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4   **Nutritional state modulates growth hormone-stimulated lipolysis**
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12   Running head: Nutritional state modulates GH-stimulated lipolysis
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ABSTRACT

Growth hormone (GH) regulates several processes in vertebrates, including two metabolically disparate processes: promotion of growth, an anabolic action, and mobilization of stored lipid, a catabolic action. In this study, we used hepatocytes isolated from continuously fed and long-term (4 week) fasted rainbow trout (*Oncorhynchus mykiss*) as a model to investigate the mechanistic basis of the anabolic and catabolic actions of GH. Our hypothesis was that nutritional state modulates the lipolytic responsiveness of cells by adjusting the signal transduction pathways to which GH links. GH stimulated lipolysis as measured by increased glycerol release in both a time- and concentration-related manner from cells of fasted fish but not from cells of fed fish. Expression of mRNAs that encode the lipolytic enzyme hormone-sensitive lipase (HSL), *HSL1* and *HSL2*, also was stimulated by GH in cells from fasted fish and not in cells from fed fish. Activation of the signaling pathways that mediate GH action also was studied. In cells from fed fish, GH activated the JAK-STAT, PI3K-Akt, and ERK pathways, whereas in cells from fasted fish, GH activated the PLC/PKC and ERK pathways. In hepatocytes from fasted fish, blockade of PLC/PKC and of the ERK pathway inhibited GH-stimulated lipolysis and GH-stimulated HSL mRNA expression, whereas blockade of JAK-STAT or of the PI3K-Akt pathway had no effect on lipolysis or HSL expression stimulated by GH. These results indicate that during fasting GH activates the PLC/PKC and ERK pathways resulting in lipolysis but during periods of feeding GH activates a different complement of signal elements that do not promote lipolysis. These findings suggest that the responsiveness of cells to GH depends on the signal pathways to which GH links and helps resolve the growth-promoting and lipid catabolic actions of GH.

Keywords: Growth hormone, lipolysis, signal transduction, rainbow trout (*Oncorhynchus mykiss*)

1. Introduction

Growth hormone (GH) coordinates numerous physiological processes in vertebrates, including various aspects of feeding, growth, metabolism, osmoregulation, immune function, and behavior (Bjornsson et al., 2004; Moller and Jorgensen, 2009; Norrelund, 2005; Reindl and Sheridan, 2012). The growth-promoting actions of GH occur during periods of feeding and are primarily mediated through insulin-like growth factor-1 (IGF-1) produced chiefly in the liver, a mode of action that is highly conserved from fish to mammals (Butler and LeRoith, 2001; Reinecke et al., 2005; Wood et al., 2005). GH stimulates hepatic IGF-1 synthesis and secretion by activating three cell signaling pathways: JAK-STAT, PI3K-Akt, and ERK (Reindl et al., 2011). Fish, which are particularly good models for the study of growth because most species have the capacity to grow throughout their life (i.e., indeterminate growth), display increases in body weight and body length when continuously feed, both of which are accelerated by GH treatment (cf. Norbeck et al., 2007; Biga and Meyer, 2009). Interestingly, some species of fish display a steepened growth trajectory (i.e., compensatory growth) compared to continuously fed animals upon refeeding following a bout of food deprivation that has been attributed to, at least in part, a sensitized GH-IGF system (Won and Borski, 2013).

During periods when food is not available, energy is diverted away from growth to sustain metabolic processes. In rainbow trout, for example, animals cease growing compared to their fed counter parts and mobilize stored lipid and carbohydrate and activate gluconeogenesis; with prolonged fasting such mobilization is reflected in animals as reduced condition and reduced body weight (Norbeck et al., 2007; Sheridan and Mommsen, 1991). Accompanying the

catabolic shift is an alteration in the endocrine profile, including depression of plasma levels of insulin and IGF-1 (Caruso and Sheridan, 2011; Norbeck et al., 2007; Sheridan and Mommsen, 1991); however, despite cessation of growth, plasma levels of GH increase, a pattern that is seen in fish as well as other vertebrates (Gomez-Requeni et al., 2005; Norbeck et al., 2007). This rather enigmatic finding most likely underlies the mobilization of lipid observed during fasting. Evidence that GH stimulated lipid breakdown in fish was first shown in liver and adipose tissue of coho salmon *in vivo* (Sheridan, 1986). GH was subsequently shown to directly stimulate lipolysis in liver of rainbow trout (O'Connor et al., 1993) and in adipose tissue of seabream (Albalat et al., 2005) *in vitro*. Using rainbow trout hepatocytes as a model, we recently showed for the first time in any species that GH promotes lipolysis by activating (via phosphorylation) the lipolytic enzyme that hydrolyzes stored lipid (e.g., triacylglycerol), hormone-sensitive lipase (HSL), and by stimulating the expression of HSL-encoding mRNAs (Bergan et al., 2013).

Despite our understanding of the role of GH in growth promotion and lipid catabolism, the mechanisms that enable the activation of such metabolically disparate processes are unknown. In this study, we used hepatocytes isolated from continuously fed and long-term (4 week) fasted rainbow trout (*Oncorhynchus mykiss*) as a model to investigate the mechanistic basis of the anabolic and catabolic actions of GH. Our hypothesis was that nutritional state modulates the lipolytic responsiveness of cells by adjusting the signal transduction pathways to which GH links. The rationale for this work extends from our previous observations in rainbow trout that selected signaling pathways are activated during periods of feeding (e.g., JAK-STAT, PI3K-Akt), whereas a different complement of signaling pathways are activated during periods of fasting (e.g., ERK, PKC) (Bergan et al., 2012) and that hepatic lipolysis results from GH activation of PLC/PKC and ERK (Bergan et al., 2013).

2. Materials and Methods

2.1. Materials

All chemicals and reagents 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, mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor U0126 (MEK1 and 2 are directly responsible for the activation of ERK), PI3K inhibitor LY294002 [PI3K produces phosphatidylinositol phosphates that are critical for activation of Akt by phosphoinositide-dependent kinase 1 (PDK1)], and cell lysis buffer were all obtained from Cell Signaling Technology (Beverly, MA, USA). The JAK2 inhibitor, 1,2,3,4,5,6-hexabromocyclohexane (Hex), the broad spectrum protein kinase C (PKC) inhibitor, chelerythrine chloride, and the broad spectrum phospholipase C inhibitor, U73122, were obtained from EMD Chemicals (Gibbstown, NJ, USA). Molecular weight markers were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Salmonid GH was generously provided by Prof. Akiyoshi Takahashi and Dr. Shunsuke Moriyama (Kitasato University, Japan).

2.2 Experimental animals and conditions

Juvenile rainbow trout of both sexes (*ca.* 1 year of age) 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 acclimated to laboratory conditions for at least 4 weeks prior to

experimentation and were routinely fed twice daily to satiety with AquaMax Grower (PMI Nutrition International, Brentwood, MO, USA) semi-floating trout grower. 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.

For experiments, fish were either fed or fasted continuously for 4 weeks; for the fed group animals were sampled 2h after feeding. Animals were anesthetized by immersion in 0.05% (v/v) 2-phenoxyethanol and euthanized by transection of the spinal cord. 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 6-8 x10⁶ cells/ml, and aliquoted into 24-well plates (6-8 x10⁶ cells/well). Cells were incubated in medium alone (control) or in medium with GH as specified in the figure legends under the same conditions as those used for recovery (14°C with gyratory shaking at 100 rpm under 100% O₂). In combination experiments involving pathway inhibition, inhibitors were added 2 h prior to GH treatment at concentrations specifically recommended by the manufacturer and/or used by us previously (Reindl et al., 2011) as follows: 20 µM LY294002, 10 µM U0126, 50 µM Hex, 10 µM

chelerythrine chloride, and 10 μ M U73122. In all cases, cells in replicate treatments came from different fish

After treatment, cells were pelleted (1,000 $\times$ g for 4 min) and the supernatant medium was removed. Cells were washed with 0.5 ml phosphate-buffered saline. Cell pellets and medium samples 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 TRI-Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA) as specified by the manufacturer's protocol. Each RNA pellet was redissolved in 35-200 μ l RNase-free deionized water and quantified by NanoDrop1000 spectrophotometry (A_{260}) (Thermo Scientific, West Palm Beach, FL, USA). RNA samples were then stored at -80°C until further analysis. mRNA was reverse transcribed in 5 μ l reactions using 150 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.

Rainbow trout possess two HSL-encoding mRNAs, *HSL1* and *HSL2*, and steady-state levels of each 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 HSL1 or HSL2 and β -actin. Reactions contained 2 μ l cDNA from the reverse transcription reactions, 5 μ l Brilliant® II QPCR Master Mix (Stratagene), 1 μ l of each 150 nM gene-specific probes, 0.5 μ l of 600 nM gene-specific forward

and reverse primers, and 0.15 μ l 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.

2.4. Western blot analysis

Cells were homogenized in 300 μ l 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 μ g) was separated by SDS-PAGE (7.5% running gel) and transferred to 0.45 μ m nitrocellulose (Bio-Rad Laboratories) for western analysis as previously described (Reindl et al., 2011; Bergan et al., 2012). 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 PKC α / 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. Glycerol Analysis

Medium samples were deproteinated (65°C for 10 min), then centrifuged (16,000 x g for 10 min at room temperature). An aliquot of the supernatant was added to a microplate well containing buffer A (0.205 M K₂CO₃, 0.205 M KHCO₃, pH 10.0, 0.65 M (NH₄)₂SO₄), NAD⁺ (10 mM in Buffer A), and glycerol dehydrogenase [7 units/ml in buffer B (4.76 mM KH₂PO₄, 4.76 mM K₂HPO₄, pH 7.6, 1.4 µM MnCl₂, 0.9 µM (NH₄)₂SO₄)], in a 4:2:1:1 ratio, respectively, in a total volume of 200 µl. Mixtures were incubated at 25°C for 1 h, after which time the absorbency was read at 340 nm.

2.6. Data analysis

Statistical differences were estimated by one-way or two-way ANOVA, as appropriate. In all cases, main effects were significant, and no significant interactions were observed between main effects in two-way ANOVAs. 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).

3. Results

3.1. GH stimulates lipolysis in hepatocytes from fasted fish but not from fed fish

The lipolysis of stored lipids by HSL results in the release of glycerol into culture medium from hepatocytes incubated *in vitro*. The effects of GH on the time course of glycerol release from hepatocytes isolated from 4-week fasted fish and from continuously fed fish are shown Fig. 1A. The initial release of glycerol from cells of fasted fish (12 ± 2 µM/10⁶ cells) was

slightly but not significantly higher than that from cells of fed fish ($10 \pm 1 \mu\text{M}/10^6$ cells). GH rapidly stimulated glycerol release from hepatocytes from fasted fish, and within 3 h of treatment glycerol levels increased significantly above initial levels. The rate of GH-stimulated glycerol release from hepatocytes from fasted fish slowed over time but continued to increase for up to 24 hours. By contrast, GH had no effect on glycerol release from hepatocytes isolated from fish fed continuously at any time period measured. The effects of GH concentration on glycerol release from hepatocytes from 4-week fasted and from continuously fed fish also were determined (Fig. 1B). Similar to the results of the time course experiment, basal glycerol release from cells from fasted fish ($12 \pm 2.5 \mu\text{M}/10^6$ cells) was slightly but not significantly higher than that from cells from fed fish ($9.7 \pm 1.0 \mu\text{M}/10^6$ cells). GH stimulated glycerol release from hepatocytes from fasted fish in a concentration-related manner. Glycerol release from fasted fish was stimulated significantly above basal levels at a concentration of GH as low as 1 ng/ml. GH continued to stimulate glycerol release from hepatocytes from fasted fish at progressively higher concentrations, with maximum release observed at 100 -1000 ng/ml. GH had no effect on glycerol release from hepatocytes from continuously fed fish at any concentration of hormone tested. Taken together, these results indicate that GH stimulates lipolysis as measured by glycerol release from hepatocytes of fasted fish but not from cells of continuously fed fish.

We next investigated the influence of GH on *HSL* mRNA expression in hepatocytes isolated from 4-week fasted fish and from fish fed continuously. Rainbow trout possess two HSL-encoding mRNAs, *HSL1* and *HSL2* (Kittilson et al., 2011); both mRNA forms were detected in all samples and treatments of hepatocytes. In hepatocytes from fasted fish, GH stimulated the expression of both HSL-encoding mRNAs in a time-dependent matter (Fig. 2A). A significantly increase in the expression *HSL1* and *HSL2* mRNAs in cells from fasted fish was

observed after 3 h of GH treatment. Maximum stimulation of *HSL* mRNA expression in cells from fasted fish occurred after 12 h of GH treatment, increasing 244% and 277% for *HSL1* and *HSL2*, respectively; after 24 h of GH treatment, mRNA expression decreased significantly from peak values but not to levels observed in controls or in cells from fed fish. *HSL1* and *HSL2* mRNAs were differentially expressed only after 3 h of GH treatment in hepatocytes from fasted fish, with *HSL2* mRNA being expressed to a greater extent than *HSL1*. GH had no effect on *HSL* mRNA expression in hepatocytes isolated from continuously fed fish at any time period measured. The effects of GH concentration on *HSL* mRNA expression in hepatocytes from 4-week fasted and from continuously fed fish also were determined (Fig. 2B). In hepatocytes from fasted fish, GH stimulated *HSL* mRNA expression in a dose-related manner. The expression of *HSL2* mRNA was significantly stimulated over control levels at a GH concentration of 1 ng/ml, whereas *HSL1* mRNA expression was significantly increased over control levels at 10 ng/ml GH. Significant differential expression of *HSL* mRNAs, with *HSL2* being expressed to a greater extent than *HSL1*, was evident in cells from fasted fish treated with 100 ng/ml and 1000 ng/ml GH. GH had no effect on *HSL* mRNA expression in hepatocytes from continuously fed fish at any concentration of hormone tested. Taken together, these results indicate that GH stimulates *HSL* mRNA expression in cells from fasted fish but not in cells from continuously fed fish.

3.2. Previous nutritional state influences the 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. Activated (i.e., phosphorylated) forms of JAK2, STAT5, Akt, ERK, and PKC were detected in lysates of hepatocytes from all treatments. The basal activation state of JAK2, STAT5, and Akt was greater in hepatocytes from

fed fish than in cells from fasted fish (Fig. 3A-3C); whereas, the basal activation state of ERK and PKC was greater in hepatocytes from fasted fish than in cells from fed fish (Fig. 3D & 3E). In cells from fed fish, GH significantly increased phosphorylation of JAK2, STAT5, Akt, and ERK (Fig. 3A-D). GH treatment of hepatocytes from fed fish did not change the abundance of phospho-PKC (Fig. 3E). In cells from 4-week fasted fish, GH significantly increased the abundance of phospho-ERK and phospho-PKC (Fig. 3D & 3E). Notably, GH increased the phosphorylation of ERK in cells from fasted fish to a greater extent than in cells from fed fish (Fig. 3D). GH had no effect on the activation of JAK2, STAT5, or Akt in hepatocytes from fasted fish (Fig. 3A-3C). Taken together, these results indicate that nutritional state influences the complement of signal pathways activated by GH. GH activates JAK2, STAT5, and ERK in hepatocytes from fed fish, whereas in cells from fasted fish, GH activated PKC and ERK; ERK is activated by GH to a greater extent in cells from fasted fish than in cells from continuously fed fish.

3.3. Linkage of signal pathway activation to GH-stimulated lipolysis

The linkage of specific cell signaling pathways to GH-stimulated lipolysis and GH-stimulated *HSL* mRNA expression in hepatocytes isolated from 4-week fasted fish and from fish fed continuously was studied using pharmacological inhibitors previously verified for use in rainbow trout (cf. Bergan et al., 2013; Reindl et al., 2011). The first series of experiments examined the effects of pathway blockade on lipolysis as measured by glycerol release (Fig. 4). As observed above, GH (100 ng/ml) stimulated the release of glycerol over basal levels from hepatocytes isolated from fasted fish but not from fish fed continuously. Pretreatment of cells from fasted fish with the PKC inhibitor, chelerythrine chloride, or with the PLC inhibitor,

U73122, completely blocked GH-stimulated lipolysis. The MEK inhibitor, U0126, partially blocked GH-stimulated lipolysis in cells from fasted fish. By contrast, inhibition of JAK2 (with Hex) or PI3K (with LY294002) had no effect on GH-stimulated glycerol release from hepatocytes from fasted fish. Blockade of the JAK-STAT, PI3K-Akt, MEK/ERK, or PLC/PKC pathways had no effect on glycerol release from hepatocytes isolated from fed fish.

The second series of experiments examined the effects of pathway blockade on GH-stimulated *HSL* mRNA expression in hepatocytes isolated from 4-week fasted fish and from fish fed continuously (Fig. 5). Similar to observations made above, GH (100 ng/ml) stimulated the expression of *HSL* mRNAs in hepatocytes from fasted fish but not in cells from fed fish. In hepatocytes from fasted fish, blockade of the ERK pathway with the MEK inhibitor (U0126) partially inhibited expression of *HSL*-encoding mRNAs. When cells from fasted fish were pretreated with the PLC inhibitor (U73122) or the PKC inhibitor (chelerythrine chloride), GH-stimulated *HSL* expression was abolished. Similar to the effects of pathways blockade on glycerol release, inhibition of JAK or PI3K had no effect on GH-stimulated *HSL* expression in hepatocytes from fasted fish. None of the pathway inhibitors affected *HSL* expression in cells from fed fish. Taken together, these results indicate that GH-stimulated lipolysis and GH-stimulated *HSL* expression are inhibited by blockade of the PLC/PKC and ERK pathways but not by blockade of the JAK-STAT or PI3K-Akt pathways.

4. Discussion

The results of this study indicate that GH stimulates lipolysis as well as *HSL* mRNA expression in hepatocytes from fasted fish but not in hepatocytes from fish fed continuously. In addition, the effects of GH on lipolysis and *HSL* expression are mediated through the PLC/PKC

and MEK-ERK signaling pathways, pathways that are not activated by GH in cells from fed fish. These findings support our starting hypothesis that nutritional state modulates the lipolytic responsiveness of cells by adjusting the signal transduction pathways to which GH links. Such findings provide new insight into the mechanisms that underlie the differential responses of cells to GH and how those responses can be modulated in a manner adaptive to an animal's physiological or developmental state.

The lipolytic action of GH depends on nutritional state. This conclusion is supported by two current observations. First, GH stimulated the release of glycerol from hepatocytes isolated from fasted fish but not from cells isolated from fish fed continuously. Second, GH stimulated the expression of HSL-encoding mRNAs in hepatocytes isolated from fasted fish but not in cells isolated from fish fed continuously. These findings are consistent with the lipolytic actions of GH observed in the adipose tissue of mammals (Chavez et al., 2006; Gorin et al., 1990) and in the liver of fish (Bergan et al., 2013; O'Connor et al., 1993) that results in elevated plasma fatty acids in both groups (Fain, 1980; Sheridan, 1994). The findings contrast with observations in adipocytes of seabream in which the lipolytic effects of GH appear more pronounced from fed animals than from fasted animals (Albalat et al., 2005). The basis for this difference is not known, but it may reflect differences in tissue, species, and/or life history. The current findings also support our recent report that the lipolytic action of GH includes activation of HSL as well as *de novo* synthesis of HSL as reflected by enhanced *HSL* mRNA expression (Bergan et al., 2013). Trout and other teleosts possess multiple *HSL* mRNAs (cf. Kittilson et al., 2011) and the functional significance of the two isoforms is unclear. However, interestingly, the responsiveness of *HSL2* to GH was greater than that of *HSL1* in cells from fasted fish. Structural differences in the intracellular domains of the trout HSLs, including the number of potential phosphorylation

sites (cf. Kittilson et al., 2011), may explain the observed differences in responsiveness to GH and contribute to distinct physiological roles. Sheridan (1994) noted that GH is not universally lipolytic and attributed the variability to route/duration of exposure, species/life history pattern, seasonal and other rhythmicity, and interaction with other factors. For example, GH has been shown to have short-term antilipolytic effects in mammalian adipose tissue not previously exposed to GH (i.e., from hypophysectomized animals or from cell of normal animals preincubated in the absence of GH); however, the physiological relevance of such findings are unclear (Carrel and Allen, 2000). In fish, chronic GH exposure was strongly lipolytic in coho salmon parr; however, following smoltification, fish were refractory to GH treatment and no lipolytic effects were observed (Sheridan, 1986).

The lipolytic action of GH in the fasting state results from selective activation of the PLC/PKC and MEK/ERK signaling pathways. This conclusion is supported by several lines of evidence. First, GH increased the abundance of phospho-ERK as well as of phospho-PKC in cells from fish fasted 4 weeks; ERK and PKC were not activated by GH in cells from continuously fed fish. Second, specific blockade of the ERK pathway or of PLC/PKC inhibited GH-stimulated lipolysis as well as GH-stimulated *HSL* expression in cells from fasted fish. Third, previous work showed that fasting of rainbow trout resulted in the activation of the PKC and ERK pathways accompanied by enhanced HSL activity and enhanced expression of *HSL* mRNAs (Bergan et al., 2012). Enhanced ERK activation also has been observed in calorie-restricted mammals (Nadeau et al., 2006). Fourth, previous work on hepatocytes from short-term (7 day) fasted trout demonstrated that PKC and ERK mediated GH-stimulated lipolysis (Bergan et al., 2013). PKC also was shown to mediate GH-stimulated lipolysis in adipose tissue from rats (Gorin et al., 1990) but not in the 3T3-L1 immortal cell line stably transfected with

hGHR (Asada et al., 2000). Lastly, crosstalk between pathways also exists, and evidence in mammalian adipose indicates that PKC can activate ERK as well as PKA (Fricke et al., 2004).

The JAK/STAT and PI3K/Akt pathways do not play a role in GH-stimulated lipolysis. This conclusion is supported by the current observation that GH increased the abundance of phospho-JAK2, phospho-STAT5, and phospho-Akt in hepatocytes not undergoing lipolysis from fish fed continuously; whereas, GH had no effect on the activation of JAK2, STAT5, or Akt in cells undergoing lipolysis that were isolated from fasted animals. In addition, specific inhibition of JAK2, PI3K, or of Akt directly had no effect on GH-stimulated lipolysis in cells from fasted fish (current data; Bergan et al., 2013). Moreover, the basal phosphorylation state of JAK, STAT, and Akt was lower in cells from fasted fish compared to cells from continuously feed fish, an observation that was consistent with our previous study that showed that the JAK-STAT and PI3K-Akt pathways were activated in tissues of fed fish that were actively growing and deactivated in tissues of fasted fish that were growth retarded (Bergan et al., 2012). Fasting also was shown to blunt activation of JAK-STAT in mammalian adipose and muscle tissues (Moller et al., 2009).

Several possible linkages between signaling pathways and specific lipolytic responses exist. Given that HSL possesses two catalytic states governed by phosphorylation sites (Sheridan, 1994; Watt and Steinberg, 2008), one possible mechanism is direct activation of HSL. The findings that GH-stimulated lipolysis in hepatocytes from fasted fish is dependent on ERK and PLC/PKC activation coupled with our previous findings that GH-stimulated phosphorylation of HSL is mediated by ERK and PLC/PKC (Bergan et al., 2013) indicate that HSL of trout is activated by ERK and PLC/PKC. Studies in mammals have shown that ERK is an immediate activator of HSL (Greenberg et al., 2001). Evidence shows that other kinases (e.g., cGMP-

dependent protein kinases) also may activate HSL (Yeaman, 2004). Enhanced lipolysis also may result from increased HSL synthesis. From early studies in rat adipocytes, GH-stimulated lipolysis was found to be blocked by inhibitors of RNA and protein synthesis (Fain, 1980). The present findings indicate that expression of HSL-encoding mRNAs was dependent on the ERK pathway and on PKC/PLC. The *HSL* promoter in mammals possesses several elements that may be recognized by transcription factors known to associate with the PKA, PKC and ERK (*SFI*, *Sp1*, C/EBPs) (Lampidonis et al., 2008; Holysz et al., 2011; Fricke et al., 2004; Piwien-Pilipuk et al., 2002; Lo et al., 2007).

The differential activation of signal pathways observed in the present study helps resolve the metabolically disparate growth-promoting and lipid catabolic actions of GH (Fig 6). During periods of feeding, GH activates the JAK-STAT, PI3K/Akt, and ERK pathways (current data), which in turn, leads to the synthesis and release of IGF-1 (Reindl et al., 2011; Reindl and Sheridan, 2012). There is concomitant repression of catabolic pathways through 1) suppression/deactivation of catabolic signaling molecules such as PKC (current data; Bergan et al., 2012) and 2) Akt-mediated activation of PDE, which in turn hydrolyzes cAMP (Chavez et al., 2006), thereby reducing HSL activation through PKA (cf. Bergan et al., 2013). During periods when food is not available, animals cease growing and begin to mobilize stored energy reserves, including a marked breakdown of stored lipids that is accompanied by an increase in the activity and synthesis of HSL (Bergan et al., 2012; Han et al., 2011; Kittilson et al., 2011; Mommsen and Sheridan, 1991; Norbeck et al., 2007; Tian et al., 2013). Despite the cessation of growth, plasma levels of GH increase during periods of fasting in fish and other animals (Gomez-Requeni et al., 2005; Norbeck et al., 2007). During this period, GH activates PLC/PKC and ERK (current data), which in turn leads to the activation and synthesis of HSL (Bergan et al.,

2013). There also is a suppression/deactivation of anabolic signaling molecules such as JAK2, STAT5, and Akt (current data; Bergan et al., 2012). Taken together, these findings indicate that the nutritional environment governs the signal pathways to which GH links and that such linkage determines whether the growth-promoting or lipid catabolic actions of GH prevail. That ERK is activated by GH under both anabolic and catabolic conditions (but to a greater extent under catabolic conditions; current data) suggests that the entire complement of signal pathways activated under a given condition may be more important in determining a physiological response than the activation of a specific pathway, possibly due to alterations in the network of downstream events that link pathways to responses.

The proximate “governor” of GH-signal pathway selection is not known. There is evidence that SOCS, which has been shown to selectively inhibit JAK, may play a role (Leung et al., 2004). Given our postulated position of Akt at the nexus of anti-lipolysis/lipogenesis and lipolysis (cf. Fig. 6), the presence (or absence) of factors that influence Akt activation, such as INS and IGF-1 (Caruso and Sheridan, 2011), also may play a role. It is possible to envision, therefore, that under nutrient-limiting conditions that the observed deficiency of INS and IGF-1 (cf. Norbeck et al., 2007) and, consequently, reduced phospho-Akt abundance, shifts the balance toward GH-stimulated lipolysis; whereas, under positive nutrient conditions, the presence of INS and IGF-1 and, consequently, increased abundance of phospho-Akt, the balance is shifted toward the anabolism, including, possibly, the insulin-like anti-lipolytic (Carrel and Allen, 2000) actions of GH. Support for such a scenario requires future work aimed at addressing the effects of INS and IGFs on GH-signal pathways linkages.

In summary, we found that nutritional state modulates the lipolytic actions of GH. GH stimulated lipolysis in hepatocytes removed from fasted rainbow trout but not in cells removed

from fish that were continuously fed. The lipolytic actions of GH include activation of lipolysis as measured by glycerol release as well as increased expression of HSL-encoding mRNAs. During fasting GH activates the PLC/PKC and ERK pathways resulting in lipolysis, but during periods of feeding GH activates a different complement of signal pathways (JAK-STAT, PI3K/Akt, ERK) that do not promote lipolysis. These findings demonstrate that nutritional state modulates the lipolytic responsiveness of cells by adjusting the cell signaling pathways to which GH links and suggest possible mechanisms that underlie the disparate growth-promoting and lipid catabolic actions of GH (cf. Fig. 6). On a more general level, the notion of specific GH-signal pathway linkages provides insight into the potential mechanisms that underlie the differential responses of cells to GH on two levels. First, cell-specific GH-signal pathway linkages may underlie the vast array of GH actions observed in animals (e.g., feeding, feeding, growth, metabolism, osmoregulation, immune function, and behavior; cf. Bjornsson et al., 2004; Moller and Jorgensen, 2009; Norrelund, 2005; Reindl and Sheridan, 2012). Second, alterations in GH-signal pathway linkages provide a means for cells to adjust, either short term or long term, their responsiveness to GH in a manner adaptive to an animal's physiological or developmental state.

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Legends to Figures

Fig. 1. Growth hormone (GH)-stimulated lipolysis as measured by glycerol release from hepatocytes isolated from rainbow trout fed continuously or fasted for 4 weeks. A: time-dependent release of glycerol from cells incubated with 100 ng/ml GH. B: concentration-dependent release of glycerol from cells incubated in the absence or presence of various concentrations of GH for 6 h. Data are presented as means $\pm$ S.E.M. (n=6). For a given nutritional state (fed or fasted), groups with different letters are significantly ($p < 0.05$) different; * indicates significant difference between nutritional states at given time or given concentration.

Fig. 2. Growth hormone (GH)-stimulated expression of hormone sensitive lipase (HSL) 1- and HSL2-encoding mRNAs in hepatocytes isolated from rainbow trout fed continuously or fasted for 4 weeks. A: time-dependent HSL mRNA expression in cells incubated with 100 ng/ml GH. B: concentration-dependent HSL mRNA expression in cells incubated in the absence or presence of various concentrations of GH for 6 h. Data are presented as % of control (time 0 or 0 ng/ml GH) and expressed as mean $\pm$ SEM (n = 6). For a given HSL isoform in a given nutritional state, groups with different letters are significantly ($p < 0.05$) different; * indicates significant difference between HSL isoforms in cells of the same nutritional state at a given time or at a given concentration; + indicates significant difference between cells from fed and fasted fish for a given HSL isoform treated with GH for a given time or at a given concentration.

Fig. 3. Effects of 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 or fasted for 4 weeks. Cells were incubated in the absence or presence of 100 ng/ml of GH for 30 min. Data are presented as % of control (cells from fed animals treated with 0 ng/ml GH) are expressed as means $\pm$ S.E.M. (n=4). Groups with different letters are significantly ($p < 0.05$) different.

Fig. 4. Effects of signaling element blockade on growth hormone (GH)-stimulated lipolysis as measured by glycerol release from hepatocytes isolated from rainbow trout fed continuously or fasted for 4 weeks. Cells were pretreated for 2 h with or without specific inhibitors for the following signaling elements: MEK (10 μ M U0126), PI3K (10 μ M LY294002), JAK (10 μ M Hex.), PKC (10 μ M chelerythrine chloride), PLC (10 μ M U73122); after which time, cells were treated with 100 ng/ml GH for 6 h (control is 0 ng/ml GH). Data are presented as means $\pm$ S.E.M. (n=6). For a given nutritional state (fed or fasted), groups with different letters are significantly ($p < 0.05$) different; * indicates significant difference between nutritional states within the same treatment.

Fig. 5. Effects of signaling element blockade on growth hormone (GH)-stimulated expression of hormone sensitive lipase (HSL) 1- and HSL2-encoding mRNAs in hepatocytes isolated from rainbow trout fed continuously (A) or fasted for 4 weeks (B). Cells were pretreated for 2 h with or without specific inhibitors for the following signaling elements: MEK (10 μ M U0126), PI3K (10 μ M LY294002), JAK (10 μ M Hex.), PKC (10 μ M chelerythrine chloride), PLC (10 μ M U73122); after which time, cells were treated with 100 ng/ml GH for 6 h. Data are presented as % of control (0 ng/ml GH) and expressed as mean $\pm$ SEM (n = 6). For a given HSL isoform, groups with different letters are significantly ($p < 0.05$) different. * indicates significant difference between HSL isoforms within the same treatment.

Fig. 6. Model of the modulation of growth hormone (GH)-signal pathway linkages and the activation of growth-promoting or lipid mobilizing (lipolysis) responses by nutritional state. When animals are in an anabolic state (e.g., during periods of feeding), GH initially activates JAK2 which, in turn, activate Raf-MEK-ERK, PI3K-Akt, and STAT5. Crosstalk between the PI3K-Akt and Raf-MEK-ERK pathways exists via Akt activation of Raf. ERK, Akt, and STAT5 all promote growth via the synthesis and secretion of IGF-1. Lipolysis is retarded in the anabolic state as a result of Akt activation of PDE and the ensuing degradation of cAMP, a factor involved in activation of the lipolytic enzyme, hormone sensitive lipase (HSL), concomitant with repression of activation of catabolic signal elements (PLC/PKC). When animals are in a catabolic state (e.g., during periods of fasting), PLC and PKC are activated, which are essential for propagation of signal to activate Ras/GRP-MEK-ERK and, possibly, PKA (not shown). Lipolytic processes involve activation (via phosphorylation) of HSL, which hydrolyzes stored triacylglycerol to fatty acids and glycerol, as well as *de novo* synthesis of HSL as reflected by increases in *HSL* mRNA expression. HSL can be directly activated by PKA, PKC, and ERK. Expression of HSL mRNAs is enhanced by PKC, ERK, and PKA via a variety of transcription factors to which these signal elements interact (e.g., SF1, Sp1, C/EBPs). GH-stimulated lipolysis is accompanied by deactivation of the JAK-STAT and PI3K-Akt pathways; lower Akt abundance results in reduced activation of antilipolytic processes (via PDE) and the favoring of lipolysis. Indeterminate number steps are indicated by dashed lines. (This model represents a synthesis of many studies; see discussion for details and references.)

Figure 1

A. Time

B. Concentration

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6