf1*: $P < 0.0001$ and *igf2*: $P < 0.0001$). For both *igfs*, mRNA levels were statistically detectably increased at $0.5 \times 10^{-9} \text{ M}$ GH, maximal response occurred at $5 \times 10^{-9} \text{ M}$ GH (*igf1* 1.92 ± 0.12 -fold 0 GH controls and *igf2* 8.36 ± 0.54 -fold 0 GH control), and the response was

reduced to control levels at $500 \times 10^{-9} \text{ M}$ GH. Based on these results, co-incubation with $5 \times 10^{-9} \text{ M}$ GH was used to assess the effects of insulin and cortisol on GH sensitivity.

The effects of insulin (10^{-6} M) alone and in combination with GH ($5 \times 10^{-9} \text{ M}$) were tested in four primary hepatocyte cultures. Although the magnitude of effect varied, insulin, GH, and GH plus insulin treatment affected *igf1* and *igf2* mRNA levels similarly in all cultures (*igf1*: insulin: 0.89, 0.75, 0.80, 0.76; GH: 2.48, 1.94, 1.48, 1.48; GH plus insulin: 1.86, 1.68, 1.45, 1.63-fold controls respectively and *igf2*: insulin: 1.34, 3.27, 1.98, 4.03; GH: 1.82, 6.97, 3.16, 3.93; GH plus insulin: 2.79, 16.87, 7.63, 17.23-fold controls respectively). The effect of GH on *igf1* and *igf2* mRNA levels was statistically detectable in all cultures (one-way ANOVA on replicate culture wells). To present data from all cultures, log₂ transformed data were normalized to the average level of stimulation of each *igf* by GH (Fig. 3, $n = 4$ fish, one-way ANOVA *igf1*: $P < 0.0001$ and *igf2*: $P < 0.0001$). Insulin slightly suppressed basal *igf1* mRNA levels (0.77 ± 0.06 -fold controls) but did not affect GH-stimulated *igf1* mRNA levels. Insulin increased basal *igf2* mRNA levels (2.44 ± 0.42 -fold controls), but to a lesser degree than GH (3.59-fold controls, calculation of s.d. precluded by normalization), and insulin plus GH strongly increased *igf2* mRNA levels (2.71 ± 0.46 -fold GH alone).

The effect of insulin alone and in combination with GH ($5 \times 10^{-9} \text{ M}$) was further examined in a concentration–response study (Fig. 4, $n = 8$ wells per point, two-way ANOVA *igf1*: insulin 1.8% of variation, $P = 0.0661$; GH 73%, $P < 0.0001$; interaction 2.9%, $P = 0.0043$ and *igf2*: insulin 30% of variation, $P < 0.0001$; GH 57%, $P < 0.0001$; interaction 12%, $P < 0.0001$). GH increased *igf1* and *igf2* mRNA levels at all insulin concentrations, including 0 insulin (*igf1* 1.73 ± 1.6 -fold controls and *igf2* 4.40 ± 0.10 -fold controls). Insulin did not affect the response of *igf1* mRNA levels to GH, and did not decrease basal *igf1* mRNA levels. An apparent slight increase in GH-stimulated *igf1* mRNA level with increasing insulin was not statistically detectable. Insulin increased basal *igf2* levels, and strongly increased the response

Figure 3 Effects of insulin (Ins, bovine, 10^{-6} M) treatment on basal and GH-stimulated (A) *igf1* and (B) *igf2* mRNA level in primary cultured tilapia hepatocytes, expressed relative to the control treatment (rel. expr.). Symbols show replicate cultures from individual fish. Cultures from individual fish were normalized to the average response to GH of all cultures. Letters indicate statistically detectable differences among treatments (Student–Newman–Keuls test, $P < 0.05$, $n = 4$ fish); error bars show s.d.

Figure 4 Effects of insulin concentration on basal and GH-stimulated (A) *igf1* and (B) *igf2* mRNA level in primary cultured tilapia hepatocytes and expressed relative to the 0 insulin 0 GH control treatment (rel. expr.). Filled circles, control; open triangles, native tilapia GH, 5×10^{-9} M. Letters indicate statistically detectable differences among insulin concentrations within each GH treatment (Student–Newman–Keuls test, $P < 0.05$, $n = 8$ wells per point). The effect of GH treatment was statistically detectable at all insulin levels for both *igf* transcripts. Error bars show s.d.

of *igf2* to GH. The increase in basal *igf2* mRNA level became statistically detectable at 10^{-9} M insulin, and was maximal at 10^{-6} M insulin (3.20 ± 0.43 -fold 0 insulin controls). The increase in GH-stimulated *igf2* mRNA level also became statistically detectable at 10^{-9} M insulin, and was maximal at 10^{-8} M insulin (2.83 ± 0.18 -fold GH alone).

The effects of cortisol (10^{-6} M) alone and in combination with GH (5×10^{-9} M) were tested in three primary hepatocyte cultures. Although the magnitude of effect varied, cortisol, GH, and GH plus cortisol treatment affected *igf1* and *igf2* mRNA levels similarly in all cultures (*igf1*: cortisol: 0.93, 1.11, 0.88; GH: 1.58, 1.52, 1.52; GH plus cortisol: 1.19, 1.31, 1.10-fold controls respectively and *igf2*: cortisol: 0.73, 0.77, 0.59; GH: 3.43, 4.35, 1.77; GH plus cortisol: 7.59, 8.85, 3.82-fold controls respectively). The effect of GH on *igf1* and *igf2* mRNA levels was statistically detectable in all cultures (one-way ANOVA on replicate culture wells).

To present data from all cultures, log₂ transformed data were normalized to the average level of stimulation of each *igf* by GH (Fig. 5, $n = 3$ fish, one-way ANOVA *igf1*: $P < 0.0001$ and *igf2*: $P = 0.0001$). Cortisol did not statistically detectably affect basal *igf1* mRNA levels, but suppressed GH-stimulated *igf1* mRNA levels (0.78 ± 0.038 -fold GH alone). There was a consistent but not statistically detectable trend toward suppression of basal *igf2* by cortisol ($P = 0.1312$; 0.65 ± 0.15 -fold controls). Cortisol strongly increased GH-stimulated *igf2* mRNA levels (2.65 ± 0.81 -fold GH alone).

The effect of cortisol alone and in combination with GH (5×10^{-9} M) was further examined in a concentration-response study (Fig. 6, $n = 7-8$ wells per point, two-way ANOVA *igf1*: cortisol 13% of variation, $P < 0.0001$; GH 48%, $P < 0.0001$; interaction 4.5%, $P < 0.0001$ and *igf2*: cortisol 2.2% of variation, $P < 0.0001$; GH 84%, $P < 0.0001$; interaction 5.8%, $P < 0.0001$). GH increased *igf1* and *igf2* mRNA levels at all cortisol concentrations, including 0 cortisol (*igf1* 1.86 ± 0.045 -fold controls and *igf2* 3.74 ± 0.089 -fold controls). Cortisol did not affect basal *igf1* mRNA levels, but reduced GH-stimulated *igf1* mRNA levels. The decrease in *igf1* mRNA level in cells co-incubated with GH became statistically detectable at 10^{-8} M cortisol and was maximal at 10^{-6} M cortisol (0.67 ± 0.028 -fold GH alone). Cortisol reduced basal *igf2* mRNA levels at levels of 10^{-8} M and above (0.54 ± 0.037 -fold control levels at 10^{-6} M). In contrast, cortisol increased GH-stimulated *igf2* mRNA levels. The increase became statistically detectable at 10^{-8} M cortisol, was maximal at 10^{-7} M (1.50 ± 0.065 -fold GH alone), and was statistically detectably reduced from maximal levels at 10^{-6} M.

In an analysis of all hepatocyte culture experiments employing 5×10^{-9} M GH, GH stimulation of *igf2* mRNA levels was statistically detectably greater than GH stimulation of *igf1* mRNA levels (*igf1* 1.7 ± 0.10 -fold control; *igf2* 4.2 ± 0.65 -fold control; $P = 0.0017$, t -test, $n = 10$).

Fish were injected with insulin and GH to confirm the results of our *in vitro* experiments (Fig. 7, $n = 7-8$ fish

Figure 5 Effects of cortisol (Cort, 10^{-6} M) treatment on basal and GH-stimulated (A) *igf1* and (B) *igf2* mRNA level in primary cultured tilapia hepatocytes, expressed relative to the control treatment (rel. expr.). Symbols show replicate cultures from individual fish. Cultures from individual fish were normalized to the average response to GH of all cultures. Letters indicate statistically detectable differences among treatments (Student–Newman–Keuls test, $P < 0.05$, $n = 3$ fish per point); error bars show s.d.

Figure 6 Effect of cortisol concentration on basal and GH-stimulated (A) *igf1* and (B) *igf2* mRNA level in primary cultured tilapia hepatocytes and expressed relative to the 0 cortisol 0 GH control treatment (rel. expr.). Filled circles, control; open triangles, native tilapia GH, 5×10^{-9} M. Letters indicate statistically detectable differences among cortisol concentrations within each GH treatment (Student–Newman–Keuls test, $P < 0.05$, $n = 7$ –8 wells per point). The effect of GH treatment was statistically detectable at all cortisol levels for both *igf* transcripts. Error bars show S.D.

per point, one-way ANOVA glucose: $P = 0.0010$; *igf1*: $P < 0.0001$; and *igf2*: $P = 0.0002$). Insulin and GH doses similar to those used in previous studies in fish were used, and plasma glucose was measured to confirm the expected hypo- and hyperglycemic effects of insulin and GH respectively (Mommensen & Plisetkaya 1991, Wood *et al.* 2005). Insulin injection (bovine, $5 \mu\text{g/g}$ body weight) reduced plasma glucose levels at 6 h. GH injection (ovine, $5 \mu\text{g/g}$ body weight) caused apparent increase in plasma glucose. However, this increase was not statistically detectable under our experimental conditions ($\alpha = 0.05$; probability of type 1 error = 0.156 and probability of type 2 error = 0.220). Nevertheless, co-injection of GH with insulin prevented the insulin-induced decrease in plasma glucose. GH injection increased liver mRNA levels of both *igfs* (*igf1*: 1.94 ± 0.17 and *igf2*: 7.08 ± 0.75 -fold controls). An apparent decrease in *igf1*

mRNA level with insulin injection was not statistically detectable, and insulin injection did not change *igf2* mRNA level; however, co-injection with GH and insulin increased mRNA levels of both *igf1* and *igf2* compared with levels produced by injection with GH alone (*igf1*: 1.43 ± 0.12 and *igf2*: 1.52 ± 0.13 -fold GH alone). The effects of GH and GH plus insulin on *igf2* mRNA levels were statistically detectably greater than effects on *igf1* mRNA levels.

Discussion

This study shows that *igf1* and *igf2* mRNA levels are differentially regulated by insulin and cortisol in tilapia hepatocytes through direct effects and differential modulation of GH sensitivity. Insulin increased basal *igf2* mRNA levels and strongly increased the response of *igf2* mRNA levels to GH, but did not increase basal *igf1* mRNA levels or the response of *igf1* mRNA levels to GH. Similar results were found in salmon hepatocytes (Pierce *et al.* 2005, 2010), suggesting that insulin and GH synergistically stimulate liver Igf2 but not Igf1 production in teleost fishes.

Both *igf1* and *igf2* mRNA levels were stimulated by GH in tilapia primary hepatocytes, as has been found in previous *in vitro* studies in teleost fishes and elasmobranchs (Shamblott *et al.* 1995, Schmid *et al.* 2000, Pierce *et al.* 2004, 2010, Leung *et al.* 2008, Moriyama *et al.* 2008a, b). Recent *in vivo* studies have shown that GH treatment increases liver *igf2* mRNA levels in a wide variety of fish species (see Introduction for citations), which is consistent with our results. Circulating GH levels in tilapia range from ~ 0.05 to 1×10^{-9} M (Weber & Grau 1999, Uchida *et al.* 2003, Fox *et al.* 2009), suggesting that physiological regulation of liver *igf1* and *igf2* by GH occurs on the left side of the concentration–response curve. In humans, GH injection increased liver *Igf2* transcripts from promoters P2 and P4, but did not change overall *Igf2* transcript levels, and GH treatment increased P2 transcripts in primary human hepatocytes (Olivecrona *et al.* 1999, von Horn *et al.* 2002). As far as we are aware, this is the only recent report of GH-dependent liver *Igf2* gene expression in mammals. This suggests that teleost fishes differ from mammals in that fish liver *igf2* gene expression is GH-dependent.

Incubation with insulin stimulated *igf2* mRNA level in tilapia primary hepatocytes, and co-incubation with insulin and GH strongly increased *igf2* mRNA levels over levels with insulin or GH alone. In contrast, basal *igf1* mRNA level was slightly suppressed by insulin, and GH-stimulated *igf1* mRNA level did not respond to insulin. A concentration–response study showed strong enhancement of the stimulation of *igf2* from 10^{-10} to 10^{-8} M insulin in the presence of GH. In the same cells, *igf1* responded reliably to GH, but the response was not modulated by insulin, suggesting that the synergistic interaction between insulin and GH in the regulation of *igf2* mRNA level is specific to this transcript. Although circulating insulin levels in tilapia have not been measured, fish plasma insulin ranges from 0.2 to 5×10^{-9} M

Figure 7 Effects of insulin (bovine, 5 µg/g body weight) and GH (ovine, 5 µg/g body weight) injection and co-injection on (A) plasma glucose concentration, (B) liver *igf1* mRNA level, and (C) liver *igf2* mRNA level in male tilapia. Plasma and liver samples were taken 6 h after injection. mRNA levels are expressed relative to the saline injected control treatment (rel. expr.). Symbols show individual fish. Letters indicate statistically detectable differences among treatments (Student–Newman–Keuls test, $P < 0.05$, $n = 6-8$ fish per point). Error bars show s.d.

(Mommensen & Plisetskaya 1991), suggesting that regulation of *igf2* occurs over the physiological range. In salmon hepatocytes, basal and GH-stimulated *igf2* responded similarly to insulin, whereas GH-stimulated *igf1* was suppressed by insulin (Pierce *et al.* 2005, 2010). Thus, insulin stimulation of basal liver *igf2* but not *igf1* gene expression, and enhancement of the response of liver *igf2* but not *igf1* to GH, appear to be conserved in teleost fishes.

Injection of hormones confirmed *in vivo* GH stimulation of liver *igf2* gene expression, and a positive interaction between insulin and GH in stimulation of liver *igf2* gene expression. However, insulin injection alone did not result in a statistically detectable increase in liver *igf2* gene expression, which differs from our *in vitro* results. This may have been caused by the decrease in blood glucose seen after insulin injection, or a cortisol response to the injection. Glucose positively regulates *igf2* gene expression in fetal rat hepatocytes (Goya *et al.* 1999), and in this study, cortisol tended to suppress basal *igf2* *in vitro*. Injection of GH-stimulated *igf1* gene expression, consistent with our *in vitro* results and previous studies (Kajimura *et al.* 2001, Eppler *et al.* 2010). Injection of insulin tended to decrease *igf1* gene expression (not statistically detectable), consistent with the slight suppression of basal *igf1* gene expression by insulin seen *in vitro*. However, co-injection of GH and insulin increased *igf1* gene expression versus GH alone, which differs from our *in vitro* results. This may have

been an indirect effect of insulin. In brown trout, insulin injection increased plasma Igf1 3 h later (Banos *et al.* 1999), even though in coho salmon hepatocytes, insulin did not affect basal *igf1* and suppressed GH-stimulated *igf1* (Pierce *et al.* 2005), suggesting that indirect stimulatory effects of insulin on liver Igf1 production may exist in salmonids. In this study, GH-stimulated hepatic *igf2* mRNA levels to a biologically significantly greater degree than *igf1* mRNA levels *in vitro* and *in vivo*, consistent with recent results in GH transgenic tilapia (Eppler *et al.* 2010). This suggests that liver-derived endocrine Igf2 may play a larger role than liver-derived endocrine Igf1 in mediating the growth-stimulating effects of GH in tilapia.

Insulin plays a central role in the regulation of liver Igf1 production in mammals. Insulin secretion into the hepatic portal vessels results in exposure of the liver to high concentrations of insulin. Maintenance of insulin levels is necessary to maintain liver GH sensitivity, liver *igf1* gene expression, blood Igf1 levels, and growth (Griffen *et al.* 1987, Rodgers *et al.* 1994, Thissen *et al.* 1994, Phillips *et al.* 1998, Butler *et al.* 2003). Insulin levels in the portal vessels are also elevated in fish (Plisetskaya & Sullivan 1989). In mammalian and avian primary hepatocyte culture, insulin increases basal, and GH-dependent *Igf1* mRNA level and protein secretion (Tollet *et al.* 1990, Boni-Schnetzler *et al.* 1991, Houston & O'Neill 1991, Denver & Nicoll 1994, Phillips *et al.* 1998). The results of the present and our previous studies suggest that the regulatory linkage between the pancreatic islets and the liver may also operate in fish, in the regulation of liver Igf2 production.

The differences found between mammals and fish in the regulation of liver *igf* mRNA levels by GH and insulin implies divergence of gene regulation. In mammals, current models suggest that GH stimulation of liver *Igf1* transcription is mediated by binding of the transcription factor Stat5b to multiple enhancer sites dispersed across at least 135 kb throughout the *Igf1* locus (Rosenfeld & Hwa 2009, Chia *et al.* 2010). In fish, GH can stimulate liver *igf1* transcription via Stat5 and the liver-specific transcription factor Hnf-1α (Meton *et al.* 1999, Vong *et al.* 2003b), suggesting that this pathway may be conserved. In salmonids, GH stimulation of *igf2* gene expression has been proposed to be mediated by transcription factors in the CCAAT/enhancer-binding protein (C/EBP) family (Shamblott *et al.* 1998, Palamarchuk *et al.* 2001). In rainbow trout, GH treatment increased liver *igf2* gene expression but reduced liver C/EBPβ2 occupancy of binding sites dispersed over at least 5 kb in the *igf2* locus, suggesting that the role of C/EBPβ2 may be negative (Lo *et al.* 2007, Lo & Chen 2010). However, in carp, an *igf2* promoter region sufficient for response to GH did not contain binding sites for C/EBP, Stat, or Hnf family transcription factors (Tse *et al.* 2008). The discrepancies between studies may be due to the operation of multiple long-range enhancers. Further studies are required to determine the transcription factors and gene regulatory elements that mediate GH stimulation of *igf2* transcription in tilapia.

In rats, an *igf1* promoter positive response element that interacts with a novel insulin-response element-binding protein (IRE-BP1) downstream of PI3-kinase has been identified (Villafuerte *et al.* 2004). Insulin regulation of fish *igf2* promoters has not been investigated.

In the presence of GH, cortisol reduced *igf1* and increased *igf2* mRNA levels in tilapia hepatocytes. Modulation of GH response occurred at 10^{-8} – 10^{-6} M cortisol, coinciding with the physiological range from basal to stressed in tilapia (Balm *et al.* 1994, Breves *et al.* 2010). Cortisol injection reduced liver *igf1* gene expression and circulating Igf1 *in vivo* in tilapia (Kajimura *et al.* 2003). In sea bream hepatocytes, cortisol reduced basal *igf1* mRNA levels at physiological concentrations (Leung *et al.* 2008), and in coho salmon hepatocytes, the glucocorticoid receptor agonist dexamethasone strongly inhibited GH-stimulated *igf1* mRNA levels (Pierce *et al.* 2005). Thus, glucocorticoid suppression of *igf1* expression, either directly or via GH resistance, appears to be conserved in teleost fishes. Glucocorticoids reduce liver IGF1 production in mammals (Rodgers *et al.* 1994, Brameld *et al.* 1995, Beauloye *et al.* 1999). This study and our previous study in coho salmon hepatocytes show glucocorticoid stimulation of *igf2* mRNA levels, either directly or via enhanced GH response (Pierce *et al.* 2010). Therefore, the most reasonable hypothesis at this point is that glucocorticoids inversely regulate liver production of Igf1 and Igf2 in fishes. This suggests that liver-derived endocrine Igf1 and Igf2 may have different functions in fishes. It is possible that the two Igfs differentially regulate organ and tissue growth. Cortisol increases in fishes during life history transitions in which tissue remodeling and differential growth occur, such as salinity adaptation, smoltification in salmonids, and reproductive maturation.

This and other studies provide evidence that liver-derived endocrine Igf2 may be a somatomedin in tilapia and other teleost fishes. Studies on the function of Igf2 in fish support this idea. In tilapia, administration of either recombinant native Igf1- or Igf2-stimulated growth (Chen *et al.* 2000). Circulating Igf2 is regulated by metabolic status in salmonids (Wilkinson *et al.* 2006). Both Igfs stimulated mitogenic endpoints in zebrafish embryos (Pozios *et al.* 2001), and rainbow trout muscle cell culture (Codina *et al.* 2008). Both Igfs exert negative feedback on pituitary GH secretion (Fruchtman *et al.* 2000). If endocrine Igf2 is a somatomedin in fishes, then both Igfs should be taken into account in applications of growth endocrinology in fisheries management, conservation, and aquaculture. Plasma levels of both Igfs may be useful indicators of growth status in wild and captive fishes (Picha *et al.* 2008a). Plasma levels of both Igfs may provide information on energetic status to neuroendocrine systems that regulate major life history transitions, such as smolting and reproductive maturation (Dickhoff *et al.* 1997, Luckenbach *et al.* 2010). Clearly, conclusions regarding Igf2 physiology based on experiments in mammalian model systems cannot be extended to teleost fishes (White *et al.* 2009). Further study of the physiology of the two Igfs

from a comparative perspective will clarify the functional evolution of the GH/Igf system (O'Neill *et al.* 2007), and may provide an answer to 'the Igf2 enigma' (Humbel 1990, Holly 1998).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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