Endocrine and orexigenic actions of growth hormone secretagogues in rainbow trout (Oncorhynchus mykiss)

Brian S. Shepherd a,⁎, Jaime K. Johnson b, Jeffrey T. Silverstein a, Ishwar S. Parhar c, Mathilakath M. Vijayan d, Alison McGuire d, Gregory M. Weber a

a USDA/ARS, National Center for Cool and Coldwater Aquaculture, Kearneysville, WV 25430, USA
b Department of Biology, University of Kentucky, Lexington, KY 40506-0225, USA
c Department of Physiology, Nippon Medical School, Tokyo 113, Japan
d Department of Biology, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

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Abstract

The effects of growth hormone secretagogues (GHSs) on the teleost somatotropic axis are poorly understood, particularly with respect to insulin-like growth factor-I (IGF-I) and the IGF-binding proteins (IGFBPs). To assess the endocrine and orexigenic responses of rainbow trout (Oncorhynchus mykiss) to GHS treatment, animals were injected with human GHRH1-29-amide, KP-102 or rat ghrelin at 0, 1 or 10 pmol/g body mass. Feed intake was tested at 2 and 5 h post-injection and plasma levels of growth hormone (GH), IGF-I and the IGFBPs were determined at 3, 6 and 12 h post-injection. Feed intake was significantly elevated by all of the GHSs tested at both post-injection time points. All GHSs elevated plasma GH levels in a time-dependent manner. Plasma IGF-I levels were elevated by all GHSs at 3 h post-injection, whereas those animals treated with KP-102 and ghrelin exhibited depressions at 6 h. Four IGFBPs were identified in the plasma by western blotting. Levels of the 20 kDa IGFBP decreased over the sampling time. Levels of the 32 kDa IGFBP were significantly depressed by all GHSs tested. Levels of the 42 kDa IGFBP were significantly elevated by all GHSs tested. Plasma levels of the 50 kDa IGFBP was decreased in some treatment groups at 3 h, but elevated by 6 h in the ghrelin-treated groups and elevated in all treatment groups by 12 h post-injection. The endocrine and orexigenic responses demonstrate that GHSs influence the teleost neuroendocrine system beyond short-term actions (<3 h post-injection) on GH release and the responses of the IGFBPs to GHS treatment support this notion and clarify their identification as functional homologues to mammalian IGFBPs.

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1. Introduction

The vertebrate somatotropic axis is principally comprised of growth hormone (GH), its anabolic intermediaries, the insulin-like growth factors-I and -II (IGF-I and -II), and their high-affinity binding proteins (insulin-like growth factor binding proteins: IGFBPs) and receptors. The somatotropic axis also consists of additional ligands (e.g., GH variants) as well as the GH receptor(s) and binding protein (Goffin et al., 1996; Rajaram et al., 1997; Butler and Le Roith, 2001).

Growth hormone is secreted by the vertebrate anterior pituitary, and its secretion is principally under the control of neuroendocrine factors (e.g., growth hormone-, gonadotropin- and thyrotropin-releasing hormones, pituitary adenylate cyclase-activating polypeptide, somatostatin and dopamine, to name a few) (Harvey, 1995; Peng and Peter, 1997; Wong et al., 2000). In addition to these well-established regulatory factors, synthetic Met-enkephalin derivatives (hexapeptides), or growth hormone releasing peptides (GHRPs, e.g., KP-102 and hexarelin), have also been shown to stimulate GH release and growth in mammals (Bowers, 1998; Camanni et al., 1998). The GHRPs...
have been shown to exert their actions via a G protein-coupled orphan receptor (GHS-R: growth hormone secretagogue receptor) which is expressed in the pituitary and hypothalamus and is distinct from the GHRH receptor (Korbonits et al., 2004; Ghigo et al., 2005). The recent cloning of GHS-R (Howard et al., 1996) enabled Kojima et al. (1999) to isolate a novel 28-amino acid residue peptide, termed ghrelin, from stomach extracts. In this landmark study, they showed that ghrelin stimulated GH release in vitro and in vivo, via the GHS-R, demonstrating that it is an endogenous ligand for this receptor.

Recent studies have shown that GHRPs and ghrelin stimulate GH release in teleost fishes (Unniappan and Peter, 2005). However, teleost ghrelin(s) have been shown to stimulate prolactin (PRL) release in tilapia (Kaiya et al., 2003b,c), but not in rainbow trout (Kaiya et al., 2003a), and luteinizing hormone (LH) release in the goldfish (Unniappan and Peter, 2004). By contrast, recent work conducted in juvenile grass carp (Xiao et al., 2002) found that GHRH and dopamine, but not GHRP-6 or hexarelin, were effective at elevating GH release in vitro and in vivo. Collectively, this range of findings suggests the presence of species-specific and developmental-specific responses to various secretagogues in teleosts and the need for further studies in this area. While GHRPs and ghrelin stimulate GH secretion and appetite in teleosts, there has been no comprehensive effort to characterize their effects on the somatotropic axis (GH, IGF-I and the IGFBPs). Against this background we have examined the in vivo effects of human GHRH, rat ghrelin and GHRP-2 (KP-102) in the rainbow trout. The aims of this study were 1) to assess whether growth hormone secretagogues (GHSs: GHRH, GHRP-2 and ghrelin), which stimulate GH release via different receptors (Korbonits et al., 2004; Unniappan and Peter, 2005), similarly affect the somatotropic axis in the rainbow trout, and 2) to ascertain whether any differences exist between the endocrine and orexigenic effects of these GHSs.

2. Materials and methods

2.1. Animal husbandry and experimental design

Juvenile rainbow trout (Oncorhynchus mykiss) (body mass 148±2.1 g; 20.0±0.9 cm fork length) were hatched and reared at the National Center for Cool and Coldwater Aquaculture (USDA/ARS) in Kearneysville, WV, USA. The fish were individually identified using PIT (passive integrated transponder) tags (American Veterinary Identification Devices, Norco, CA) implanted at least a month prior to the initiation of the study. Several weeks before the study, animals (n=27/tank) were randomly assigned to 1 of 15 tanks (200 L) equipped with fresh, flow-through, well water at 13.5±0.3 °C, and a 12L:12D photoperiod. The study was composed of two side-by-side experiments designed to 1) assess the effects of GHS treatment on feed intake (Experiment 1) at 2 and 5 h post-injection, and 2) to determine the effects of GHSs on endocrine parameters (Experiment 2) at 3, 6 and 12 h post-injection. The tanks were blocked by sampling time (Expt. 1: 2 and 5 h post-injection and Expt. 2: 3, 6 and 12 h post injection), with each block consisting of 3 replicate tanks. This design permitted for complete randomization of animals across 3 replicate tanks within a single sampling time (block). For Experiment 1, the time-course for feed intake evaluation was based upon reports (Markert et al., 1977; Johnsson and Björnsson, 1994; Vega-Rubin de Celis et al., 2004) demonstrating long-term effects of GH on feed intake as well as the desire to assess the short-term effects of GHSs on feed intake, but keeping within the overall experimental time-course used for Experiment 2. For Experiment 2, the time-course for sampling (3, 6 and 12 h post-injection) was chosen based upon work showing elevated GH levels (6 and 12 h post-injection) in tilapia treated with GHRH and KP-102 (Shepherd et al., 2000) or native ghrelin(s) (Riley et al., 2005) as well as the need for measuring GH-dependent IGF-I and -BP production which typically occurs within 3–24 h following GH treatment. All work was done in accordance with approved animal care protocols with the University of Kentucky and the National Center for Cool and Coldwater Aquaculture/ARS/USDA.

2.2. Injection of GH secretagogues

The various GH secretagogues were weighed out and dissolved in sterile-filtered vehicle (0.75% saline with 0.1% bovine serum albumin) to a final concentration of 1 and 10 pmol/μL. Rainbow trout were anesthetized with 3-amino-benzoic acid ethyl ester (MS-222, 100 mg/liter, Sigma chemical, St. Louis, MO), weighed, and injected (intraperitoneal) with either sterile-filtered vehicle, or vehicle containing the following GH secretagogues: 1) KP-102 (GHRP) (Kaken Pharmaceuticals, Tokyo, Japan), 2) human GHRH1-29-amide (Sigma-Aldrich Chemical, St. Louis, MO, USA), and 3) synthetic n-octanoyl rat ghrelin (Peptide Institute, Inc., Tokyo, Japan: Lot #510124). Injection volume was 1 μL/g body mass.

At the time of this study (October, 2003), teleost ghrelin(s) were not commercially available, therefore, rat ghrelin, which has been shown to be equally active as homologous ghrelin(s) in a number of teleosts (Riley et al., 2002; Kaiya et al., 2003a,b; Ran et al., 2004), was used in this study. Additionally, mammalian GHRH was used because it has been proven to be an effective and specific GH secretagogue in a number of teleost species (Melamed et al., 1995; Kelly and Kohler, 1996; Lescroart et al., 1996; Shepherd et al., 2000; Rousseau et al., 2001; Xiao et al., 2002; Drennon et al., 2003) and is also commercially available. Fish used for the injection and feed intake studies were fasted for 24 h prior to injection and throughout the study, unless indicated otherwise, and sampled at similar times.

2.3. Feed intake determination

Labeled and unlabeled control feeds (42% crude protein, 16% crude fat floating trout grower diet) were prepared at the National Fish Technology Center in Bozeman, MT, USA. For the labeled diet, leaded glass ballotini beads of 0.4 to 0.6 mm diameter (Sigmund Lindner GmBH, type H) were mixed into the feed at the rate of 1%, prior to pelleting. The unlabeled control diet replaced wheat flour for the beads. Prior to feeding the labeled diet, unlabeled feed was fed to the experimental groups for 1 week to insure that a change in feed would not bias
feed intake results. Labeled feed was distributed to animals, over a 1-h interval, starting at 2 and 5 h post injection. After feeding, the fish were left undisturbed for an additional hour and then netted, anesthetized and identified by PIT tag number for radiography and tissues collected at 4 and 7 h post-injection, however, we shall refer to these fish as the 2 and 5 h post-injection groups. Radiographs (9 fish/treatment within each time point) were taken at a distance of 60 cm with a Soeye SY-31-90P (Soeye, South Korea) X-ray unit (settings were 20 mA, 70 kV p for 0.1 s, the film used was Dupont/Sterling UVL 100 speed, with a detail screen). A standard curve for the number of radio-opaque particles per gram of feed was produced from radiographs of duplicate samples of 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 g of labeled feed. Radiographic images were digitized using the Alpha Innotech Corporation ChemiImager 4400 Low Light Imaging System (San Leandro, CA, USA) and radio-opaque particles were counted with the Metamorph image analysis program (version 6.0, Universal Imaging Corporation, Downingtown, PA, USA) and compared with the standard curve to estimate the mass of feed consumed.

2.4. Sampling

Prior to sampling, animals from each tank were anesthetized with MS-222 (100 mg/L), identified by PIT tag, weighed and length determined. Following these measurements, blood was collected from each animal via caudal puncture using a 3 mL heparinized syringe. For animals in Experiment 1, three fish (representing a single treatment group of \( n = 9 \)) from one of three replicate tanks were sampled for tissues and X-rayed to determine feed intake (see above) at 4 and 7 h post-injection and feeding. For animals used in Experiment 2, three fish (representing a single treatment group of \( n = 9 \)) from one of three replicate tanks were sampled for tissues and X-rayed to determine feed intake at 2 and 5 h post-injection and feeding. Prior to sampling, animals from each tank were anesthetized with MS-222 (100 mg/L), identified by PIT tag, weighed and length determined. Following these measurements, blood was collected from each animal via caudal puncture using a 3 mL heparinized syringe. For animals in Experiment 1, three fish (representing a single treatment group of \( n = 9 \)) from one of three replicate tanks were sampled for tissues and X-rayed to determine feed intake (see above) at 4 and 7 h post-injection and feeding. For animals used in Experiment 2, three fish (representing a single treatment group of \( n = 9 \)) from one of three replicate tanks were sampled for tissues and X-rayed to determine feed intake at 2 and 5 h post-injection and feeding.

2.5. Measurement of plasma GH and IGF-I in rainbow trout

Plasma growth hormone (GH) levels were determined using \(^{125}\)I-labeled recombinant salmon GH (rsGH: Lot # DAB-GHB1) (GroPep, S. Australia, Australia) for label, unlabeled rsGH as standards and anti-salmon GH primary antibody (Lot # AJ1-PAN1) (GroPep, S. Australia, Australia) as previously described (Shepherd et al., 2005). Inter- and intra-assay coefficients of variation were 7.0% and 7.6%, respectively. The detection limit of the assay, defined as the 90% binding, was 0.45±0.14 ng/mL. Samples were measured in duplicate.

Plasma IGF-I levels were determined according to the method of Shimizu et al. (2000), using \(^{125}\)I-labeled recombinant salmon IGF-I (Lot #: AJ1-PAN1) (GroPep) for label and standards and the anti-barramundi IGF-I primary antibody (Lot # DAA-PAF1) (GroPep). Inter- and intra-assay coefficients of variation were 5% and 4%, respectively. The detection limit of the assay, defined as the 90% binding, was 0.1±0.01 ng/mL. Samples were measured in triplicate.

2.6. Western ligand blotting using digoxigenin (DIG)-labeled IGF-I

Western ligand blotting, using DIG-recombinant human (rh) IGF-I (USB Corp., Cleveland, OH, USA), was done according to the method of Shimizu et al. (2000) as modified and validated by Johnson et al. (2003) and Shepherd et al. (2005) for rainbow trout. Samples were measured in duplicate. Plasma protein was determined by the bicinchoninic acid method using bovine serum albumin as standards.

2.7. Statistical analysis

Differences among groups were evaluated by two-way analysis of variance (ANOVA) with sampling time and secretagogue treatment as independent variables (Minitab version 12.1, State College, PA, USA). Where significant differences occurred \( (p < 0.05) \), comparisons between groups were performed using Fisher’s Least Significant Test (FPLSD) for predetermined pair-wise comparisons (Steele and Torrie, 1980) using the SPSS (Chicago, IL, USA, version 11.0.1) statistical package. Stated \( p \)-values for individual comparisons are general cut-off values.

3. Results

3.1. Feed consumption (Experiment 1)

There was a significant \( (p = 0.009, \text{two-way ANOVA}) \) effect of GHS treatment on feed intake in animals presented feed at 2 and 5 h post injection and sampled 2 h later (4 and 7 h post-injection) \( (\text{Fig. 1}) \). No significant \( (p > 0.05, \text{two-way ANOVA}) \) effect of sampling time on feed intake was noted. There was no significant \( (p > 0.05, \text{two-way ANOVA}) \) interaction of sampling time \( \times \) GHS treatment on feed intake. Feed intake was significantly elevated in all GHS-treated animals given food at 2 h \( (p < 0.05, \text{FPLSD}) \) and 5 h \( (p < 0.01, \text{FPLSD}) \) post injection, with exception of the ghrelin high dose group given food at 2 h post injection. Feed intake in the control groups was not significantly \( (p > 0.05, \text{FPLSD}) \) different \( (\text{Fig. 1}) \). Feed intake was not measured for animals in Experiment 2.

3.2. Plasma GH levels (Experiment 2)

There was a significant effect of time \( (p < 0.001, \text{two-way ANOVA}) \) and GHS treatment \( (p < 0.006, \text{two-way ANOVA}) \) on plasma GH levels in fish from this experiment \( (\text{Fig. 2}) \). Compared with their time-matched control groups, plasma GH levels were elevated in the GHRH high dose group at 3 h \( (p < 0.01, \text{FPLSD}) \) and 6 h \( (p < 0.05, \text{FPLSD}) \) post-injection, in the low dose KP-102 group at 6 h post-injection \( (p < 0.05, \text{FPLSD}) \) and the high dose KP-102 and ghrelin groups at 12 h.
post-injection \((p<0.001, \text{FPLSD})\). There was also a significant interaction of time × GHS treatment \((p<0.05, \text{two-way ANOVA})\) on plasma GH levels which tended to increase over time with the highest levels occurring 12 h after initial GHS injection. There were no significant \((p>0.05, \text{FPLSD})\) differences between plasma GH values in the control groups sampled at 3, 6 and 12 h post-injection. Plasma GH levels are not presented for animals from Experiment 1 (feeding study).

3.3. Plasma IGF levels (Experiment 2)

There was a significant effect of time \((p<0.001, \text{two-way ANOVA})\), but no effect \((p>0.05, \text{two-way ANOVA})\) of GHS treatment on plasma IGF-I levels (Fig. 3). However, there was a significant interaction of time × GHS treatment \((p=0.005, \text{two-way ANOVA})\) on plasma IGF-I levels. At 3 h post injection, plasma IGF-I levels were significantly elevated \((p<0.05, \text{FPLSD})\) in all GHS treatment groups compared with the 3 h control group. At 6 h post-injection, plasma IGF-I levels tended to decrease in all treatment groups, with significantly \((p<0.01, \text{FPLSD})\) lower levels occurring in those animals injected with KP-102 (both doses) or ghrelin at the low dose, compared with the 6 h control group (Fig. 3). There were no significant \((p>0.05; \text{FPLSD})\) differences among the control animals sampled at 3, 6 and 12 h post-injection. Plasma IGF-I levels are not presented for animals from Experiment 1 (feeding study).

3.4. Plasma IGFBPs (Experiment 2)

Four IGFBPs of 20, 32, 42 and 50 kDa in size, that we have previously found to be highly specific \((\text{Shepherd et al., 2005}),\)

were identified in the plasma of rainbow trout from this experiment. There were no significant differences in plasma protein levels among the treatment groups, so equal volumes of plasma were used for IGFBP determinations. Plasma IGFBPs were not measured in animals from Experiment 1 (feeding study).

There was a significant \((p<0.001, \text{two-way ANOVA})\) effect of time, but no significant \((p=0.189, \text{two-way ANOVA})\) effect of GHS treatment on plasma levels of the 20 kDa IGFBP.
levels were significantly (\(p<0.001\); one-way ANOVA) different among the sampling time points, with significantly (\(p<0.05\), FPLSD) elevated levels occurring at the 3 h sampling time-point and decreasing to their lowest levels by the 12 h sampling time. There was a significant (\(p<0.001\), two-way ANOVA) effect of GHS treatment, but no significant (\(p>0.05\)) effect of sampling time on plasma levels of the 32 kDa IGFBP (IGFBP-32K) (Fig. 5). However, a significant (\(p<0.001\), two-way ANOVA) sampling time\(\times\)GHS treatment interaction was apparent for plasma IGFBP-32K levels. Plasma IGFBP-32K levels were significantly (\(p<0.01\), FPLSD) decreased in all of the GHS treatment groups and sampling times except for the GHRH-H treatment (\(p>0.05\), FPLSD) group sampled at the 3 h time point. Mean levels of the IGFBP-32K increased over time in the control groups, with significantly elevated (\(p<0.01\), FPLSD) levels occurring in those animals sampled at the 12 h time-point, compared with values in the 3 h and 6 h control groups (Fig. 5).

Plasma levels of the 42 kDa binding protein (IGFBP-42K) were significantly affected by sampling time (\(p<0.001\), two-way ANOVA) and GHS treatment (\(p<0.001\), two-way ANOVA) (Fig. 6). There was also a significant (\(p<0.001\), two-way ANOVA) interaction of sampling time\(\times\)GHS treatment with the highest levels occurring in those groups sampled at 12 h post-injection. More specifically, mean plasma IGFBP-42K levels were elevated in all the groups sampled at 12 h except for the GHRH high dose and ghrelin low dose groups. At the 6 h sampling time point, plasma IGFBP-42K levels were significantly (\(p<0.05\), FPLSD) elevated in the ghrelin injected groups and a significant (\(p<0.001\); two-way ANOVA) effect of GHS treatment was observed. Plasma levels of IGFBP-42K were not affected by sampling time (\(p>0.05\); two-way ANOVA), however, there was a significant (\(p<0.001\); two-way ANOVA) interaction of sampling time\(\times\)GHS treatment on plasma IGFBP-32K levels. **\(P<0.01\) and ***\(P<0.001\) compared with corresponding, time-matched, controls (FPLSD).

![Fig. 4. Time-course of changes in plasma levels of the 20 kDa IGFBP (IGFBP-20K) in rainbow trout injected with GHRH, KP-102 or ghrelin and sampled at 3, 6 and 12 h after injection. The “L” and “H” letters for each GHS on the x-axis represent the “Low (1.0 pmol/g bw)” and “High (10.0 pmol/g bw)” doses, respectively. Values are arbitrary densitometry units (ADU) and are represented as means±S.E.M. (n=4–6 duplicate determinations). There was a significant (\(p<0.001\); two-way ANOVA) effect of time, no significant (\(p=0.189\); two-way ANOVA) effect of GHS treatment nor a significant (\(p=0.189\); two-way ANOVA) interaction of sampling time\(\times\)GHS treatment on plasma IGFBP-20K levels. Inset: when values from each treatment were combined by sampling time (treatments collapsed within sampling time), plasma IGFBP-20K levels were significantly (\(p<0.001\); one-way ANOVA) different among the sampling time points (FPLSD). Groups with different alpha characters (figure inset) are significantly (\(p<0.05\); FPLSD) different from other groups.](image1)

![Fig. 5. Time-course of changes in plasma levels of the 32 kDa IGFBP (IGFBP-32K) in rainbow trout injected with GHRH, KP-102 or ghrelin and sampled at 3, 6 and 12 h after injection. The “L” and “H” letters for each GHS on the x-axis represent the “Low (1.0 pmol/g bw)” and “High (10.0 pmol/g bw)” doses, respectively. Values are arbitrary densitometry units (ADU) and are represented as means±S.E.M. (n=4–6 duplicate determinations). Plasma levels of IGFBP-32K were not affected by sampling time (\(p>0.05\); two-way ANOVA), however, there was a significant (\(p<0.001\); two-way ANOVA) effect of GHS treatment and a significant (\(p<0.001\); two-way ANOVA) sampling time\(\times\)GHS treatment interaction on plasma IGFBP-32K levels. **\(P<0.01\) and ***\(P<0.001\) compared with corresponding, time-matched, controls (FPLSD).](image2)

![Fig. 6. Time-course of changes in plasma levels of the 42 kDa IGFBP (IGFBP-42K) in rainbow trout injected with GHRH, KP-102 or ghrelin and sampled at 3, 6 and 12 h after injection. The “L” and “H” letters for each GHS on the x-axis represent the “Low (1.0 pmol/g bw)” and “High (10.0 pmol/g bw)” doses, respectively. Values are arbitrary densitometry units (ADU) and are represented as means±S.E.M. (n=4–6 duplicate determinations). Plasma levels of IGFBP-42K were not significantly different among the control groups sampled at 3, 6 and 12 h post-injection (Fig. 6).](image3)
Plasma levels of the 50 kDa IGFBP (IGFBP-50K) were significantly affected by sampling time \((p<0.001,\) two-way ANOVA) and GHS treatment \((p<0.001,\) two-way ANOVA) (Fig. 7). There was also a significant \((p<0.001;\) two-way ANOVA) interaction of sampling time \(\times\) GHS treatment, with the highest levels occurring at the 12 h time point (Fig. 7). When compared to the time-matched controls, there were significant increases in plasma IGFBP-50K levels in the KP-102 high dose \((p<0.01,\) FPLSD) and the two ghrelin (low and high dose) groups \((p<0.001,\) FPLSD) at the 6 h sampling time point. By contrast, plasma levels of the IGFBP-50K were significantly \((p<0.001,\) FPLSD) depressed in the GHRH low dose and ghrelin low dose groups \((p<0.01,\) FPLSD) at the 3 h sampling time point. Plasma IGFBP-50K levels were not significantly \((p>0.05;\) FPLSD) different among the control groups sampled at 3, 6 and 12 h post-injection (Fig. 7).

4. Discussion

We demonstrated that plasma GH levels are stimulated, in a time-dependent manner, by low doses of peripherally administered GHSs (GHRH, KP-102 and ghrelin) in rainbow trout. We also report for the first time, that peripheral components of the somatotropic axis, including IGF-I and its binding proteins, were measurably altered by all of the GHSs tested. In addition to having measured the endocrine response to GHS treatment, we determined that administration of all of the GHSs tested increased feed intake. The doses of the GHSs used \((1.0\) and \(10\) pmol/g body mass) in this study are comparable to doses of ghrelin, GHRH and KP-102 used in the tilapia \((Shepherd et al., 2000; Riley et al., 2005)\) which are the lowest tested \((in vivo)\) in a teleost model, thus suggesting that the observed responses are true physiological and endocrine responses to the GHSs tested.

4.1. Orexigenic response to GHS treatment

Given the orexigenic effects of exogenous GHSs \((Korbonits et al., 2004; Unniappan and Peter, 2005)\), we were particularly interested in determining whether the GHSs tested would stimulate feed intake, a conserved physiological action for ghrelin in teleosts and ghrelin and GH-releasing peptides \((e.g.,\) KP-102) in mammals \((Korbonits et al., 2004; Unniappan and Peter, 2005)\). We found that the GHSs tested stimulated feed intake at 2 and 5 h post-injection. The single exception is the 2 h time point in the high dose ghrelin treatment group. This appears to be the first report of an orexigenic effect of GHRH in a teleost, an effect that may involve the stimulation of GH which has been shown to increase feed intake in rainbow trout \((Markert et al., 1977; Johnsson and Björnsson, 1994; Johansson et al., 2005)\) and in the gillhead sea bream \((Vega-Rubín de Celis et al., 2004)\).

The precise neuroendocrine mechanisms responsible for the orexigenic actions of peripherally and centrally administered ghrelin and GHRPs are not known in teleosts. Studies in mammals indicate that orexigenic neurons, which produce neuropeptide-Y \((NPY)\) and agouti-related protein \((AgRP)\) and are located in the arcuate nucleus \((teleost homologue=\) nucleus lateralis tuberis) and the paraventricular nucleus \((teleost homologue=\) hypothalamic nucleus preopticus), are activated \((increased c-fos expression)\) following ghrelin or GHRP treatment \((Lawrence et al., 2002; Bernier, 2006)\). Given the expression of NPY and its receptors in homologous areas of the teleost central nervous system \((reviewed by Volkoff et al., 2005)\), it could be proposed that the GHS activation of neurons in mammals may be analogous to the effects of centrally administered NPY \((likely AgRP as well)\) which has been shown to stimulate feed intake in teleosts \((Volkoff et al., 2005; Bernier, 2006)\). As for GHRH, a possible mechanism for its orexigenic actions in rainbow trout may involve its specific actions on GH release. Reports of the orexigenic actions of GH are few in teleosts, however, studies have shown concurrent changes in monoamine levels within the central nervous system and feeding and behavior following GH treatment in rainbow trout \((Jönsson et al., 2003; Johansson et al., 2004, 2005)\). While our findings add to recent reports describing the orexigenic effects of native ghrelin(s) in goldfish \((Unniappan et al., 2002; Unniappan et al., 2004)\) and in the Mossambique tilapia \((Riley et al., 2005)\), it is evident that more studies are needed to elucidate the neuroendocrine mechanisms by which GHSs, particularly GHRH, regulate feed intake in teleosts.

4.2. Plasma GH and IGF-I

The time-course of the responses to treatment by various GHSs in this study is of particular interest. Studies using higher levels of ghrelin(s) in goldfish \((100\) ng/g body mass) \((Unniappan and Peter, 2004)\), rainbow trout \((25\) and \(250\) ng/g body mass) \((Kaiya et al., 2003a)\) and channel catfish \((Kaiya et al., 2006)\) show that intraperitoneal injection of ghrelin elicits an increase in plasma GH levels within 15 min in goldfish, up to 3 h in trout and up to 6 h in tilapia \((Riley et al., 2005)\). The only
Four IGFBPs have been identified in teleosts, based upon their physiological regulation and sequence (DNA and protein) similarities to mammalian homologues (Kelley et al., 2002; Duan and Xu, 2005). As for regulation in teleosts, it is becoming increasingly clear that the low molecular mass IGFBPs (<32 kDa) are associated with catabolic states such as stress or fasting (Duan et al., 1999; Maures and Duan, 2002; Shimizu et al., 2005, 2006), whereas the higher molecular mass binding proteins (>32 kDa) are associated with anabolic states such as positive nutritional status or following salinity- or dietary-induced elevations in plasma GH or IGF-I (Shimizu et al., 2003a; Beckman et al., 2004; Shepherd et al., 2005).

Presently, there is a single report describing the effects of a hypophysiotropin (bovine GHRH₁₋₄₀-amide) on IGFBPs in fish, the channel catfish (Johnson et al., 2003); however, the responses in this teleost were unlike that previously described for any vertebrate species. Consequently, we have expanded our investigation to include examining the effects of GHRH, ghrelin and KP-102, which exert their actions via a distinctly different receptor/signaling mechanism from that of GHRH (Harvey, 1995; Korbonits et al., 2004), on plasma IGFBPs in rainbow trout. In this study, IGFBPs with molecular mass of 20 kDa, 32 kDa, 42 kDa and 50 kDa, were detected. Plasma levels of the 20 kDa binding protein (IGFBP-20K) decreased over the sampling time interval in all the treatment groups. This decrease may be associated with handling stress. While cortisol levels were not measured in animals from this study, the cortisol response to handling stress is brief in trout and typically decreases within 3 to 6 h (Gamperl et al., 1994), but is also accompanied by temporal changes in the central nervous system that cannot be easily separated from changes in cortisol alone (Gamperl et al., 1994; Wendelaar Bonga, 1997; Reid et al., 1998). Therefore, it is unlikely that knowledge of cortisol levels in animals from this study would alter our understanding or interpretation of the experimental outcomes. Indeed, studies have consistently shown increased levels of the lower molecular mass IGFBPs in response to stressors such as fasting and hypoxia (Maures and Duan, 2002; Shimizu et al., 2005), and we recently demonstrated that levels of 21 kDa IGFBP were upregulated in rainbow trout during salinity challenge wherein no differences in plasma cortisol levels were noted (Shepherd et al., 2005). Pursuing this further, Davis and Peterson (2006) recently reported that stress-induced increases in the low molecular mass IGFBPs were independent of cortisol levels in hybrid striped bass. As for the identity of the trout IGFBP-20K protein, a recent study (Shimizu et al., 2005) has shown that a 22-kDa IGFBP in chinook salmon, that has high protein sequence similarity to mammalian IGFBP-1, is similarly upregulated by stress (fasting and salinity challenge). Collectively, our findings support the notion that the IGFBP-20K protein is the rainbow trout equivalent of the chinook salmon IGFBP-1.

Plasma levels of the 32 kDa binding protein (IGFBP-32K) increased over the sampling interval in the control groups, suggesting that this protein may be influenced by handling stress in direction opposite to that of the IGFBP-20K protein. More important, however, is the complete reduction of the IGFBP-32K levels by all of the GHSs tested. This reduction is similar to that reported for IGFBPs of similar molecular mass in this study that exhibited increased plasma GH levels by 3 h post-injection was the GHRH low dose group (1 pmol/g body mass). By contrast, plasma GH levels were significantly elevated at the later time points (6 and 12 h post-injection) in the KP-102 (low and high doses), GHRH (high dose) and ghrelin treatment groups. While this delayed response differs from the aforementioned studies, a similar (longer) time-course of the GH response to GHRH, KP-102 and ghrelin and has been reported for tilapia (Shepherd et al., 2000; Riley et al., 2003). The basis for these differences is unknown but may be related to 1) the lower doses (1 and 10 pmol/g body mass) of GHSs used in this study compared to other studies, 2) a prolonged effect of these GHSs on pulsatile GH, or 3) to differences in the half-life of these compounds, which have not yet been examined in a teleost system. There are, however, other reasons for differences in time-course responses to GHS treatment such as species specific differences, the sex and size (maturity) of the animals used (Xiao et al., 2002; Parhar et al., 2003), environmental conditions in which the animals were held, or the dose, route and type of GHS(s) used (Korbonits et al., 2004; Unniapan et al., 2004; Unniapan and Peter, 2004).

In addition to seeing increased GH levels following GHS treatment in rainbow trout, we show that there was a time-dependent increase in plasma IGF-I levels. The increase in plasma IGF-I levels, within specific treatment groups, appears to be the first such report in a teleost system. While all of the GHS treatments elicited an increase in plasma IGF-I at 3 h post-injection, there was also a decrease at 6 h post-injection in those animals treated with KP-102 and ghrelin. Plasma IGF-I levels were elevated (3 h) before observed increases in GH, but like that of GH, mean levels were elevated at the 12 h sampling time-point in almost all of the GHS treatment groups. Thus, plasma IGF-I levels, which are generally GH-dependent, did not appear to conform to the expected elevation which typically follows increases in blood GH levels such as those following salinity challenge (Shepherd et al., 2005) or exogenous GH treatment in rainbow trout (Moriyama, 1995; Moriyama et al., 1995; Biga et al., 2004). As for the elevated IGF-I levels at 3 h post-injection, this could be attributed to an undetected transient release of GH, occurring before 3 h, as described by Kaiya et al. (2003a). This possibility is supported by the concurrent increases in feed intake, plasma IGF-I and decreased IGFBP-32K observed at 3 h post-injection in our study. The decrease in IGF-I at 6 h may be the result of negative feedback at the pituitary following the increase in IGF-I observed at 3 h (Perez-Sanchez et al., 1992; Fruchtman et al., 2000). Thus, the apparent uncoupling may be an artifact of the sampling schedule employed in this study. Nevertheless, instances of uncoupling between GH and IGF-I levels have been reported in the literature (Riley et al., 2005; Shepherd et al., 2005).

4.3. Plasma IGFBPs

In all vertebrates examined to date, plasma IGF-I and -II circulate in the blood bound to high affinity binding proteins, termed insulin-like growth factor-binding proteins (IGFBPs). Four IGFBPs have been identified in teleosts, based upon their
(30–33 kDa) which have been shown to be negatively regulated by GH in teleosts (Park et al., 2000) or upregulated by cortisol treatment or acute stress (Duan et al., 1999; Kajimura et al., 2003; Davis and Peterson, 2006). Given the similarities in molecular size and regulation, the IGFBP-32K protein is likely the rainbow trout homologue to zebrafish IGFBP-2 which has been recently characterized and found to be similar in size (31 kDa), function and regulation to mammalian IGFBP-2 (Duan et al., 1999). While designation of this protein as the trout IGFBP-2 is plausible, Bauchat et al. (2001) isolated, and partially characterized, a 30 kDa IGFBP secreted by a trout hepatoma cell-line (RTH-149) with sequence identity to that of human IGFBP-1. While definitive answers await further study, the similarities in molecular size of the trout 30–32 kDa IGFBPs, and negative regulation by GHSs (essentially GH) seen in this study and others (Park et al., 2000), which are characteristics similar to that of the zebrafish IGFBP-2 (Duan et al., 1999), suggest that this protein is a putative rainbow trout IGFBP-2 homologue. Lastly, the differences in the sizes of the 20 kDa and 32 kDa IGFBPs seen in this study, and in their differential responses over the sampling time, suggest that these are different IGFBPs.

Additional IGFBPs of 42 kDa and 50 kDa were up-regulated by GHS treatment (as early as 6 h post-treatment) in the rainbow trout from this study and coincide with the GH-dependent regulation of the IGFBP-3 doublet reported in salmonids (Kelley et al., 1992; Shimizu et al., 2003a; Shepherd et al., 2005). While these two high molecular mass IGFBPs have been recently shown in chinook salmon to be the same protein(s), with varying degrees of glycosylation (Shimizu et al., 2003b), we have seen slight differences in their response to GHS treatment in rainbow trout. Specifically, at 12 h post-injection, plasma IGFBP-42K levels were elevated in all of the treatment groups except for the GHRH-L group, whereas plasma IGFBP-50K levels were elevated at 12 h post-injection by all GHSs tested. Additionally, there appears to be a significant depression in IGFBP-50K levels, but not IGFBP-42K levels, in the low dose ghrelin and GHRH groups sampled at 3 h post injection. There is no apparent explanation for this depression at 3 h post-injection, but these depressions do coincide with lower mean GH levels seen in all the treatment groups sampled at 3 h post-injection.

5. Perspective

We have shown that peripherally administered GHRH, ghrelin and KP-102 stimulate feed intake and plasma GH levels over a wider time course than previously reported for a single teleost species. Additionally, this appears to be the first report to show that GHS treatment (particularly ghrelin) stimulates plasma IGF-I and IGFBP levels in a teleost.

Given that GHSs and GH stimulate IGF-I and IGFBP production and feed intake, one is left to question the relative contributions of GH and GHSs to appetite regulation, body composition and growth in teleosts and other vertebrates. It is quite interesting that GHRH (Kelly and Kohler, 1996), KP-102 (Piñeiro et al., 1997; Bowers, 1998; Mericq et al., 1998; Phung et al., 2000) and GH (Donaldson et al., 1979; Scanes and Baile, 1993; Collier and Byatt, 1996) can stimulate lean body growth, whereas ghrelin has been shown to be adipogenic in teleosts and other vertebrates (Korbonits et al., 2004; Riley et al., 2005). One possible explanation for the adipogenic effects of ghrelin may reside in the trophic effect of this peptide on the pituitary in various species. Ghrelin and synthetic GHSs have been shown to affect pituitary hormones with known adipogenic actions in mammals, i.e., stimulation of ACTH (and cortisol) and prolactin (PRL), and a reduction of thyroid-stimulating hormone (TSH) (Korbonits et al., 2004). Similarly, ghrelin has been shown to stimulate pituitary PRL release in tilapia (Riley et al., 2002; Kaiya et al., 2003b,c), but not in rainbow trout (Kaiya et al., 2003a), and LH in goldfish (Unniappan and Peter, 2004). Consequently, it is tempting to hypothesize that the lipogenic actions of ghrelin in teleosts, and other vertebrates, may reside in the effects of ghrelin on the release of pituitary hormones other than GH (Clark et al., 1997; Thomas et al., 1997; Camanni et al., 1998; Carmignac et al., 1998; Korbonits et al., 2004). Given the range of actions of GHSs in teleosts, additional studies are needed to further our understanding of the central and peripheral actions of ghrelin, and the contrasting mechanisms by which GHRH and GH stimulate feed intake and growth.

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