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4 **Insulin and insulin-like growth factor-1 modulate the lipolytic action of growth hormone**
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6 **by altering signal pathway linkages**
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20 Running head: modulation of growth hormone actions
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ABSTRACT

Growth hormone (GH) has many actions in vertebrates, including the regulation of two disparate metabolic processes: growth promotion (anabolic) and the mobilization of stored lipids (catabolic). Our previous studies showed that GH stimulated IGF-1 production in hepatocytes from fed rainbow trout, but in cells from fasted fish GH stimulated lipolysis. In this study, we used rainbow trout (*Oncorhynchus mykiss*) to elucidate regulation of the mechanisms that enable cells to alter their lipolytic responsiveness to GH. In the first experiment, cells were removed from either fed or fasted fish, conditioned in medium containing serum (10%) from either fed or fasted fish, then challenged with GH. GH stimulated the expression of hormone sensitive lipase (HSL), the primary lipolytic enzyme, in cells from fasted fish conditioned with “fasted serum” but not in cells from fasted fish conditioned in “fed serum.” Pretreatment of cells from fed fish with “fasted serum” resulted in GH-stimulated HSL expression, whereas GH-stimulated HSL expression in cells from fasted fish was blocked by conditioning in “fed serum.” The nature of the conditioning serum governed the signaling pathways activated by GH irrespective of the nutritional state of the animals from which the cells were removed. When hepatocytes were pretreated with “fed serum,” GH activated JAK2, STAT5, Akt, and ERK pathways; when cells were pretreated with “fasted serum,” GH activated PKC and ERK. In the second study, we examined the direct effects of insulin (INS) and insulin-like growth factor (IGF-1), two nutritionally-regulated hormones, on GH-stimulated lipolysis and signal transduction in isolated hepatocytes. GH only stimulated HSL mRNA expression in cells from fasted fish. Pretreatment with INS and/or IGF-1 abolished this lipolytic response to GH. INS and/or IGF-1 augmented GH activation of JAK2 and STAT5 in cells from fed and fasted fish. However, INS and/or IGF-1 eliminated the ability of GH to activate PKC and ERK from fasted cells. These results indicate

67 that INS and IGF-1 determine the signaling pathways activated by GH and whether or not a
68 lipolytic response ensues. Such hormone-receptor-signal pathway linkages provide insight into
69 the molecular basis of GH multifunctionality and into how cellular responses to GH can be
70 adjusted to meet physiological (e.g., nutritional), developmental, or other conditions.

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72 *Keywords:* Signal transduction, rainbow trout (*Oncorhynchus mykiss*), hepatocytes, hormone
73 sensitive lipase, insulin, insulin-like growth factor

1. Introduction

The brain-pituitary-liver axis and endocrine pancreas play major roles in coordinating growth and metabolism. When food is available, anabolic conditions prevail and growth ensues. Plasma insulin (INS) peaks in the circulation after a meal to facilitate the uptake of glucose and amino acids into cells through a receptor tyrosine kinase and downstream activation of PI3K, SHP2, fyn, and several adaptors (SOCS1 and 3) (Mommensen and Plisetsakya, 1991; White, 2003). Secretion of growth hormone (GH) also increases following feeding and results in growth-related responses in target cells (e.g. increased amino acid uptake, increased RNA synthesis, increased protein synthesis, increased cartilage synthesis, and increased muscle growth); some of these resulting from direct actions of GH and others mediated by insulin-like growth factor-1 (IGF-1), which is produced by the liver and other cells (Butler and LeRoith, 2001; Reinecke et al., 2005). GH stimulates hepatic production and release of IGF-1 in fish through activation of the JAK-STAT, PI3K/Akt, and ERK signaling pathways (Reindl et al., 2011).

By contrast, during periods of fasting, catabolic conditions prevail and growth is suspended and energy stores are mobilized to fuel metabolic functions. In all groups of vertebrates studied, from fish to humans, plasma levels of INS and IGF-1 decline whereas glucagon levels increase (Norbeck et al., 2007; Pierce et al., 2005; Salgin et al., 2012). The cessation of growth appears to result from the reduced signaling pathways associated with INS and IGF-1 (Duan and Xu, 2005; Taniguchi et al., 2006). The decrease in IGF-1 occurs despite the enigmatic observation that plasma GH is elevated during fasting (Norbeck et al., 2007; Pierce et al., 2005; Salgin et al., 2012). The mobilization of energy reserves (glycogen, lipid) results from the actions of glucagon and GH (Albalat et al., 2005; Harmon and Sheridan, 1992;

O'Connor et al., 1993; Sheridan and Mommsen, 1991; Sheridan, 1994; Sos et al., 2011). Hormone sensitive lipase (HSL) is a major lipolytic enzyme that breaks down stored lipids (Watt and Spriet, 2010). It has been well characterized in adipose tissue of mammals (Lafontan and Langin, 2009) and in adipose tissue and liver of fish (Sheridan, 1994). Uniquely, fish possess two HSL-encoding mRNAs that are differentially expressed among tissues (Kittilson et al., 2011). Enhanced lipolysis results from increased HSL expression along with post-translational phosphorylation of HSL (Bergan et al., 2013; Watt and Spriet, 2010). We recently demonstrated that the lipolytic actions of GH on HSL are mediated through activation of the ERK and PKC/PLC pathways (Bergan et al., 2013).

The major question arising from these foregoing observations is how does GH have growth-promoting actions during periods of feeding and lipolytic actions during periods of fasting. In recent experiments we found that JAK-STAT and Akt pathways are activated concomitantly with deactivation of the PKC and ERK pathways during periods of feeding (and growth) whereas the PKC and ERK pathways were activated concomitant with deactivation of the JAK-STAT and Akt pathways during periods of fasting (and growth cessation) (Bergan et al., 2012). We also found that GH activated the JAK-STAT and Akt pathways in hepatocytes from fed trout resulting in enhanced IGF-1 expression but no effect on HSL expression (Bergan et al., 2015; Walock et al., 2011). By contrast, GH activated the PKC/PLC and ERK pathways in hepatocytes from fasted fish resulting in enhanced HSL expression but no effect on IGF-1 expression (Bergan et al., 2015; Walock et al., 2011). Moreover, blockade of the PKC/PLC and ERK pathways abolished GH-stimulated HSL expression (Bergan et al., 2015). From these findings, we conclude that nutritional state acts to “reprogram” the responsiveness of hepatocytes to GH.

The present study was designed to determine how nutritional state alters hepatocyte responsiveness to GH. Given previous observations that plasma levels of INS and IGF-1 differ with nutritional status (cf. Norbeck et al., 2007), we developed the working model that INS and IGF-1 alter the signal pathways to which growth hormone receptors (GHR) link to influence the specific cellular response. The hypothesis of this study was that INS and IGF-1 redirects GHR linkage away from the PKC/PLC and ERK pathways to the JAK/STAT and PI3K/Akt pathways and results in the inactivation of lipolysis.

2. Materials and Methods

2.1. Materials

All chemicals and reagents used were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise. Antibodies for the phospho-specific and total (recognizing both phosphorylated and nonphosphorylated protein) forms of Akt, ERK1/2, JAK2, PKC α/β II, and STAT5, horseradish peroxidase (HRP)-linked anti-rabbit IgG antibody, biotinylated molecular weight marker, anti-biotin-HRP antibody, and cell lysis buffer were all obtained from Cell Signaling Technology (Beverly, MA, USA). Bovine INS was obtained from Sigma, ovine IGF-1 was obtained from the NIH National Hormone and Pituitary Program, and salmonid GH was generously provided by Prof. Akiyoshi Takahashi and Dr. Shinsuke Moriyama (Kitasato University, Japan).

2.2. Experimental animals and conditions

Juvenile rainbow trout of both sexes (*ca.* 1 year of age; 88.1 ± 5.3 g) were obtained from Dakota Trout Ranch (Carrington, ND). The animals were transported to North Dakota State University where they were maintained in well-aerated, 800-L circular tanks supplied with

recirculated (10% make-up volume per day) dechlorinated municipal water at 14°C under a 12:12 hour light:dark photoperiod. Fish were fed twice daily to satiety with AquaMax Grower (PMI Nutrition International, Brentwood, MO, USA; 45% crude protein, 16% crude fat, 3% crude fiber) semi-floating trout grower until commencing experiments where fish were fasted continuously for 4 weeks or fed continuously for 4 weeks. Fed fish were fasted 2 hours before experimental manipulations. Animals were acclimated to laboratory conditions for at least 4 weeks prior to experimentation and sampling occurs at the same time of day. All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*, 8th edition (National Research Council, Washington, DC) and were approved by the North Dakota State University Institutional Animal Care and Use Committee.

At the time of sampling, fish were anaesthetized in 0.05% (v/v) 2-phenoxyethanol, measured (body weight and fork length) and euthanized by transection of the spinal cord. With some fish, whole blood was collected by caudal venipuncture (Galt et al., 2014), incubated at 4°C for 1 hour to allow clotting, then centrifuged (11,000 g, 3 min) to secure only the serum. Serum was either used that day to treat cells or stored at -80° C for later analysis. With other fish, hepatocytes were isolated by *in situ* perfusion (Mommsen et al., 1994). The isolated cells were incubated in recovery medium [in mM: 137.8 NaCl, 5.4 KCl, 0.80 MgSO₄, 0.4 KH₂PO₄, 0.34 Na₂HPO₄, 4.2 NaHCO₃, and 10 HEPES, 0.65 glucose, pH 7.6, with 2% defatted BSA, 2 ml MEM amino acid mix (50X)/100 ml, and 1 ml nonessential amino acid mix (100X)/100 ml] for 2 h at 14°C with gyratory shaking (100 rpm) under 100% O₂. The viability of the cells was assessed by trypan blue dye exclusion and ranged between 93-97% for all experiments. After the recovery period, hepatocytes were collected by centrifugation (550 g for 8-10 min) and resuspended in

incubation media (recovery media with 1.5 mM CaCl₂) to a final concentration of 2.3 x10⁶ cells/ml and aliquoted into 24-well plates (2.3 x10⁶ cells/well).

For the first experiment, cells were incubated in medium containing serum (10 % v/v) from fed ([INS] ~ 12 ng/ml; [IGF-1] ~ 212 ng/ml) or fasted fish ([INS] ~ 1.4 ng/ml; [IGF-1] ~ 107 ng/ml) and without (control) or with exogenous GH (100 ng/ml) under the same conditions as those used for recovery (14°C with gyratory shaking at 100 rpm under 100% O₂). The eight resulting experimental groups were as follows: fed cells fed serum without GH (fed-fed control), fed-fed with GH, fed cells fasted serum control, fed-fasted with GH, fasted cells fed serum control, fasted-fed with GH, fasted cells fasted serum control, and fasted-fasted with GH.

For the second experiment, fed or fasted cells were incubated in GH, INS and GH, IGF-1 and GH, or INS, IGF-1 and GH. The ten resulting experimental groups were as follows: fed cells alone, fed cells with GH, fed cells with INS and GH, fed cells with IGF-1 and GH, fed cells with INS, IGF-1, and GH, fasted cells alone, fasted cells with GH, fasted cells with INS and GH, fasted cells with IGF-1 and GH, fasted cells with INS, IGF-1, and GH. Concentrations of 100 ng/ml for INS, 200 ng/ml for IGF-1, and 100 ng/ml of GH were used; concentrations were selected to mimic “fed” conditions based on previous data (Mommensen and Plisetskaya, 1991; Pierce et al., 2005; Norbeck et al., 2007). For all experiments, serum, INS, or IGF treatments lasted for 24 h then another 30 min (for Western blot analysis) or 6 h (for mRNA expression) with or without GH. After incubation, cells were pelleted (1,000 x g for 4 min) and the supernatant medium was removed. Cells were washed with 0.5 ml phosphate- buffered saline. Cell pellets were immediately frozen on dry ice then stored at -80°C until further analysis.

2.3. *Hormone-sensitive lipase mRNA expression.*

Total RNA was extracted using RNeasy Lysis Reagent® (Qiagen, Crawfordsville, IN, USA) as specified by the manufacturer's protocol. Each RNA pellet was redissolved in 35-100 µl RNase-free deionized water and quantified by NanoDrop1000 spectrophotometry (A₂₆₀) (Thermo Scientific, West Palm Beach, FL, USA). RNA samples were then stored at -80°C until further analysis. mRNA was reverse transcribed in 5 µl reactions using 200 ng total RNA and AffinityScript QPCR cDNA Synthesis kit reagents (Master Mix, random primers, oligo-dT primers, and reverse transcriptase with block) according to the manufacturer's protocol (Stratagene, La Jolla, CA, USA). Reactions without reverse transcriptase were included as negative controls to exclude the possibility of contamination from genomic DNA; no amplification was detected in negative controls.

Steady-state levels of HSL1 (HQ225622) and HSL2 (HQ225623) mRNAs were determined by quantitative real-time PCR as described previously (Kittilson et al., 2011). Briefly, real-time reactions were carried out for samples, standards, and no-template controls in multiplex reactions with β -actin and either HSL1 or HSL2. Reactions contained 2 µl cDNA from the reverse transcription reactions, 5 µl Brilliant® II QPCR Master Mix (Stratagene), 1 µl of each 150 nM gene-specific probes, 0.5 µl of 600 nM gene-specific forward and reverse primers, and 0.15 µl reference dye (Stratagene, Agilent Technologies). Cycling parameters were set as follows: 95°C for 10 min and 45 cycles of 95°C for 30 s and 58°C for 1 min. Cross reaction was assessed by substituting alternate primer/probe sets in assays for each standard; no amplification was observed under these conditions. Sample copy number was calculated from the threshold cycle number (C_T) and relating C_T to a gene-specific standard curve, followed by normalization to β -actin (the expression of which was not affected by experimental treatments).

2.4. Western blot analysis

Cells were homogenized in 300µl 1x cell lysis buffer (Cell Signaling Technology, Beverly, MA) with 1mM PMSF, 1x protease inhibitor (Calbiochem, San Diego, CA, USA), and 1x phosphatase inhibitor (G-Biosciences, St. Louis, MO, USA). The homogenate was incubated on ice for 5 minutes then centrifuged at 16,000 x g for 10 min at 4°C. The protein concentration of the supernatant was determined by the Bio-Rad (Hercules, CA, USA) dye-binding method for microplates. Protein (50 µg) was separated by SDS-PAGE (7.5% running gel) and transferred to 0.45 µm nitrocellulose (Bio-Rad Laboratories) for western analysis as previously described (Bergan et al., 2012; Reindl et al., 2011). Membranes were washed and visualized with chemiluminescence according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK); chemiluminescence was detected directly and the bands quantified with a FluorChem FC2 imager (Alpha Innotech Corp, San Leandro, CA, USA). The abundance of phosphorylated ERK 1/2, Akt, JAK2, STAT5, and PKCα/β II was normalized to total ERK 1/2, Akt, JAK2, STAT5, and β-actin, respectively. The use of these commercial antisera to detect signal elements in rainbow trout was previously validated (Bergan et al., 2012; Reindl et al., 2011).

2.5. Data analysis.

Statistical differences were estimated by one-way ANOVA. Pairwise comparison of simple effects was assessed by Duncan's multiple range test; statistical notations on the faces of the figures reflect such comparisons. A probability level of 0.05 was used to indicate significance. All statistics were performed using SigmaStat v. 1.0 (SPSS, Chicago, IL, USA), and graphs and curve fitting models were constructed with SigmaPlot v8.0 (SPSS). Quantitative data are shown relative to control ease of comparison and are expressed as means ± S.E.M. For

experiment one, the fed cells fed serum control group was designated as the control for both fed and fasted cells.

3. Results

3.1. Serum alters HSL mRNA expression and GH pathway activation

Serum affected the expression of HSL mRNA. In fed-fed samples, GH failed to stimulate expression of HSL mRNA (Fig 1A). When pretreated with fasted serum, in the fed-fasted control group, HSL2 mRNA was significantly expressed over the fed-fed control. When the fed-fasted group was treated with GH, expression of both HSL1 and HSL2 mRNAs was greater than the fed-fed control, with HSL2 mRNA being expressed to a greater extent than HSL1 mRNA. Levels of HSL mRNAs from the fasted-fasted control group did not differ from the fed-fed control (Fig. 1B). However, when the fasted-fasted group was treated with GH, expression of HSL mRNAs were greater than the fed-fed controls but not the fasted-fasted controls. Interestingly, when fasted cells were pretreated with fed serum, in the fasted-fed group, the response to GH was abolished such that expression of HSL mRNAs in the fasted-fed control and fasted-fed GH groups were similar to the fed-fed control.

Serum influenced signaling pathways activated by GH. Activation of cell signaling pathways by GH was studied in lysates of hepatocytes isolated from 4-week fasted fish and from fish fed continuously and treated with serum. Activated (i.e., phosphorylated) forms of JAK2, STAT5, Akt, ERK, and PKC were detected in lysates of hepatocytes from all treatment groups. In the fed-fed group, GH significantly increased phosphorylation of JAK2 (Fig. 2A), STAT5 (Fig. 2C), Akt (Fig. 2E), and ERK (Fig. 2G) but not PKC (Fig. 2I). However, when either cell type (fed and fasted) was pretreated with fasted serum, GH-stimulated phosphorylation of JAK2,

STAT5, and Akt was abolished. Additionally the fed-fasted control and the fasted-fasted control groups had significantly less phosphorylation of JAK2, STAT5, and Akt than fed-fed control (Figs. 2A-2F). GH augmented phosphorylation of ERK and PKC in both fed and fasted cell types when pretreated with fasted serum above that of the fed-fed control, fed-fasted control, and fasted-fasted control (Figs 2G-2J). In this study, serum treatments modified the expression of HSL mRNAs and activated signaling pathways to reflect the serum type (fed or fasted) and not the cell type (fed or fasted).

3.2. INS and IGF-1 alter HSL mRNA expression and GH pathway activation

INS and IGF-1 are tightly regulated by nutritional state; both hormones peak postprandially and are diminished in fasting vertebrates (Mommensen and Plisetskaya, 1991; Pierce et al., 2005; Norbeck et al., 2007; Shimizu et al., 2009). We investigated INS and IGF-1 as possible factors mediating the altered expression of HSL mRNAs and activation of signaling pathways caused by serum described in the previous section. Like serum, INS and IGF-1 altered expression of HSL mRNAs. In experimental groups with fed cells, GH, INS, and IGF-1 pretreatments did not affect expression of HSL mRNAs (Fig. 3A). Basal expression of HSL mRNAs was slightly greater in fasted cells compared to fed cells. In fasted cells, GH stimulated expression of HSL mRNAs above fasted control (Fig. 3B). However, pretreatment of fasted cells with INS and/or IGF-1 abolished GH-stimulated expression of HSL mRNAs.

INS and IGF-1 influenced signaling pathways activated by GH. In fed cells, GH significantly increased phosphorylation of JAK2 (Fig 4A), STAT5 (Fig. 4C), and ERK (Fig. 4E) but not PKC (Fig. 4G). Similarly in fasted cells, pretreatment with INS and/or IGF-1 augmented GH-stimulated phosphorylation of JAK2 (Fig 4A) and STAT5 (Fig 4C). Additionally in fasted cell, GH alone stimulated phosphorylation of ERK (Fig. 4F) and PKC (Fig. 4H). However, GH-

stimulated phosphorylation of ERK and PKC was abolished when cells were pretreated with INS and/or IGF-1. Taken together, these results suggest that INS and IGF-1 are key factors in serum that regulate the metabolic mechanisms of action of GH including expression of HSL mRNAs and activation of signaling pathways.

4. Discussion

Previous work from our laboratory demonstrated that nutritional state acts as a switch to “reprogram” the responsiveness of hepatocytes to GH (Bergan et al., 2015). The results of the current study elucidates the mechanisms through which nutritional state alters the responsiveness of hepatocytes to GH and confirms our starting hypothesis that INS and IGF-1 redirects GHR linkage away from the PKC/PLC and ERK pathways to the JAK/STAT and PI3K/Akt pathways and results in the inactivation of lipolysis. The findings provide demonstrate that GHR differentially link to signal pathways that provides new insight into the mechanistic basis of the pleiotropic actions of GH and that helps resolve the disparate growth-promoting (anabolic) and lipolytic (catabolic) actions of GH.

Nutritional state modulates the lipolytic responsiveness of hepatocytes to GH. This conclusion is based on several observations. First, hepatocytes isolated from either fed fish or from fasted fish displayed higher basal rates of lipolysis (as measured by HSL expression) than hepatocytes incubated in plasma from fed fish—an observation consistent with our previous findings that basal lipolysis is higher in hepatocytes from fasting fish than in cells from fed fish (Bergan et al., 2015). Second, pretreatment of hepatocytes with serum from fasting fish augmented HSL mRNA expression regardless of the nutritional state from which the cells came. Moreover, only when cells were treated with fasting serum was GH able to stimulate HSL

mRNA expression over levels observed in fed controls. GH-stimulated lipolysis has been reported previously in fish and mammals (Albalat et al., 2005; Bergan et al., 2013, 2015; Chavez et al., 2006; Gorin et al., 1990; O'Connor et al., 1993); however, GH also has been reported to have anti-lipolytic effects in mammals (Richelsen, 1997). The antilipolytic, insulin-like effects in mammals are short-term and are observed in adipose tissue not previously exposed to GH (i.e., from hypophysectomized animals or from cells of normal individuals preincubated in the absence of GH for 2-4h). Given the conditions under which it is brought, the biological significance of the antilipolytic, insulin-like effects of GH has been questioned (Carrel and Allen, 2000). Our current and previous findings (Bergan et al., 2015) indicate that the lipolytic actions of GH can be modulated in a physiological manner by nutritional state. This work is consistent with findings in the liver of mice where nutritional state has been shown to modulate (reduced by high fat diet) the lipolytic effects of GH (Yang et al., 2013).

INS and IGF-1 underlie the nutritional regulation of GH-stimulated lipolysis. This contention is supported by several observations. First, both INS and IGF-1 are closely regulated by nutritional state: plasma levels of INS and IGF-1 peak postprandially but are diminished during fasting (Caruso and Sheridan, 2011; Norbeck et al., 2007; Shimizu et al., 2009; Stone, 2003). Second, this work indicated that cells pretreated with serum from fasted animals (when levels of INS and IGF-1 are reduced) resulted in enhanced HSL expression compared to cells treated with serum from fed animals (when levels of INS and IGF-1 are elevated). Third, INS and IGF-1 had no effect on GH-stimulated expression of HSL mRNAs in cells from fed animals, whereas INS and IGF-1 blocked GH-stimulated expression of HSL mRNAs in cells from fasted animals. It should be noted that the nature of serum is undefined and it may be that factors in addition to GH or IGF-1 contribute to the observed effects. Regardless, these findings are consistent with the

anabolic actions of INS and IGF-1 (Caruso and Sheridan, 2011) as well as with the specific anti-lipolytic actions of INS in mammals. For example, INS inhibits activation of HSL in fish (Sheridan, 1994) and attenuated fasting-stimulated lipolysis in mammals to control levels (Schneider et al., 2011).

Nutritional state dictates to which signaling pathways GHR link. Under fed conditions, GH activates JAK2, STAT5, Akt, and ERK. Evidence for this comes from the current observation that GH stimulated increased abundance of phospho-JAK2, STAT5, Akt, and ERK1/2 when cells were pretreated with serum from fed fish despite the nutritional environment from which the cells came. These pathways have been linked to GH-stimulated growth promotion previously (Reindl et al., 2011). By contrast, under fasting conditions, GH links to ERK and PKC. This is evident from our current observations that when cells were pretreated with serum collected from fasted fish, despite the nutritional environment from which they came (fed or fasted), GH increased the abundance of phospho-PKC; this did not occur in cells pretreated with serum from fed fish. These results are consistent with our previous observations that nutrition influences the activation of signaling pathways (Bergan et al., 2012) as well as on signal pathways known to link to GHR and to pathways known to activate HSL in fish and mammals (Bergan et al., 2013, 2015; González-Yanes and Sánchez-Margalet, 2006; Gorin et al., 1990).

INS and IGF-1 alter the activation of GH-stimulated pathways to reflect a fed-like state. This conclusion is supported by the observation that INS and/or IGF-1 pretreatment augmented GH-stimulated increases in phospho-JAK2 and STAT5 in both cell types (fed and fasted)—pathways associated with the fed state and known to mediate growth promotion (Reindl et al., 2011; Walock et al., 2011). Moreover, GH-stimulated phosphorylation of PKC (which has been shown

to link to lipolysis in fish and mammals, cf. Bergan et al., 2013; Gorin et al., 1990) was abolished when cells were pretreated with INS and/or IGF.

The mechanisms by which INS and IGF-1 alter pathway linkages to modulate the actions of GH are not fully known. INS and IGF-1 signaling involves numerous, interacting pathways (Caruso and Sheridan, 2011), some of which are in common with pathways that influence growth in fish as well as in mammals, such as JAK/STAT, ERK, and Akt/PI3K (Martinez et al., 2013; Reindl et al., 2011). Our current findings suggest that when GH is present with INS and/or IGF-1 (which occurs during periods of feeding) intracellular signaling becomes aligned with growth-promoting processes. This is supported by the observation in mammals that IGF-1R was required for GH to activate STAT5 in osteoblasts—an action that was mediated by inhibition of protein tyrosine phosphatase 1B (PTP1B)(which degrade JAK/STAT)(Gan et al., 2013). Concomitantly, INS activates pathways that prevent GHR linkage to pathways that activate lipolysis. For example, INS inhibits catecholamine-stimulated lipolysis in mammals by blocking signal transduction from adrenergic receptors through PKC β I (Nakamura, 2007). Additionally, insulin can work through PI3K/Akt to degrade cAMP and therefore block activation of PKA, which phosphorylates HSL and results in its activation (González-Yanes and Sánchez-Margalet, 2006). In the absence of INS (as during periods of fasting), cAMP is not degraded, PKC β I signaling is not inhibited, and GH-signaling shifts away from JAK/STAT- and Akt/PI3K-stimulated growth (Reindl et al., 2011) to PKC- activated lipolysis. Moreover, in the absence of IGF-1 (also as during periods of fasting), the inhibition of PTP1B is lifted, resulting in the degradation of JAK/STAT and contributing further to the shift away from GH-stimulated growth to GH-stimulated lipolysis.

In summary, the current findings provide novel insight into the molecular basis of the multifunctionality of GH and help resolve its disparate anabolic (growth promoting) and catabolic (lipolytic) actions. Figure 5 illustrates the pathways involved with the activation of lipolysis and the influence of insulin and IGF-1 on these pathways. During periods of feeding, the anabolic, growth-promoting actions of GH results from GHRs linking to JAK/STAT and Akt/PI3K—linkages that are enabled by the presence of INS and IGF-1. During periods of fasting, the absence of INS and IGF-1 “reprograms” cells such that GHR linkages to JAK/STAT and Akt/PI3K are disabled and linkage of GHR to PKC is enabled and the activation of HSL and lipolysis ensues. Such metabolic switching is adaptive because it provides a means of promoting growth when nutrients are available and inhibiting growth when nutrients are not available while concomitantly mobilizing the stored energy reserves necessary for sustaining maintenance metabolism. It also helps explain the apparent enigma of growth cessation in the face of sustained (or elevated) plasma levels of GH during periods of fasting (cf. Norbeck et al., 2007).

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 498

Legends to Figures

Figure 1. Effects of serum and growth hormone (GH) treatment on expression of hormone sensitive lipase (HSL) 1- and HSL2-encoding mRNAs from isolated hepatocytes of rainbow trout (A) fed continuously or (B) fasted for 4 weeks. Cells were pretreated for 24 hours with 10% serum harvested from the blood plasma of fed or fasted fish then treated with or without 100 ng/ml of GH for an additional 6 hours. Steady-state levels of mRNA were determined by quantitative real-time RT-PCR as described in the materials and methods section. Data are presented as % control from fed cells-fed serum control and expressed as mean $\pm$ SEM (n = 6). Groups with different letters are significantly (p < 0.05) different.

Figure 2. Effects of serum and growth hormone (GH) on the abundance of phosphorylated Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), protein kinase B (Akt), extracellular signal-regulated kinase (ERK), and protein kinase C (PKC) in isolated hepatocytes of rainbow trout fed continuously (A, C, E, G, I) or fasted for 4 weeks (B, D, F, H, J). Cells were incubated for 24 hours with 10% serum harvested from the blood plasma of fed or fasted fish and with or without 100 ng/ml of GH for an additional 30 minutes. Cell lysates were separated by SDS-PAGE followed by Western immunoblotting, and the blots were quantified with a FluorChem imager. The abundance of phosphorylated JAK2, STAT5, Akt, ERK1/2, and PKC α/β II was normalized to total JAK2, STAT5, Akt, ERK1/2, and β -actin respectively. Data are presented as % control from fed cells-fed serum control and expressed as mean $\pm$ SEM (n = 6). Groups with different letters are significantly (p < 0.05) different.

Figure 3. Effects of insulin (INS), insulin-like growth factor (IGF-1), and growth hormone (GH)

treatment on expression of hormone sensitive lipase (HSL) 1- and HSL2-encoding mRNAs from isolated hepatocytes of rainbow trout (A) fed continuously or (B) fasted for 4 weeks. Cells were pretreated for 24 hours with or without 100 ng/ml INS and/or 200 ng/ml IGF-1 then treated with or without 100 ng/ml of GH for an additional 6 hours. Steady-state levels of mRNA were determined by quantitative real-time RT-PCR as described in the materials and methods section. Data are presented as % control from fed cell control and expressed as mean $\pm$ SEM (n = 6). Groups with different letters are significantly (p < 0.05) different.

Figure 4. Effects of insulin (INS), insulin-like growth factor (IGF-1), and growth hormone (GH) on the abundance of phosphorylated Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), extracellular signal-regulated kinase (ERK), and protein kinase C (PKC) in isolated hepatocytes of rainbow trout (A, C, E, G) fed continuously or (B, D, F, H) fasted for 4 weeks. Cells were pretreated for 24 hours with or without 100 ng/ml INS and/or 200 ng/ml IGF-1 then treated with or without 100 ng/ml of GH for an additional 30 minutes. Cell lysates were separated by SDS-PAGE followed by Western immunoblotting, and the blots were quantified with a FluorChem imager. The abundance of phosphorylated JAK2, STAT5, ERK1/2, and PKC α/β II was normalized to total JAK2, STAT5, ERK1/2, and β -actin respectively. Data are presented as % control from fed cell control and expressed as mean $\pm$ SEM (n = 6). Groups with different letters are significantly (p < 0.05) different.

Figure 5. Model of differential growth hormone (GH)-signal pathway linkages and the activation of growth-promoting or lipid mobilizing (lipolysis) responses. Nutritional state (anabolic or catabolic), insulin (INS), and insulin-like growth factor (IGF)-1 modulate pathway

545 linkages. GH-stimulated lipolysis (as reflected by hormone-sensitive lipase, HSL,
546 activation/expression) prevails with activation of PLC/PKC/ERK pathways under
547 catabolic (e.g., fasting, absence of INS and/or IGF-1) conditions. Lipolysis is deactivated
548 under anabolic (e.g., feeding, presence of INS and/or IGF-1) conditions as signal linkages
549 switch away from the PLC/PKC/ERK pathways to the JAK-STAT and PI3K-Akt
550 pathways. In addition, crosstalk via Akt activation of phosphodiesterase, PDE, further
551 suppresses lipolysis by degrading cAMP during anabolic conditions

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5