NUTRITIONAL REGULATION OF GROWTH HORMONE-STIMULATED LIPOLYSIS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Growth hormone (GH) regulates several physiologic processes in vertebrates, including the promotion of growth, an anabolic process, and mobilization of stored lipid, a catabolic process. Here, we used rainbow trout (*Oncorhynchus mykiss*) as a model to examine the nutritional programming required for the disparate metabolic actions of GH, specifically lipolysis. Juvenile trout were exposed to fed and fasting regimens *in vivo* and subsequent hormone treatment *in vitro*. We used real-time quantitative-PCR to measure levels of mRNA expression of Hormone-sensitive lipase 1 (HSL1) and HSL2 in liver, muscle, and adipose tissue. We used Western blotting to investigate the signaling pathways affected by nutritional state and activated by GH (e.g., JAK-STAT, MAPK, PI3K-AKT, PKC-PLC).

In vivo, fasting retarded growth and activated lipolysis through enhanced HSL mRNA expression and protein activation. Moreover, fasting resulted in phosphorylation of ERK and PKC but not Akt, JAK2, and STAT5 in adipose tissue, liver, and muscle. *In vitro*, GH stimulated glycerol release, HSL mRNA expression, and HSL phosphorylation in a time- and concentration-related manner but only in hepatocytes isolated from fasted and not fed fish. Moreover, these actions were dependent upon PKC-PLC and MAPK-ERK activation but not JAK-STAT or PI3K-Akt action. Nutritional state, insulin, and insulin-like growth factor I (IGF-I) pretreatments affect lipolytic responsiveness in hepatocyte. When in a fed state, with high levels of insulin and IGF, GH links to JAK-STAT pathways to promote growth. In a fasted state, with low levels of insulin and IGF, GH links to lipolysis through PKC and ERK activation. The findings of this dissertation indicate that nutritional status of an organism may mediate the pleiotropic actions of GH by linking it to unique intracellular signaling pathways. In the circumstances of fasting, GH stimulates lipolysis through PKC and ERK activation.

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LIST OF ABBREVIATIONS

AC……..……..…..… ……..……...…..…….……Adenylate Cyclase

Akt..……..……..Protein Kinase B

ATGL ….……... Adipose Triacyglyceride Lipase

βA….…….……...…… β-adrenergic

cAMP…….……..…..… ……..……Cyclic Adenosine Monophosphate

cDNA……..……Complimentary Deoxyribonucleic Acid

CHO……..……Chinese Hamster Ovarian

ERK……..……...…Extracellular Signal-Regulated Kinase

FAK…..……..……..……..……...Focal Adhesion Kinase

GH……..……...Growth Hormone

GHR……..…… Growth Hormone Receptor

GHRH……..…… Growth Hormone Releasing Hormone

GPCR ……..……..…..G Protein-Coupled Receptor

GRF……..……..……..…..… ……..……Growth Hormone Releasing Factor

HRP……..……Horseradish Peroxidase

HSL……..……...…Hormone-Sensitive Lipase

IGF……..……Insulin-like Growth Factor

IGFR……..……..…… Insulin-like Growth Factor Receptor

IMTG…..……..……..…..… ……..……Intramuscular Triglyceride

IP3…..……..……..……1,4,5-Trisphosphate

IRS……..……...Insulin Receptor Substrates

JAK……..……..Janus Tyrosine Kinase

LD…..……...Lipid Droplet

MAPK…..……..……..…..… ……..Mitogen-Activated Protein Kinase

MEK…….. …….MAPK kinase

mRNA……..……Messenger Ribonucleic Acid

PACAP…..……..……..…..… Pituitary Adenylate Cyclase Activating Polypeptide

CRH ….……..……..…..… ……..…….....Corticotropin-Releasing Hormone

PCR……..……..Polymerase Chain Reaction

PI3K…..……..…….Phosphoinositide 3-Kinase

PIP2….. ……..……..Phosphatidylinositol 4,5-bisphosphate

PKC……..……..Protein Kinase C

PLC……..…….....Phospholipase C

PP1……..……...Protein Phosphatase 1

PP2A…..…. … ….……..……..…..… ….……..……..Protein Phosphatase 2A

PRIP… ….……..……..….Phospholipase C-Related Catalytically Inactive Protein

RT……..……..…Reverse Transcription

RT-QPCR……..…….Real-Time Quantitative Polymerase Chain Reaction

SOCS…… ….……..……..……..…Suppressor of Cytokine Signaling

SRIF…..……..……..…..… ……..…… …..... Somatostatin

STAT……..……...Signal Transducer and Activation of Transcription

TBS-T……..……Tris-Buffered Saline with Tween20

TRH…..……..……..…..… ……..……..…Thyrotropin-Releasing Hormone

UTR…..……..……..…..… ….……..…… ……Untranslated Region

WAT…..……..……White Adipose Tissue

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Growth hormone (GH) is an important regulator of growth, metabolism, reproduction, immunity, osmoregulation, and other physiological functions of vertebrates (Bjornsson, 1997; Bjornsson et al., 2002; Moller and Jorgensen, 2009; Norrelund, 2003). GH elicits its cellular effects by binding to and activating GH receptors (GHR). This activation triggers intracellular transduction through several signaling cascades and pathways. Many reviews have described the characterization, evolution, and mechanisms through which GH exerts its actions. However, a comprehensive explanation of what influences may be directing the disparate actions of GH has yet to be concluded. Therefore, this review will take a portion out of this lofty endeavor by presenting a comprehensive overview of GH, GHR, and its signaling related to lipolytic functions.

Growth hormone

GH was first examined in the 1920s and demonstrated that pituitary extracts stimulated growth in rats (Handelsman and Gordon, 1930; Lee and Gagnon, 1930). Since then, the polypeptide hormone has been shown to play a fundamental role in several other physiologies like metabolism, reproduction, and immunity in several vertebrates (Bjornsson, 1997; Bjornsson et al., 2002; Moller and Jorgensen, 2009; Norrelund, 2003). GH belongs to a structural and functional superfamily with prolactin (PRL), the fish hormone somatolactin (SL), and mammalian placental hormones (Forsyth et al., 2002; Lioupis et al., 1999). GH is primarily secreted from the pituitary gland and is regulated by the hypothalamus, peripheral feedback, and other external factors.

GH gene and protein structure

The gene structure of GH is conserved among most vertebrates. GH genes are comprised of five exons and four introns (Rajesh and Majumdar, 2007) with the exception of three orders of teleost fish, which have six exons and five introns (Moriyama et al., 2008). One copy of the GH gene exists in the majority of vertebrates; however, higher primates, caprine ruminants, and tetraploid isospondylid teleosts have multiple copies of the GH gene (Chuzhanova et al., 2000; Wallis, 1996). Specifically, higher order primates, like humans, have a group of five GH-like genes, two of which (*gh1* and *gh2*) differentially express GH through somatotroph cells in the pituitary and placenta, respectively (Baumann, 2009; Rodriquez-Sanchez et al., 2010). Some teleosts, like salmonids, have multiple GH genes due to recent tetraploidization events (Volff, 2005; Forbes et al., 1994; Venkatesh and Brenner, 1997). Despite being discovered long ago, GH continues to be characterized in novel models (Wallis et al., 2006; Wallis, 2008; Perez-Maya et al., 2012; Panicz et al., 2012; Rong-qing et al., 2013). Therefore, GH remains a topic of interest within the field of comparative endocrinology.

Transcriptional regulation of the GH gene primarily occurs in the promoter region. The GH promoter contains different *cis*-acting elements. A TATA box and Pit-1 binding site (GHF-1) have been highly conserved in all species examined. Other transcription factor binding sites are evident in the deduced promoter regions of some species and include cAMP (CRE), thyroid hormone (TRE), Vitamin D, and estrogen (ERE) response elements (Chuzhanova et al., 2000; Moriyama et al., 2008; Ip et al., 2004;). Additionally, negative regulatory elements are present in the promoter of GH (Theill and Karin, 1993). Therefore, transcriptional regulation of GH is well conserved but does show species-specific variation depending on promoter content. Transcription results in the production of an mRNA strand that is susceptible to alternative

splicing; however, only the mammalian lineage has been shown to undergo alternative splicing to produce different GH isoforms (DeNoto et al., 1981; Baumann, 2009).

Translation of the GH-encoding mRNA results in a prehormone that includes a hydrophobic signal peptide, 22-27 amino acids in length, which is later cleaved to produce a mature polypeptide ranging from 176-191 amino acids, depending on the species and isoform (Poen and Pornbanlualap, 2013; Ip et al., 2004). Interestingly, the isolated C-terminus of human GH (hGH) (resides 177-191) has been shown to stimulate lipolysis through hormone-sensitive lipase (HSL) activation (Ng et al., 2000; De Palo et al., 2006). Amino-acylation, deamidation, and glycosylation are post-translation modifications of GH; however, the biological significance of the later has yet to be elucidated (Lewis et al., 1979; Baumann, 2009). GH protein folding includes the formation of two disulfide bonds (Youngman et al., 1995; Vestling et al., 1991). In all species examined, GH contains 50-60% alpha helices (Clackson et al., 1998) and exists in a four-helix bundle motif with the two pairs of helixes parallel within the pair but antiparallel against the other pair (Schiffer et al., 2002; Poen and Pornbanlualap, 2013). GH molecules can dimerize with non-covalent bonds in circulation (Baumann, 2009).

GH in circulation

A dynamic balance of GH secretion and clearance determines circulating levels of GH. GH is primarily secreted from somatotroph cells in the pituitary (adenohypophysis), although other tissues produce GH like the placenta in mammals. Pituitary secretion is pulsate in mammals (Hall et al., 1986) and birds (Anthony et al., 1990) but secretion patterns in other groups, like fish (Bjornsson et al., 2002), are less clear. Upon stimulation, GH is released into circulation and detected at concentrations ranging from 1-10 ng/ml in most species (Salgin et al., 2012; Norbeck et al., 2007; Einarsdottir et al., 2002; Fox et al., 2010; Anthony et al., 1990) but

are as high as 300 ng/ml in cockerel birds (Harvey et al., 1978) and fine flounder (Fuentes et al., 2012). While in the bloodstream, the GH polypeptide can circulate freely or bound by one of two proteins, α2-macroglobulin or GH binding protein (GHBP) (Fisker, 2006; Kratzsch et al., 1996). GHBP is the extracellular domain of GHR and is generated either by alternative splicing or cleavage off of GHR, depending on species, and may include glycosylation (Leung et al., 1987; Martini et al., 1997; Baumann, 2002). It is soluble in circulation, but a membrane-bound version has also been characterized and shown GH-binding capability (L. González et al., 2007; Calduch-Giner et al., 2001). Work on the effects of GHBP on GH half-life has produced contradictory results (Salgin et al., 2012). GH half-life is impacted by the ability of GHBP to affect GH clearance. GH is removed from circulation through renal clearance or through interactions with GHRs (Bjornsson et al., 2002; Baumann, 2002). However, when GH is bound to GHBP, the compound is too large for glomerular filtration; moreover, GH-GHBP dimers can prevent GH from binding GHR or assist in the delivery (Fisker et al., 2006). Thus many factors determine levels of circulating GH.

GH regulation

GH regulation is complex and depends on numerous factors including species, developmental stage, and environmental conditions. Nevertheless, a brief overview of major GH regulation is important in understanding the role of GH signaling in lipid metabolism. In vertebrates, GH is regulated by direct and indirect means; the hypothalamus and peripheral tissues secrete factors and/or hormones which target pituitary cells responsible for GH production, while environmental factors and nutritional state indirectly regulate GH.

Direct GH regulation

The hypothalamus is a major controller of pituitary GH production and release. However, when hypothalamic control over the pituitary is relinquished, by physical or chemical means, the pituitary is still able to release GH into circulation and have physiological impacts in many vertebrates; this phenomenon is labeled basal GH secretion (Hall et al., 1986). When hypothalamic control is present, the route of influence varies along with the disparate anatomies of different vertebrates; anatomical features such as dispersed versus grouped somatotroph cells, portal vein presents or absents, and neurological innervations all influence how the hypothalamus governs release and production of GH from the pituitary (Hall et al., 1986).

Hypothalamic regulation is both stimulatory and inhibitory. GH releasing factors (GRF) stimulate GH secretion and have been found in all major vertebrate groups (Hall et al., 1986). However, the specific compounds acting as GRFs vary among groups (Harvey, 1993); for example, GH releasing hormone (GHRH) is a major GRF in mammals; however, it has very little affect on GH in teleosts (Peng and Peter, 1997). Key GRFs in lower vertebrates, like pituitary adenylate cyclase activating polypeptide (PACAP) and corticotropin-releasing hormone (CRH), do not have GRF activity in mammals (Rousseau and Dufour, 2007). Regardless of species or compounds, GRFs generally activate the same signal transduction cascades when triggering GH synthesis and secretion. The most is know about mammals, where GH is secreted after adenylate cyclase (AC)/cyclic AMP (cAMP)/ protein kinase A (PKA) and phosphoinositide 3-kinase (PI3K)/ mitogen-activated protein kinase (MAPK) pathway activation. Moreover, GRFs use those same pathways to upregulate and activate GH-related transcription factors, Pit-1 and CREB respectively, hence inducing GH expression (Wong et al., 2006). Somatostatin (SRIF) is a major endogenous GH inhibitor in all vertebrate groups, albeit in different mature forms ranging

from 14 to 28 amino acids in length (Rousseau and Dufour, 2007; Hall et al., 1986). In mammals, SRIF works to inhibit AC and therefore impede GH section but does not affect GH expression (Fukata et al., 1985). SRIF can also prevent GHRH release the hypothalamus (West et al., 1997).

Along with hypothalamic control, GH is regulated by peripheral tissues. Long loop feedback mechanisms are a result of GH release into systemic circulation, acting upon target tissues (e.g., liver, gonads) that discharge hormones which circulate back to impact GH at the level of the pituitary and/or hypothalamus. Insulin-like growth factor (IGF) is a major downstream hormone released by the liver upon GH stimulation. In fact, many actions promoted by GH, especially growth, are actually mediated through IGF-II and/or IGF-I, depending on life stage and species (Nakae et al., 2000; Moriyama et al., 2008; Wong et al., 2006). In regulating GH, IGF-I binds cognate receptors on the pituitary to diminish GH production and secretion (Fruchtman et al., 2002; Morita et al., 1987; Bjornsson, 1997). Moreover, IGF-I has been shown to affect hypothalamic control of GH by stimulating and diminishing SRIF and GHRH, respectively, in rat (Ghigo et al., 1997).

Besides IGFs from the liver, GH can also stimulate steroid synthesis in the gonads, which circulate back to regulate GH synthesis and secretion (Chowen et al., 2004); the mechanisms of this control are not well understood across species but can be regulated at the level of the hypothalamus and the pituitary, depending on species and compound (Wong et al., 2006; Peng and Peter, 1997). Lastly, thyroid hormones (T_3 and T_4) can be stimulated by GH and then feedback to regulate GH; however, the effect again varies with species. For example, in most fish and mammals, T_3/T_4 treatments stimulate GH synthesis and secretion, where as in eel (Rousseau,

et al., 2002), turtle, and birds, thyroid hormones attenuate GH release (Harvey, 1993; Wong et al., 2006).

Other peripheral tissues, specifically those that monitor energy, produce hormones that regulate GH. For example, ghrelin is peptide hormone produced in all major groups of vertebrates (Kaiya et al., 2011). It is multifunctional but its orexigenic actions are most studied. Ghrelin stimulates GH secretion by acting on the somatotroph cells of the pituitary (Li and Lin, 2010) and/or as a GRF synergist, depending on species (Kaiya et al., 2011; Li and Lin, 2010).

Like ghrelin, leptin is another peptide hormone that responds to overall energy storage, usual secreted from adipocytes in mammals and liver in piscine species (Rousseau and Dufour, 2007). It has been found in all major classes of vertebrates except birds, although they do posses a receptor (Denver et al., 2011), and despite dramatic amino acid reside substitutions, secondary and primary structures are well conserved. The actions of leptin, especially on GH regulation are less clear, even though leptin has been shown to regulate metabolism similarly to GH (Rousseau and Dufour, 2007). For now, all we can say is that leptin may impact GH through indirect influence on hypothalamic neuropeptides (Denver et al., 2011). More work will need to be done across vertebrates in order to generate clear trends on the regulation of GH by leptin and other peripheral factors.

Recently, some have speculated that GH could act in an autocrine/paracrine manner to self-regulate within the pituitary. However, evidence for this ultrashort loop feedback mechanism has been controversial. No clear patterns have emerged to explain the presence of GHRs on somatotroph cells and the occasional downstream regulation of GH expression and release (Wong et al., 2006). The story is further complicated when looking across species

(Bjornsson, 1997) and much more work needs to be done to understand how GH directly influences its own regulation.

Indirect GH regulation

Moving outside of the body, environmental factors can also influence GH secretion. In fish, increasing day length increases plasma GH levels; this photoperiod-response is related to developmental smoltification in anadromous species. Increased temperature has also been correlated with increased GH production in salmon, again during smoltification (Handeland et al., 2013; McCormick et al., 2000; Bjornsson, 1997; Hevrøy et al., 2013). Temperature is also important in reptiles, where thyrotropin-releasing hormone (TRH) mediates the regulation of GH (Harvey, 1993). Other environmental influences on GH in piscine species include salinity, pollutants, and handling (Deane and Woo, 2009). Environmental factors are carefully monitored by poikilotherms, and some homeotherms can also regulate GH with external influencers like photoperiod, stress, and temperature; however, the mechanisms of these actions are poorly understood and are mediated indirectly through other hormones like melatonin or cortisol (Jin et al., 2013; Brown and Martin, 1974; Harvey, 1993).

GH is strongly influenced by nutritional state. Several studies across vertebrates have shown that fasting induces heightened levels of GH. In humans, evidence of this has been available since the late 1970s. Plasma GH can swell in as little as 3 hours of fasting (Salgin et al., 2012) and remained inflated through 7 days until dropping to fed control levels even without a meal (Palmblad et al., 1977). Some have indicated that the elevated levels of GH were due to increased frequency and inflated amplitude of peaks (Ho et al., 1988; Hartman et al., 1992). This is similar in avian species. Specifically, cockerels experienced plasma GH increases after 12 hours of fasting and remained elevated through 72 hours (Harvey et al., 1978); whereas in toms,

fasting-induced elevated plasma GH was attributed to heighted amplitude during pulsate secretion but not increased frequency or duration (Anthony et al., 1990).

In piscine species, fasting induced heighted levels of GH in plasma. Specifically in rainbow trout, plasma GH levels rose after 2 weeks of fasting and continued to rise through 6 weeks of fasting, yet growth and IGF-I levels were suppressed significantly during these times (Norbeck et al., 2007). Similar results have been seen in juvenile grass carp (Wei-Min et al., 2001), fine flounder (Fuentes et al., 2012), juvenile halibut (Imsland et al., 2008), Chinook salmon (Pierce et al., 2005), coho salmon (Shimizu et al., 2009), gilthead seabream (Company et al., 1999), and common carp (Sinha et al., 2012). Tilapia has produced conflicting results in which some have shown that fasting did not affect plasma GH (Fox et al., 2010; Fox et al., 2009; Uchida et al., 2003), while other have shown a significant increase in plasma GH with fasting (Fox et al., 2009; Weber and Grau, 1999; Pierce et al., 2007; Fox et al., 2006). Moreover, other exceptions were found in rainbow trout where fasting did not boost plasma GH and could either increase (Jonsson et al., 2007) or had no effect on plasma levels of IGF-I (Kling et al., 2012). Therefore, more work needs to be done to clarify the affects of fasting on GH.

Some have proposed that GH levels rise and IGF-I levels fall due to a desensitization of the liver through changes with the GHR to diminish the negative feedback IGF-I has on GH (Bjornsson et al., 1997; Yamamoto et al., 2013; Salgin et al., 2012). These studies and others indicate that the increased plasma GH and reduced IGF-I levels associated with fasting enables energy to be allocated away from growth and focused on survival. We suspect that the fasted liver is still sensitive to GH but stimulates lipolysis and not growth promotion through IGF-1.

GH affects lipid metabolism

GH is multifunctional, being an important regulator of growth, metabolism, reproduction, immunity, osmoregulation, behavior, and other physiological functions in vertebrates (Bjornsson, 1997; Bjornsson et al., 2002; Moller and Jorgensen, 2009; Norrelund, 2003). This review is focused on the affects GH has on lipid metabolism.

Lipids play many roles in animals, but their most significant use is as an energy reserve. The breakdown of stored lipids (lipid mobilization or lipolysis) is a critical aspect of vertebrate metabolism. Generally, lipids are stored as neutral triacylglycerides (TG) surrounded by a phospholipid monolayer in a lipid droplet. The droplet is surrounded by proteins, commonly perilipins, which physically mediate enzyme access to lipids. In mammals, primary storage is in adipose tissue. By contrast, fish store lipids in a more diverse manner, which also includes the liver and red muscle (Sheridan, 1988). Enzymatic hydrolysis is required to access the lipid stores, thus releasing fatty acids and glycerol into circulation available for energy production in other cells via glycolytic/gluconeogenic pathways or oxidation, respectively (Jaworski et al., 2007).

Enzymes responsible for TG breakdown include adipose triacylglyceride lipase (ATGL), hormone-sensitive lipase (HSL) (previously known as TG lipase or lipe), and monoacylglycerol lipase, each responsible for the sequential hydrolysis of fatty acids (FA) from the glycerol backbone of the triacylglycerides with some overlapping of substrates (Watt and Spriet, 2010; Jaworski et al., 2007). Recently, novel lipases have surfaced; however, their exact role in lipolysis is less understood. Lipases have been recently reviewed (Quiroga and Lehner, 2012; Zimmermann et al., 2009; Lass et. al., 2011; Watt and Steinberg, 2008). Balancing the lipid pool is lipogenesis or lipid deposition mediated by lipoprotein lipase (LPL), which frees glycerol and

FFAs from transport proteins in the blood to be taken up by cells for use or storage (Wang and Eckel, 2009).

GH stimulates lipolysis *in vivo* as is evident by increased FA (Zhao et al., 2011; N. Moller et al., 2009; Sos et al., 2011; Clasen et al., 2013) or glycerol (Sakharova et al., 2008; L. Moller et al., 2009) in circulation after GH treatments in mammals. Similar results have also been seen in piscine species (Leatherlands and Nuti, 1981). Specifically, carp with GH transgenes underwent enhanced lipolysis and resulted in overall fewer stored lipids (C. Zhong et al., 2012). Moreover, in humans, pathological GH deficiency is associated with increased lipid deposits; GH treatment of these individuals led to reduced fat masses toward ranges normal for non-GH deficient patients (Chaves et al., 2013). GH affects a number of tissues and cell types to stimulate overall lipolysis. Below is the most recent and important literature on how GH affects lipid-storing tissues and cells.

Adipose

GH regulates lipolysis in adipose of vertebrates by stimulating lipolysis and inhibiting lipogenesis and anti-lipolysis. Recently in mice, visceral fat masses were shown to be significantly reduced and HSL mRNA and protein levels were increased with GH treatment (Yang et al., 2013). However, in other mammalian studies, the expression of HSL in response to GH is less clear (Vijayakumar et al., 2010); lipolysis may result from an increase in HSL activity through phosphorylation and not due to altered lipase expression. In primary porcine adipocytes, stored TGs were reduced by 8 hours of GH treatment and lasted through 24 hours. Also, GH stimulated HSL and ATGL mRNA expression after 15 minutes, lasting through 24 hours (Yang et al., 2012). These responses are similar across most mammals (Vijayakumar et al., 2010). Human studies generally indicate that GH has stronger lipolytic effects with prolonged exposure

to GH versus acute treatment (Carmean et al., 2014). Conversely, lactating cows showed reduced expression and phosphorylation of lipases and perilipins, when treated with feed reduction or GH, despite increased plasma non-esterified fatty acids (NEFA) (Faylon et al., 2014). LPL activity in adipose was diminished with GH treatment although the mechanisms of the action are not well understood. Interestingly, levels of LPL mRNA increase with fasting; however, the resultant protein was inactive until refeeding commenced to quickly activate latent LPL protein. Moreover, GH treatment on rat adipocytes causes a downregulation of $G\alpha_i$ expression, which is known to mediate anti-lipolytic signaling (Doris et al., 1994).

Comparatively, rainbow trout underwent GH-stimulated lipolysis *in vivo*, where mesenteric fat was reduced as measured by the mesenteric somatic index (Kling et al., 2012). Similarly, isolated adipocytes from fed and fasted seabream underwent lipolysis upon GH stimulation with more glycerol released by cells from fasted fish (Albalat et al., 2005). Unfortunately, little work has been done on the effects of GH on lipolytic gene expression in adipocytes of non-mammalian species possibly because only a handful of these genes have been characterized (Kittilson et al., 2011). However, recently it was shown that LPL mRNA expression was significantly reduced in adipose tissue of rainbow trout with fasting and GH could be the mediator of this response (Sanchez-Gurmaches et al., 2012).

Liver

In mammals, it has been shown that GH does not trigger lipolysis in the liver but instead activates TG uptake and storage (Vijayakumar et al., 2010); although, a recent study showed enhanced HSL mRNA expression with GH treatment (Yang et al., 2013). ATGL expression is very low in hepatic tissue but has been shown to contribute significantly to hepatic lipolysis in mammals (Quiroga et al., 2012). Generally, healthy mammalian livers generally do not store an

appreciable amount of lipids, whereas the lipid content in fish liver can be 10-20% and up to 67% wet weight in some species (Sheridan, 1994). These lipid stores are accessed during extended fasting periods (e.g., spawning and migration) that occur naturally throughout some piscine life histories (Magnoni et al., 2006; Sheridan, 1994). The liver is also an interesting model because it is the sight of GH-stimulated IGF-I production, which is the key mediator of several actions of GH including growth. Recently, we investigated the balance between lipolysis and IGF production in the liver of rainbow trout. GH stimulated HSL mRNA expression and glycerol release in hepatocytes isolated from fish fasted 7 days and 4 weeks but not those that were recently fed (Bergan et al., 2013; Bergan et al., in press). Moreover, HSL became phosphorylated, an indicator of activation, upon GH treatment (Bergan et al., 2013). These results agree with previous research in rainbow trout (O'Connor et al., 1993; Sheridan, 1986) and tilapia (Han et al., 2011; Tian et al., 2013). Moreover, GH-stimulated IGF mRNA expression only occurred in hepatocytes from fed and not fasted fish (Walock et al., in preparation)

The debate on whether the lipolytic effects and other physiologies regulated by GH are direct or through IGF-I continues. In mammals, some have proposed a dual-effector hypothesis in which GH and IGF-I work together in achieving physiological effects (Green et al., 1985; Isaksson et al., 1987). More recently, it has been amended to include GH-stimulated paracrine/autocrine actions of IGF-I (Bjornsson et al., 2002).

However, we and others believe that the lipolytic actions of GH are direct and not through IGF-I (L. Moller et al., 2009). In our recently work, we show that IGF-I and –II levels are not affected or are diminished by fasting and/or *in vitro* GH treatment (Walock et al., in preparation); this agrees with previous reports in coho salmon (Larsen et al., 2001), tilapia (Uchida et al., 2003), and mammals (Clasen et al., 2013; Norrelund, 2003; Chen et al., 2005).

Moreover, alone IGF-I has insulin-like effects (Simpson et al., 2004), which activate lipogenesis and arrest lipolysis (Vijayakumar et al., 2010; Sanchez-Gurmaches et al., 2012). Nevertheless, the picture is not completely clear, as others have reported that implantation of GH to the body wall of rainbow trout followed by a 6 week fast resulted in enhanced plasma IGF-I levels (Kling et al., 2012); however, Chen et al., (2005) determined that only free and not total circulating IGF levels are bioactive, and thus reports of total plasma IGF may be skewed. Therefore, this topic will require further investigation to decipher the distinct yet overlapping roles of GH and IGF.

Muscle

Muscle tissue is variable among vertebrates. In fish, dark muscle stores appreciable amounts of lipids within the fiber, whereas light muscle contains adipocytes dispersed between fibers (Sheridan, 1994). Mammalian skeletal muscle was regarded to be most like light muscle with extramyocellular adipocytes; however, within the past 20 years, intramuscular triglyceride (IMTG) stores in skeletal muscle have been a topic of interest because of their involvement with insulin resistance. Most recently, technological advances in immunofluorescence microscopy have allowed researchers to investigate these IMTG stores without contamination from extramyocellular adipocytes. It is noteworthy that IMTG are not released into circulation but instead used locally for oxidation to fuel muscle contraction during exercise (Badin et al., 2013). Contraction-stimulated lipolysis is dependent on ATGL and possibly other lipases but not HSL (Shaw et al., 2013). Fasting decreased IMTG, and HSL and ATGL were activated to a greater extent with intact GH signaling in skeletal muscle of fasted mice (Vijayakumar et al., 2010). Also, GH stimulated upregulation of ANGPTL4 expression, a protein that suppresses LPL, in human skeletal muscle *in vivo* (Clasen et al., 2013). Yet, fasting increased LPL mRNA expression after 35 days of fasting in red muscle of rainbow trout (Sanchez-Gurmaches et al.,

2012). By contrast, studies in humans and rodents indicate increased LPL activity in skeletal and cardiac muscle when fasted or treated with GH (Wang and Eckel, 2009; Oscarsson et al., 1999). Although it is known that GH stimulates muscle lipolysis and lipogenesis, the specific mechanisms have to be elucidated.

In many of the aforementioned studies, GH treatments are given on top of fasting regimens and augmented lipolysis. This could provide some insight on the multifunctionality of GH, specifically explaining how GH can trigger disparate metabolic actions (e.g., lipid breakdown and protein accretion). Moreover, upon fasting the hormonal profile in circulation changes dramatically. Some have hypothesized that GH plays a secondary metabolic role behind insulin, whose levels are diminished with fasting. It is possible that the nutritional and subsequent hormonal environment programs cells to alter GH responsiveness thus explaining the disparate metabolic actions of GH.

Growth hormone receptor

GH initiates it action by binding to GHR present on the plasma membrane of target cells. GHRs belong to the class I cytokine receptor family along with prolactin, erythropoietin, leptin, interleukins, and the piscine somatolactin receptor (Zhu et al., 2001). The single transmembrane protein is comprised of extracellular, transmembrane, and intracellular domains that homodimerize (Zhu et al., 2001; De Vos et al., 1992). Interaction with ligands activates the receptor, passing the signal internally to elicit biological function. GHRs have been characterized in over 100 species, including 25 species of fish (Reindl et al., 2009; Ellens et al., 2013).

GHR gene and protein structure

Although the GHR gene(s) has been characterized in a number of vertebrates from diverse groups, most of what is known about the gene and proteins comes from mammals.

Specifically in humans, GHR is encoded by a single copy with a 10-exon coding region. The extracellular, transmembrane, intracellular domains are encoded by exons 2-7, 8, and 9-10, respectively. Upstream, the 5' untranslated region (UTR) contains several more exons but remains poorly understood (Baumann, 2002; Pantel et al., 2000).

Expression of the GHR gene results in variable proteins. In mammals, two GHRs are known and result from alternative mRNA processing (Talamantes and Ortiz, 2002), and several GHRs are produced from distinct mRNAs in fish (Perez-Sanchez et al., 2002). Recently, Walock et al., (2014) described a third GHR-encoding cDNA in rainbow trout that has caused a re-organization of GHR nomenclature. Recent discoveries like these continue to change views on the evolution of GHRs. Nevertheless, most GHRs have a well conserved structure, from 593 to 638 amino acids in length (fish, Bjornsson et al., 2002; human, Herrington and Carter-Su, 2001).

Briefly, the extracellular domain is cysteine-rich with several sites specific for ligand binding; post-translational modifications like glycosylation and ubiquitination can influence this interaction (Bjornsson et al., 2002). The intracellular domain is tyrosine-rich and contains proline-rich regions, Box 1 and Box 2; these properties are vital for proper signal transduction (Herington and Lobie, 2012; Zhu et al., 2001).

GHR tissue distribution

GHRs are present on the plasma membrane of several tissues; across vertebrates, the most abundant is liver (Reindl and Sheridan, 2012). Other tissue types with appreciable levels of GHRs are spleen, pancreas, heart, skeletal muscle, gill, gonads, adipose, kidney, and pituitary (Very et al., 2005; Walock et al., 2014; Hirano et al., 2011; Pierce et al., 2006; Higashimoto et al., 2000). In some piscine species, which have multiple GHR-encoding mRNAs, GHR isoforms

are clearly differentially expressed among tissues (Reindl and Sheridan, 2012) raising the possibility that GHRs may play a role in the multifunctionality of GH.

Interestingly, in the past 15 years, GHRs have been found in the nuclei of some cells, usually highly proliferative cells like cancers and during liver regeneration (reviewed in Herington and Lobie, 2012; Swanson and Kopchick, 2007; Brooks et al., 2007). GHRs and GHBPs only enter the nucleus by associating with a carrier protein. Once inside, GHRs and GHBPs have been shown to act as transcription factors, associate with other TFs (Conway-Campbell et al., 2008), and are able to affect expression of reporter genes (Graichen et al., 2003). Notably, effects were only present with GH activation through an autocrine source (Brooks et al., 2008). Nuclear GHR effects could be another route of GH multifunctionality. Unfortunately, all of this work has been done *in vitro* using mammalian cell lines and sequence constructs; however, the field is in its infancy but will hopefully expand to provide new insights on the effects of nuclear GHR across vertebrates.

GHR activation

GH is the only endogenous ligand of GHR, although several synthetic compounds have been developed to activate GHR (Birzneice et al., 2009). It was previously thought that GHR-GH binding initiated recruitment of a second GHR and step-wise activation of downstream signal transduction; however, recently this idea has been changed to reflect that GHR is a constitutive dimer, associated with inactive kinases at the C-terminal intracellular domain that are activated upon GH binding through conformational changes of one of the involved GHRs (Brown et al., 2005). This process has been reviewed extensively by others albeit primarily covering mammalian models (Herington and Lobie, 2012; Brooks et al., 2008; Zhu et al., 2001);

nevertheless, we suspect the mechanisms are very similar across vertebrates due to the well-conserved actions of GH through GHRs.

GHR regulation

Regulation of GHR synthesis and activation has been recently reviewed (Reindl and Sheridan, 2012) and includes influences from hormones (e.g., GH, insulin, IGF, SRIF, estradiol) and environmental factors (e.g., nutritional state, photoperiod, temperature, salinity, etc.); however, here, we want to bring special attention to nutritional regulation as it pertains to our topic: the effects of GH on lipolysis. As mentioned earlier, levels of plasma GH rise with fasting (Salgin et al., 2012; Palmblad et al., 1977; Harvey et al., 1978; Norbeck et al., 2007; Wei-Min et al., 2001; Fuentes et al., 2012; Imsland et al., 2008; Pierce et al., 2005; Shimizu et al., 2009; Company et al., 1999), with tilapia producing conflicting results (Fox et al., 2010; Fox et al., 2009; Uchida et al., 2003; Fox et al., 2009; Weber and Grau, 1999; Pierce et al., 2007; Fox et al., 2006). However, expression of GHRs on the plasma membrane of target cells is altered in a tissue-specific manner upon fasting in many vertebrates. For example, in rat, liver GHR expression decreases with fasting (Kaji et al., 1994); this is also true for several piscine species (Fukada et al., 2004; Deng et al., 2004; Small et al., 2006; Peterson et al., 2009; Seara-Vila et al., 2005; Norbeck et al., 2007; Reindl and Sheridan, 2012) including recently rainbow trout (Walock et al., 2014), common carp (Sinha et al., 2012a), crucian carp (H. Zhong et al., 2012), and goldfish (Sinha et al., 2012b); dissimilarly, Zanzibar tilapia experienced increased hepatic GHR expression when fasted (Gao et al., 2011). In muscle of fasted species, GHR expression was amplified in tilapia, gilthead seabream, and striped bass but diminished in Mozambique tilapia (Peirce et al., 2007), rainbow trout (Walock et al., 2014), and fine flounder (Fuentes et al., 2011).

Furthermore, the multiple and distinct GHR-encoding mRNAs in some species clouds the picture. When rainbow trout were fasted 6 weeks, only GHR2a on adipose experienced augmented expression and binding capacity (Norbeck et al., 2007; Walock et al., 2014). GHRs are differentially affected by fasting in other species as well, (Seara-Vila et al., 2005; Picha et al., 2008) and we suspect this to be true across species with multiple GHRs. During fasting, tissue-specific expression of GHR subtypes could mediate the pleiotropic actions of growth hormone, i.e., type 2 receptors may better mediate lipolysis and hence are present on adipose and hepatic tissues when fasted, whereas type1 may promote growth and be more prevalent on hepatic and muscle in times of feeding. More work needs to be done on differential GHR expression, ligand binding, and linkages with signaling cascades during various nutritional states to test whether GHRs play a key role in differentiating the diverse metabolic effects of GH during distinct metabolic states.

In addition to expression of GHRs, inactivation also attributes to GHR regulation. GHR can be inactivated by ligand-mediated receptor internalization, degradation, receptor cleavage, and interruption of signaling cascades. Again, this has been recently covered (Reindl and Sheridan, 2012); however, its role in mediating the multifarious actions of GH has not been made explicit. Specifically, several ligands have been shown to trigger GHR internalization: GH, insulin, and SRIF. Insulin is said to be the key metabolic regulator; however, upon fasting, insulin levels drop and it is thought that GH takes over metabolic control. Moreover, GHR subtypes are differentially internalized upon GH treatment with more GHR2 being internalized than GHR1 (Reindl et al., 2009); this too helps support the possibility that certain GHR subtypes may be responsible for the metabolic responses to GH stimulation and others the growth-promoting actions. Nevertheless, this would only be true in those vertebrates which have

separate GHR subtypes; however, using these model organisms can still provide insight into organisms which only have a single GHR.

Tissue-specific GHR expression and activation may provide the disparate mechanism to mediate GH multifunctionality, but let us also consider the possibility that these different GHRs may be connecting to separate intracellular signaling mechanisms to mediate the pleiotropic actions of GH.

Signaling

Lipolytic signaling, specifically in mammalian adipocytes, has been recently reviewed (Chaves et al., 2011); however, the role of GH in triggering lipolysis has only been briefly mentioned. Interestingly, Chaves et al. (2011) claim that G-coupled protein receptors are the key mediator. Furthermore, the signaling mechanisms through which GH exert its actions have been recently reviewed (Herington and Lobie, 2012), but the multifunctionality of GH was not addressed. Here, we focus on the lipolytic mechanisms of GH signaling; specifically, we argue that GH acts through its cognate receptor, GHR, to activate downstream signaling events regulated by nutritional state.

Classic GH signaling

GH elicits its actions through GHR and subsequent activation of effector pathways. Classically, GH actions are mediated through Janus tyrosine kinases (JAK); however, other signaling pathways can be activated by GH. Moreover, signaling can also be influenced by negative regulators and cross talk among these pathways is frequent.

JAK2 plays an important role in GH signaling and has been the focus of much investigation for many years. Most of what is known comes from mammalian species and cell lines and has been reviewed recently (Herington and Lobie, 2012). Briefly, prior to GH/GHR

binding, JAK2 is inactively bound to each GHR. Ligand binding triggers JAK2 autophosphorylation and trans-phosphorylation of GHR and the associated JAK2 molecule (Kiu and Nicholson, 2012). The activated GHR complex can then recruit other signaling molecules.

In the canonical pathway, signal transducer and activator of transcription (STAT) 5a and 5b, and to a lesser extent STAT1 and 3, are recruited, phosphorylated, dimerized, and translocated into the nucleus to impact transcription (Mohr et al., 2012). Non-canonical pathways allow STAT to act independently of JAK2 in the cytoplasm to influence the cytoskeleton and/or cellular respiration (Mohr et al., 2012).

Besides STAT, JAK2 can interact with other signaling molecules thus triggering alternative signaling pathway activation. For example, JAK2 association with Shc can activate MAPK pathways (Grb/Sos/Ras) and associated transcription factors (Elk and CEBPβ) to influence cellular function. Also, JAK2 can hijack insulin signaling pathways (IRS1, 2, 3) to impact PI3K/protein kinase B (Akt) activity (Xu and Menssina, 2009). Lastly, JAK2 may cause alterations in the cytoskeleton through interactions with focal adhesion kinases (FAK). In fact, FAK may be used by GH to interact with PI3K (Zhu et al., 2001). FAK can also be regulated by GH but independently of JAK2 (Herington and Lobie, 2012).

Independently of JAK2, other kinases can dimerize with GHR and mediate signal transduction. Src family kinases (c-Src, c-Fyn, Lyn), for example, have been shown to transmit the GH signal to MAPKs, small GTPases, and FAK. Many of these signaling molecules have been shown to participate in crosstalk with other pathways, including canonical JAK/STAT, EGF Receptors, and IGF-I Receptor (Herington and Lobie, 2012).

GH signaling is negatively regulated by suppressors of cytokine signaling (SOCS) and protein tyrosine phosphatases (PTP). SOCS regulate all forms of cytokine signaling, but SOCS1,

2, 3, and cytokine inducible SH2 protein (CIS) are best understood and are known to regulate GH-specific signaling, albeit through different mechanisms in fish (Studzinski et al., 2009) and mammals (Ahmed and Farquharson, 2010). All SOCS induce proteosomal degradation of targets through ubiquitination (Ahmed and Farquharson, 2010). However, there is some controversy with the exact mechanism and effects of individual SOCS proteins, as cell type and concentration influence the outcomes (Herington and Lobie, 2012; Ahmed and Farquharson, 2010). SOCS1 binds inactive JAK thus preventing GHR phosphorylation. SOCS2 and 3 bind the activated receptor through SH2 domains, which prevents recruitment of subsequent signaling molecules and downstream activation (Krebs and Hilton, 2000; Ahmed and Farquharson, 2010).

SOCS regulates GH signaling as a negative feedback mechanism as GH has been shown to stimulate SOCS expression. In skeletal muscle from human males, a single *in vivo* GH bolus caused enhanced expression of SOCS1-3 and CIS (Clasen et al., 2013). The same response was seen *in vitro* in a GH-treated 3T3-L1 adipocytes cell line (Fleenor et al., 2006). And GH stimulated expression of SOCS2 and 3 in porcine primary adipocytes (Yang et al., 2012). Moreover, GH-stimulated SOCS2 expression in liver was STAT5b-dependent (Vidal et al., 2007). Again, this relationship is not completely understood. For example, SOCS displayed concentration-dependent influence on the growth-promoting actions of GH. Specifically, low concentrations of SOCS inhibited GH-stimulated growth promotion, while high concentrations augmented these actions in the liver of mice (Greenhalgh et al., 2005).

PTPs also negatively regulate classic GH signaling. PTPs in animals, from sponges (Ono et al., 1999; Muller et al., 2001) to fish (Van Eekelen et al, 2010) and mammals, are highly conserved. Around 100 PTPs have been characterized, of which PTP-1 and -2 (SHP-1 and -2), PTP-1B, TC-PTP, and PTP-H1 have been shown to impact GH signaling, again by different

mechanisms (Pilecka et al., 2007). SHP-1 and PTP-1B can mediate dephosphorylation by binding with JAK2 and/or GHR, respectively (Gu et al., 2003) possibly through IGF-1R signaling (Gan et al., 2013). PTP-H1 prevents recruitment of downstream signaling molecules by binding active GHR (Pasquali et al., 2003). SHP-2 can act as a positive or negative regulator and associate with signal regulatory protein α, which is a negative GH regulator itself (Stofega et al., 2000; Kim et al., 1998). The mechanistic details of PTP action are not clear and continue to be investigated; however, based on recent work (Gan et al., 2013), it is reasonable to suggest that during well-fed states, when IGF is in abundant circulation, IGFR signaling inhibits PTP repression of GH/JAK/STAT signaling, while in fasting conditions, when plasma IGF is low, PTPs are free to inhibit classical GH signaling and possibly shift GH signaling to lipolytic actions.

Lipolytic signaling

Intracellular signaling used to mediate lipolysis does so by targeting expression, activation, and localization of lipases and LD-associated proteins (e.g., perilipins). Classically, HSL is activated with β-adrenergic (βA) signaling through cAMP/PKC pathways. Specifically, catecholamines stimulate G protein-coupled receptors (GPCRs) that trigger heterotrimeric G proteins, specifically Gαs to activate adenylyl cyclase to increase cAMP production. cAMPs bind and activate PKA, which phosphorylates and thereby activates HSL and translocates it to the lipid droplet (Chaves et al., 2011; Lampidonis et al., 2011). Specific phosphorylation sites have been shown to be vital for HSL activity. Mutagenesis studies of mammalian HSLs have demonstrated that phosphorylation at Ser650 in humans and Ser660 in rats trigger HSL activation. Likewise, Ser559/600 is important for both activation and translocation, and Ser565 inhibits HSL activity in rats (Yeaman 2004, Watt and Steinberg 2008; Krintel et al., 2008; Choi

et al., 2010; Su et al., 2003; Zechner et al., 2012). Work in rainbow trout has confirmed conservation of classic HSL-activating signaling mechanisms, although specific phosphorylation patterns have not been investigated in fish (Harmon et al., 1993; Michelsen et al., 1994).

ATGL was only discovered in 2004 but has been heavily investigated since and is activated similarly to HSL (through βA signaling) but is not a target of PKA (Zimmerman et al., 2004). Human ATGL is phosphorylated at two sites, but the meaning of this is not yet clear (Bartzt et al., 2007). In *C. elegans*, ATGL-1 phosphorylation leads to inactivation and is related to life span regulation, but the mechanisms behind this have not been elucidated (Narbonne and Roy, 2009). Unlike HSL and ATGL, MGL does not seem to undergo regulation on any level. Other lipases are present across tissues but only account for about 10% of lipolysis, whereas HSL and ATGL are responsible for the other 90% (Jaworski et al., 2007; Duncan et al., 2007).

In addition to lipases, LD-associated proteins, like perilipins, physically guard the lipid droplet and also serve as lipase coactivators. Perilipins are regulated by βA-stimulated kinases that also target lipases. Specifically, inactivated perilipin-1 (formerly perilipin A) patrols the perimeter of the LD, preventing lipase access. Upon stimulation, perilipin-1 is phosphorylated by PKA, relinquishes its adherence to the LD and releases its binding partner CGI-58. CGI-58 moves on to binds with ATGL to activate its lipase activity in WAT. In addition, with free access to the LD, activated HSL can then hydrolyze TG inside the LD (Lass et al., 2011; Lampidonis et al., 2011; Miyoshi et al., 2007). Less is known about perilipin activity in other tissues with less robust lipid stores. Although several other kinases can phosphorylate HSL, evidence suggests that only PKC and PKG can do so for perilipin-1 (Gonzalez-Yanes and Sanchez-Margalet, 2006; Lampidonis et al., 2011).

Signaling crosstalk and nutritional influence

Although the pathways that are triggered by GH and regulate lipolytic action seem distinct, they in fact converge under certain circumstances. These context-specific actions are necessary as GH is a multifunctional hormone that is responsible for the regulation of several physiologies including osmoregulation, growth promotion, behavior, reproduction, and metabolism. GH commonly triggers lipolysis in major lipid depots (e.g., adipose) but can do so in other tissues (e.g., liver and skeletal muscle). We suspect that the ability of GH to be multifarious is due to a cellular programming that dictates which intracellular signaling pathways GH activates and the physiological outcomes of those pathways. Here, we describe evidence that GH triggers lipolytic action through distinct signaling pathways under certain nutritional states.

In well-fed fish, GH promotes growth through hepatic IGF-1 production and release through JAK-STAT, PI3K/Akt, and ERK signaling pathways (Reindl et al., 2011); however, when fish were fasted, no such connection was present (Walock et al., in preparation). Instead, GH triggered hepatic lipolysis through HSL activation and *de novo* synthesis through PKC/PLC and MAPK/ERK but not JAK/STAT activation (Bergan et al., 2012; Bergan et al., in press). A similar nutritional paradigm on GH action has been demonstrated in mammals (L. Moller et al., 2009; Gorin et al., 1990; Rhoads et al., 2007).

This dual activation of the MAPK pathway by GH under fed and fasted conditions suggests that the entire set of signal pathways activated under a given condition may be more important in determining a physiological response than the activation of a single pathway (Bergan et al., in press). This is exemplified further by Greenberg et al., (2001) who demonstrated that extracellular signal-regulated kinase (ERK), through MAPK kinase (MEK) activation, directly phosphorylated HSL at Ser600 to increase its lipase activity in the 3T3-L1

adipocyte line. Reduced caloric intake also triggered ERK activation in skeletal muscle of monkeys (Nadeau et al., 2006).

Although GH is classically regarded to work through JAK-STAT signaling, this is not always the case, especially in nutritionally-restricted models. Fasting was shown to blunt activation of JAK-STAT in human adipose and muscle tissues (L. Moller et al., 2009). Similarly, GH-stimulated lipolysis in the adipose and muscle tissue of fasted human males was accompanied by a deactivation of STAT5b (N. Moller et al., 2009). Fasting seems to remove the connection between GH and its fed-associated actions (e.g., growth promotion) by inhibiting JAK2 signaling. PTP-H1 then may be a key molecule used to mediate these shift of GH signaling from growth-promotion to lipolysis. The protein-tyrosine phosphatase (PTP)-H1 has been shown to inhibit GH-stimulated IGF-1 expression in HEK293 cells through inactivating JAK/STAT (Pileck et al., 2007). Conversely, intact JAK2 was necessary for GH-stimulated lipolysis in the liver (Sos et al., 2011) and adipose of fed mice (Nordstrom et al., 2013). Likewise, in adipocytes of mammals, STAT5 seems to play an essential role in GH-stimulated lipolysis (Richard and Stephens, 2014). Moreover, only in differentiated adipocytes does GH stimulate lipolysis; however, GH induced STAT5a and 5b activation in mature and preadipocytes (Fleenor et at., 2006). Therefore, STAT molecules, stimulated by GH independent of JAK2, may mediate lipolytic action through alterations in the cytoskeleton via FAKs.

PKC activation mediates GH-stimulated lipolysis. This is evident in adipose tissue from rats (Gorin et al., 1990) and hepatocytes from fasted trout (Bergan et al., 2013; Bergan et al., in press). By contrast, the 3T3-L1 immortal cell line stably transfected with hGHR did not require PKC activation for GH-stimulated lipolysis (Asada et al., 2000). Little work has been conducted to elicit the complete signaling mechanisms GH uses to mediate lipolysis, especially concerning

PKC. Besides the aforementioned studies, only parts of the pathway have been elucidated. For example, Nivet et al. (1993) demonstrated that GH activated PKC in rat hepatocytes. And PKA and PKC/MAPK have been shown to stimulate lipolysis independently of one another in 3T3-L1 adipocytes (Fricke et al., 2004). By contrast, PKC seems to mediate adipogenesis by attenuating PKA inhibition on LPL translation in the 3T3-F442A adipocyte line (Unal et al., 2008).

The diverse actions of PKC may stem from the multiple isoforms that exist in vertebrates. At least fifteen isoforms exist and are organized into conventional, novel, and atypical based on their activating partners (Rosse et al., 2010). Here, we briefly described the role of PKC in regulating GH-stimulated lipolysis; however, the individual roles of those PKC isoforms, within a cell-type context are not complete. Therefore, we describe below the antilipolytic actions of insulin through PKC, which is stimulated by PLC-generated diacylglycerol.

Research on PLC**,** a proximate signaling molecule, has produced little information regarding lipolytic action possibly due to its indirect actions; however, in regard to GH signal transduction, it may serve as a key molecule for crosstalk between pathways. Classically, PLC is activated by $G\alpha_q$ and converts membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol and 1,4,5-trisphosphate (IP3). Diacylglycerol helps activate PKC, while IP3 acts as a second messenger for subsequent signaling. PLC activation via GH is tissue specific. For example, GH-stimulated activation of PLC is seen in canine kidney (Rogers and Hammerman, 1989). In addition, PLC, like PKC, has several isoforms. PLCγ interacts with GHR and seems to be dependent upon JAK2 tyrosine phosphorylation (Argetsinger, L.S. and Carter-Su, 1996). Zhu et al., (2001) suggests that PI3K and PKCδ activity are required for GH-stimulated MEK activation. Moreover, PKCζ can phosphorylate Raf and MEK when activated by PI3K (Zhu et

al., 2001). However, these actions were not examined under different nutritional states and likely reflect GH signaling in well fed, anabolically programmed models.

Upon re-feeding, the link between GH and lipolysis may be severed by insulin. Insulin peaks in circulation after a meal to facilitate blood glucose and amino acid uptake and impede lipolysis. The mechanisms mediating insulin attenuation of lipolysis have been investigated, but a clear picture has yet to be resolved. Generally, activation of insulin's cognate receptor, tyrosine kinase, triggers a cascade of activation involving IRS, PI3K, Akt, and several downstream elements including SHP2, and fyn, (Caruso and Sheridan, 2011; White, 2003). Upon refeeding, insulin has been shown to activate STAT5a and STAT5b to enhance SOCS mRNA expression independently of ERK and PI3K (Sadowski et al., 2001). The importance of this is discussed below. Insulin has been shown to attenuate classic lipolytic signaling by Akt-activated phosphodiesterase 3B (PDE3B), which degrades cAMP, thus preventing PKA-activated HSL phosphorylation at Ser600 (Berggreen et al., 2009; Choi et al., 2010). Furthermore, in rat adipocytes it was shown that insulin reorganizes β1-adrenergic receptor (AR) to couple with $G\alpha_{i/o}$ instead of $G\alpha_s$ through PI3K-activated PCKβI independently of Akt; this removed the ability of catecholamines to stimulate lipolysis (Nakamura et al., 2007; Nakamura et al., 2008). By contrast, in subcutaneous cultures of adipocytes from dairy cows, insulin could not attenuate isoproterenol-stimulated lipolysis, as measured by glycerol and NEFA release and HSL phosphorylation (Kenez et al., 2014).

Besides affecting cAMP levels, insulin inhibits lipolysis in other ways. In adipose and skeletal muscle in rats, insulin activates protein phosphatase-1 (PP1), which dephosphorylates HSL and perilipins (Olsson et al., 1987; Ragolia et al., 1998; Stralfors et al., 1989; Clifford et al., 1998). Moreover, insulin and refeeding have also been shown to inhibit expression of ATGL in

3T3-L1 adipocytes (Kershaw et al., 2006). Therefore, insulin triggers several downstream events that work to inhibit lipolysis.

One of those events is SOCS signaling, which may be a key pathway mediating the multifarious actions of GH. It is well established that SOCS inhibits GH signaling by inactivating and degrading GHR and JAK as a part of negative feedback. However, the role of SOCS in lipolytic GH signaling is still under investigation. Research shows that GH-stimulated expression of ATGL and HSL was annulled when SOCS2 was overexpressed in porcine adipocytes; this was accompanied by reduced STAT3 and STAT5 activation (Yang et al., 2012). However, no changes in SOCS mRNA were seen in adipose or fat of fed and fasted human males when comparing for 37.5 hours (L. Moller et al., 2009). Moreover, SOCS displayed concentration-dependent actions on GH signaling with low concentrations of SOCS inhibiting the growth-promoting actions of GH, while high concentrations augmenting those actions; unfortunately, lipolytic ends were not measured (Greenhalgh et al., 2005). Further work needs to be done to understand the role of SOCS in regulating GH signaling. But it seems that SOCS may play a key role in shifting GH action from growth-promotion to lipolysis.

Although we are describing how insulin inhibits lipolysis, also be aware that the resulting elements of lipolysis, FAs and subsequent metabolites, can inhibit insulin signaling and can lead to insulin resistance (Zechner et al., 2012). Moreover, GH and fasting separately stimulated lipolysis and decreased insulin sensitivity by half in skeletal muscle of humans through inhibitory pyruvate dehydrogenase phosphorylation (Nelleman et al., 2014). Therefore, physiological outcomes are not dictated by a single hormone, but instead a complex collection of inputs and feedback mechanisms play a role.

Conclusion

GH regulates a wide array of processes including growth, reproduction, osmoregulation, immunity, behavior, and metabolism. GH and its regulation have been studied for many years. The lipolytic actions of GH seem to take a backseat to GH-stimulated growth promotion and catecholamine-stimulated lipolysis. Moreover, there is a lack of understanding of GH paracrine and autocrine function. Basic GHR understanding exists; however, little is known about the functional significance of GHR subtypes. In particular, further investigation is needed on nutritional regulation of GHR expression, binding, and internalization as well as linkages among GHR subtypes, effector pathways, and resulting physiological processes. Overall, a comparative understanding of GH-stimulated lipolysis may provide insight into human health, like obesity and diabetes, as well as aquacultural benefits, like feed content and meat quality.

Objectives

Two main questions that face the field of chemical signaling are 1) what mechanisms underlie hormone multi-functionality and 2) can a specific response to a hormone be altered? My research addressed these questions and resolves two disparate actions of growth hormone (GH): growth promotion and stored lipid breakdown (lipolysis). The hypothesis of this dissertation is that nutritional state modulates GH responsiveness of cells by impacting effector pathways that GH links. We focused on signaling pathways that have previously shown to either be activated by GH or are involved in mediating lipolysis in order to link GH stimulation to lipolysis by specific signaling pathways. We investigated this hypothesis by examining 1) how nutrition regulates lipolytic processes *in vivo*, 2) the role of GH stimulating lipolysis *in vitro*, 3) signaling cascades involved in nutritional and GH-mediated lipolysis, 4) the ability of nutritional state, and associated hormones, to alter cellular responsiveness to GH.

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CHAPTER 2. NUTRITION-REGULATED LIPOLYSIS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) IS ASSOCIATED WITH ALTERATIONS IN THE ERK, PI3K-AKT, JAK-STAT, AND PKC SIGNALING PATHWAYS[1]

Abstract

Previous studies have shown that food deprivation, which occurs naturally in the life cycle of many species of fish, results in cessation of growth and catabolism of stored energy reserves, including lipids. In this study, we used rainbow trout (*Oncorhynchus mykiss*) to identify the cellular mechanisms involved with this metabolic shift. Fish were placed on one of five dietary regimes—fed continuously for 2 or 4 weeks, fasted continuously for 2 or 4 weeks, or fasted 2 weeks then refed 2 weeks—and the effects on organismal growth and lipid catabolism and on the activation state of signaling elements (*e.g.,* Akt, ERK, JAK-STAT, PKC) in selected tissues were measured. Fasting for either 2 or 4 weeks significantly retarded growth in terms of body weight, body length, and body condition; refeeding restored growth such that body length and body condition were similar to measures seen in continuously fed fish. Fasting activated lipid catabolism by stimulating the mRNA expression and catalytic activity of hormone-sensitive lipase (HSL). Two HSL-encoding mRNAs have been characterized, and the expression of both forms of mRNA in 2- and 4-week fasted fish was significantly elevated over levels in fed fish in all tissues. In adipose tissue, liver, and white muscle, HSL activity was significantly elevated in 2- and 4-week fasted fish compared to fed animals; whereas in red muscle, HSL activity was significantly elevated compared to fed fish after 4 weeks of fasting. Refeeding reversed both fasting-associated HSL mRNA expression and HSL activity. Fasting resulted in the deactivation

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of Akt, JAK2, and STAT5 in adipose tissue, liver, and red and white muscle. By contrast, fasting activated ERK and PKC in all tissues measured. Refeeding reversed fasting-associated alterations in the activation state of all signal elements. These findings suggest that deactivation of Akt and JAK-STAT in conjunction with activation of ERK and PKC underlie fasting-associated growth retardation and lipolysis.

Introduction

Fish have evolved elaborate life history patterns involving morphological, physiological, and behavioral adaptations in order to adjust to the vast array of gaseous, thermal, osmotic, and other conditions presented in their environments. Many species of fish experience periodic food deprivation that results in a shift from anabolic to catabolic processes, a shift that may be accentuated in the face of high metabolic demand such as during gonad development or in anadromous species during periods of migration or seawater preadaptation (Jobling, 1994; Love, 1979; Sheridan, 1988). The tolerance to food deprivation varies with species and can be modified by gender, salinity, season, and temperature (Love, 1979; Sheridan and Mommsen, 1991).

The shift to a catabolic state by fish in response to food deprivation results in the cessation of growth and the breakdown and utilization of stored energy reserves. Fasting-associated growth retardation has been observed in rainbow trout by us (Norbeck et al., 2007; Sheridan and Mommsen, 1991) and others (Chauvigne et al., 2003), as well as in other species of teleosts (Deng et al., 2004; Fox et al., 2006; Picha et al., 2006; Small et al., 2006; Uchida et al., 2003). Depending on species and their life history, fish catabolize stored glycogen, lipids, and proteins, and activate gluconeogenetic mechanisms (Moon et al., 1989; Sheridan and Mommsen, 1991). The mobilization of fat, which, unlike mammals, is stored among many tissues in fish

(*e.g.*, mesenteric adipose tissue, liver, red muscle) (Sheridan, 1988), is particularly important during long-term fasts and for carnivorous species, which have high metabolic rates and deplete glycogen rapidly (Jobling, 1994; Sheridan and Mommsen, 1991). In fish and mammals, the mobilization of stored lipids, primarily triacylglycerols (TG), proceeds from their hydrolysis primarily by hormone-sensitive lipase (HSL) and the release of fatty acids and, with subsequent enzymatic catalysis, glycerol into the plasma for use by other tissues (Duncan et al., 2007; Gonzalez-Yanes and Sanchez-Margalet, 2006; Sheridan, 1988). Hormone-sensitive lipase is one member of a multi-gene family that includes HSL, lipoprotein lipase, hepatic lipase, pancreatic lipase, and endothelial lipase; interestingly, two HSL-encoding genes are present in teleosts, including rainbow trout, which appear to have arisen during the teleost-specific gene duplication event ca. 350 MYA (Kittilson et al., 2011).

The mechanisms stimulated by fasting to trigger cellular reprogramming from an anabolic state to a catabolic state remain elusive. A number of intracellular signaling cascades are candidates for transducing external signals and mediating cellular responses. In particular, the Janus kinase (JAK)/signal transducer and activator of transcription (STAT), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), and extracellular signal-regulated kinase (ERK) signaling pathways have been implicated as key growth-mediating pathways in fish, as triggered by growth hormone (GH) and insulin-like growth factors (IGF) (Fuentes et al., 2011; Reindl et al., 2011). Lipolysis-mediating pathways, primarily targeting HSL, have been shown to include cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), protein kinase C (PKC), and ERK (Gonzalez-Yanes and Sanchez-Margalet, 2006; Greenberg et al., 2001; Sheridan, 1994); however, these investigations have been limited to mostly mammalian adipose tissue and cell lines.

In this study, we used rainbow trout to examine the nutritional regulation of ERK, PI3K-Akt, JAK-STAT, and PKC signaling pathways in different tissues in order to understand the catabolic shift teleosts undergo during fasting conditions. The specific hypothesis of this study was that fasting induces altered intracellular signaling that leads to cessation of growth and to enhanced expression and activation of lipolysis.

Materials and methods

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 fed twice daily to satiety with AquaMax Grower (PMI Nutrition International, Brentwood, MO, USA) semi-floating trout grower, except 24-36 hours prior to commencing experiments in order to prevent any postprandial responses that feeding may cause. Animals were acclimated to laboratory conditions for at least 4 weeks prior to experimentation.

For experiments, fish were assigned randomly to one of six treatment groups (initial, fed continuously for 2 weeks, fasted for 2 weeks, fed continuously for 4 weeks, fasted for 4 weeks, and fasted for 2 weeks then refed for 2 weeks) in 100-L circular tanks (approximately 18-24 fish per tank) with a flow-through water supply at 14°C under a 12:12 L:D photoperiod. Fish were allowed to acclimate for 2 days in their experimental tank prior to beginning their respective nutritional regime. For those animals receiving food, feeding was suspended 24-36 hours before sampling. At the time of sampling, fish were anaesthetized in 0.05% (v/v) 2-phenoxyethanol, measured (body weight, length, and liver weight), bled via the severed caudal vessels, and

euthanized by transection of the spinal cord. Samples of liver, mesenteric adipose tissue, white skeletal muscle, and red skeletal muscle were removed, frozen on dry ice, and stored at -80°C for later analyses. These tissues were selected as each store appreciable amounts of lipid and serve as lipid depots (Sheridan, 1988).

RNA extraction and analysis

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 µl 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 µl 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 with genomic DNA; no amplification was detected in negative controls.

Levels of HSL1 and HSL2 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 HSL1 or HSL2 and β-actin. cDNA standards were generated using HSL1 and HSL2, the PCR products were cloned into the pGEM-T Easy Vector, and the sequences were verified as described previously (Kittilson et al., 2011). 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

[HSL1: 5'-FAM-CAAGGACCTCCGGT-3' (150 nM); HSL2: 5'-FAM-TCACCACTTCTATCCC-3' (150 nM); β-actin: 5'-VIC-TGCTTGCTGATCCACAT-3' (150 nM)], 0.5 µl of gene-specific forward [HSL1: 5'-GTCCTAGGTCATGGTCATCGT-3' (600 nM); HSL2: 5'-CATCGTCAAGAACCCGTTTC-3' (600 nM); β-actin: 5'-GGCTTCTCTCTCCACCTTCCA-3' (900 nM)] and reverse primers [HSL1: 5'-TCTCTGGTGGGCCTTGTTGT-3' (600 nM); HSL2: 5'-GCGGTAGTCCTCTCAGTAGGTCAT-3' (600 nM); β-actin: 5'-AGGGACCAGACTGTCGTAACTC-3' (900 nM)], 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.

Western blot analysis

Tissues 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. Proteins (typically 50 µg) and molecular weight marker (Cell Signaling Technology, Beverly, MA; catalog no. 7727) were separated by SDS-PAGE (7.5% running gel) and transferred to 0.45 µm nitrocellulose (Bio-Rad Laboratories) for western analysis as previously described (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. All antisera were obtained from Cell Signaling Technology (Beverly, MA); antisera for ERK 1/2, Akt, JAK2, and STAT5 were validated for the detection of signaling elements in rainbow trout previously (Reindl et al., 2011). The ability of the commercial antisera to PKCα/β II and β-actin to detect these elements in rainbow trout compared to Chinese hamster ovary-K1 cells (American Type Culture Collection, Rockville, MD) was assessed by Western analysis (Fig. 1). Bands of the predicted size for phospho-PKCα/β II (80-82 kDa and 135-140 kDa, the later resulting from hyperphosphorylation) and β-actin (45 kDa) were observed. Treatment of isolated rainbow trout hepatocytes with 100 ng/ml for 5 min increased the abundance of both the 80-82 kDa and 135-140 kDa form of phospho-PKCα/β II; therefore, the bands were combined for all quantitative analyses in this study.

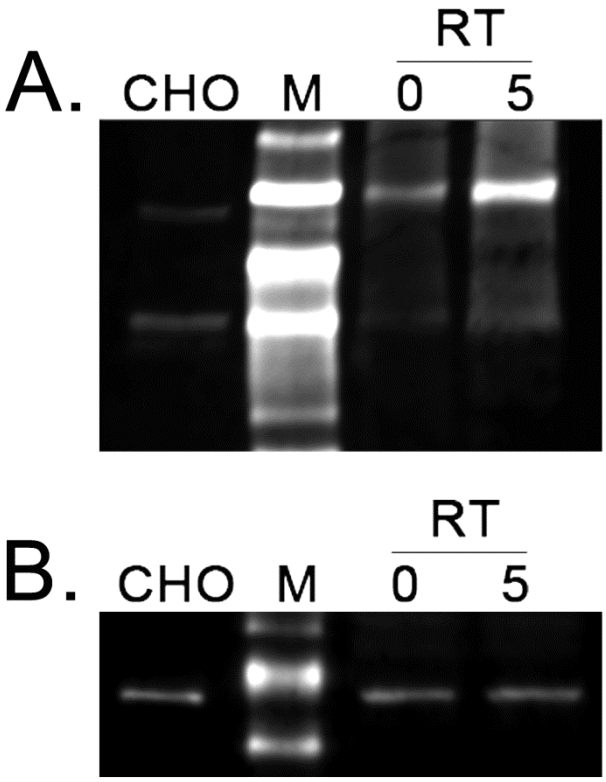

Figure 1. Western immunoblot analysis of lysates from Chinese hamster ovarian-K1 (CHO) cells and rainbow trout hepatocytes (RT) using antisera to (A) phospho-PKCα/β II and (B) β-actin. Rainbow trout hepatocytes were isolated and incubated as described previously [37] with salmonid growth hormone for 0 or 5 min. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and hybridized individually with the specific primary sera. Blots were then hybridized with anti-rabbit IgG-horse-radish peroxidase (HRP) secondary antibody and anti-biotin-HRP antibody for the detection of signal elements and biotin-labeled molecular weight marker (M; ranging from, top to bottom, 200 kDa to 60 kDa in panel A, and 60 kDa to 40 kDa in panel B), respectively. Blots were visualized with chemiluminescence and a FluorChem FC2 imager.

Hormone-sensitive lipase isolation and activity

Hormone-sensitive lipase (HSL) was partially purified essentially as described previously (Harmon et al., 1991) with minor modifications. Briefly, tissues were homogenized in 300 µl 25 mM Tris-HCl with 0.25 M sucrose, 4.5 mM β-mercaptoethanol, 5 mM EDTA, 5 mM EGTA, 100 mM KF, 0.18 mM PMSF, pH 7.4. The homogenate was incubated on ice for 5 minutes then centrifuged at 20,000 x g for 20 min at 4°C. The infranatant was centrifuged at 110,000 x g for

1.15 h at 4°C. The pH of resulting supernatant was then brought to 5.2 with the addition of 2.0 N acetic acid. The tubes were incubated on ice for 15 min, and the precipitate was collected by centrifugation at 3,500 x g for 45 min at 4°C. The pellets were dried, frozen on dry ice, and then stored at -20°C for later analysis. The pH 5.2 pellets were resuspended in 300 µl 25 mM Tris-HCl, pH 7.4. Approximately 100 µg of protein was assayed for HSL catalytic activity as described previously (Sheridan and Harmon, 1994). Briefly, isolated HSL protein was incubated with [^{3}H]-triolein substrate. HSL was allowed to hydrolyze the [^{3}H]-triolein into glycerol and free [^{3}H]-oleic acid. An organic solvent was used to separate [^{3}H]-oleic acid product from [^{3}H]-triolein substrate. And the [^{3}H]-oleic acid fraction was assessed for radioactivity by liquid scintillation counting.

Data analysis

Data are expressed as means ± S.E.M. Statistical differences were estimated by one-way or two-way ANOVA, as appropriate, followed by Duncan's multiple range test using SigmaStat (SPSS, Chicago, IL). A probability level of 0.05 was used to indicate significance.

For ease of comparison, mRNA and protein abundance data are expressed as % of initial control; statistics were performed on untransformed data. Control groups (initial, fed continuously groups) were compared and were found to be indistinguishable for all parameters.

Results

Fasting retards growth

Fish that were continuously fed grew significantly over the course of the experiment, whereas rainbow trout that were fasted experienced retarded growth (Table 1). After 2 weeks, food-deprived fish failed to grow in length and displayed significantly reduced body weight and condition compared to their continuously fed counterparts. After 4 weeks, body weight and

condition factor of fasted fish continued to decline, being significantly lower than their 4-week fed counterparts as well as lower than 2-week fasted fish. Refeeding fish for 2 weeks after a 2 week fast restored growth such that refed fish had significantly greater length, weight, and condition than 4-week fasted fish; in fact, refed fish displayed body lengths and condition similar to those of fish that had been fed continuously for 4 weeks.

Table 1.

Effects of nutritional state on selected body characteristics of rainbow trout.[A]

Characteristic	Initial	2 weeks		4 weeks		
		Fed	Fasted	Fed	Fasted	Refed
Body weight (g)	100 ± 6^{a}	144 ± 11^{bc}	81 ± 5^{d}	161 ± 7^{c}	70 ± 5^{d}	130 ± 12^{b}
Body length (cm)	20 ± 0.6^{a}	22.7 ± 0.5^{b}	19.5 ± 0.6^{a}	24 ± 0.6^{b}	19.6 ± 0.7^{a}	22.2 ± 0.6^{b}
Condition factor[B]	1.22 ± 0.04^{a}	1.2 ± 0.06^{a}	1.06 ± 0.03^{b}	1.2 ± 0.02^{a}	0.95 ± 0.02^{c}	1.15 ± 0.09^{ab}

[A] Fish were either fed or fasted continuously for the period indicated; refed animals were fasted for 2 weeks then refed for 2 weeks. Data are presented as means $\pm$ SEM (n = 3-6). For a given characteristic, groups with different letters are significantly ($p < 0.05$) different from initial fed control.

[B] Calculated as [body weight/(body length)^3] x 100.

Fasting activates lipolysis

Rainbow trout possess two HSL-encoding mRNAs, HSL1 and HSL2, and both forms were detected in each of the tissues examined (mesenteric adipose tissue, liver, and red and white muscle). Fish that were fed continuously either for 2 or 4 weeks displayed levels of HSL mRNAs comparable to those of initial control fish. A 2-week fast significantly increased steady-state levels of both HSL1 and HSL2 mRNA (Fig. 2). Notably, the expression of HSL1 mRNA was greater than that of HSL2 mRNA in all tissues except mesenteric adipose tissue. The increase in HSL expression was most pronounced in liver and white muscle, with levels of HSL1 mRNA increasing in these tissues 225% and 362%, respectively.

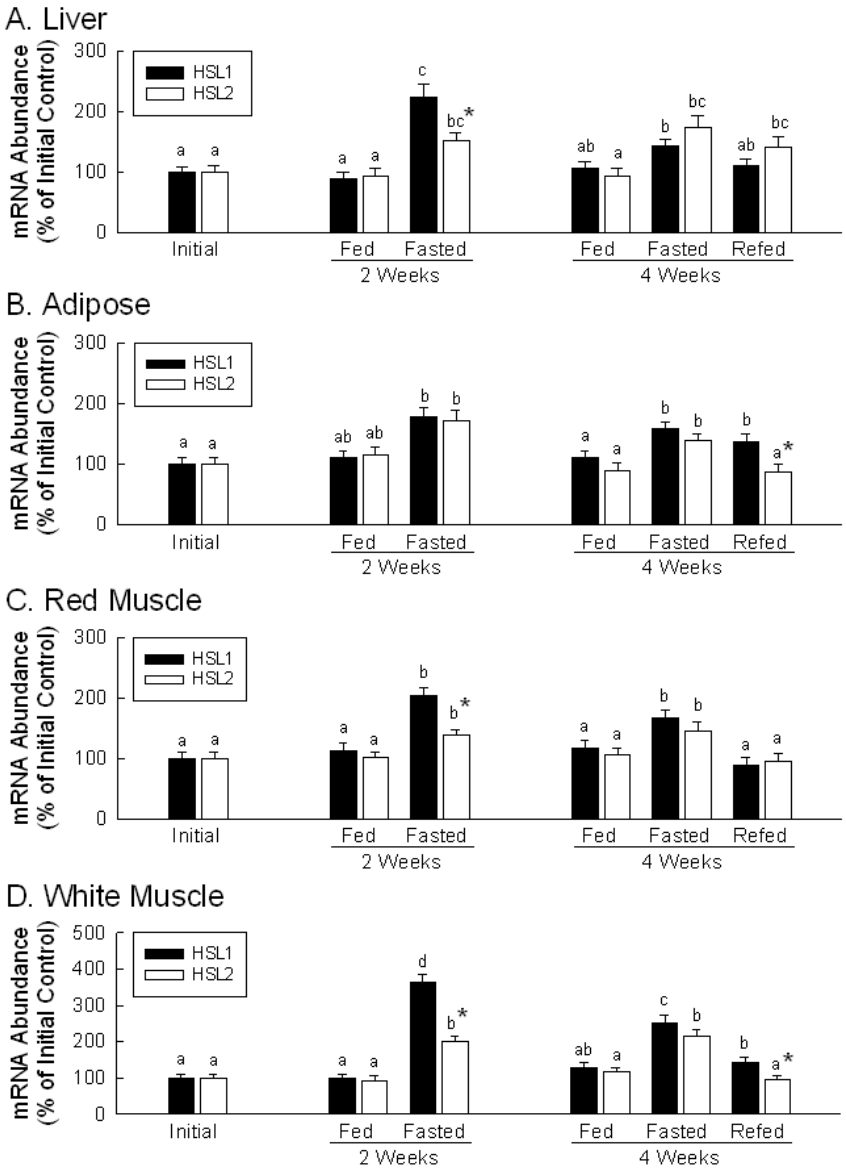

Figure 2. Expression of hormone-sensitive lipase (HSL) 1 and HSL2 mRNAs in (A) liver, (B) mesenteric adipose tissue, (C) red muscle, and (D) white muscle of rainbow trout on various nutritional regimes. Fish were fed or fasted continuously for the period indicated; refed fish were fasted for 2 weeks then refed for 2 weeks. mRNA levels were determined by quantitative real-time RT-PCR as described in the materials and methods section. Data are presented as % control from initial fed controls and expressed as mean $\pm$ SEM (n = 6-8). For a given HSL isoform, groups with different letters are significantly ($p < 0.05$) different; * indicates significant difference between HSL subtypes in tissues removed from fish on a given nutritional regime.

difference in expression between the two HSL subtypes within any of the tissues examined. Fasting fish for 4 weeks also resulted in elevation of both HSL mRNAs in all tissues (Fig. 2). White muscle displayed the most striking response to fasting, with levels of HSL1 mRNA increasing 252% and those of HSL2 mRNA increasing 226%. Refeeding fish for 2 weeks after a 2-week fast lowered expression of HSL1 and HSL2 mRNAs to levels seen in initial control fish only in red muscle. In liver, refeeding resulted in reduced expression of HSL1 mRNA, but expression of HSL2 mRNA remained elevated. In mesenteric adipose tissue and white muscle, refeeding reduced levels of HSL2 mRNA, but HSL1 mRNA expression remained at heightened levels.

Catalytic activity of the principal lipolytic enzyme, HSL, also was detected in all tissues examined (mesenteric adipose tissue, liver, and red and white muscle). HSL activity in initial control fish was similar to levels seen in fish fed continuously for 2 or 4 weeks. Fasting for two weeks increased HSL activity in liver (1.6 fold), mesenteric adipose tissue (2.6 fold), and white muscle (1.8 fold) as compared to their continuously fed counterparts; however, HSL activity in the red muscle of 2-week fasted fish was similar to that of continuously fed fish (Fig. 3). Fasting fish for 4 weeks resulted in increased HSL in all tissues (increased by 3.2 fold in liver; 3.5 fold in adipose, 3.3 fold in red muscle, and 2.7 fold in white muscle); moreover, there was significant augmentation of HSL activity in liver, mesenteric adipose tissue, and white muscle over levels observed in 2-week fasted fish. Refeeding fish restored HSL activity to initial control levels in all tissues.

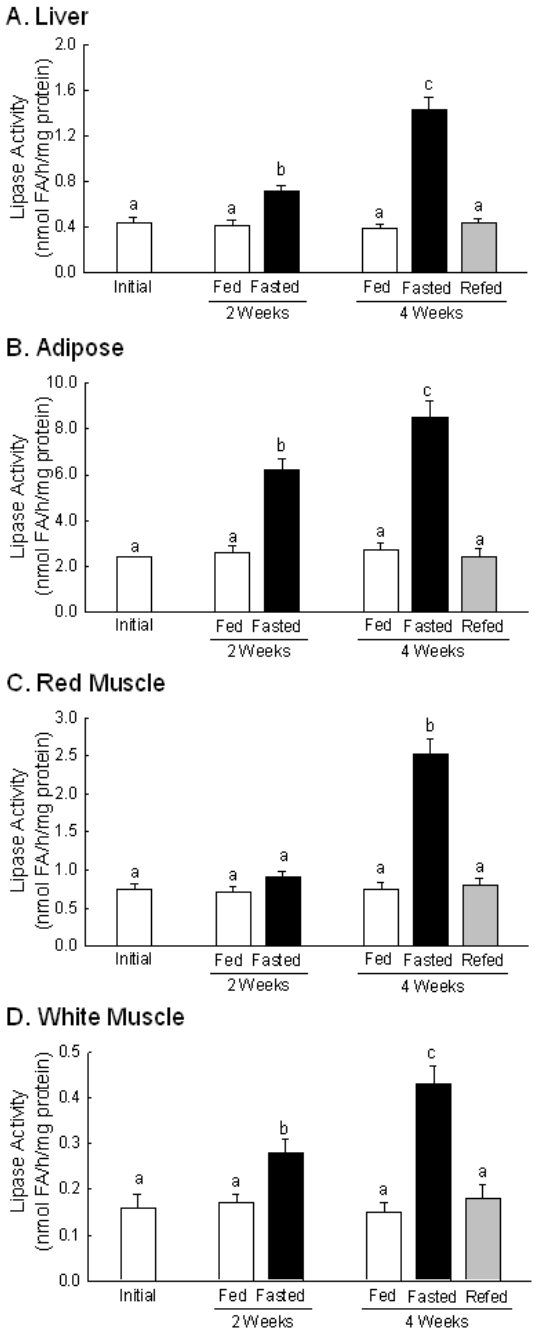

Figure 3. Catalytic activity of hormone-sensitive lipase (HSL) in (A) liver, (B) mesenteric adipose tissue, (C) red muscle, and (D) white muscle of rainbow trout on various nutritional regimes. Fish were fed or fasted continuously for the period indicated; refed fish were fasted for 2 weeks then refed for 2 weeks. Enzyme activity was determined by release of [^{3}H]-oleic acid from [^{3}H]-triolein as described in the materials and methods section. Data are expressed as mean $\pm$ SEM (n = 6-8). Groups with different letters are significantly ($p < 0.05$) different.

Fasting differentially deactivates/activates signaling pathways

Phospho-JAK2, phospho-STAT5, phospho-Akt, phospho-ERK, and phosphor-PKC were detected in all of the tissues examined, and their abundance in initial control fish was similar to that observed in fish fed continuously for 2 or 4 weeks. When fasted for 2 weeks, levels of phospho-JAK2 decreased significantly in the liver (Fig. 4A), mesenteric adipose tissue (Fig. 4B), and red muscle (Fig. 4C); however, the abundance of phospho-JAK2 in white muscle (Fig. 4D) was not different from that in fish that were fed continuously. A 4-week fast resulted in reduced levels of phospho-JAK2 in mesenteric adipose tissue, red muscle, and white muscle; in fact, phospho-JAK2 abundance decreased further than that observed after a 2-week fast in red muscle. The abundance of phospho-JAK2 in the liver of 4-week fasted fish rose slightly and was not significantly different from that in their continuously fed counterparts.

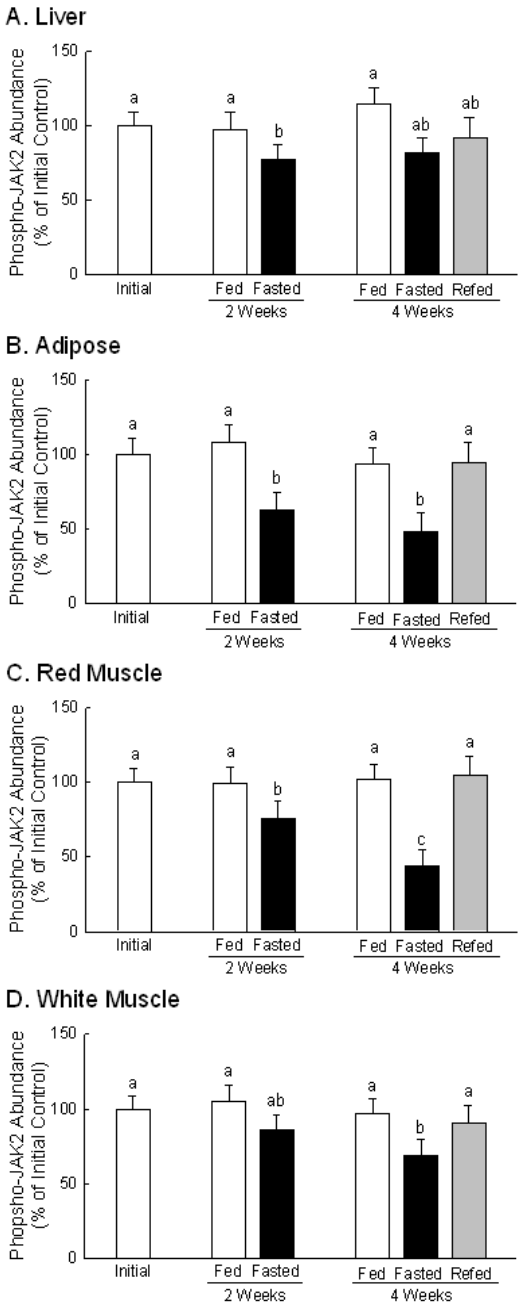

Figure 4. Abundance of phosphorylated Janus kinase 2 (phospho-JAK2) in (A) liver, (B) mesenteric adipose tissue, (C) red muscle, and (D) white muscle of rainbow trout on various nutritional regimes. Fish were fed or fasted continuously for the period indicated; refed fish were fasted for 2 weeks then refed for 2 weeks. The abundance of phospho-JAK2 was determined by western blotting as described in the materials and methods section. Data are presented as % control from initial fed controls and expressed as mean $\pm$ SEM (n = 3-6). Groups with different letters are significantly ($p < 0.05$) different.

Similarly, levels of phospho-STAT5 were reduced in liver and mesenteric adipose tissue after 2 or 4 weeks of fasting (Fig. 5). Most notably, hepatic phospho-STAT5 levels dropped to 53% and 46% of initial fed levels after 2 and 4 weeks, respectively. The abundance of phospho-STAT5 declined in white muscle only after 4 weeks of fasting. The abundance of phospho-STAT5 in red muscle did not change with any feeding regimen.

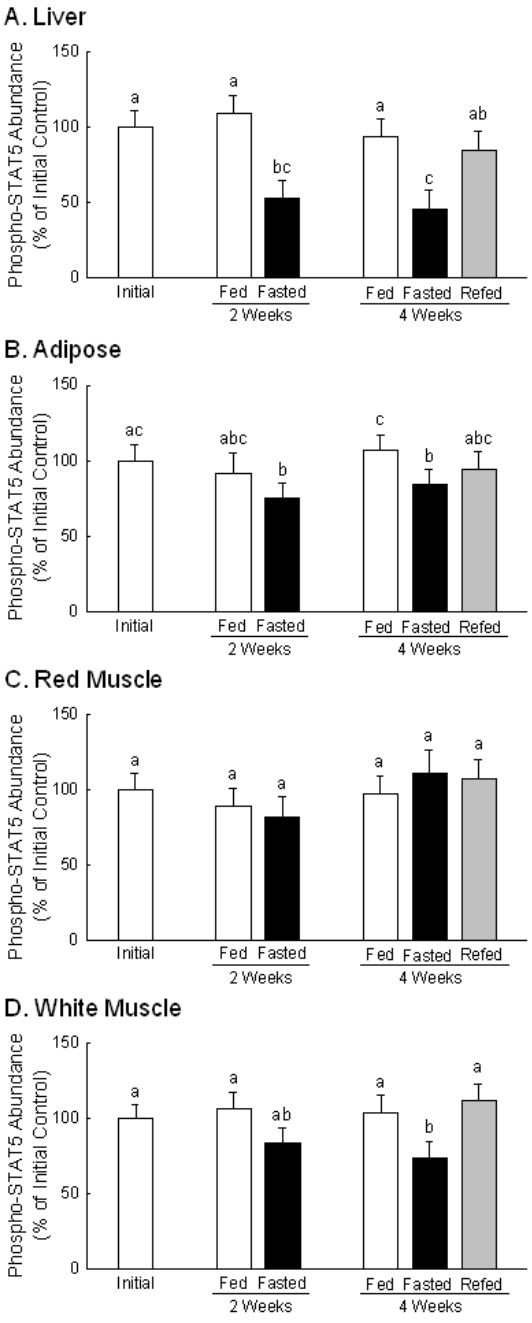

Figure 5. Abundance of phosphorylated signal transducer and activator of transcription 5 (phospho-STAT5) in (A) liver, (B) mesenteric adipose tissue, (C) red muscle, and (D) white muscle of rainbow trout on various nutritional regimes. Fish were fed or fasted continuously for the period indicated; refed fish were fasted for 2 weeks then refed for 2 weeks. The abundance of phospho-STAT5 was determined by western blotting as described in the materials and methods section. Data are presented as % control from initial fed controls and expressed as mean $\pm$ SEM (n = 3-6). Groups with different letters are significantly ($p < 0.05$) different.

Nutritional state also affected the abundance of phospho-Akt in a tissue specific manner. Specifically, a 2-week fast lead to reductions in the levels of phospho-Akt in liver and red muscle (Fig. 6A, 6C). The abundance of phospho-Akt in liver and red muscle continued to drop after a 4-week fasted, declining to 37% and 32% of initial controls, respectively. The levels of phospho-Akt in mesenteric adipose tissue and white muscle were not affected by a 2-week fast, but after 4 weeks of fasting, levels were significantly reduced compared to their fed counterparts (Fig. 6B, 6D).

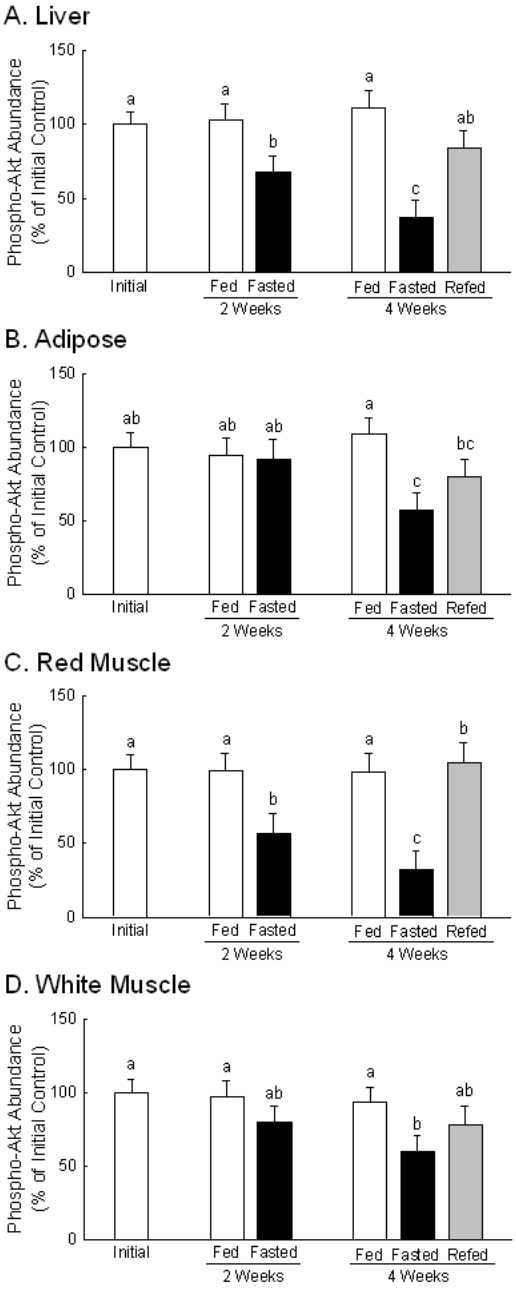

Figure 6. Abundance of phosphorylated protein kinase B (phospho-Akt) in (A) liver, (B) mesenteric adipose tissue, (C) red muscle, and (D) white muscle of rainbow trout on various nutritional regimes. Fish were fed or fasted continuously for the period indicated; refed fish were fasted for 2 weeks then refed for 2 weeks. The abundance of phospho-Akt was determined by western blotting as described in the materials and methods section. Data are presented as % control from initial fed controls and expressed as mean $\pm$ SEM (n = 3-6). Groups with different letters are significantly ($p < 0.05$) different.

In contrast to alterations in the abundance of phospho-JAK2, phospho-STAT5, and phospho-Akt in response to fasting, the abundance of phospho-ERK increased in tissues of rainbow trout following periods of food deprivation. Specifically, levels of phospho-ERK1/2 increased significantly in liver, adipose, and red and white muscle after a 2-week fast and remained elevated compared to their fed counterparts after 4 weeks of fasting (Fig. 7).

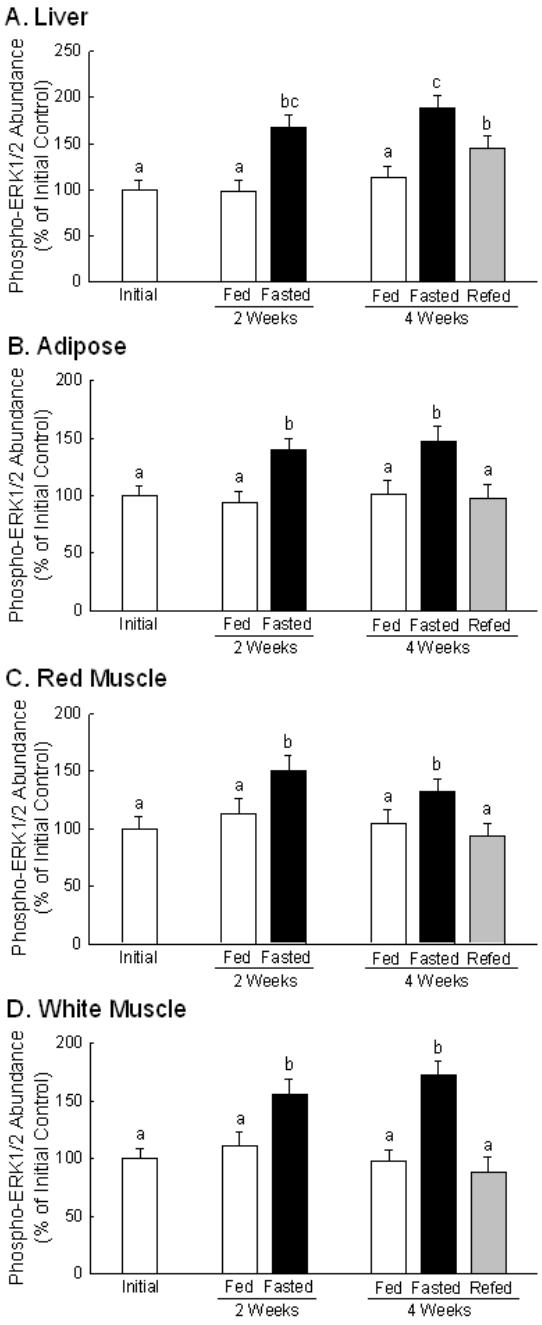

Figure 7. Abundance of phosphorylated extracellular signal-regulated kinase 1/2 (phospho-ERK 1/2) in (A) liver, (B) mesenteric adipose tissue, (C) red muscle, and (D) white muscle of rainbow trout on various nutritional regimes. Fish were fed or fasted continuously for the period indicated; refed fish were fasted for 2 weeks then refed for 2 weeks. The abundance of phospho-ERK1/2 was determined by western blotting as described in the materials and methods section. Data are presented as % control from initial fed controls and expressed as mean $\pm$ SEM (n = 3-6). Groups with different letters are significantly ($p < 0.05$) different.

Similarly, PKC also was activated in tissues of rainbow trout by fasting. After a 2-week fast, phospho-PKC levels increased in each of the tissues examined: liver, mesenteric adipose tissue, red muscle, and white muscle (Fig. 8). After 4 weeks of fasting, levels of phospho-PKC in red muscle remained similar to those in fish fasted for 2 weeks; however, levels in liver, mesenteric adipose tissue, and white muscle increased further to 226%, 205%, and 179%, respectively, of initial fed control animals.

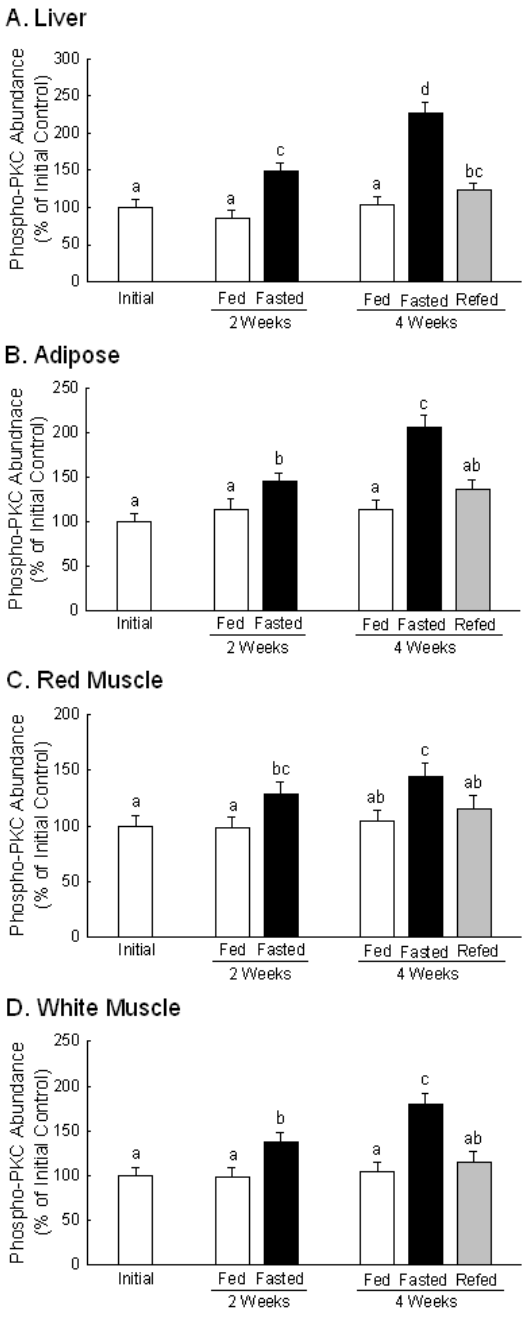

Figure 8. Abundance of phosphorylated protein kinase C (phospho-PKC) in (A) liver, (B) mesenteric adipose tissue, (C) red muscle, and (D) white muscle of rainbow trout on various nutritional regimes. Fish were fed or fasted continuously for the period indicated; refed fish were fasted for 2 weeks then refed for 2 weeks. The abundance of phospho-PKC was determined by western blotting as described in the materials and methods section. Data are presented as % control from initial fed controls and expressed as mean $\pm$ SEM (n = 3-6). Groups with different letters are significantly ($p < 0.05$) different.

Refeeding fish for 2 weeks following a 2-week fast generally returned levels of phospho-proteins to those levels observed in initial fed control animals except the abundance of phospho-ERK in liver; the abundance of which remained elevated to levels similar to those seen in 2-week fasted fish.

Discussion

Fish experience a variety of metabolic demands that are influenced by both internal (*e.g.*, stage of sexual maturations) and external (*e.g.*, food availability) factors. Therefore, the ability to regulate metabolic shifts is vital for their survival and fitness. In this study, we examined the cellular mechanisms that underlie an anabolic to catabolic shift. Our findings supported our starting hypothesis that fasting induces altered intracellular signaling which leads to the cessation of growth and the activation of lipolysis.

The influence of nutritional state on the growth of rainbow trout was supported by several observations. First, animals that were fed *ad libitum* grew significantly in terms of body weight; whereas growth was impeded by fasting of fish for either 2 or 4 weeks, which lead to significant reductions in body weight, body length, and body condition factor. Second, rainbow trout fasted for 2 weeks then refed for 2 weeks displayed compensatory growth, with body length and condition factor being equivalent to fish that were continuously fed. Similar influences of nutritional state on growth of rainbow trout have been observed by us (Norbeck et al., 2007; Sheridan and Mommsen, 1991) and others Chauvigne et al., 2003), and likewise in other species of teleosts previously (Deng et al., 2004; Fox et al., 2006; Gabillard et al., 2006; Picha et al., 2006; Small et al., 2006; Uchida et al., 2003). Alterations in several elements of the growth hormone (GH)-insulin-like growth (IGF) system appear to underlie fasting-associated growth retardation of fish. For example, several studies in fish have demonstrated a positive correlation

between growth and levels of plasma IGF-1 and hepatic IGF-1 mRNA (Beckman et al., 2005; Cruz et al., 2006; Perez-Sanchez et al., 1995; Picha et al., 2006). Fasting has been shown to reduce plasma IGF-I by diminishing both IGF-1 production and IGF-1 secretion, a dual action that was evident in rainbow trout (Chauvigne et al., 2003; Gabillard et al., 2006; Norbeck et al., 2007). Other studies have reported fasting-associated reduction of hepatic IGF-1 mRNA expression in coho salmon (Duan et al., 1998; Pierce et al., 2005) and grouper (Pedroso et al., 2006) and of plasma IGF-I in catfish (Small et al., 2006), coho salmon (Duan et al., 2004), flounder (Fuentes et al., 2011), and freshwater- and seawater-adapted tilapia [Fox et al., 2006; Uchida et al., 2003).

Paradoxically, fasting-associated growth retardation of fish often occurs in the face of elevated plasma levels of GH [Fox et al., 2006; Gomez-Requeni et al., 2005; Norbeck et al., 2007; Pedroso et al., 2006). Notably, however, fasting results in tissue-specific alterations in the sensitivity to GH. For example, in the liver of several species of fish, binding of ^{125}I-GH and the mRNA expression of growth hormone receptors (GHR) were reduced by fasting (Deng et al., 2004; Fukada et al., 2004; Norbeck et al., 2007; Picha et al., 2008; Small et al., 2006). By contrast, in adipose tissue of rainbow trout, binding of ^{125}I-GH and mRNA of one of the GHR subtypes, GHR1, increase after 6 weeks of fasting (Norbeck et al., 2007). Fasting also alters target organ sensitivity to IGF-1 in a tissue-specific manner. For example, in rainbow trout, fasting decreased mRNA expression of IGF-1 receptors in gill, whereas its expression was increased in cardiac muscle and not altered in white skeletal muscle (Norbeck et al., 2007). Such findings suggest that cellular processes are reprogrammed so as to divorce the growth-promoting actions of GH from other actions (*e.g.*, protein sparing, lipolysis).

The influence of nutritional state on lipolysis in rainbow trout also was supported by several observations. First, fasting augmented the expression of both of the two HSL-encoding mRNAs in each of the tissues studied (mesenteric adipose tissue, liver, red and white muscle). Notably, this effect was observed within 2 weeks, and levels of HSL1 mRNA were more abundant than levels of HSL2 mRNA in liver, red and white muscle. Second, fasting increased the catalytic activity of the HSL, the principal lipolytic enzyme, in liver, mesenteric adipose tissue, and white muscle after 2 weeks and in all of these tissues in addition to red muscle after 4 weeks. Third, refeeding fish for 2 weeks that had previously been fasted for 2 weeks lowered expression of the two HSL-encoding mRNAs as well as HSL activity to levels observed in animals that had been fed continuously. Taken together, these results extend previous findings on nutrition-regulated HSL activity (Sheridan, 1989) and HSL mRNA expression (Kittilson et al., 2011) in rainbow trout, and suggest that fasting increases the biosynthesis and activation of the principal lipolytic enzyme, HSL. Enhanced lipolytic processes helps to explain reduced lipid content/tissue mass and increased plasma levels of fatty acid and glycerol observed previously in fasted rainbow trout (Norbeck et al., 2007; Sheridan and Mommsen, 1991) and in fasted seabream (Albalat et al., 2005). Moreover, it appears that adjustments in lipid metabolism of fish associated with the anabolic to catabolic shift consist of enhanced lipolysis concomitant with reduced lipogenesis (Sheridan and Mommsen, 1991).

In an attempt to elucidate the cellular mechanisms that underlie the anabolic to catabolic shift, we examined the influence of nutritional state on signaling cascades known to be operative in the rainbow trout. Our findings indicated that JAK2, STAT5, and Akt were activated (*i.e.*, increased abundance of the phosphorylated protein) in all tissues examined (*e.g.*, liver, mesenteric adipose tissue, red and white muscle) during periods of feeding and that JAK2,

STAT5, and Akt were deactivated (*i.e.*, reduced abundance of the phosphorylated protein) during periods of fasting. By contrast, ERK1/2 and PKC were activated to a greater extent in tissues during periods of fasting than in tissues during periods of feeding. Moreover, during periods of compensatory growth, JAK2, STAT5, and Akt reverted to their more activated state, whereas ERK1/2 and PKC reverted to their less activated state. To our knowledge, this is the first report of the abundance of signal elements in tissues other than white muscle in fish on differing nutritional regimes. Our findings of reduced Akt abundance in white muscle are consistent with those in fasting fine flounder (Fuentes et al., 2011) and rainbow trout (Seiliez et al., 2008). Our findings differ, however, from those in fine flounder with regard to the influence of long term-fasting on the abundance of ERK in white muscle (Fuentes et al., 2011). The reason for this difference is unknown, but as fish in both studies were sexually immature, it may be due to species and/or life history.

The role of the JAK/STAT and PI3K/Akt pathways in mediating growth-related processes in fish also is supported by several other previous observations. First, IGF-1 activates Akt in the skeletal muscle *in vivo* and *in vitro* in several species of fish (Castillo et al., 2006; Codina et al., 2008; Fuentes et al., 2011; Montserrat et al., 2007). Second, GH stimulated IGF-1 expression in isolated hepatocytes is mediated by JAK-STAT and Akt (Reindl et al., 2011). Given the importance of the GH-IGF system to the growth of fish (Bjornsson et al., 2004,Wood et al., 2005), it is reasonable to conclude that the deactivation of the JAK/STAT and PI3K/Akt pathways observed in the tissues of fasting fish contributes to their growth retardation. The role of the ERK pathway in fasting-associated growth retardation is more difficult to resolve. Given that IGF-1 results in activation of ERK in skeletal muscle of fish *in vivo* and *in vitro* (Castillo et al., 2006; Codina et al., 2008; Fuentes et al., 2011; Montserrat et al., 2007) and that hepatic GH-

stimulated IGF-1 expression in rainbow trout also is mediated to some extent by ERK (Reindl et al., 2011), on first blush, a role for ERK appears tenable. This supposition is supported by findings in fine flounder in which ERK activation accompanies fasting; yet, in rainbow trout, our findings that ERK is activated to a lower extent in tissues of fasting fish than in those of fed fish is contradictory to this notion. As already mentioned, it is possible that nutritional regulation of ERK is species and/or life history specific; indeed, in calorie-restricted monkeys, ERK activation in skeletal muscle is increased compared to animals on a normal diet (Nadeau et al., 2006). It also is possible that responses to nutritional challenge alters the web of signal cascades in a complex manner in order to activate adaptive adjustments to competing (*e.g.*, anabolic versus catabolic) cellular processes. Future studies that examine the effects of IGF and GH on signaling cascades in cells isolated from fish on varying nutritional regimes will help to resolve this issue.

The role of the ERK and PKC in mediating lipolytic processes in fish also is supported by several observations. First, activation of ERK and PKC was coincident with enhanced lipolysis in fasting fish. Second, in 3T3-L1 preadipocytes, ERK was shown to phosphorylate specific serine residues on HSL that led to the activation of the enzyme and to lipid breakdown (Greenberg et al., 2001). Third, PKC also was shown to activate HSL activity in BDF-1 mice (Galvin-Paron, 1997). In addition, cross talk was demonstrated between ERK and PKC in the activation of HSL, and these pathways were shown to synergize with cAMP/PKA to promote lipolysis in mammals (Duncan et al., 2007; Gonzalez-Yanes and Sanchez-Margalet, 2006). Given that the HSL of trout possess regulatory domains similar to those of mammalian HSLs (including serine phosphorylation sites) (Kittilson et al., 2011) and that the HSL of rainbow trout is activated by phosphorylation (Harmon et al., 1993; Michelsen et al., 1994), it is reasonable to suggest that activation of ERK and PKC contributes to the enhanced lipolysis we observed in

fasting fish. Moreover, given that GH was shown to stimulate lipolysis *in vivo* and *in vitro* in rainbow trout and in seabream (Albalat et al., 2005; O'Connor et al., 1993; Sheridan, 1986) and that GH activates ERK and PKC signaling cascades in fish and mammals (Piwien-Pilipuk et al., 2002; Reindl et al., 2011), it is likely that the elevated levels of GH observed in fasting fish (Fox et al., 2006; Gomez-Requeni et al., 2005; Norbeck et al., 2007; Pedroso et al., 2006) plays a role in stimulating fasting-associated lipolysis. Such a role of GH helps to resolve the apparent paradox of elevated plasma levels of the hormone during periods of growth cessation. Indeed, alterations in the cell signaling pathways that we observed may explain the rather disparate effects of GH on growth-promotion (anabolic) and lipolysis (catabolic). Future studies that examine the effects GH on activation of signaling cascades in cells isolated from fish on varying nutritional regimes will provide important insight into this issue.

In summary, the findings of this study indicate that nutritional state differentially activates intracellular signaling pathways in the liver, mesenteric adipose tissue, and red and white muscle of rainbow trout. During periods of feeding, the JAK/STAT and PI3K/Akt pathways were activated, and anabolic processes prevailed and growth was promoted. During periods of fasting, the ERK and PKC pathways were activated, and catabolism of lipids prevailed and growth was retarded.

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CHAPTER 3. PKC AND ERK MEDIATE GH-STIMULATED LIPOLYSIS[2]

Abstract

Growth hormone (GH) regulates several physiologic processes in vertebrates, including the promotion of growth, an anabolic process, and mobilization of stored lipid, a catabolic process. In this study, we used hepatocytes isolated from rainbow trout (*Oncorhynchus mykiss*) as a model to examine the mechanism of GH action on lipolysis. GH stimulated lipolysis as measured by increased glycerol release in both a time- and concentration-related manner. Promotion of lipolysis was accompanied by GH-stimulated phosphorylation of the lipolytic enzyme, hormone-sensitive lipase (HSL). GH-stimulated lipolysis also was manifested by increased expression of the two HSL-encoding mRNAs, HSL1 and HSL2. The signaling pathways that underlie GH-stimulated lipolysis also were studied. GH resulted in the activation of PLC/PKC and the MEK/ERK pathways, whereas JAK-STAT and the PI3K-Akt pathway were deactivated. Blockade of PLC/PKC and of the MEK/ERK pathway inhibited GH-stimulated lipolysis and GH-stimulated phosphorylation of HSL as well as GH-stimulated HSL mRNA expression, whereas blockade of JAK-STAT or of the PI3K-Akt pathway had no effect on the activation of lipolysis or HSL expression stimulated by GH. These results indicate that GH promotes lipolysis by activating HSL and by enhancing the *de novo* expression of HSL mRNAs via activation of PKC and ERK. These findings also suggest molecular mechanisms for activating the lipid catabolic actions of GH while simultaneously deactivating anabolic processes such as anti-lipolysis and the growth promoting actions of GH.

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Introduction

Lipids play many roles in animals, but their most significant use is as an energy reserve; therefore, the breakdown of stored lipids (lipolysis) is a critical aspect of their metabolism (Sheridan 1988). The main lipolytic enzyme is hormone-sensitive lipase (HSL) (Watt & Spriet 2010). HSL has been characterized in adipose tissue of mammals (Lafontan & Langin 2009) and in adipose tissue and liver of fish (Sheridan 1994). Interestingly, fish possess two HSL-encoding mRNAs that are differentially expressed among tissues (Kittilson et al. 2011). In mammals (Watt & Steinberg 2008) and fish (Sheridan 1994) HSL exists in two catalytic states, and upon activation by phosphorylation, hydrolyzes triacylglycerol (TG) to glycerol and fatty acids (FA).

Numerous hormones stimulate HSL in mammals and fish, most notably glucagon and catecholamines, which act through PKA (Sheridan, 1994, Gonzales-Yanes & Sanchez-Margalet 2006). By contrast, insulin is lipogenic in mammals and fish as well as directly anti-lipolytic (via dephosphorylation of HSL) (Harmon et al. 1993, Albalat et al. 2007, Chaves et al. 2011). The effects of GH on lipid metabolism are complex, and short-term insulin-like (antilipolytic) and long-term anti-insulin-like (lipolytic) effects have been reported in mammals (Carrel & Allen 2000, Chaves et al. 2011). Insulin-like effects 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), whereas continued exposure to GH results in lipolysis (Carrel & Allen 2000). Given the conditions under which it is brought, the biological significance of the insulin-like effects has been questioned (Carrel & Allen 2000). The lipolytic effect of GH in fish was first demonstrated in adipose tissue and liver of salmon *in vivo*. Growth hormone implantation stimulated HSL activity; whereas, hypophysectomy reduced HSL activity, an effect that was reversed by GH replacement (*cf.* Sheridan 1994). Subsequently, GH was

shown to directly stimulate lipolysis by us in liver isolated from rainbow trout (O'Connor et al. 1993) and others in adipose tissue isolated from sea bream (Albalat et al. 2005).

Despite extensive knowledge of GH signal transduction (Waters et al. 2006), the mechanism(s) by which GH enhances lipolysis as well as how these mechanisms integrate with other actions of GH (e.g., growth, reproduction, osmoregulation) (Bjornsson et al. 2004, Norrelund 2005) in any species is (are) unclear. In this study, we used rainbow trout hepatocytes as a model to examine the mechanism of GH action on lipolysis. This system is particularly advantageous because trout liver cells possess a high abundance of GH receptors (GHR) (Reindl & Sheridan 2012) and are a significant lipid storage site with a well characterized lipolytic system (Sheridan 1994). Our specific hypothesis was that ERK and PKC mediate GH-stimulated lipolysis. The rationale for this hypothesis extends from our previous observations that hepatic lipolysis and plasma GH levels increased during periods of food deprivation (Norbeck et al. 2007) and that fasting-induced lipolysis in trout liver was accompanied by deactivation of Akt, JAK2, and STAT5 and by activation of ERK and PKC (Bergan et al. 2012).

Materials and methods

Materials

All chemicals were 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 (#4060, #9272), ERK1/2 (#9101, #9102), JAK2 (#3776, #3230), and STAT5, phospho-PKCα/β II (#9375), B-actin (#4967) horseradish peroxidase (HRP)-linked anti-rabbit IgG antibody (#7074), biotinylated molecular weight marker (#7727), anti-biotin-HRP antibody (#7075), mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor U0126 (#9903) (MEK1 and 2 are directly responsible for the activation of

ERK), PI3K inhibitor LY294002 (#9901) [PI3K produces phosphatidylinositol phosphates that are critical for activation of Akt by phosphoinostide-dependent kinase 1 (PDK1)], protein kinase A (PKA) inhibitor H-89 dihydrocholoride (#9844) (selective for cAMP-dependent PKA), and cell lysis buffer (#9803) were all obtained from Cell Signaling Technology (Beverly, MA, USA). The JAK2 inhibitor, 1,2,3,4,5,6-hexabromocyclohexane (Hex), as well as the STAT5 inhibitor, N'-((4-oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (Nico), the Akt inhibitor, 1L6-hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-sn-glycerocarbonate (Carb), the broad spectrum protein kinase C (PKC) inhibitor, chelerythrine chloride (CC), 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, obtained from Drs. Akiyosi Takahasi and Shiunsuke Moriyama, were used for all experiments.

Experimental animals and conditions

Juvenile rainbow trout of both sexes (*ca*. 1 year of age) were obtained from Dakota Trout Ranch (Carrington, ND). Animals were transported to North Dakota State University where they were maintained in 800-L circular tanks supplied with recirculated (10% make-up volume per day) 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), except 7 days prior to experiments. Seven-day fasted animals were used because we previously showed they were in a catabolic state and capable of undergoing lipolysis (Bergan et al. 2012). Animals were acclimated to laboratory conditions for at least 4 weeks. All procedures performed were in accordance with the *Guide for Care and Use of Laboratory Animals* (National Research Council,

Washington, DC) and approved by the North Dakota State University Institutional Animal Care and Use Committee Protocol #A13001.

For experiments, fish 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 $MgSO_4$, 0.4 KH_2PO_4, 0.34 Na_2HPO_4, 4.2 $NaHCO_3$, 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% O_2. Cell viability was assessed by trypan blue dye exclusion and ranged between 93-97% for all experiments. After recovery, hepatocytes were collected by centrifugation (550 g for 8-10 min) and resuspended in incubation medium (recovery medium with 1.5 mM $CaCl_2$) to a final concentration of 6-8 $x10^6$ cells/ml, and aliquoted into 24-well plates (6-8 $x10^6$ 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% O_2). 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, 25 μM Carb, 10 μM U0126, 50 μM Hex, 200 μM Nico, 10 μM chelerythrine chloride, 10 μM U73122, 2.5 μM H-89 dihydrocholoride. After incubation, cells were pelleted (1,000 x g for 4 min) and the supernatant 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.

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 µl RNase-free deionized water and quantified by NanoDrop1000 spectrophotometry (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 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.

Steady-state levels of HSL1 and HSL2 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 HSL1 or HSL2 and β-actin. 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 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.

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. 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 (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 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 and of pharmacological pathway inhibitors in rainbow trout was previously validated (Reindl et al. 2011, Bergan et al. 2012).

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 K_2CO_3, 0.205 M $KHCO_3$, pH 10.0, 0.65 M $(NH_4)_2SO_4$), NAD^+ (10 mM in Buffer A), and glycerol dehydrogenase [7 units/ml in buffer B (4.76 mM KH_2PO_4, 4.76 mM K_2HPO_4, pH 7.6, 1.4 µM $MnCl_2$, 0.9 µM $(NH_4)_2SO_4$)], in a 4:2:1:1 ratio, respectively, in a total volume of 200 ul. Mixtures were incubated at 25°C for 1 h, then A_{340} was measured.

Hormone-modulated phosphorylation

GH-regulated phosphorylation of HSL was determined in hepatocytes according to Harmon et al. (1993). Four million cells were preincubated in 0.46 ml Hanks' buffer [in mM: 137.8 NaCl, 5.37 KCl, 0.8 $MgSO_4$, 0.4 KH_2PO_4, 0.337 Na_2HPO_4, 4.17 $NaHCO_3$, 10.0 HEPES, 4 Glucose, pH 7.6] with 4μCi [^{32}P]-monopotassium phosphate (specific activity 1000 mCi/mM; MP Biomedicals, Santa Ana, CA) in the presence or absence of pathway inhibitors at the concentrations described above for 3 h at 14°C with gyratory shaking at 100 rpm under 100% O_2. Cells were collected by centrifugation (550 rpm for 8-10 min), washed twice with Hanks' buffer, then dispersed and incubated in Hanks' buffer with or without GH at100 ng/ml for 3 h at 14°C with gyratory shaking at 100 rpm under 100% O_2. Incubations were stopped by centrifugation (1,000 g for 4 min at 14°C). The cells were resuspended in Hanks' buffer and homogenized. Following centrifugation (16,000 x g for 10 min at 14°C), the supernatant was removed, separated and subjected to 20% ammonium sulfate fractionation; the HSL-containing precipitate (Harmon et al. 1991) was collected after ice incubation (30 min) and centrifugation (16,000 x g for 15 min). HSL samples were resuspended in buffer (25 mM Tris-HCl, pH 7.4) and subjected to SDS-PAGE. [^{32}P]-phosphorylated-HSL was detected by direct phosphor imaging (Packard Cyclone) of gels.

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. After determining that data were normally distributed and displayed equal variance, 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 on untransformed data using SigmaStat v. 1.0 (SPSS, Chicago, IL, USA), and graphs and curve fitting models (for concentration response and computation of ED_{50s}) were constructed with SigmaPlot v8.0 (SPSS). Quantitative data are shown relative to control for ease of comparison and are expressed as means ± S.E.M.

Results

GH stimulates lipolysis in isolated hepatocytes

The lipolysis of stored lipids by HSL results in glycerol release into culture medium. GH stimulated lipolysis was assessed by the release of glycerol in a time-related manner (Fig. 9A). Initial levels of glycerol in the medium were 8.4±1.3 μM/10^6 cells, and within 1 h of GH exposure glycerol levels increased significantly. Maximum glycerol release was observed after 6 h; thereafter, glycerol concentration declined. GH also stimulated the release of glycerol in a concentration-related manner (Fig. 9B). Basal glycerol release was 8.7±1.3 μM/10^6 cells. GH stimulated a significant increase in glycerol release at 10 ng/ml, and maximum glycerol release was observed at a GH concentration of 1000 ng/ml.

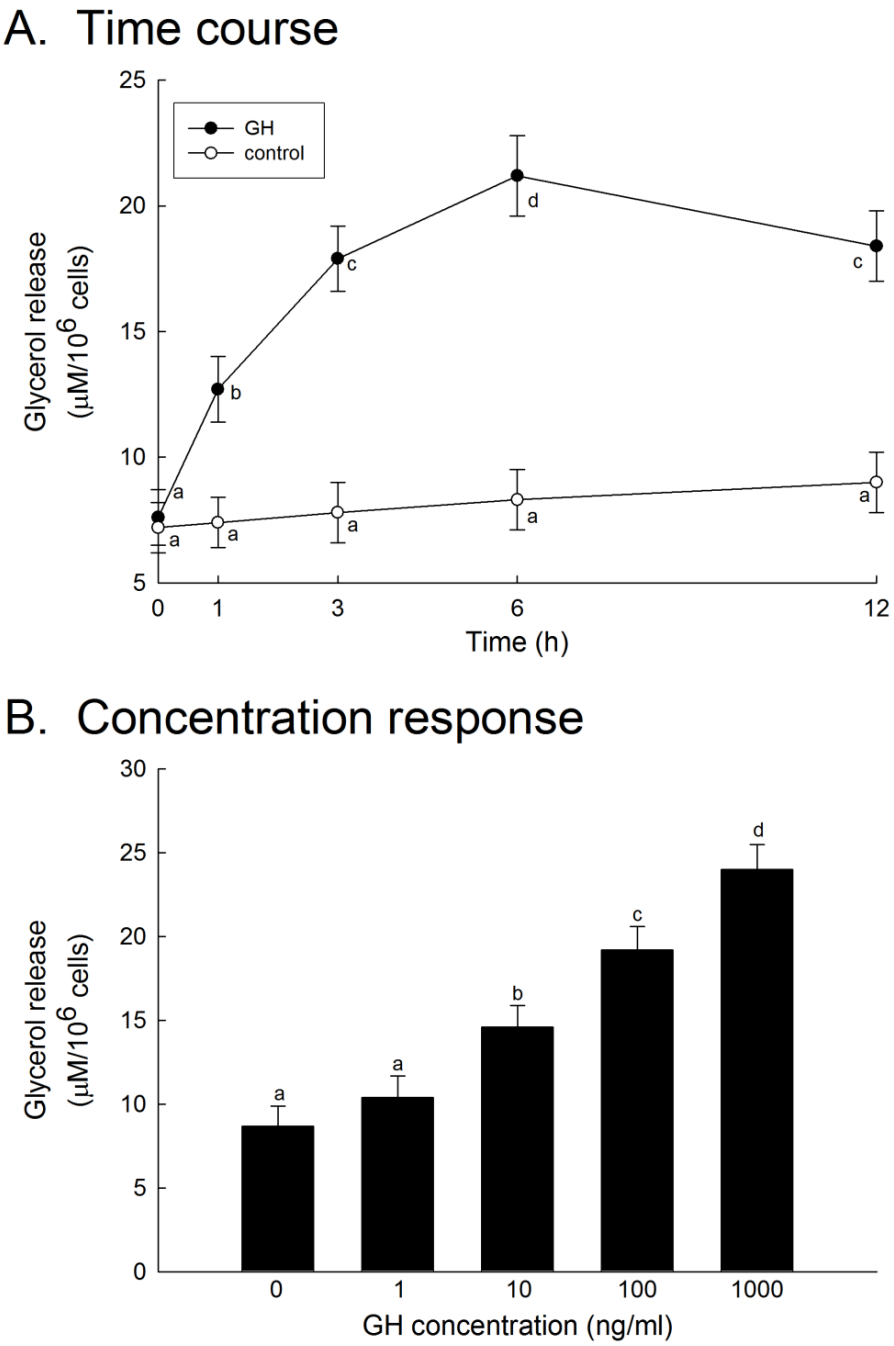

Figure 9. Growth hormone (GH)-stimulated lipolysis as measured by glycerol release from isolated hepatocytes of rainbow trout fasted for 7 days. A: time-dependent glycerol release of cells incubated with 100 ng/ml GH. B: concentration-dependent glycerol release of cells incubated in the absence or presence of various concentrations of GH for 6 h. Data are presented as means ± SEM (n=8). Groups with different letters are significantly ($p < 0.05$) different.

The effect of GH on the activation of HSL was studied in isolated hepatocytes that were preincubated with [^{32}P]-monopotassium phosphate. GH stimulated the phosphorylation of HSL as evidenced by the increased radioactive signal in the HSL fraction when compared to the untreated control (Fig. 10). The increased HSL phosphorylation came after a 3 h incubation of GH at 100 ng/ml. Taken together, these findings suggest that GH stimulates lipolysis through phosphorylation of HSL.

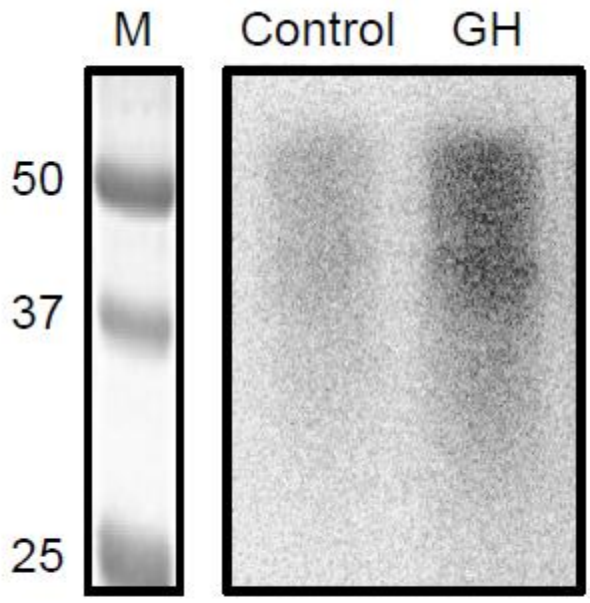

Figure 10. Growth hormone (GH)-stimulated phosphorylation of hormone-sensitive lipase (HSL) in isolated hepatocytes of rainbow trout fasted for 7 days. Hepatocytes were preincubated with [^{32}P]-monopotassium phosphate for 3 h, after which time cells were treated with or without (control) growth hormone (GH) at 100 ng/ml for another 3 h. HSL was purified from isolated hepatocytes by ammonium sulfate fractionation, and the phosphorylated enzyme was separated by SDS-PAGE and detected by autoradiography as described in the materials and methods section. ^{32}P- phosphorylation of HSL was stimulated by incubation with GH. Lane 1: control; Lane 2: GH; M: molecular weight marker. The image shown is a representative autoradiogram from three independent experiments.

The possibility that GH promotes lipolysis by stimulating expression of HSL mRNAs also was investigated. Rainbow trout possess two HSL-encoding mRNAs, HSL1 and HSL2; both forms were detected in all samples of hepatocytes. GH stimulated the expression of both HSL1 and HSL2 mRNAs in a time-dependent matter (Fig. 11A). GH significantly increased the expression of HSL mRNAs within 3 h. Maximum stimulation occurred after 12 h of GH treatment, increasing 275% and 300% for HSL1 and 2 mRNAs, respectively. By 24 h of GH treatment, expression decreased significantly from peak values, but not to levels observed in controls. The pattern of expression of HSL1 and HSL2 mRNAs differ significantly only after 3 h of GH treatment, with HSL2 mRNA being expressed to a greater extent than HSL1. GH also stimulated both HSL1 and HSL2 mRNAs in a concentration-related manner (Fig. 3B). Notably, GH was more potent in stimulating expression of HSL2 mRNA than of than HSL1. The expression of HSL2 mRNA was significantly stimulated over control levels at a concentration of 1 ng/ml, whereas HSL2 expression was significantly increased over control levels at10 ng/ml GH. There also was a significant difference between HSL2 and HSL1 mRNA expression evoked by GH at 10, 100, and 1000 ng/ml; and at a GH concentration of 1000 ng/ml, expression of HSL1 mRNA declined from the maximum level observed at 100 ng/ml.

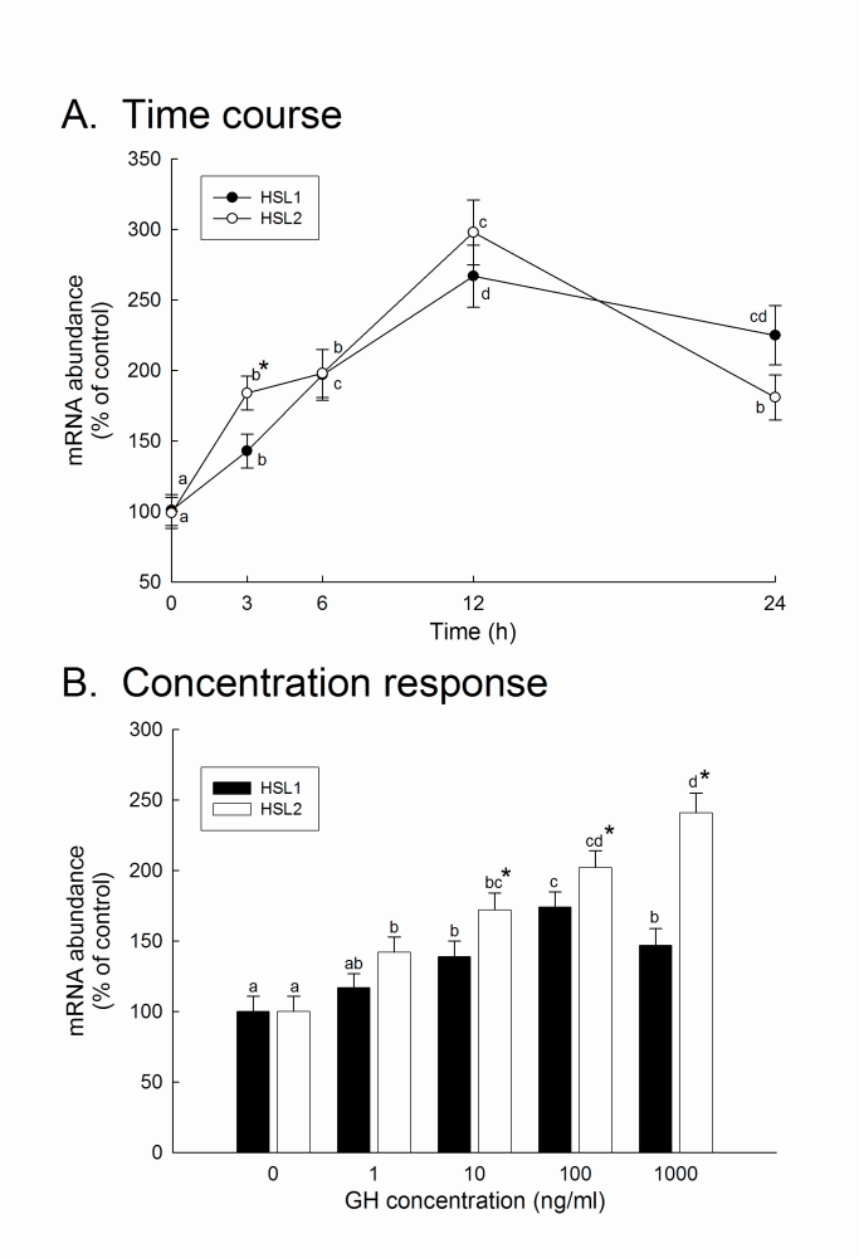

Figure 11. Growth hormone (GH)-stimulated expression of hormone-sensitive lipase (HSL) 1- and HSL2-encoding mRNAs from isolated hepatocytes of rainbow trout fasted for 7 days. A: time-dependent HSL mRNA expression in cells incubated with100 ng/ml GH. B: concentration-dependent HSL mRNA expression in cells incubated in the absence or presence of various concentrations of GH for 6 h. 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 percent of control (Panel A: 0 ng/ml GH at each respective time; Panel B: 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 subtypes in cells treated with GH for a given time or at a given concentration.

GH selectively deactivates/activates signaling pathways

The activation state of cell signaling pathways activated by GH was studied in lysates from hepatocytes isolated from fish fasted for 7 days probed with phospho-specific and control antibodies. Phospho-JAK2, phospho-STAT5, phospho-Akt, phospho-ERK, and phospho-PKC were detected in all treatments of hepatocytes. GH deactivated JAK2, STAT5, and Akt in a time-related manner (Fig. 12A, 12C, 12E). Significant deactivation of both JAK2 and Akt, a downstream target of PI3K, occurred within 5 min, then returned to control levels by 1 h of GH treatment. STAT5 was significantly deactivated within 10 min, an effect that persisted through 3 h. GH also deactivated JAK2, STAT5, and Akt in a concentration-related manner (Fig. 12B, 12D, 12F). Significant deactivation of JAK2, STAT5, and Akt occurred initially at a concentration of 10 ng/ml GH, and progressively lower degrees of phosphorylation were observed as GH concentration increased. Maximum deactivation occurred at 1000 ng/ml GH, resulting in a reduction in phosphorylation state of JAK2, STAT5, and Akt to 25%, 35%, and 58% of control, respectively.

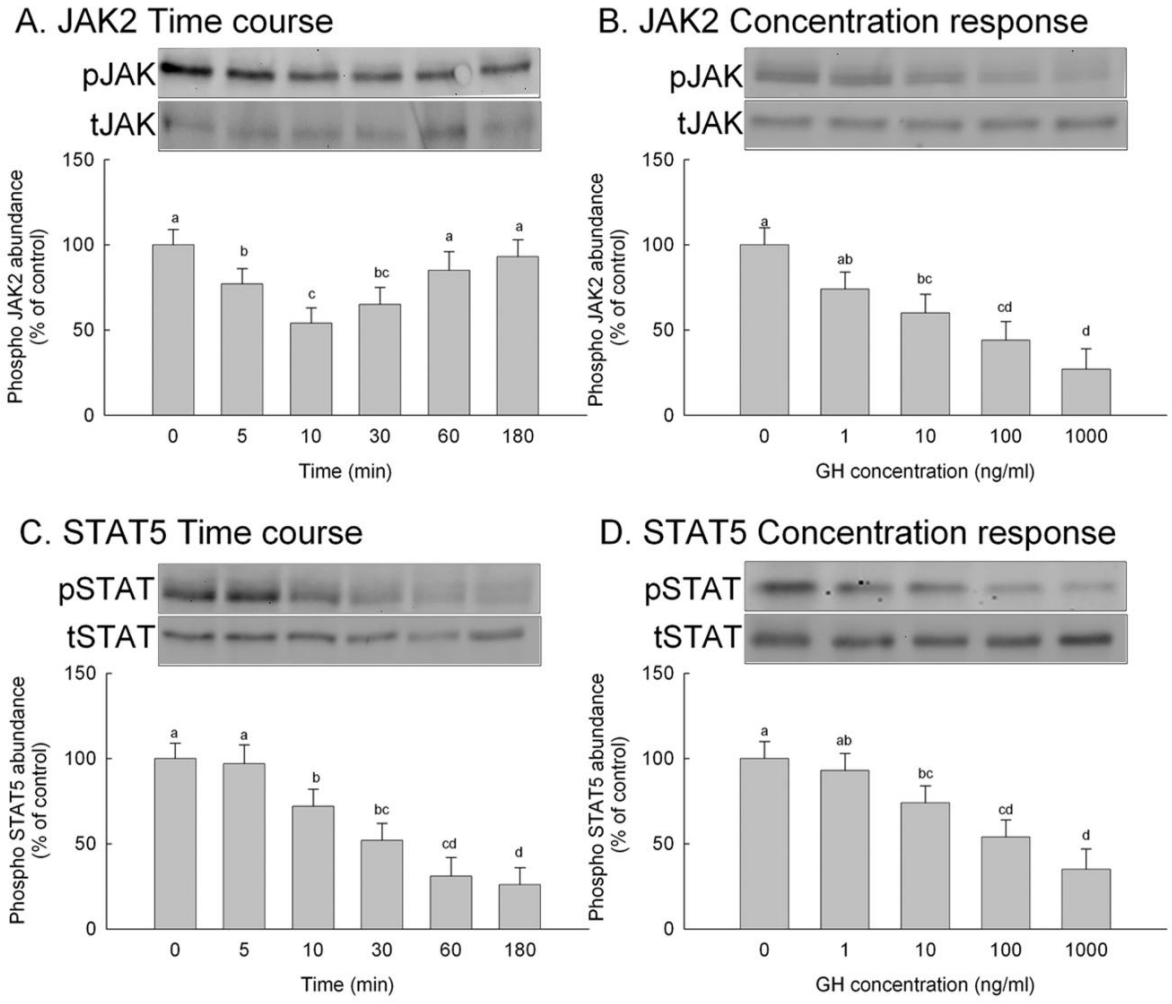

Figure 12. Effects of growth hormone (GH) on the abundance of phosphorylated Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), and protein kinase B (Akt) in isolated hepatocytes of rainbow trout fasted for 7 days. A, C, E: time-dependent phosphorylation of JAK2, STAT5, and Akt, respectively, in cells incubated with100 ng/ml GH. C, D, F: concentration-dependent phosphorylation of JAK2, STAT5, and Akt, respectively, in cells incubated in the absence or presence of GH for 30 min (control is 0 ng/ml GH). 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, and Akt was normalized to total JAK2, STAT5, and Akt, respectively. Data are presented as representative immunoblots (insets) and as means $\pm$ SEM. (n=4). Groups with different letters are significantly ($p < 0.05$) different.

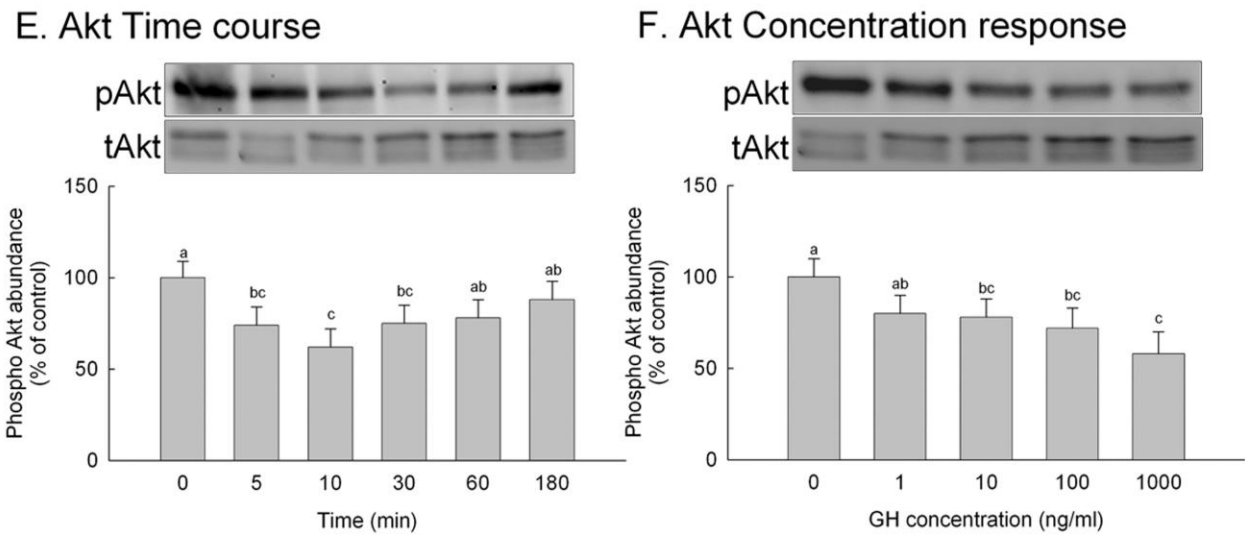

Figure 12. Effects of growth hormone (GH) on the abundance of phosphorylated Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), and protein kinase B (Akt) in isolated hepatocytes of rainbow trout fasted for 7 days (continued). A, C, E: time-dependent phosphorylation of JAK2, STAT5, and Akt, respectively, in cells incubated with100 ng/ml GH. C, D, F: concentration-dependent phosphorylation of JAK2, STAT5, and Akt, respectively, in cells incubated in the absence or presence of GH for 30 min (control is 0 ng/ml GH). 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, and Akt was normalized to total JAK2, STAT5, and Akt, respectively. Data are presented as representative immunoblots (insets) and as means $\pm$ SEM. (n=4). Groups with different letters are significantly ($p < 0.05$) different.

By contrast to the response on JAK2, STAT5, and Akt, GH resulted in the activation ERK and PKC. Significant activation of ERK by GH occurred within 10 min and persisted through 3 h (Fig. 13A). GH also significantly induced the phosphorylation of PKC within 10 min, an effect that peaked after 60 min, then subsided back to control levels by 3 h (Fig. 13B). GH also activated ERK and PKC in a concentration-related manner (Fig. 13B, 13C). Significant phosphorylation was observed at a GH concentration of 10 ng/ml GH; maximal activation, increasing to 183% of control levels, was observed at 1000 ng/ml. A similar profile was seen with PKC activation, for which significant phosphorylation was induced by a GH concentration of 10 ng/ml and maximal phosphorylation was achieved at 1000 ng/ml.

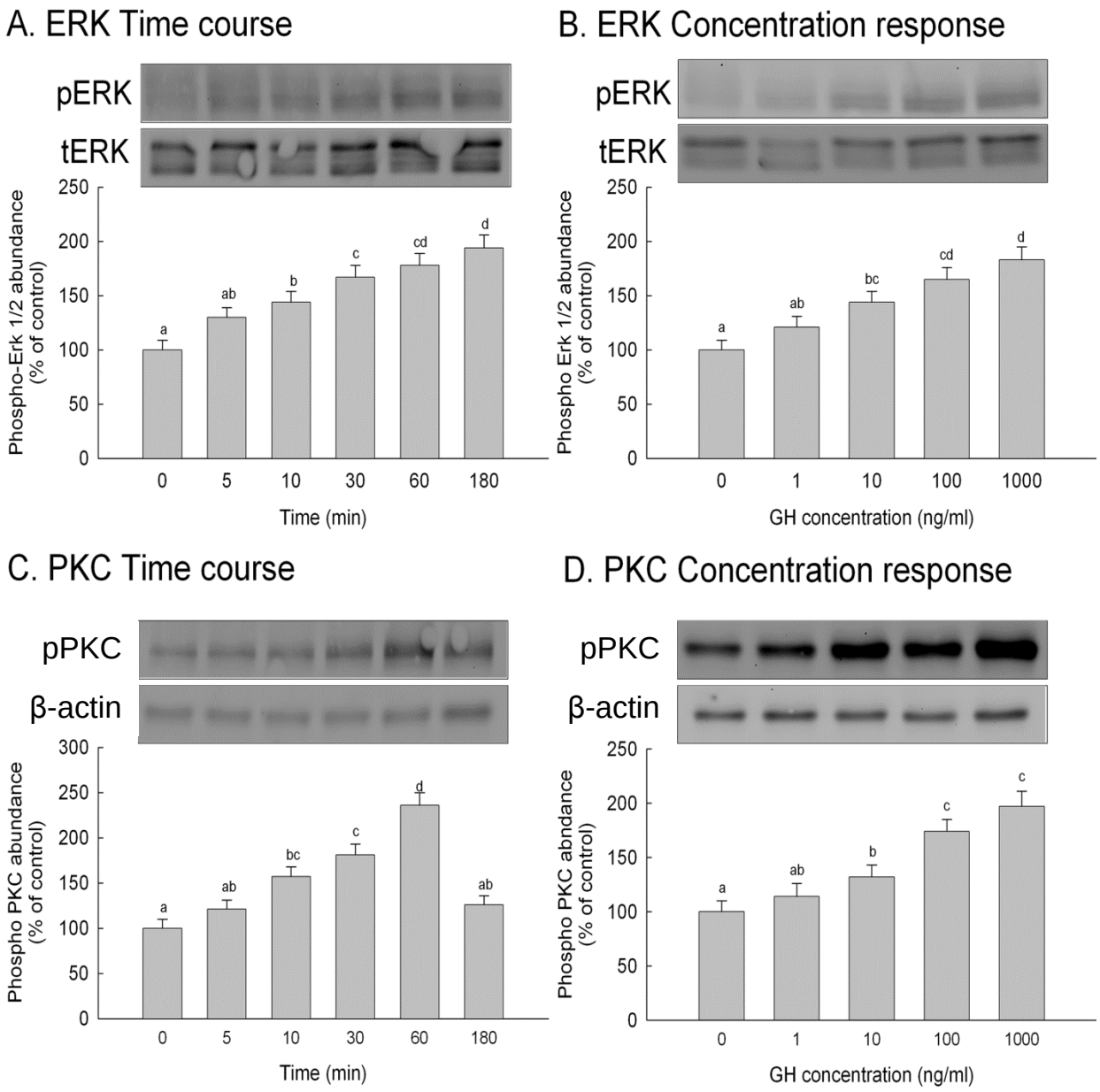

Figure 13. Effects of growth hormone (GH) on the abundance of phosphorylated extracellular signal-regulated kinase (ERK) and protein kinase (PKC) in isolated hepatocytes of rainbow trout fasted for 7 days. A, C: time-dependent phosphorylation of ERK and PKC, respectively, in cells incubated with 100 ng/ml GH. B, D: concentration-dependent phosphorylation of ERK and PKC, respectively, in cells incubated in the absence or presence of GH for 30 min (control is 0 ng/ml GH). Cell lysates were separated by SDS-PAGE followed by Western immunoblotting, and the blots were quantified with a FluorChem imager. The abundance of phosphorylated ERK 1/2 and PKCα/β II was normalized to total ERK 1/2 and β-actin, respectively. Data are presented as representative immunoblots and as means $\pm$ SEM. (n=4). Groups with different letters are significantly ($p < 0.05$) different.

Linkage of cell signaling elements to GH-stimulated lipolysis

The linkage of specific cell signaling pathways to GH-stimulated lipolysis and GH-stimulated HSL expression in isolated hepatocytes was studied using pharmacological inhibitors. The first series of experiments examined the effects of pathway blockade on lipolysis as measured by glycerol release (Fig. 14). As noted previously, GH (100 ng/ml) stimulated glycerol release over basal levels. Pretreatment of hepatocytes 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. By contrast, inhibition of JAK2 (with hex), PI3K (with LY294002), or of Akt directly (with Carb) had no effect on GH-stimulated glycerol release from hepatocytes.

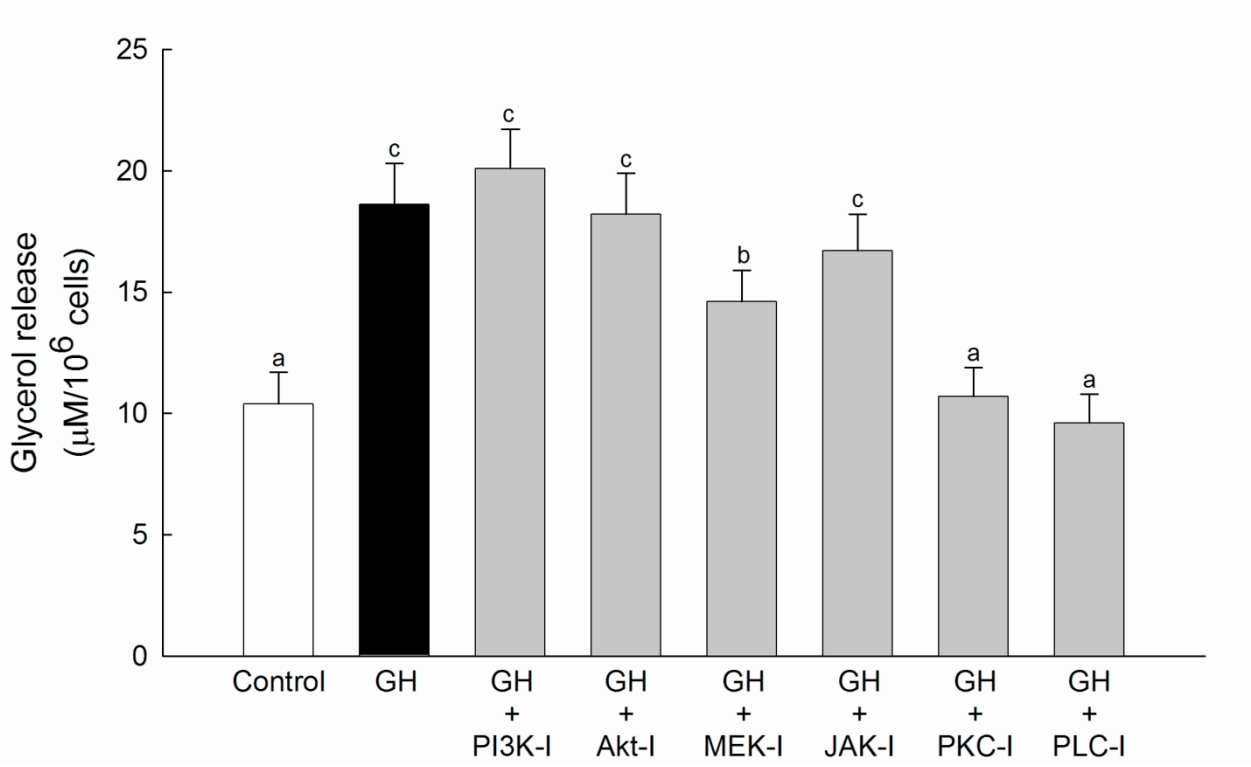

Figure 14. Effects of signaling element blockade on growth hormone (GH)-stimulated lipolysis as measured by glycerol release from isolated hepatocytes of rainbow trout fasted for 7 days. Cells were pretreated for 2 h with or without specific inhibitors for the following signaling elements: PI3K (10 μM LY294002), Akt (10 μM Carb.), MEK (10 μM U0126), JAK2 (10 μM Hex.), PKC (10 μM chelerythrine chloride), PLC (10 μM 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 $\pm$ SEM. (n=6). Groups with different letters are significantly ($p < 0.05$) different.

In the second series of experiments, we examined how signaling pathways mediate the activity state of HSL by evaluating their role in the phosphorylation of the enzyme. GH stimulated ^{32}P-phosphorylation of HSL (Fig. 15). Blockade of JAK (with Hex) had no effect on GH-stimulated phosphorylation of HSL, whereas blockade of ERK (with U0126) or of PKC (with chelerythrine chloride) blocked GH-stimulated phosphorylation of HSL (Fig. 15). In addition, inhibition of PKA, a known regulator of HSL phosphorylation (Harmon et al. 1993; therefore used as positive control), partially blocked GH-stimulated phosphorylation of HSL.

Inhibition of PI3K (with LY294002) or of Akt directly (with Carb) also had no effect on GH-stimulated phosphorylation of HSL (data not shown).

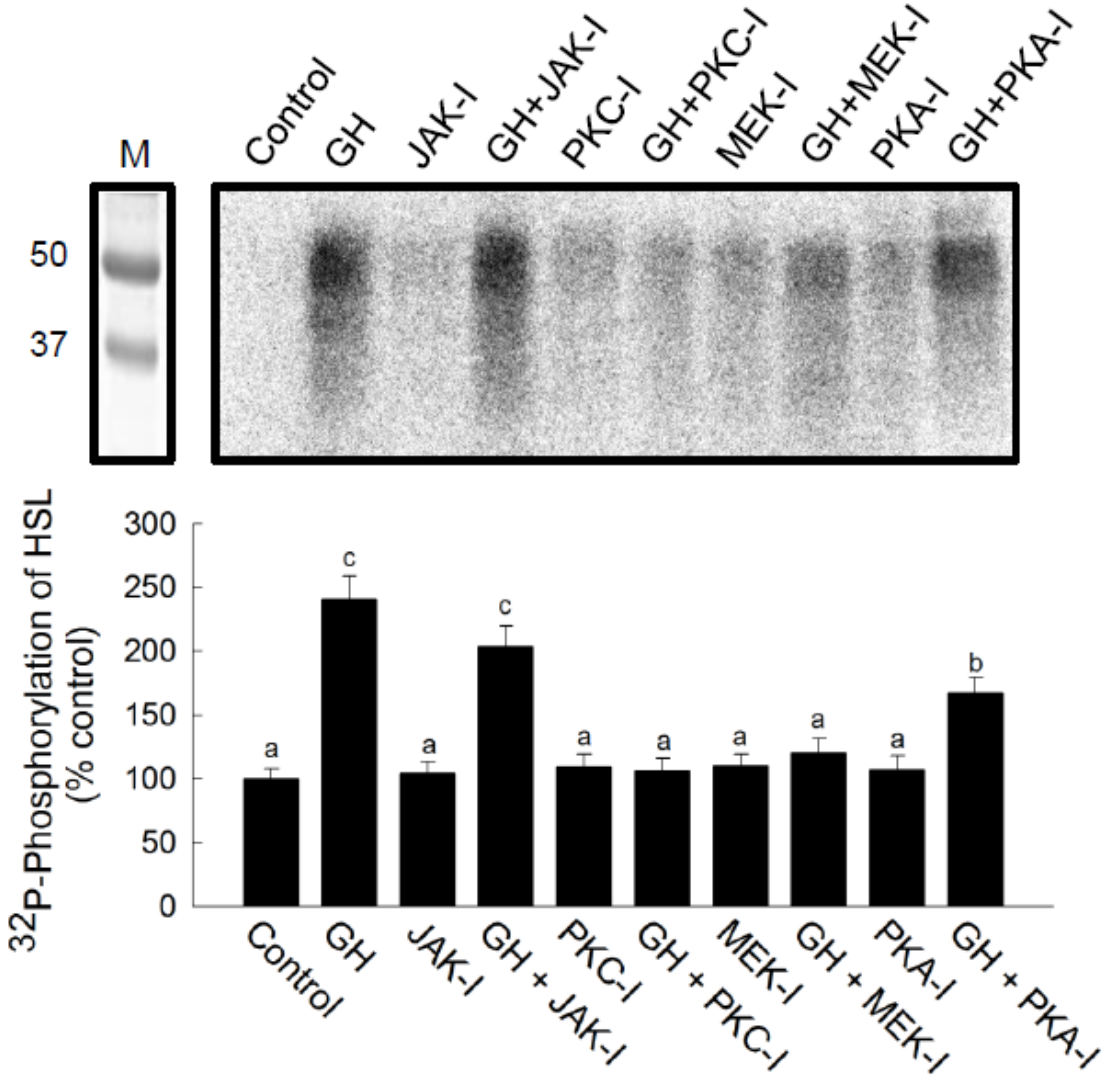

Figure 15. Effects of signaling element blockade on growth hormone (GH)-stimulated phosphorylation of hormone-sensitive lipase (HSL) in isolated hepatocytes of rainbow trout fasted for 7 days. Hepatocytes were preincubated for 3 h with [^{32}P] monopotassium phosphate in the presence or absence of specific inhibitors for the following signaling elements: JAK2 (10 μM Hex=JAK-I), PKC (10 μM chelerythrine chloride=PKC-I), MEK (10 μM U0126=MEK-I), PKA (10 μM H-89 dihydrochloride=PKA-I); after which time, cells were treated with or without (control) GH at 100 ng/ml for another 3 h. HSL was purified from isolated hepatocytes by ammonium sulfate fractionation, and the phosphorylated enzyme was separated by SDS-PAGE and detected by autoradiography as described in the materials and methods section. Data are presented as a representative autoradiogram (upper panel) and as means ± SEM (n=3).

The third series of experiments examined the pathways mediating GH-stimulated mRNA expression (Fig. 16). Blockade of the ERK pathway with a MEK inhibitor (U0126) and blockade of PLC (with U73122) partially reduced expression of both HSL mRNAs. When cells were pretreated with the PKC inhibitor (chelerythrine chloride) followed by treatment with GH, HSL mRNA expression decreased below control levels. Blockade of P13K, Akt, JAK2, and STAT5 signaling elements did not affect GH-stimulated HSL mRNA expression.

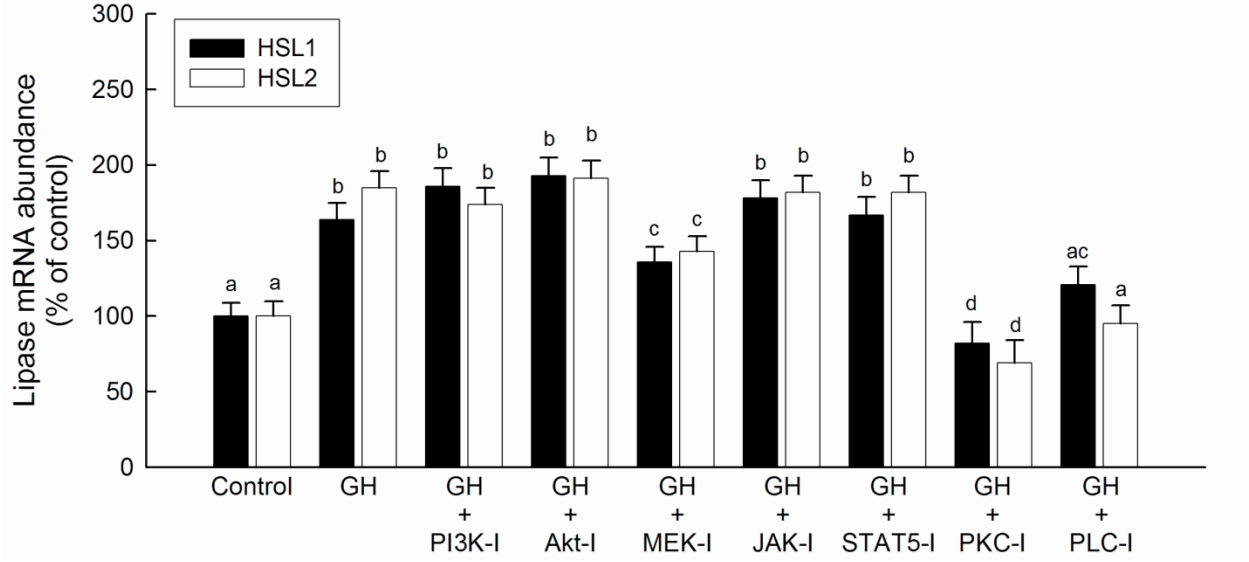

Figure 16. Effects of signaling element blockades on growth hormone (GH)-stimulated expression of hormone-sensitive lipase (HSL) 1- and HSL2-encoding mRNAs from isolated hepatocytes of rainbow trout fasted for7 days. Hepatocytes were preincubated for 2 h with or without specific inhibitors for the following signaling elements: PI3K (10 μM LY294002=PI3K-I), Akt (10 μM Carb=Akt-I.), MEK (10 μM U0126=MEK-I), JAK2 (10 μM Hex=JAK-I), STAT5 (10 μM Nico=STAT-I), PKC (10 μM chelerythrine chloride=PKC-I), PLC (10 μM U73122=PLC-I); and in the absence or presence of GH at 100 ng/ml for 6 h (control is 0 ng/ml GH); after which time, cells were treated with 100 ng/ml GH for 6 h (control is 0 ng/ml GH). 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 controls and expressed as mean $\pm$ SEM (n = 6). For a given HSL isoform, groups with different letters are significantly ($p < 0.05$) different.

Discussion

The liver of teleost fish is a particularly opportune system in which to investigate the influence of GH on lipid mobilization because it is a significant lipid storage site with a well characterized lipolytic system (Sheridan 1994) and it possesses a high abundance of GH receptors (GHR) (Reindl & Sheridan 2012). The present results demonstrate that GH, at concentrations in the physiologic range in the plasma of trout (Norbeck et al. 2007), stimulates lipolysis by activating HSL and by enhancing the *de novo* expression of HSL mRNAs. The results also confirm our starting hypothesis that GH-stimulated HSL activity and GH-stimulated HSL mRNA expression are mediated through the ERK and PKC signaling pathways. These findings establish the mechanisms through which GH exerts comprehensive lipolytic actions and provides insight into how such actions may be integrated with other actions of GH.

GH stimulates lipolysis by activation of HSL. This is supported by the present observation that GH directly stimulated lipolysis in isolated hepatocytes resulting in the release of glycerol. This observation is consistent with previous studies in mammalian adipose tissue (Gorin et al. 1990, Chavez et al. 2006) and in liver and adipose tissue of fish (Sheridan 1994, Albalat et al. 2005) that showed the GH-stimulated hydrolysis of stored TG and the release of glycerol and FA and with studies in fish showing that GH increased the specific activity of hepatic HSL *in vivo* and *in vitro* (O'Connor et al. 1993, Sheridan 1994). Such lipolytic action underlies the increase in plasma FA following GH injection of mammals and fish (Fain 1980, Lafontan & Langin 2009). The present study also reveals for the first time to our knowledge that GH-stimulated lipolysis is accompanied by phosphorylation of HSL. In mammals (Watt & Steinberg 2008) and fish (Sheridan 1994), HSL exists in two catalytic states, and upon activation by phosphorylation hydrolyzes TG. Mutagenesis studies of mammalian HSLs have

demonstrated that phosphorylation of (in rat) Ser^{563}, Ser^{600}, Ser^{659}, and Ser^{660} result in activation of HSL, whereas phosphorylation of Ser^{565} inhibits HSL activity (Yeaman 2004, Watt & Steinberg 2008). The conservation of these residues in the two HSLs of rainbow trout (Kittilson et al. 2011) most likely explains the activation of hepatic HSL by phosphorylation observed in trout previously (Harmon et al.1993) as well as the GH-stimulated activation of HSL observed in the current study.

GH signaling involves the activation of numerous signaling cascades, including JAK-STAT, ERK, PI3K/Akt and PLC/DAG/PKC (Water et al. 2006). Of these pathways, several current observations suggest that the activation of HSL by GH involves ERK and PKC. First, GH increased the abundance of phospho-ERK as well as of phospho-PKC. Second, specific blockade of the ERK pathway or of PLC/PKC inhibited GH-stimulated lipolysis. Third, blockade of the ERK pathway or of PKC inhibited GH-stimulated phosphorylation of HSL. Taken together, these findings indicate that ERK and PKC mediate GH-stimulated activation of HSL. These findings are consistent with previous studies in mammals showing that GH activated PKC to promote lipolysis in fat pads isolated from rats (Gorin et al. 1990). Previous work in mammals also showed that PKC could activate the ERK pathway (Gonzales-Yanes & Sanchez-Margalet 2006) and that ERK is a proximate activator of HSL by phosphorylation of Ser^{600} (Greenberg et al. 2001). In light of the current findings, it is reasonable to suggest that GH-stimulated lipolysis results from ERK activation of HSL mediated by PLC/PKC. The present findings also suggest that PKA mediates GH-stimulated lipolysis as evidenced by partial inhibition of GH-stimulated phosphorylation of HSL in the presence of a specific PKA inhibitor. These findings are consistent with previous observations in mammals (Lafontan and Langin 2009) and fish (Harmon et al. 1993) that PKA is a proximate activator of HSL. Mutagenesis

studies in rats revealed that PKA phosphorylates HSL at Ser^{563}, Ser^{659}, and Ser^{660} (Yeaman 2004, Waters et al 2008). These observations coupled with the previous observation that PKC can activate PKA (Fricke et al. 2004) and our current findings suggest that GH-stimulated lipolysis also results from PKA activation of HSL mediated by PLC/PKC. There is some suggestion that GH may accentuate the action of PKA by increasing the cytoplasmic pool of cAMP through inhibition of $Gi\alpha_{2;}$ however, the specific effector(s) that transduce such action is (are) not known (Lafontan & Langin 2009). It also should be noted that the possibility exists that PKC could directly activate HSL (as well as other lipases), but there is no evidence of this; however, other kinases such as cGMP-dependent protein kinase have been implicated in HSL activation (Yeaman 2004).

The current findings also suggest that GH-stimulated lipolysis is accompanied by deactivation of JAK/STAT and PI3K/Akt. This conclusion is supported by GH reducing transiently the abundance of phospho-JAK2, Phospho-STAT5, and phospho-Akt and by specific inhibition of JAK2, PI3K or Akt having no effect on GH-stimulated lipolysis or GH-stimulated phosphorylation of HSL. These findings provide new insight into the signaling mechanisms that underlie the antagonism between lipolysis and antilipolysis/lipogenesis. At the nexus of this antagonism is Akt. Akt has been found to stimulate phosphodiesterase activity which, in turn, reduces the cytoplasmic pool of cAMP and leads to the deactivation of PKA, dephosphorylation of HSL, and reduced lipolysis (Baragali et al. 2011). In addition, Akt activates AMP-activated protein kinase (Berggreen et al. 2009) the latter of which negatively regulates HSL by phosphorylation at Ser^{565} (Watt & Steinberg 2008). Akt also regulates several lipogenic enzymes, including activation of the acetyl-CoA carboxylase (Berggreen et al. 2009), the rate-limiting enzyme of FA synthesis. Given that Akt is a chief element of insulin signaling (Caruso

& Sheridan 2011), it is through Akt that insulin exerts its lipogenic and antilipolytic actions (Albalat et al. 2005 Chaves et al. 2011). The present findings suggest that by reducing Akt activation, GH shifts the balance of the antilipolysis/lipogenesis-lipolysis antagonism toward lipolysis by suppressing antilipolytic/lipogenic processes.

The current findings also indicate that GH stimulates lipolysis by increasing expression of HSL-encoding mRNAs. Interestingly, the responsiveness of HSL 1 to GH treatment appeared greater than that of HSL 2. The existence of multiple HSL isoforms appears to be unique to teleost fish and the functional significance of the different forms is not clear; however, differences do exist in the intracellular domain of the predicted proteins (Kittilson et al. 2011)--differences that may be important for activation by different signal transductions systems and sufficient to explain the differential responsiveness to GH. The ability of GH to promote lipase expression is consistent with previous observations in isolated rat adipocytes that GH-stimulated lipolysis was abolished by inhibitors of RNA and protein synthesis (Fain 1980). The current findings showing that blockade of the ERK pathway or of PLC/PKC inhibit GH-stimulated HSL expression indicate for the first time to our knowledge the mechanism(s) through which GH affects *de novo* HSL synthesis. The HSL promoter in mammals was found to possess several elements that could be recognized by general transcription factors such as SF-1, Sp1, and C/EBPs as well as several other response elements, including glucose response element, fat response element, and a cAMP response element (Lampidonis et al. 2008). Given the previous observations that PKC activated PKA (Fricke et al. 2004) and that PKA increased HSL transcription via SF-1 (Holsyz et al. 2011), it is reasonable to suggest that GH-stimulated HSL expression proceeds through a PLC/PKC-PKA-SF-1 pathway. Given that PKC activated ERK (Fricke et al., 2004) and that ERK activated c/EBPβ (Piwien-Pilipuk et al. 2002) and GH

increases expression c/EBPβ, including in rainbow trout (Lo et al. 2007), it also is reasonable to suggest that GH-stimulated HSL expression proceeds through a PLC/PKC-ERK-c/EBPβ pathways. The deactivation of Akt following GH treatment observed in the present study also may influence GH-stimulated HSL expression. For example, Akt plays a role in dephosphorylation of c/EBPβ (Piwien-Pilipuk et al. 2002); therefore, by reducing the activation of Akt, the phosphorylation of c/EBPβ and the ensuing enhancement of HSL expression would be favored. Akt also leads to the activation of PDE (Chavez et al. 2006); by reducing Akt activation, there would be an increase in the cAMP pool and an increase in HSL transcription via the cAMP response element.

The results of the present study help to explain the mechanisms that underlie fasting-associated lipid depletion. During periods of food deprivation, lipid depletion is attended by increases in plasma levels of GH in the face of reduced plasma levels of insulin and IGF-1 in mammals and fish (Norbeck et al. 2007). We recently showed that fasting-induced lipolysis in trout liver (as well as in adipose tissue and red and white skeletal muscle) was accompanied by deactivation of Akt, JAK2, and STAT5 and by activation of ERK and PKC (Bergan et al. 2012). In light of the present findings that GH-stimulated lipolysis was mediated by activation of PKC and ERK in conjunction with deactivation of PI3K-Akt and JAK-STAT, it is reasonable to conclude that GH promotes lipolysis during fasting by activation of PKC and ERK and by deactivation PI3K/Akt and JAK2-STAT. In should be noted that the hepatocytes used in the present study were isolated from fish that were fasted for 7 days, a period of time previously shown to result in reduced plasma levels of insulin and IGF-1 and in elevated plasma levels of GH, which underscores the relevance of the findings to interpreting the actions of GH in the fasting state. The findings also may help to explain the mechanisms that underlie smoltification-

and premature transfer to seawater-associated depletion of lipids observed in salmonids—both of which occur in association with increases in plasma GH (Sheridan 1989).

In summary, these results indicate that GH promotes lipolysis by enhancing expression of HSL-encoding mRNAs as well as by phosphorylating HSL via activation of PKC and ERK.

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CHAPTER 4. NUTRITIONAL STATE MODULATES GROWTH HORMONE-STIMULATED LIPOLYSIS[3]

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

[3] This chapter is in press as follows: Bergan, H.E., Kittilson, J.D., Sheridan, M.A. 2013. *General and Comparative Endocrinology,*

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.

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).

Materials and methods

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, PKCα/β II, and STAT5, horseradish peroxidase (HRP)-linked anti-rabbit IgG antibody, biotinylated molecular weight marker, anti-biotin-HRP antibody, mitogen-activated protein 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).

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 $MgSO_4$, 0.4 KH_2PO_4, 0.34 Na_2HPO_4, 4.2 $NaHCO_3$, 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% O_2. 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 $CaCl_2$) to a final concentration of 6-8 $x10^6$ cells/ml, and aliquoted into 24-well plates (6-8 $x10^6$ 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% O_2). 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 μM U73122 . 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 and medium samples were immediately frozen on dry ice then stored at -80°C until further analysis.

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 μl 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 μl 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 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.

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 (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 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).

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 K_2CO_3, 0.205 M $KHCO_3$, pH 10.0, 0.65 M $(NH_4)_2SO_4$), NAD^+ (10 mM in Buffer A), and glycerol dehydrogenase [7 units/ml in buffer B (4.76 mM KH_2PO_4, 4.76 mM K_2HPO_4, pH 7.6, 1.4 μM $MnCl_2$, 0.9 μM $(NH_4)_2SO_4$)], in a 4:2:1:1 ratio, respectively, in a total volume of 200 ul. Mixtures were incubated at 25°C for 1 h, after which time the absorbency was read at 340 nm.

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).

Results

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 in Figure 17A. The initial release of glycerol from cells of fasted fish (12 ± 2 μM/10^6 cells) was slightly, but not significantly, higher than that from cells of fed fish (10 ± 1 μM/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. 17B). Similar to the results of the time course experiment, basal glycerol release from cells from fasted fish (12 ± 2.5 $\mu M/10^6$ cells) was slightly but not significantly higher than that from cells from fed fish (9.7 ± 1.0 $\mu M/10^6$ 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.

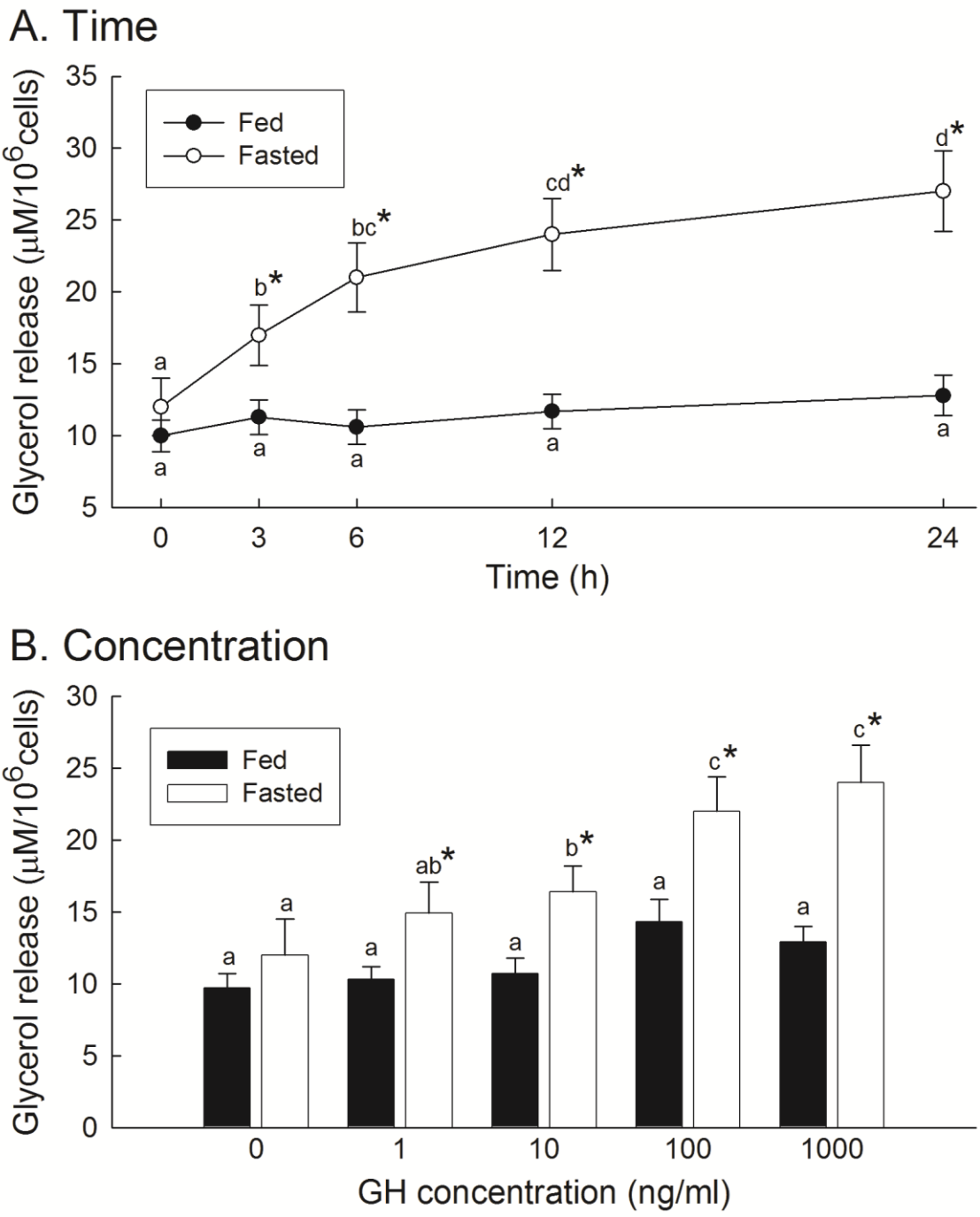

Figure 17. Growth hormone (GH)-stimulated lipolysis as measured by glycerol release from hepatocytes isolated from rainbow trout fed continuously or fasted for 4 weeks. A: time-dependent release of glycerol from cells incubated with 100 ng/ml GH. B: concentration-dependent release of glycerol from cells incubated in the absence or presence of various concentrations of GH for 6 h. Data are presented as means ± 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.

We next investigated the influence of GH on *HSL* mRNA expression in hepatocytes isolated from 4-week fasted fish and from fed fish. 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. 18A). 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. 18B). 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.

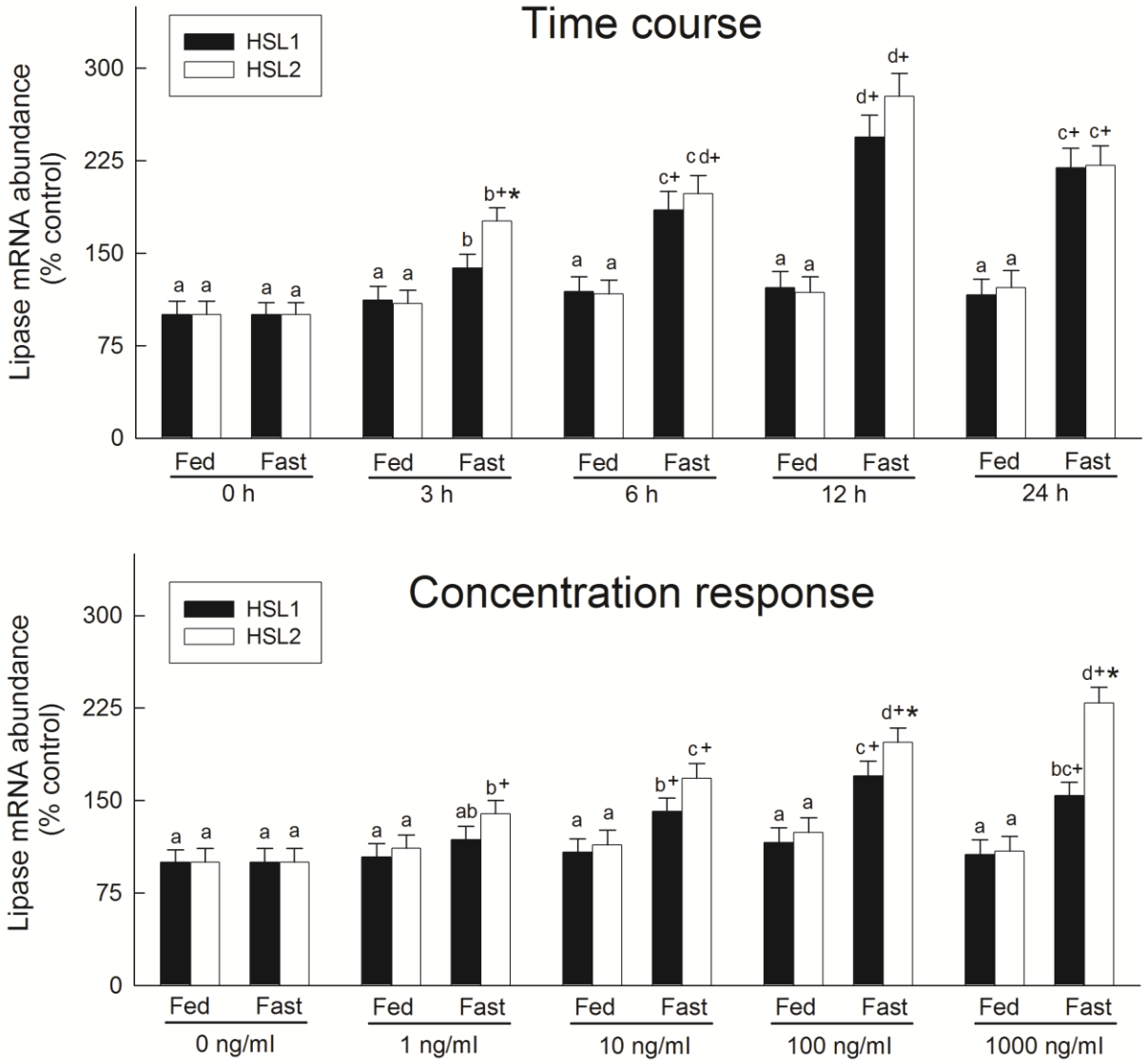

Figure 18. Growth hormone (GH)-stimulated expression of hormone-sensitive lipase (HSL) 1- and HSL2-encoding mRNAs in hepatocytes isolated from rainbow trout fed continuously or fasted for 4 weeks. A: time-dependent HSL mRNA expression in cells incubated with 100 ng/ml GH. B: concentration-dependent HSL 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.

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. 19A-19C); whereas, the basal activation state of ERK and PKC was greater in hepatocytes from fasted fish than in cells from fed fish (Fig. 19D & 19E). In cells from fed fish, GH significantly increased phosphorylation of JAK2, STAT5, Akt, and ERK (Fig. 19A-19D). GH treatment of hepatocytes from fed fish did not change the abundance of phospho-PKC (Fig. 19E). In cells from 4-week fasted fish, GH significantly increased the abundance of phospho-ERK and phospho-PKC (Fig. 19D & 19E). Notably, GH increased the phosphorylation of ERK in cells from fasted fish to a greater extent than in cells from fed fish (Fig. 19D). GH had no effect on the activation of JAK2, STAT5, or Akt in hepatocytes from fasted fish (Fig. 19A-19C). 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.

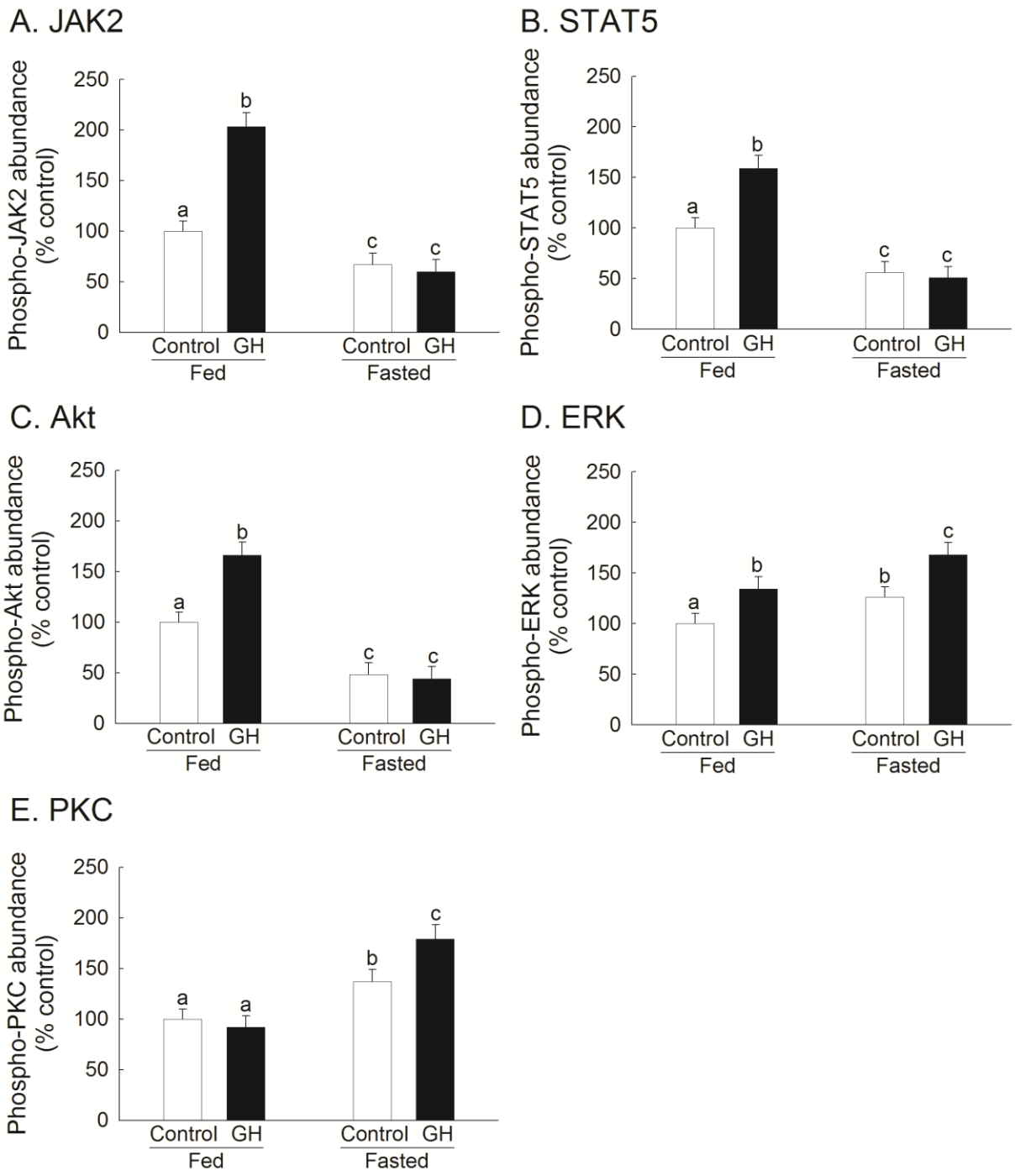

Figure 19. Effects of growth hormone (GH) on the abundance of phosphorylated Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), protein kinase B (Akt), extracellular signal-regulated kinase (ERK), and protein kinase C (PKC) in isolated hepatocytes of rainbow trout fed 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.EM. (n=4). Groups with different letters are significantly ($p < 0.05$) different.

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. 20). As observed above, GH (100 ng/ml) stimulated the release of glycerol over basal levels from hepatocytes isolated form 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 form hepatocytes isolated form fed fish.

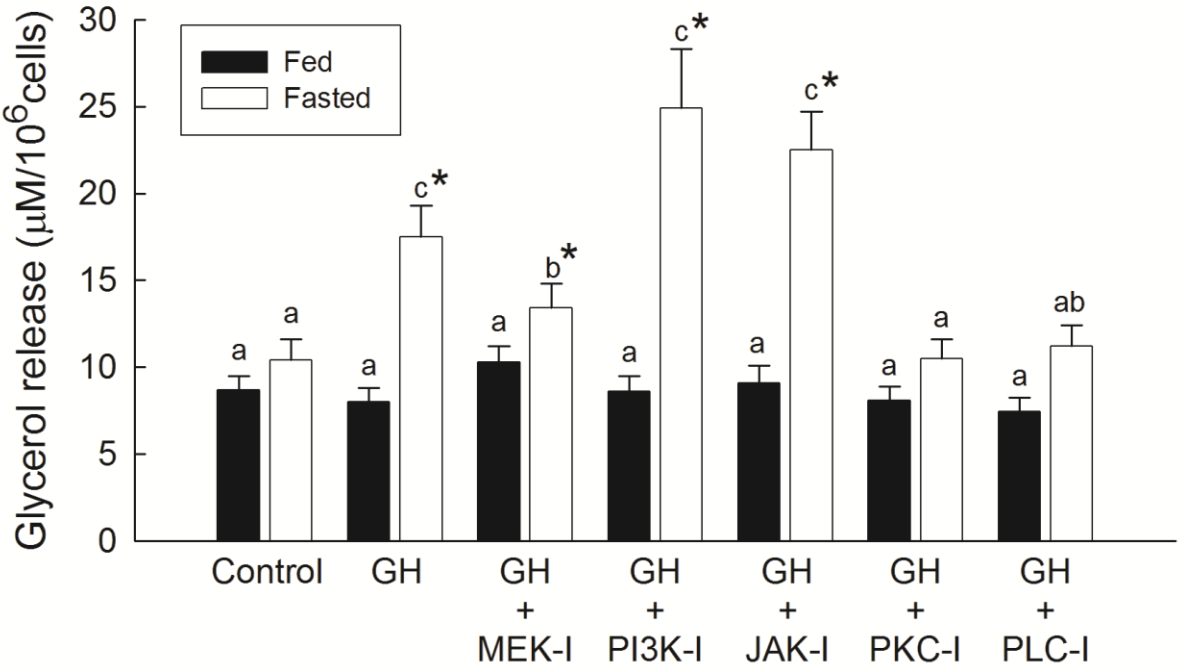

Figure 20. Effects of signaling element blockade on growth hormone (GH)-stimulated lipolysis as measured by glycerol release from hepatocytes isolated from rainbow trout fed continuously or fasted for 4 weeks. Cells were pretreated for 2 h with or without specific inhibitors for the following signaling elements: MEK (10 μM U0126), PI3K (10 μM LY294002), JAK (10 μM Hex.), PKC (10 μM chelerythrine chloride), PLC (10 μM 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 ± 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.

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. 21). 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 form 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.

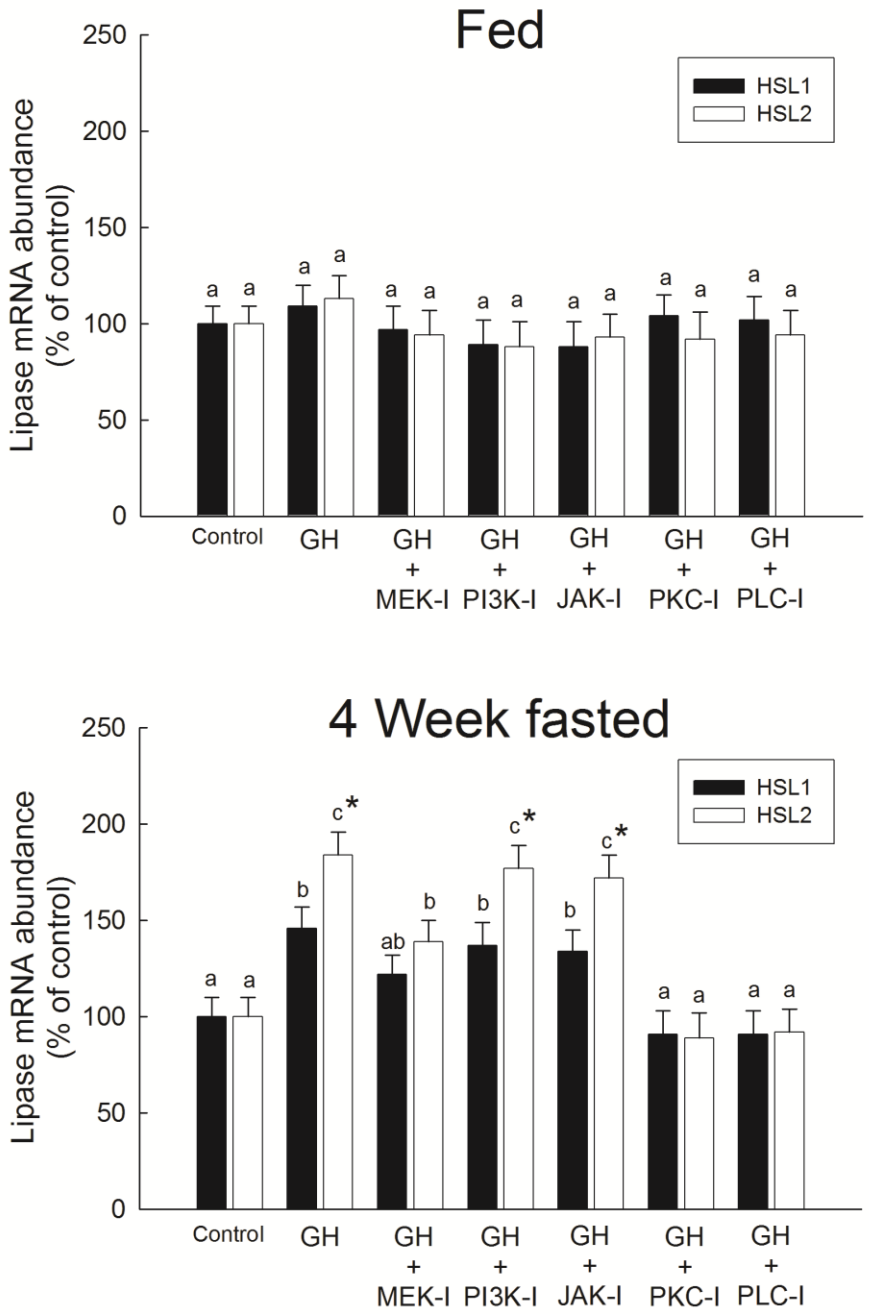

Figure 21. Effects of signaling element blockade on growth hormone (GH)-stimulated expression of hormone-sensitive lipase (HSL) 1- and HSL2-encoding mRNAs in hepatocytes isolated from rainbow trout fed continuously (A) or fasted for 4 weeks (B). Cells were pretreated for 2 h with or without specific inhibitors for the following signaling elements: MEK (10 μM U0126), PI3K (10 μM LY294002), JAK (10 μM Hex.), PKC (10 μM chelerythrine chloride), PLC (10 μM 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 ± 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.

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 fasted 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 or PI3K had no effect on GH-stimulated lipolysis in cells from fasted fish. Moreover, the basal phosphorylation state of JAK, STAT, and Akt was lower in cells from fated 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 (*SF1*, *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. 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 the position of Akt at the nexus of anti-lipolysis/lipogenesis and lipolysis, the presence (or absence) of factors that influence Akt activation, such as insulin 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 insulin and IGF-1 (cf. Norbeck et al., 2007) and, consequently, reduced phosphor-Akt abundance, shifts the balance toward GH-stimulated lipolysis; whereas, under positive nutrient conditions, the presence of insulin and IGF-1 and, consequently, increased abundance of phosphor-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 insulin 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. On a more general level, the notion of specific GH-signal pathways 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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CHAPTER 5. NUTRITIONAL STATE OF SERUM, INSULIN, AND IGF-1 INFLUENCE LIPOLYTIC ACTIONS AND GH PATHWAY LINKAGES IN HEPATOCYTES

Abstract

Growth hormone (GH) regulates several processes in vertebrates, including growth and the mobilization of stored lipids. In the first study described here, we used hepatocytes isolated from long-term fasted and well fed rainbow trout (*Oncorhynchus mykiss*) and attempted to reprogram their metabolic response to GH by pretreating the cells with 10% serum from the blood of fed or fasted fish. Serum did alter the cellular responses. When cells were pretreated with fasted serum, HSL mRNA expression slightly increased and glycerol presence in the media decreased compared to fed serum-treated cells. Under no serum conditions did GH significantly affect lipolytic measures. Conversely, when hepatocytes were pretreated with fed serum, GH activated JAK2, STAT5, Akt, and ERK pathways; while when cells were pretreated with fasted serum, GH activated PKC and ERK. In the second study described here, we again used hepatocytes isolated from fed and fasted trout but looked at the influence of the nutritionally-regulated serum factors insulin and insulin-like growth factor (IGF-1) on GH-stimulated lipolysis and signal transduction. GH could only stimulate HSL mRNA expression in cells from fasted fish. Pretreatment with insulin and/or IGF-1 abolished this lipolytic response to GH. GH triggered a drop in medium glycerol presence in fed cell samples. Insulin and/or IGF-1 augmented GH activation of JAK2 and STAT5 in cells from fed and fasted fish. However, insulin and/or IGF-1 eliminated the ability of GH to activate PKC and ERK from fasted cells. These results suggest that insulin and IGF-1 in the serum programs hepatocytes in accordance with the nutritional state of the organism. These findings also support that GH activates disparate

signaling mechanisms, depending upon its pre-programming via nutritional state, to mediate the divergent metabolic actions of GH.

Introduction

The balance of growth and metabolism is a responsibility of the endocrine system and involves several factors of the hypothalamic-pituitary axis and pancreas that target the liver. Growth is facilitated through the growth hormone (GH)-stimulated expression and release of insulin-like growth factor (IGF-1) from the liver, while insulin and glucagon act on the liver to mediate major anabolic and catabolic activities, respectively (Mommsen, 1998; Huang et al., 2014). The major environmental influence on these factors is nutritional state. In well-fed conditions, GH stimulates hepatic IGF-1 production and release through JAK-STAT, PI3K/Akt, and ERK signaling pathway activation (Reindl et al., 2011.) Moreover, insulin peaks in circulation after a meal to facilitate blood glucose and amino acid uptake into hepatocytes through a receptor tyrosine kinase and downstream activation of PI3K, SHP2, fyn, and several adaptors (SOCS1 and 3) (Mommsen et al., 1991; White, 2003). All together, these cellular events lead to tissue and organismal growth.

By contrast, fasted vertebrates from humans to fish experience diminished circulating insulin and IGF-1 despite increased plasma GH (Salgin et al., 2012; Norbeck et al., 2007; Pierce et al., 2005). Growth is suspended and energy stores are mobilized to fuel metabolic functions. Interestingly, GH also plays a stimulatory role in catabolism through the activation of lipid breakdown and mobilization (lipolysis) (Sos et al., 2011; Sheridan, 1994; O'Connor et al., 1993; Albalat et al., 2005). And we have demonstrated previously that these lipolytic actions of GH are mediated by hormone-sensitive lipase (HSL) through activated ERK and PKC/PLC pathways in fasted trout (Bergan et al., 2013; Bergan et al., in press).

HSL is a major lipolytic enzyme that breaks down stored lipids (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). Expression along with post-translational phosphorylation is necessary to activate HSL to hydrolyze triacylglycerols into glycerol and free fatty acids (Watt and Spriet, 2010; Bergan et al., 2013).

Salmonids are advantageous for studying the balance of growth and metabolism because their livers are major sites for both functions, albeit during different nutritional states. Recently, we described how the liver is sensitive to GH stimulation under both fed and fasted conditions, although producing dramatically different physiological responses. When well fed, hepatocytes from rainbow trout responded to exogenous GH treatment by increasing IGF-1 production through JAK2, STAT5, Akt, and ERK signaling pathway activation *in vitro* (Reindl et al., 2011; Walock et al., in preparation). By contrast, cells from fasted trout responded to GH lipolytically, via activation of ERK and PKC signaling pathways, but was unable to effect IGF-1 expression *in vitro* (Bergan et al, in press; Walock et al., in preparation).

From this work, we suggest that hepatocytes are programmed by the nutritional environment to respond appropriately to GH by linking to different intracellular signaling pathways that lead to divergent metabolic effects. To test this, we used hepatocytes isolated from continuously fed and long-term (4 week) fasted rainbow trout (*Oncorhynchus mykiss*) immersed in blood serum or serum factors insulin and IGF-1 to investigate the conditions that enable the diverse metabolic actions of GH and the associated mechanisms used to do so. Our hypothesize was that the nutritional status of the blood serum, with low levels of insulin and IGF in the fasted

state and high levels in the fed state, programs cells to respond appropriately to GH by stimulating lipolytic actions or inhibiting them, respectfully.

Materials and methods

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, PKCα/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). Salmonid GH was generously provided by Prof. Akiyoshi Takahashi and Dr. Shunsuke Moriyama (Kitasato University, Japan).

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 fed twice daily to satiety with AquaMax Grower (PMI Nutrition International, Brentwood, MO, USA) semi-floating trout grower, until commencing experiments where fish were fasted continuously for 4 weeks or fed for the same time until 2 hours before experimental manipulations. Animals were acclimated to laboratory conditions for at least 4 weeks prior to experimentation. 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. In 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 and stored at -80° C for later analysis. In 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 $MgSO_4$, 0.4 KH_2PO_4, 0.34 Na_2HPO_4, 4.2 $NaHCO_3$, 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% O_2. 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 $CaCl_2$) to a final concentration of 2.3 $x10^6$ cells/ml and aliquoted into 24-well plates (2.3 $x10^6$ cells/well).

For the first experiment, cells were incubated in serum from fed or fasted fish with or without GH under the same conditions as those used for recovery (14°C with gyratory shaking at 100 rpm under 100% O_2). Concentrations of 10% serum and 100 ng/ml of GH were used. For the second experiment, cells were incubated in insulin, IGF-1, or insulin and IGF-1 with or without GH. Concentrations of 100 ng/ml for insulin, 200 ng/ml for IGF-1, and 100 ng/ml of GH were used. For all experiments, serum, insulin, or IGF treatments lasted for 24 h then another 30 min (for Western blot analysis) or 6 h (for mRNA expression and glycerol analysis) 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 and medium samples were immediately frozen on dry ice then stored at -80°C until further analysis.

Hormone-sensitive lipase mRNA expression.

Total RNA was extracted using RNAzol-Reagent® (Molecular Research Center, Inc., Cincinnati, OH, 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 (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 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 and HSL2 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 HSL1 or HSL2 and β-actin. 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.

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 (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 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).

Glycerol analysis

Media 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 K_2CO_3, 0.205 M $KHCO_3$, pH 10.0, 0.65 M $(NH_4)_2SO_4$), NAD^+ (10 mM in Buffer A), and glycerol dehydrogenase [7 units/ml in buffer B (4.76 mM KH_2PO_4,

4.76 mM K_2HPO_4, pH 7.6, 1.4 μM $MnCl_2$, 0.9 μM $(NH_4)_2SO_4$)], in a 4:2:1:1 ratio, respectively, in a total volume of 200 ul. Mixtures were incubated at 25°C for 1 h, after which time the absorbency was read at 340 nm.

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 for ease of comparison and are expressed as means ± S.E.M.

Results

Serum reprograms hepatocytes to alter lipolysis and GH pathway activation

In an attempt to reprogram cells, and specifically their response to GH, we took hepatocytes from one nutritional state (e.g., fed) and treated them with serum of the opposing nutritional state (e.g., fasted), and *vise versa*. We examined glycerol abundance in the culture medium and HSL mRNA expression. Rainbow trout have two HSL-encoding mRNAs, HSL1 and HSL2 (Kittilson et al., 2011); both forms were detected in all samples and treatments. Upon activation through phosphorylation, HSL hydrolyzes the fatty acid chains from the glycerol backbone of triacyglycerides, and the products are subsequently released from the cell into culture medium (Bergan et al., 2013; Harmon et al., 1991). Together, HSL mRNAs and glycerol release are used as indications of lipolytic action.

Fed fish that were pretreated with fed serum were designated as control (fed-fed control); as expected, GH failed to elicit a lipolytic response (Fig. 22A, 23A) in these samples. By

contrast, when fed cells were incubated with serum from fasted fish, HSL2 from no-GH samples and both isoforms of HSL in GH-treated cells experienced increased mRNA expression compared to fed-fed control with HSL2 mRNA being expressed to a greater extent than HSL1 mRNA. Moreover, GH elicited a slight but not significant increase in HSL mRNA expression compared to non-GH treated fed cell-fasted serum samples.

Additionally, we isolated hepatocytes from fish fasted for 4 weeks then treated with serum from other fish fasted for 4 week or continuously fed. When fasted cells were treated with fasted serum, GH stimulated increased HSL mRNA expression compared to fed-fed controls although non-GH samples did not (Fig. 22B). Interestingly, when fasted cells were treated with fed serum, the response to GH was abolished with both non-GH and GH-treated cells experiencing HSL mRNA expression similar to the fed-fed control.

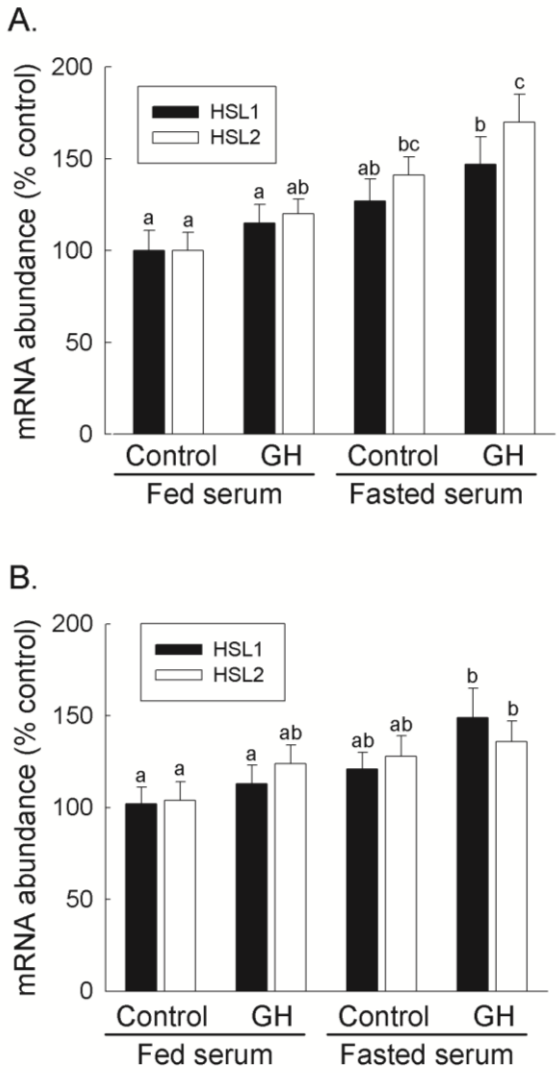

Figure 22. Effects of serum and growth hormone (GH) treatment on expression of hormone-sensitive lipase (HSL) 1- and HSL2-encoding mRNAs from isolated hepatocytes of rainbow trout (A) fed continuously or (B) fasted for 4 weeks. Cells were treated for 24 hours with 10% serum harvested from the blood plasma 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 + SEM (n = 6). Groups with different letters are significantly ($p < 0.05$) different.

Glycerol abundance in culture medium was affected by serum treatment but not GH (Fig. 23). When cells, from both fed and fasted fish, were treated with fed serum medium glycerol abundance was significantly higher than cells treated with fasted serum. Under no circumstances did GH affect medium glycerol abundance.

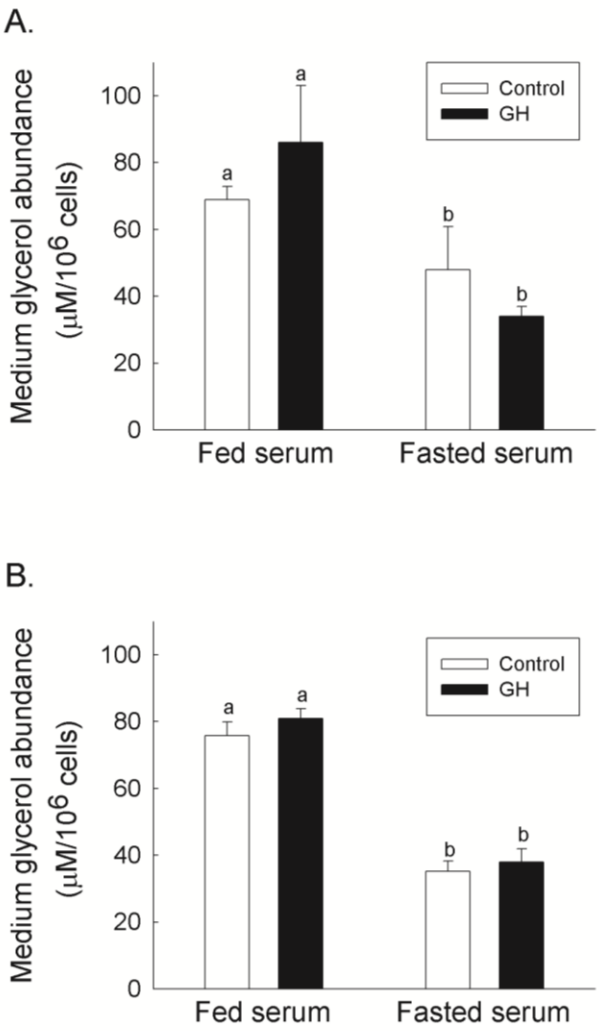

Figure 23. Effects of serum and growth hormone (GH) on medium glycerol abundance from isolated hepatocytes of rainbow trout (A) fed continuously or (B) fasted for 4 weeks. Cells were treated for 24 hours with 10% serum harvested from the blood plasma of fed or fasted fish then treated with or without 100 ng/ml of GH for an additional 6 hours. Data are presented as means ± S.E.M. (n=6). For a given nutritional state (fed or fasted), groups with different letters are significantly ($p < 0.05$) different.

Taken together, these results indicate that serum affects lipolytic ends. However, the ability of serum to alter GH responsiveness does not seem to be evident, and more work needs to be done to understand the circumstances which GH can impart disparate metabolic effects.

In addition to measuring lipolytic actions, we investigated how serum treatment would influence signal transduction in response to GH. Activated (i.e., phosphorylated) forms of 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) were detected in lysates of hepatocytes from all treatments. In fed cells treated with fed serum (fed-fed control), GH increased phosphorylation of JAK2, STAT5, Akt, and ERK but not PKC (Fig. 24A, 24C, 24E, 24G, 24I). However, when either cell type (fed or fasted) was treated with fasted serum, GH-stimulated phosphorylation of JAK2, STAT5, and Akt was abolished; in fact, both non-GH and GH-treated cells experienced diminished JAK2, STAT5, and Akt phosphorylation to levels significantly below fed-fed controls in both fed and fasted cells that where treated with fasted serum (Fig. 24A-24F). By contrast, GH augmented ERK and PKC phosphorylation in both fed and fasted cells treated with fasted serum above fed-fed controls and non-GH samples (Fig. 24G-24J). Taken together, these results indicate that serum reprograms cells to respond to GH according to the nutritional state of the serum and not that of the cell type.

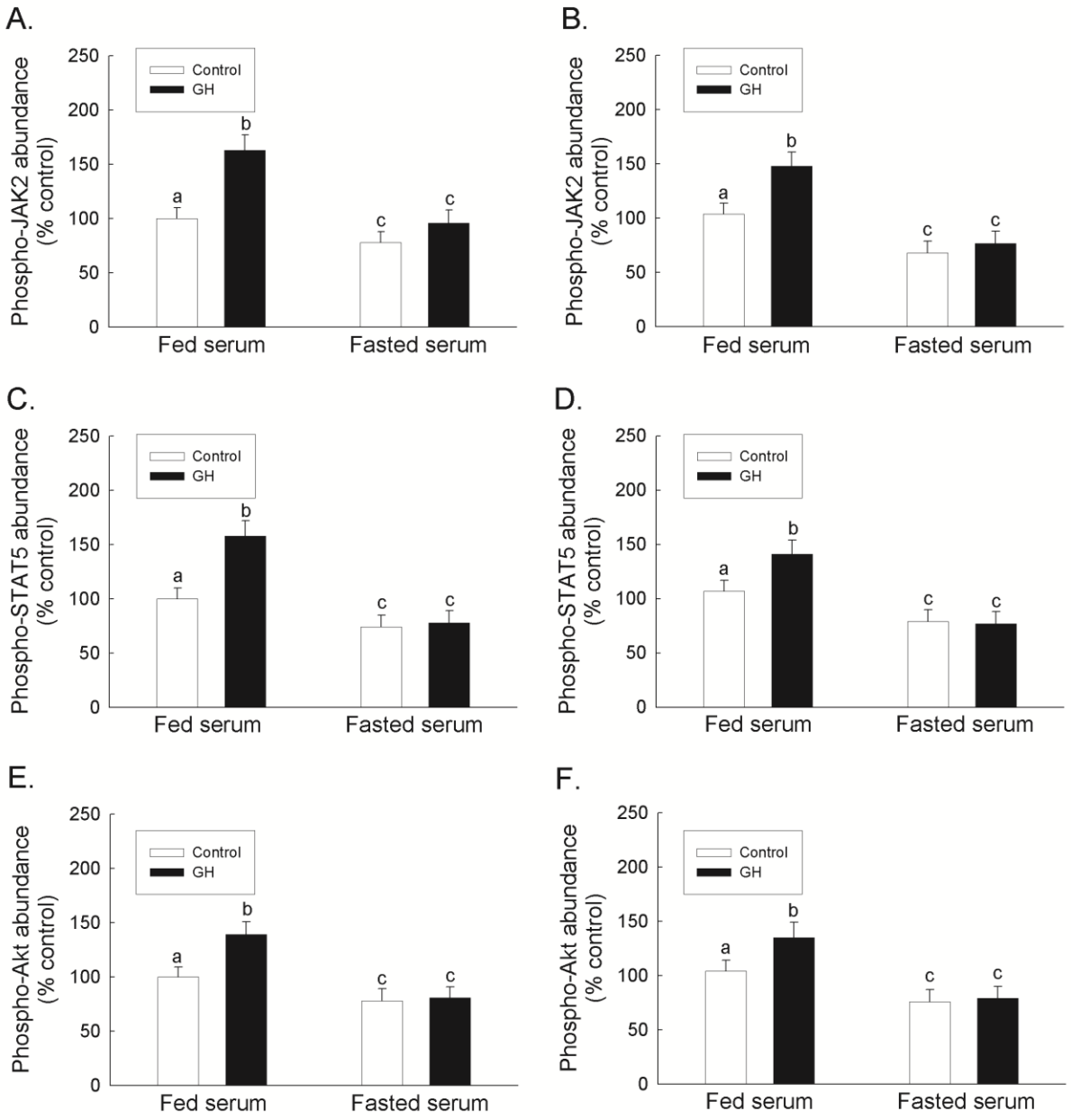

Figure 24. Effects of serum and growth hormone (GH) on the abundance of phosphorylated Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), protein kinase B (Akt), extracellular signal-regulated kinase (ERK), and protein kinase C (PKC) in isolated hepatocytes of rainbow 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 PKCα/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 + SEM (n = 6). Groups with different letters are significantly ($p < 0.05$) different.

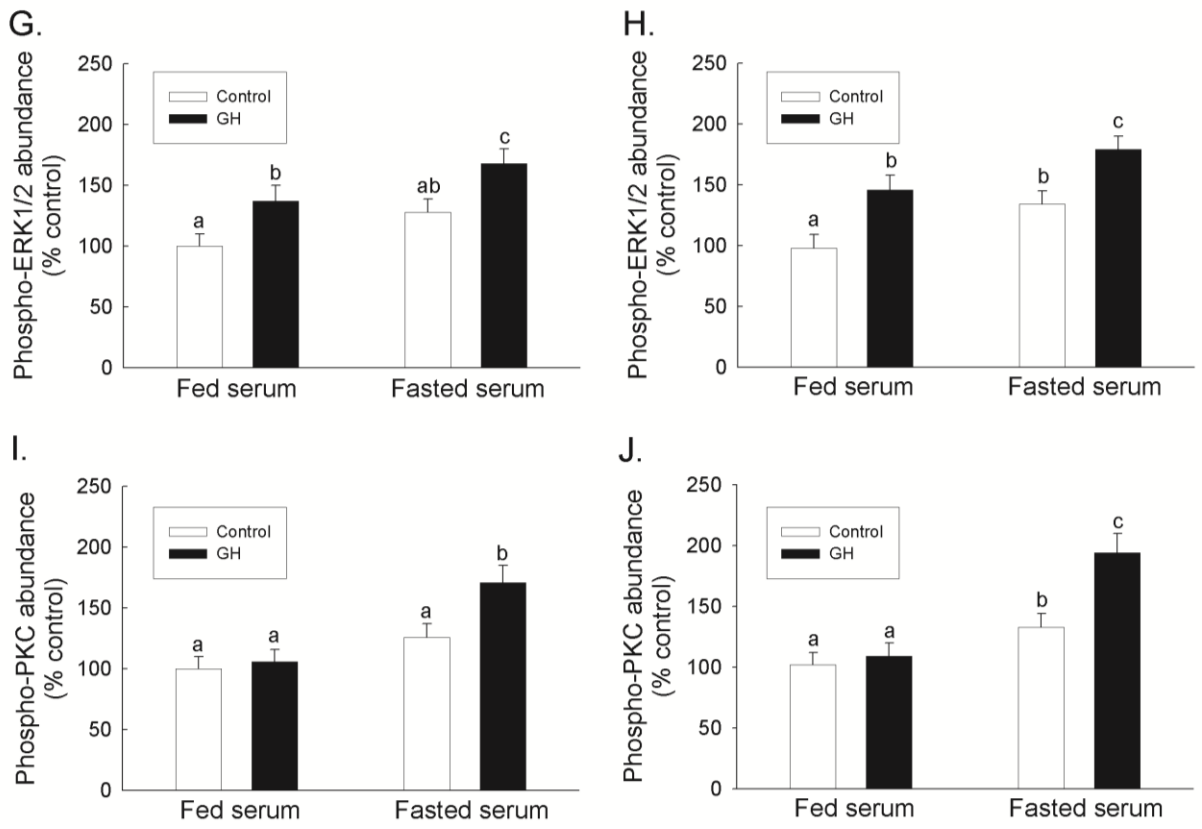

Figure 24. Effects of serum and growth hormone (GH) on the abundance of phosphorylated Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), protein kinase B (Akt), extracellular signal-regulated kinase (ERK), and protein kinase C (PKC) in isolated hepatocytes of rainbow trout fed continuously (A, C, E, G, I) or fasted for 4 weeks (B, D, F, H, J) (continued). 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 PKCα/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 + SEM (n = 6). Groups with different letters are significantly ($p < 0.05$) different.

Insulin and IGF-1 reprogram hepatocytes to alter lipolysis and GH pathway activation

To understand what elements in the serum may be responsible for effecting lipolytic actions in hepatocytes, we repeated our previous experiments but with insulin and IGF-1 in place of serum. Insulin and IGF-1 are hormones that are tightly regulated by the nutritional state of the organism. Insulin and IGF-1 peak postprandially but are diminished in fasted vertebrates

(Norbeck et al., 2007; Shimizu et al., 2009; Stone, 2003; Caruso and Sheridan, 2011). Because of this, we looked to mimic fed and fasted serum, using only these two factors, and investigate how insulin and IGF-1could influence lipolysis and GH responsiveness.

Specifically, we took cells from fish fasted 4 weeks or fed continuously and treated them with or without insulin, IGF-1, and/or GH. In fed cells, no treatment had an effect on HSL mRNA expression (Fig. 25A). Basal HSL mRNA expression was slightly higher, although not significant, in fasted over fed cells. Among fasted cells, GH stimulated HSL mRNA expression above that of control (Fig. 25B). However, when insulin and/or IGF-1 were pretreated with fasted cells, GH failed to have an effect on HSL mRNA expression.

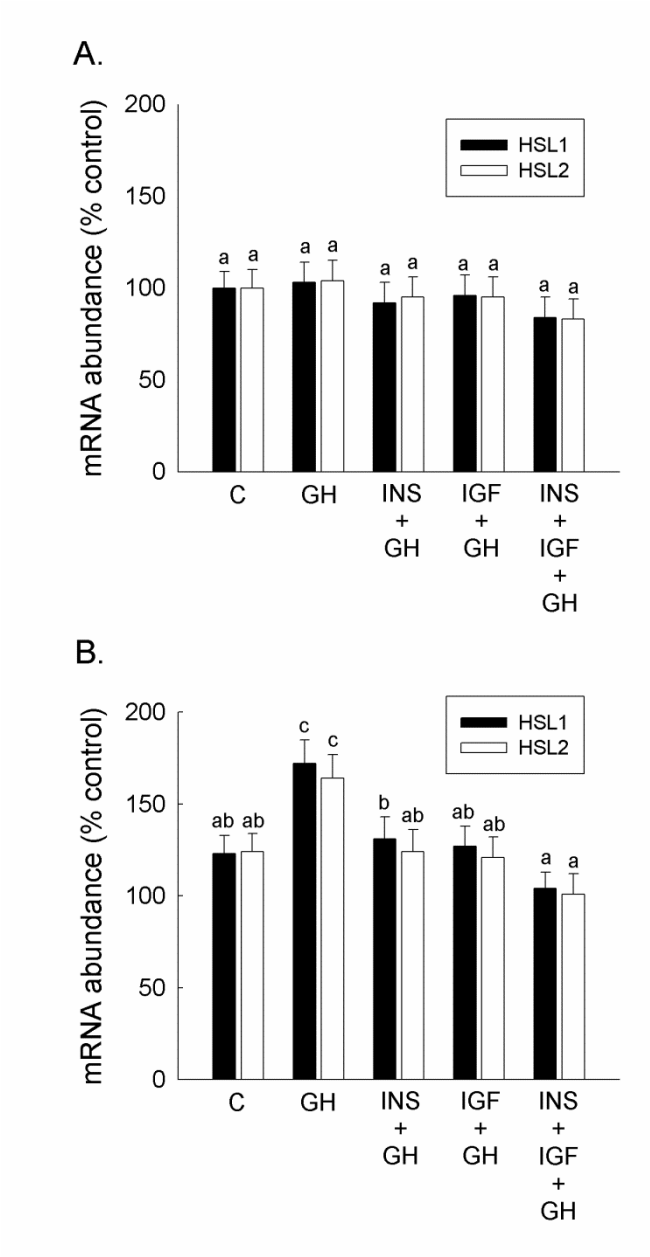

Figure 25. Effects of insulin (INS), insulin-like growth factor (IGF-1), and growth hormone (GH) treatment on expression of hormone-sensitive lipase (HSL) 1- and HSL2-encoding mRNAs from isolated hepatocytes of rainbow trout (A) fed continuously or (B) fasted for 4 weeks. Cells were treated for 24 hours 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 cells-fed and expressed as mean + SEM (n = 6). Groups with different letters are significantly ($p < 0.05$) different.

Medium glycerol abundance was affected by GH treatment. In fed cells, GH significantly decreased medium glycerol abundance (Fig. 26A). Samples pretreated with insulin and/or IGF-1 experienced reduced to medium glycerol abundance similar to GH-only treated. IGF-1 with insulin pretreatment followed by GH treatment led to slightly reduced medium glycerol abundance although statistically not significant. In fasted cells, no treatment caused a significant change in medium glycerol content (Fig. 26B). However, GH did cause a slight, although not significant, increase in medium glycerol concentration over basal levels when combined with insulin and IGF-1. Taken together, these results indicate that the presents of insulin and IGF-1 can affect cellular responsiveness to GH.

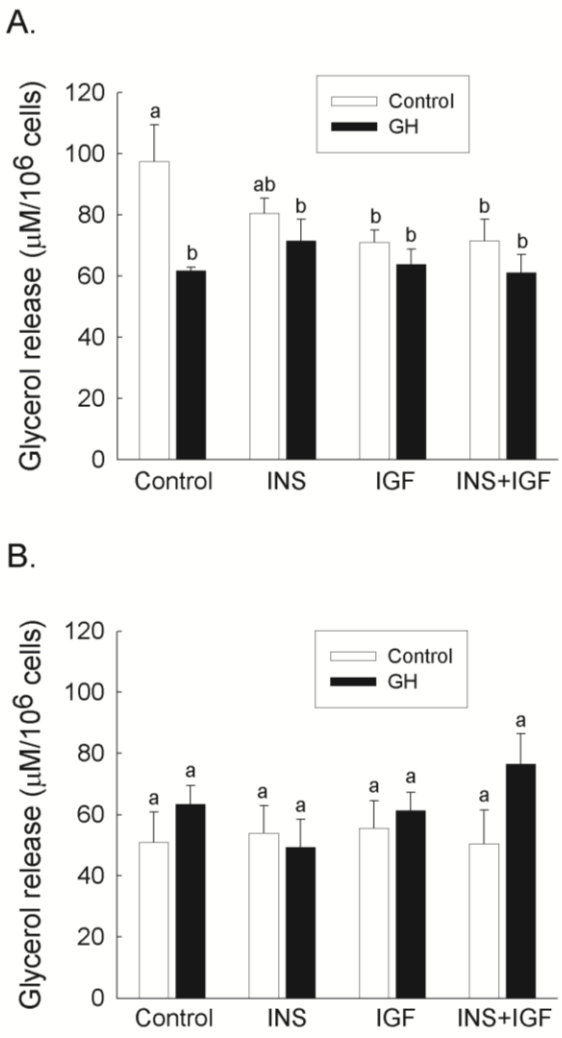

Figure 26. Effects of insulin (INS), insulin-like growth factor (IGF-1), and growth hormone (GH) on medium glycerol abundance from isolated hepatocytes of rainbow (A) trout fed continuously or (B) fasted for 4 weeks. Cells were treated for 24 hours with or without 100 ng/ml INS and 200 ng/ml IGF-1 then treated with or without 100 ng/ml of GH for an additional 6 hours. Data are presented as means ± S.E.M. (n=6). For a given nutritional state (fed or fasted), groups with different letters are significantly ($p < 0.05$) different.

Lastly, we investigated how insulin and IGF-1 pretreatment would impact GH-stimulated signaling. In fed cells, JAK2, STAT5, and ERK but not PKC were activated by GH (Fig. 27A, 27C, 27E, 27G); additionally, pretreatment of fed and fasted cells with insulin and/or IGF-1 augmented GH-stimulated phosphorylation of JAK2 and STAT5. GH alone stimulated activation of ERK and PKC in fasted cells (Fig. 27F and 27H). However, GH-stimulated activation of ERK and PKC was abolished when cells were pretreated with insulin and IGF-1. Taken together, these results indicate that insulin and IGF-1 influence GH activation of pathway elements in hepatocytes.

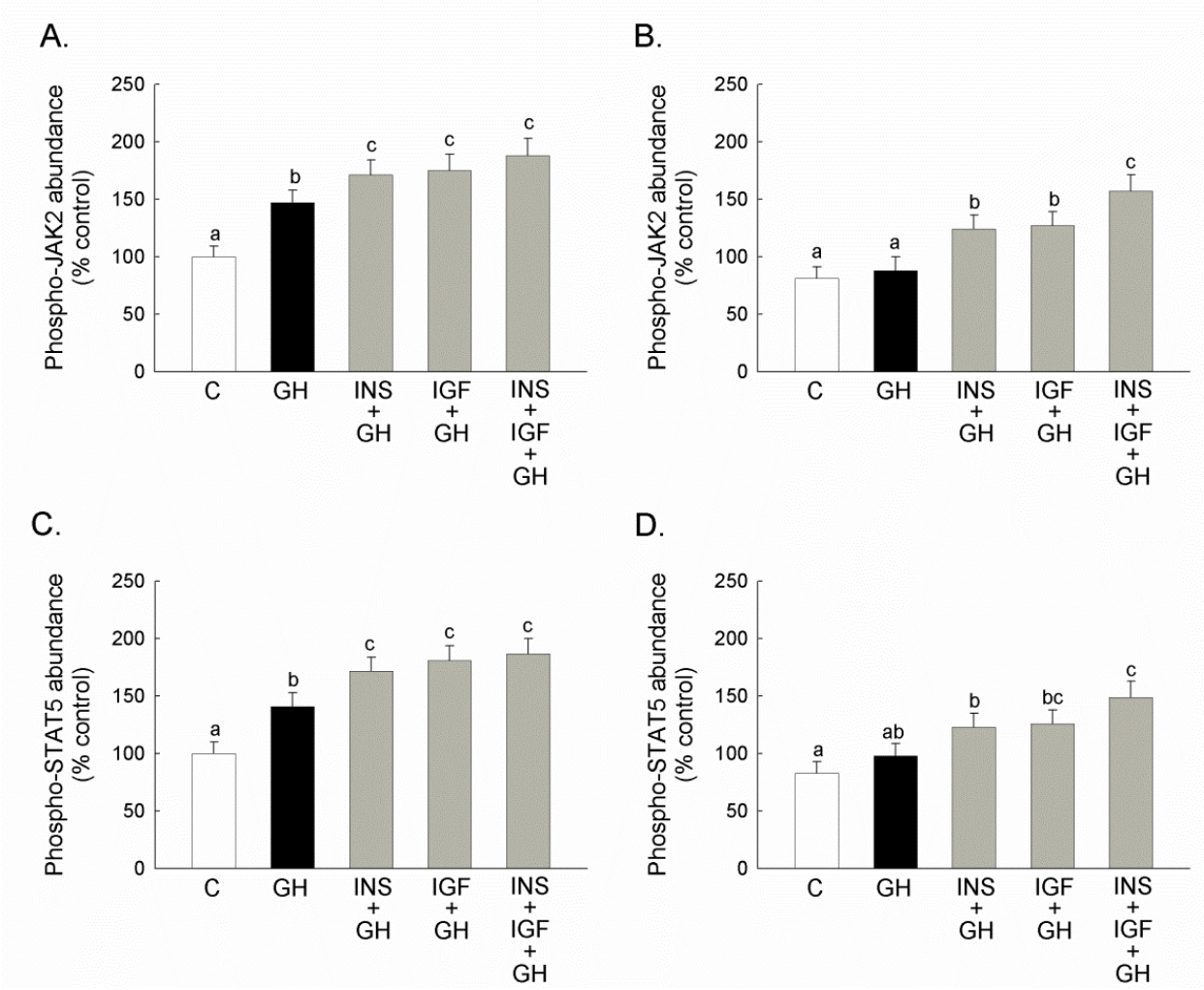

Figure 27. Effects of insulin (INS), insulin-like growth factor (IGF-1), and growth hormone (GH) on the abundance of phosphorylated Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), extracellular signal-regulated kinase (ERK), and protein kinase C (PKC) in isolated hepatocytes of rainbow trout (A, C, E, G) fed continuously or (B, D, F, H) fasted for 4 weeks. Cells were treated 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 PKCα/β II was normalized to total JAK2, STAT5, ERK1/2, and β-actin respectively. Data are presented as % control and expressed as mean + SEM (n = 6). Groups with different letters are significantly ($p < 0.05$) different.

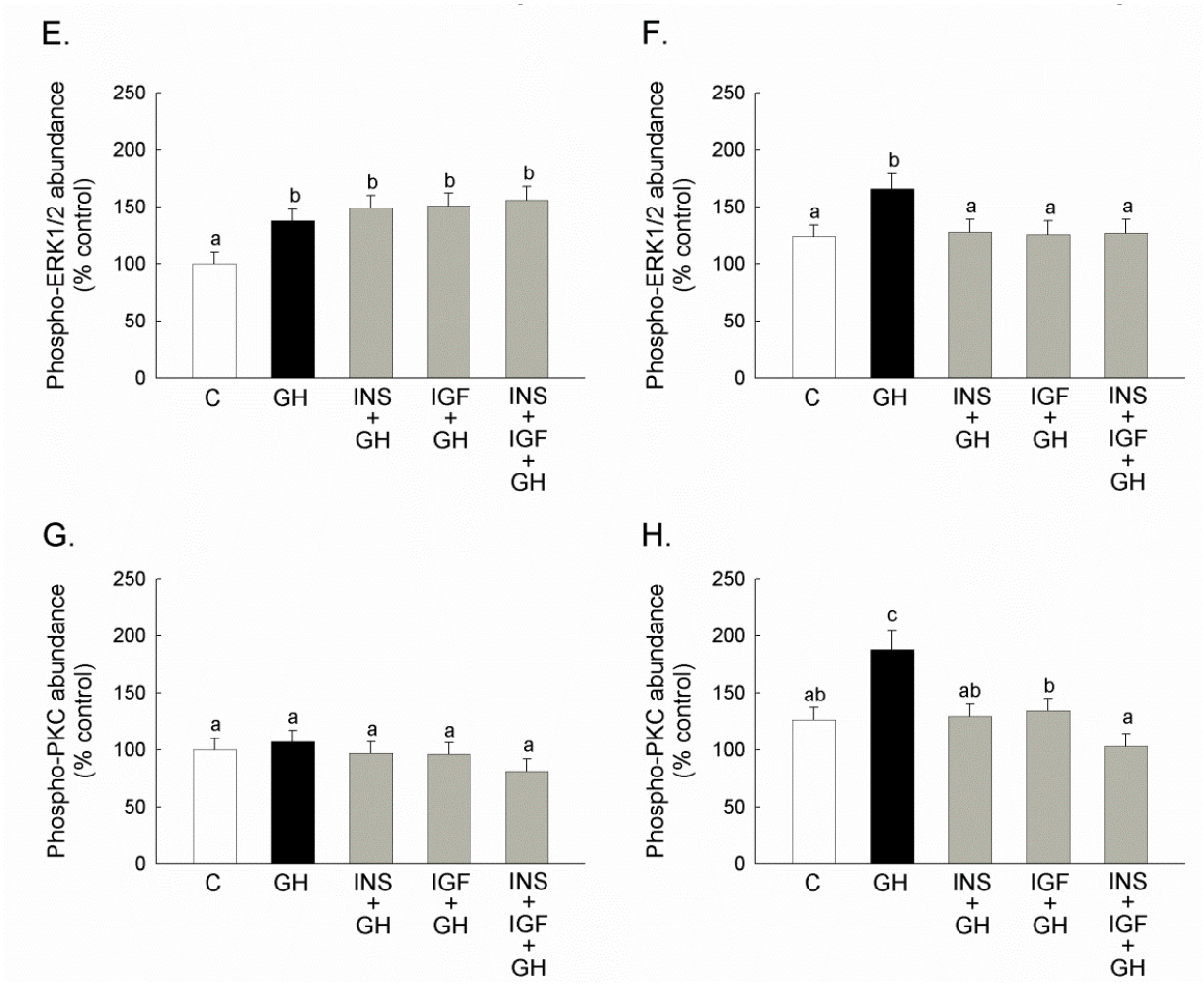

Figure 27. Effects of insulin (INS), insulin-like growth factor (IGF-1), and growth hormone (GH) on the abundance of phosphorylated Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), extracellular signal-regulated kinase (ERK), and protein kinase C (PKC) in isolated hepatocytes of rainbow trout (A, C, E, G) fed continuously or (B, D, F, H) fasted for 4 weeks (continued). Cells were treated 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 PKCα/β II was normalized to total JAK2, STAT5, ERK1/2, and β-actin respectively. Data are presented as % control and expressed as mean + SEM (n = 6). Groups with different letters are significantly ($p < 0.05$) different.

Discussion

The results of this study indicate that nutritional programming alters lipolytic actions and GH-activated signaling in hepatocytes from rainbow trout. Treatment of cells with serum from

fasting fish augmented HSL mRNA expression but reduced medium glycerol abundance. GH stimulated HSL mRNA expression in hepatocytes from fasted fish but this effect was knocked down by pretreatment with insulin and/or IGF. In cells from fed fish, GH demonstrated antilipolysis as measured by reduced medium glycerol abundance; however, GH did not have an effect on medium glycerol abundance in cells from fasted fish. Additionally, GH activated JAK2, STAT5, Akt, and ERK under fed conditions but ERK and PKC under fasted conditions. Therefore, when hepatocytes are pretreated with disparate nutritional environments, GH has proximal (intracellular signaling and mRNA expression) and functional (glycerol release) effects. These findings continue to provide insight into the mechanistic basis of the pleiotropic actions of GH, specifically the metabolic actions tied to nutritional state.

Blood serum programs cellular responsiveness. This conclusion is supported by two current observations. First, treatment of hepatocytes with serum from fasting fish augmented HSL mRNA expression slightly 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 fed controls. Similar results have been seen by us in rainbow trout (Bergan et al., in press; Bergan et al., 2013; Bergan et al., 2012) and others in the livers of mice (Yang et al., 2013) although we are the first to attempt to reprogram cellular responsiveness with serum treatments. Moreover, little work has been done on the expression of lipolytic gene in non-mammalian and non-adipocyte tissues possibly due to a lack of characterization of these genes (Kittilson et al., 2011).

Second, fasted serum treatment decreased medium glycerol abundance in both cell types (fed and fasted). Although this second observation was the opposite of what we expected, we suspect it was due to 1) the serum collected from rainbow trout used to treat isolated hepatocytes

already had glycerol in it (Magnoni et al., 2008) and 2) hepatocytes could take up glycerol in order to fuel gluconeogenesis. Although fasting can cause an increase in circulating glycerol (Sakharova et al., 2008; Leatherlands and Nuti, 1981) we suspect that prolonged fasting may shift hepatic energy production from lipid-based to glucose-centered in order to maintain blood glucose levels for the brain, skeletal muscle, cardiac muscle, and other tissues that primarily rely on glucose for energy. This idea is supported by work in carnivorous fish showing that that blood glucose levels are maintained with prolonged fasting (Costas et al., 2011) and gluconeogenic enzymes increase their activity under fasting conditions (Kirchner et al., 2008). Furthermore, fasted fish treated with fasting serum and GH failed to elicit increase medium culture glycerol abundance. We suspect that the fasted cells may have been depleted of lipid reserves; therefore GH stimulation could not induce a lipolytic response when not substrates were available. Regardless of the mechanism of these unexpected glycerol results, these data still provide evidence that blood serum can reprogram cells despite the state through which the cells were isolated.

Insulin and IGF-1 may be key molecules that trigger cellular reprogramming according to nutritional state. Both hormones are closely regulated by nutritional state. Insulin and IGF-1 peak postprandially but are diminished by fasted (Norbeck et al., 2007; Shimizu et la., 2009; Stone, 2003; Caruso and Sheridan, 2011). We hypothesized that lipolysis stimulated by GH under fasting conditions (no insulin and IGF-1) would be would be blocked by insulin and/or IGF-1 treatment because they would be a fed signal. This hypothesis was supported by the HSL mRNA expression data where no change in expression was seen in cells from fed fish. This is consistent with previous reports (Bergan et al., in press). In hepatocytes from fasted fish, HSL mRNA

expression was indeed augmented by GH treatment; this effect was inhibited by insulin and/or IGF-1 treatment.

By contrast, the medium glycerol abundance was unaffected by insulin, IGF-1, and GH, in fasted cells. Interestingly, in fed cells, GH stimulated a decrease in medium glycerol abundance. GH-stimulated antilipolysis has been documented before (Carrel and Allen, 2000). Moreover, this is logical because GH would be stimulating growth promotion under fed conditions. GH continued to stimulate antilipolysis in cells pretreated with insulin and/or IGF-1 but not to a significant degree. This is reasonable as others have shown that although acute GH treatment triggers lipolysis, chronic GH, like those seen during extended fasting, is directly related to increased hepatic gluconeogenesis (Yuen et al., 2013).

Previously, we and other have demonstrated the ability of GH to stimulate lipolysis as measured by enhanced HSL mRNA expression and glycerol release from cells to culture medium (Chavez et al., 2006; Gorin et al., 1990; O'Connor et al., 1993; Bergan et al., 2013; Bergan et al., in press; Albalat et al., 2005; Sheridan, 1994). We suspect the reasons why GH failed to have a strong down-stream effect on HSL mRNA expression and certainly medium glycerol may have been due to competing programming. That is, 4 weeks of continuous feeding or fasting may not have been able to be completely reversed by 24 hours of serum or insulin/IGF treatment. It is possible that residual programming still lingered despite acute serum and hormone treatments and thus prevented GH from eliciting lipolytic responses.

Additional differences in reports may be due to differences in tissue, species, life history, developmental stage, diet, and timing of sampling. Specifically, the work done in this chapter was conducted with fish of dramatically smaller size (ca. 80 g) compared to previous chapters (ca. 130 g). Moreover fish utilized here were also younger (less than 1 year versus as old as 2

years). This considerable difference in age and size may have implications on the transferability of this work to other studies. Nevertheless this work is the first of its kind in fish and should be replicated in other species to identify if this reprogramming is a trend or an exception.

Nutritional state dictates to which signaling pathways GH links. Under fed conditions, GH activates JAK2, STAT5, Akt, and ERK. Evidence for this is provided here by GH stimulating 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). Furthermore, insulin and/or IGF-1 pretreatment augmented GH-stimulated increases in phospho-JAK2 and STAT5 in both cell types (fed and fasted).

Under fasting conditions, GH links to ERK and PKC. This is evident with our current observations that when cells were treated 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 treated with serum from fed fish. These results are consistent with what we have seen earlier (Bergan et al., 2013; Bergan et al., in press). Moreover, GH-stimulated phosphorylation of PKC was also seen in hepatocytes isolated from fasted fish. However, this actions was eliminated when insulin and/or IGF-1 was administered to these cells prior to GH treatment.

Insulin and IGF-1 reorganizes GH-stimulated pathway activation to reflect a fed-like state. This conclusion is supported by several lines of evidence. As mentioned from this study, we demonstrated that cells were programmed according to serum treatment and not the nutritional environment from which the cells came. This also is case with insulin and IGF treatment. For example, GH-stimulated JAK2 and STAT5 activation that was seen in cells

treated with fed serum was also observed in cells (from both fed and fasted origins) that were treated with insulin and/or IGF-1. GH stimulation of JAK/STAT pathways has been reported previously and is associated with the fed state and growth-promotion (Walock et al., in preparation; Reindl et al., 2011). Furthermore, insulin and IGF-1 peak postprandially but diminish when fasting (Norbeck et al., 2007; Shimizu et la., 2009; Stone, 2003; Caruso and Sheridan, 2011). And others have demonstrated that during low doses of insulin (like those experienced during fasting) GH stimulated JAK2 phosphorylation, whereas during high doses of insulin (like those during a fed state) GH inhibited JAK2 phosphorylation in an immortal hepatoma cell line (Leung et al., 2004; Ji et al., 1999; from Birzniece et al., 2009).

Likewise, GH-stimulated PKC phosphorylation that was mediated by fasting serum was abolished when cells were pretreated with insulin and/or IGF. Previously**,** PKC also was shown to mediate GH-stimulated lipolysis by us and others (Bergan et al., 2013; Gorin et al., 1990). However, we are the first attempt to alter lipolytic responsiveness of cells from serum of hormone treatments.

ERK shows a unique pattern. ERK is stimulated by GH in both a fed and fasted state, as shown here and previously (Bergan et al., in press; Bergan et al., 2013; Walock et al., in preparation; Reindl et al., 2011). Interestingly, when we treated fed cells, GH-stimulated ERK phosphorylation was maintained by insulin and/or IGF pretreatment. Yet when we treated fasted cells, GH-stimulated ERK phosphorylation was reduced by insulin and/or IGF pretreatment to control levels. We speculate that this may be due to residual cellular programming.

Insulin serving as a fed signal to alter signaling and inhibit lipolysis has been reported previously. In regard to classically stimulated lipolysis, insulin inhibits catecholamine-stimulated lipolysis by blocking signal transduction from adrenergic receptors through PKCβI (Nakamura et

al., 2007). Additionally, insulin can work through PI3K/Akt to degrade cAMP and therefore block PKA activation, a key molecule for HSL protein phosphorylation (González-Yanes and Sánchez-Margalet, 2006). Additionally, in ewes, fasting-stimulated lipolysis was attenuated by insulin to control levels (Schneider et al., 2011).

IGF also plays an important role to alter cells to a fed state. In fact, IGF-1R was required for GH to stimulate STAT5 activation in osteoblasts by inhibiting protein tyrosine phosphatase 1B (PTP1B) (Gan et al., 2013). From this work, it is reasonable to suggest that during well-fed states, when IGF is in abundant circulation, IGFR signaling inhibits PTP repression of GH/JAK/STAT signaling, while in fasting conditions, when plasma IGF is low, PTPs are free to inhibit GH-stimulated JAK/STAT signaling. GH-stimulated JAK/STAT is the main pathway in which GH exerts its growth-promoting actions (Martinez et al., 2013). By inhibiting that pathway, GH-stimulated signaling can shift from anabolic growth promotion to catabolism, specifically lipolysis.

Previously it has been thought that the liver becomes GH-resistant under fasting conditions, as levels of IGF-1 drop despite increasing plasma GH concentrations. However, we argue that hepatocytes are still sensitive to GH but disconnected from the growth-promoting and redirected to lipolytic pathways. Here, we have shown for the first time that serum programs cells to respond according to the nutritional state of the serum and not that of the cell type. Moreover, serum and its elements, insulin and IGF, influence to which signaling pathways GH links. We suspect that different isoforms of GH receptors (GHR) may connect differentially to downstream pathways to mediate the pleiotropic actions of GH but have yet to perfect the techniques to investigate this hypothesis. Work needs to be done on how GHR subtypes may play a role in mediating the pleiotropic actions of GH.

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CHAPTER 6. GENERAL CONCLUSIONS

Growth hormone (GH) is an important regulator of growth, metabolism, reproduction, immunity, osmoregulation, and other physiological functions of vertebrates. The mechanisms which modulate multifarious GH functionality have yet to be understood. We have focused our work on the lipolytic functions of GH and the nutritional influence that mediates the lipolytic responsiveness to GH in rainbow trout. We have shown that GH directly stimulates lipolysis by modulating gene expression and protein activation, and that fasting programs cells to respond lipolytically to GH by utilizing intracellular signaling cascades that are different from well-fed models where GH functions to promote growth.

Fasting alone impacts lipolytic measures in several tissues of rainbow trout. Fasting for either 2 or 4 weeks significantly retarded growth in terms of body weight, body length, and body condition; refeeding restored growth such that body length and body condition were similar to measures seen in continuously fed fish. Fasting activated lipid catabolism by stimulating the mRNA expression and catalytic activity of HSL in adipose tissue, liver, red and white muscle. Refeeding reversed both fasting-associated HSL mRNA expression and HSL activity. Fasting resulted in the deactivation of Akt, JAK2, and STAT5 in adipose tissue, liver, and red and white muscle. Whereas fasting activated ERK and PKC in all tissues measured. Refeeding reversed fasting-associated alterations in the activation state of all signal elements. These findings suggest that deactivation of Akt and JAK-STAT in conjunction with activation of ERK and PKC underlie fasting-associated growth retardation and lipolysis.

GH is responsible for mediating the lipolytic response, *in vitro*, in hepatocytes isolated from rainbow trout fasted for 7 days. GH stimulated lipolysis as measured by increased glycerol release in both a time- and concentration-related manner. Lipolysis was accompanied by GH-

stimulated phosphorylation of HSL. GH-stimulated lipolysis also was manifested by increased expression of the two HSL-encoding mRNAs. Moreover, similar to the fasting effects on signal transduction pathways, GH resulted in the activation of PLC/PKC and the MEK/ERK pathways, whereas JAK-STAT and the PI3K-Akt pathway were deactivated. Blockade of PLC/PKC and of the MEK/ERK pathway inhibited GH-stimulated lipolysis and GH-stimulated phosphorylation of HSL as well as GH-stimulated HSL mRNA expression, whereas blockade of JAK-STAT or of the PI3K-Akt pathway had no effect on the activation of lipolysis or HSL expression stimulated by GH. These results indicate that GH promotes lipolysis by activating HSL and by enhancing the *de novo* expression of HSL mRNAs via activation of PKC and ERK. These findings also suggest molecular mechanisms for activating the lipid catabolic actions of GH while simultaneously deactivating anabolic processes such as anti-lipolysis and the growth promoting actions of GH.

Nutritional state in directs the metabolic actions of GH. GH stimulated lipolysis, as measured by increased glycerol release in both a time- and concentration-related manner, in cells of fasted fish but not from cells of fed fish. Expression of HSL mRNAs was also stimulated by GH in cells from fasted fish and not in cells from fed fish. Moreover, 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. that nutritional state modulates the lipolytic responsiveness of cells by adjusting the signal transduction pathways to which GH links.

Nutritional state, insulin, and insulin-like growth factor I (IGF-I) pretreatment affect lipolytic actions in hepatocyte. When in a fed state, with high levels of insulin and IGF, GH links to JAK-STAT pathways to promote antilipolysis as measured by unaffected HSL mRNA expression and decreased medium glycerol abundance. In a fasted state, with low levels of insulin and IGF, GH links to lipolysis through PKC and ERK activation as measured by HSL mRNA expression; this action was blocked by insulin and IGF-1 pretreatment. These results indicate that nutritional state regulates GH responsiveness through nutritionally-regulated factors in blood serum, specifically insulin and IGF-1, despite the disconnect between proximate signaling activation and distal expression and functional activity regulation. These findings also support that GH activates disparate signaling mechanisms, depending upon its pre-programming via nutritional state, to mediate the divergent metabolic actions of GH.

Based on the collective results of this dissertation, GH stimulates lipolysis in the liver by triggering HSL activation and *de novo* synthesis through activation of PKC/PLC and MAPK/ERK pathways. These effects are only seen when in a fasted state, and therefore GH action is regulated by nutritional programming, possibly through circulating insulin and IGF-1 (cf. Fig. 28). This is plausible given the position of Akt at the nexus of anti-lipolysis/lipogenesis and lipolysis (cf. Fig. 28), and the known activation of Akt by insulin and IGF-1 (Caruso and Sheridan, 2011). The results in this dissertation support that that under nutrient-limiting conditions that the observed deficiency of insulin 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 insulin and IGF-1 and, consequently, increased abundance of phospho-Akt, the balance is shifted toward the anabolism, including the insulin-like anti-lipolytic (Carrel and Allen, 2000) actions of GH.

Variability within the results described here and among other literature may be due to variations in species, developmental stage, time of year of sampling, size, diet, and life histories. Moreover, although the liver is a unique model to study the divergent metabolic actions of GH because of the localization of both responses (lipolysis and growth-promotion), its wide range of metabolic activities may have hindered our ability to gain a clear picture of the environmental influences that may delineate the lipolytic actions of GH.

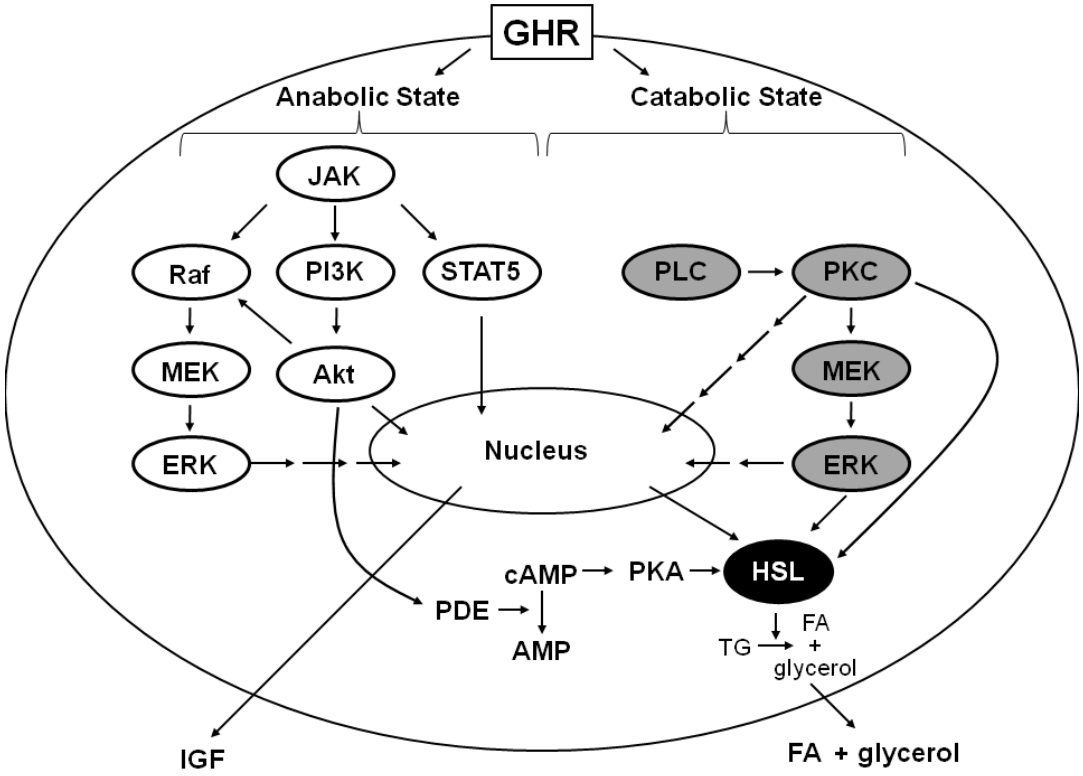

Figure 28. Model of the modulation of growth hormone (GH)-signal pathway linkages and the activation of growth-promoting or lipid mobilizing (lipolysis) responses by nutritional state. When animals are in an anabolic state (e.g., during periods of feeding), GH initially activates JAK2 which, in turn, 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.

Future studies

The multifarious activities of GH may be mediated by differential GHR subtype regulation. Multiple GHR subtypes exist in fish (Perez-Sanchez et al., 2002; Walock et al., 2014) and mammals (Talamantes and Ortiz, 2002), but limited work has been done to understand the unique roles GHR subtypes may have in mediating GH action. Some correlative work has been done on the nutritional regulation of GHR subtypes (Reindl and Sheridan, 2012); however, this area of research need to continue in order to develop clearer patterns of how GHR subtype expression, functional expression, internalization, and degradation are affected by nutritional state and therefore mediate GH action.

Moreover, controlled experiments isolating a single subtype would help determine individual roles. We suspect, in organisms with multiple GHR subtypes, one GHR is more responsible for transmitting the lipolytic signal of GH, while a different GHR subtype is more active in transmitting the growth-promoting signal of GH. It is possible that one GHR mediates *de novo* lipase expression while another GHR subtypes directs lipase and/or perilipin activation. Additionally, with three know GHR subtypes in rainbow trout, it is possible that a single GHR may not be solely responsible for a set of physiological responses; instead, a certain combination of GHR subtypes would mediate lipolysis or growth promotion.

We have already shown that GH differentially activates disparate signaling pathways when stimulating growth (Norbeck et al., 2007; Reindl et al., 2011; Walock et al., in preparation) and lipolysis (Bergan et al., 2012; Bergan et al., 2013; Bergan et al., in press;). However, more work needs to be done to directly link the different GHR subtypes to unique signaling pathways and the mechanisms leading to disparate physiologies. Overall, work should continue on the mechanisms of nutritional regulation of GH signaling, especially that concerning GHRs.

These data focused on a unique model for both GH-stimulated growth promotion and lipolysis, the salmonid liver. However, the actions of GH on lipolysis impact several tissues, and GH control is directly sourced in the brain. Therefore, the effects of nutritional programming should be examined among more tissues. Additionally, ATGL and perilipins have yet to be characterized in many vertebrates, including rainbow trout. Other studies have shown that HSL, ATLG, and perilipins are differentially regulated and do not respond uniformly. Therefore, work needs to be done to understand every piece of the lipolysis puzzle in order to understand lipolysis as a whole and specifically the regulatory actions of GH on lipolysis.

Summary

Our understanding of the complex mechanisms by which GH modulates its diverse metabolic effects is still rudimentary. By furthering our understanding, we can contribute to many fields including health and aquaculture. In regard to adult human health, GH is not needed for organismal growth; however, GH has been linked to obesity and insulin resistance. Obesity results in the reduction of GH secretion (Rasmussen et al., 2010) thus limiting the ability of GH to stimulate lipolysis, creating a dangerous cycle of fat gain. Short-term fasting can cause augmented GH secretion but only in healthy-weight individuals (Hartman et al., 1992). Only exercise, weight loss, and surgical intervention can restore GH secretion to normal levels (Rasmussen et al., 2010; Thomas et al., 2013). Here, we have argued the mechanisms by which GH stimulates lipolysis and the nutritional environment required to do so. Understanding these mechanisms may help provide pharmacological targets and therapies to combat obesity.

In GH deficient adults, increased fat accumulation can be remedied by exogenous GH treatment (Shadid and Jensen, 2003; Chaves et al., 2013). However, excess and deficient GH and can lead to insulin resistance. It has been proposed that GH uncouples IRS-activated PI3K from

Akt as a mechanism of GH-induced insulin sensitivity (Yuen et al., 2013). With our work on the cellular programming of GH responsiveness, we hope to have provided some insight on how we might treat, manage, predict, and/or prevent diseases like obesity and insulin resistance; however, more work needs to be done to understand these dynamic processes.

Beyond human health, our work in rainbow trout provides additional benefits. The aquacultural benefits of understanding the balance of growth and metabolism can be used to feed the world. Additionally, our fish model provides a unique opportunity to address broad issues in the field of chemical mediation: mechanistic basis of multifunctionality and functional significance of multiple genes. Furthermore, salmonids co-localize growth and metabolic regulation in the liver, which provides a unique opportunity to study how the two functions coordinate with one another.

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