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Nutritional state modulates growth hormone-stimulated lipolysis

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ABSTRACT

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

1. Introduction

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

During periods when food is not available, energy is diverted away from growth to sustain metabolic processes. In rainbow trout, for example, animals cease growing compared to their fed counter parts and mobilize stored lipid and carbohydrate and activate gluconeogenesis; with prolonged fasting such mobilization is reflected in animals as reduced condition and reduced body weight (Norbeck et al., 2007; Sheridan and Mommsen, 1991). Accompanying the catabolic shift is an alteration in the endocrine profile, including depression of plasma levels of insulin and IGF-1 (Caruso and Sheridan, 2011; Norbeck et al., 2007; Sheridan and Mommsen, 1991); however, despite cessation of growth, plasma levels of GH increase, a pattern that is seen in fish as well as other

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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 weeks) fasted rainbow trout (*Oncorhynchus mykiss*) as a model to investigate the mechanistic basis of the anabolic and catabolic actions of GH. Our hypothesis was that nutritional state modulates the lipolytic responsiveness of cells by adjusting the signal transduction pathways to which GH links. The rationale for this work extends from our previous observations in rainbow trout that selected signaling pathways are activated during periods of feeding (e.g., JAK–STAT, PI3K–Akt), whereas a different complement of signaling pathways are activated during periods of fasting (e.g., ERK, PKC) (Bergan et al., 2012) and that hepatic lipolysis results from GH activation of PLC/PKC and ERK (Bergan et al., 2013).

2. Materials and methods

2.1. Materials

All chemicals and reagents were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise. Antibodies for the phospho-specific and total (recognizing both phosphorylated and nonphosphorylated protein) forms of Akt, ERK1/2, JAK2, 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 phosphoinostide-dependent kinase 1 (PDK1)], and cell lysis buffer were all obtained from Cell Signaling Technology (Beverly, MA, USA). The JAK2 inhibitor, 1,2,3,4,5,6-hexabromocyclohexane (Hex), the broad spectrum protein kinase C (PKC) inhibitor, chelerythrine chloride, and the broad spectrum phospholipase C inhibitor, U73122, were obtained from EMD Chemicals (Gibbstown, NJ, USA). Molecular weight markers were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Salmonid GH was generously provided by Prof. Akiyoshi Takahashi and Dr. Shunsuke Moriyama (Kitasato University, Japan).

2.2. Experimental animals and conditions

Juvenile rainbow trout of both sexes (*ca.* 1 year of age) were obtained from Dakota Trout Ranch (Carrington, ND). The animals were transported to North Dakota State University, where they were maintained in well-aerated, 800-L circular tanks supplied with recirculated (10% make-up volume per day) dechlorinated municipal water at 14 °C under a 12:12 h 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 2 h 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% and 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 $\times$ 10^6 cells/ml, and aliquoted into 24-well plates (6–8 $\times$ 10^6 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. In all cases, cells in replicate treatments came from different fish.

After treatment, cells were pelleted (1000×*g* for 4 min) and the supernatant medium was removed. Cells were washed with 0.5 ml phosphate-buffered saline. Cell pellets and medium samples were immediately frozen on dry ice then stored at −80 °C until further analysis.

2.3. Hormone-sensitive lipase mRNA expression

Total RNA was extracted using TRI-Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA) as specified by the manufacturer's protocol. Each RNA pellet was redissolved in 35–200 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