

ABSTRACT

THE EFFECT OF GHRELIN ON GLUCOSE METABOLISM AND THE GROWTH HORMONE/INSULIN-LIKE GROWTH FACTOR-I (GH/IGF-I) AXIS IN THE TILAPIA, *OREOCHROMIS MOSSAMBICUS*

The peptide hormone ghrelin has been shown to stimulate growth hormone (GH) release, appetite, and fat deposition in vertebrates. More recently, ghrelin has been shown to play a role in glucose metabolism in mammals. Ghrelin acts through the growth hormone secretagogue receptor (GHS-R). The GHS-R codes for two gene transcripts, GHS-R1a and GHS-R1b. In tilapia, two forms of ghrelin have been identified, ghrelin-C8 and ghrelin-C10. This study measured the effect of ghrelin on the hormonal regulators involved in glucose metabolism, appetite, and growth (GH/IGF-I axis) in the tilapia, *Oreochromis mossambicus*. Fish were injected with two doses of ghrelin-C8 and ghrelin-C10. Blood and tissue samples were collected at 2, 4, and 8 h post-injection. The low dose of ghrelin-C8 elevated blood glucose levels at 4 h post-injection. Both ghrelin-C8 and -C10 reduced plasma IGF-I levels and ghrelin-C10 elevated IGF-I mRNA levels in the liver. Ghrelin-C8 and -C10 elevated liver GHR2 mRNA levels, however GHR1 mRNA levels remained unaffected. In the brain, ghrelin-C8 and -C10 treatment elevated both GHS-R1a and GHS-R1b mRNA levels. NPY mRNA levels in the brain, a regulator of appetite, were elevated by ghrelin-C10. These data confirm ghrelin's role in appetite and glucose metabolism and show that ghrelin up-regulates certain components of the GH/IGF-I axis in tilapia.

Sara E. Nilmeier
May 2010

THE EFFECT OF GHRELIN ON GLUCOSE METABOLISM
AND THE GROWTH HORMONE/INSULIN-LIKE
GROWTH FACTOR-I (GH/IGF-I) AXIS IN THE
TILAPIA, *OREOCHROMIS MOSSAMBICUS*

by

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APPROVED

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INTRODUCTION

Ghrelin is a stomach peptide that was identified in 1999 and was originally shown to stimulate growth hormone (GH) release from the pituitary (Kojima et al., 1999). Since then, ghrelin has been found to play a role in appetite, metabolism, reproduction, cell proliferation, growth, and gastric motility in vertebrates (c.f. Kaiya et al., 2008). Ghrelin also plays a role in glucose metabolism in mammals. Ghrelin can induce hyperglycemia in humans (Broglia et al., 2001), stimulate the release of glucose from cultured liver cells (Gauna et al., 2005), and modulate insulin and glucagon release (Smith et al., 2005). In many vertebrates, glucose metabolism is tightly regulated, relying on the ability of cells to transport glucose into or out of the cell. Glucose transporters (GLUTs) are a group of facilitative transport proteins that function in transporting glucose across the cell membrane. While many GLUTs have been identified in vertebrates, only GLUT4 is insulin-responsive, meaning it is recruited to the plasma membrane for glucose uptake following insulin signaling (Watson and Pessin, 2006). It is known that GLUT4 is a key player in glucose metabolism and that ghrelin influences glucose metabolism, yet there currently are no reports describing the effect of ghrelin on GLUT4 expression in any vertebrate. And while the effects of ghrelin on glucose metabolism are fairly well established in mammals, the exact role of ghrelin in glucose metabolism in fish is unclear.

The growth hormone/insulin-like growth factor-I (GH/IGF-I) axis is an important regulator of vertebrate growth (Rousseau and Dufour, 2007). Ghrelin has been shown to stimulate GH release from the pituitary in all vertebrates studied thus far (Kaiya et al., 2008). Ghrelin appears to possess anabolic actions

that are mediated through GH. Growth hormone acts through IGF-I in activating a variety of anabolic and lipolytic actions (Moller et al., 2003). Recent evidence suggests that ghrelin also elicits direct peripheral metabolic actions involved in preventing catabolism (Janssen et al., 2004), which are antagonistic to GH (Broglia et al., 2006).

Using the tilapia (*Oreochromis mossambicus*) as a model organism, my thesis examined the effects of ghrelin on glucose metabolism and the GH/IGF-I axis. The results from this study will help broaden the understanding of ghrelin's role in growth and glucose metabolism in vertebrates. The potential knowledge gained by this study could be applied to the field of medicine for treatment of metabolic disorders such as type-2 diabetes and cachexia as well as the aquaculture industry in terms of farming fish more efficiently.

Ghrelin

Ghrelin has been identified in all vertebrate classes including sharks (Kaiya et al., 2008). Ghrelin peptides in mammals are 27- or 28- amino acids long, whereas, in fish, peptide lengths vary from 12- to 23-amino acids (Kaiya et al., 2008). All ghrelins have a unique acyl modification on the third amino acid, typically a serine, with the exception of frog, shark, and goldfish ghrelins, in which serine has been substituted by a threonine (Kaiya et al., 2008). This fatty acid modification is essential for ghrelin's biological activity, allowing it to bind to the ghrelin receptor; growth hormone secretagogue receptor (GHS-R) (Ghigo, 2004) and cross the blood brain barrier (Banks et al., 2002). Because of this, the N-terminal portion of the ghrelin sequence, including the acylation, is thought to be the "active core" of ghrelin and is highly conserved across a variety of vertebrates (Table 1). Two forms of ghrelin have been identified in the

Mozambique tilapia (Kaiya et al., 2003). Both isoforms share the same amino acid sequence, but have different acyl modifications. Ghrelin-C8 has an octanoyl modification, and ghrelin-C10, a decanoyl modification (Kaiya et al., 2003). In tilapia, ghrelin-C10 is found in higher abundance than ghrelin-C8.

Table 1. Similarity among vertebrate ghrelin peptides

Vertebrate Species	Ghrelin Amino Acid Sequence
Mozambique tilapia	GSSFLSP-SQKPQ-----NKVKSSRIG
Nile tilapia	GSSFLSP-SQKPQ-----NKVKSSRIG
Wami tilapia	GSSFLSP-SQKPQ-----NKVKSSRIG
Hammerhead shark	GVSF-HPRLKEKD-DNSSGNSRKSKNP
Goldfish	GTSFLSP-AQKPQ-----G-RRPPRMG
Bullfrog	GLTFLSPADMQKIAERQSQNKL RHGNMN
Turtle	GSSFLSPEYQNTQQRKDP--KKHT-KLN
Chicken	GSSFLSPTYKNIQQKDT--RKPTARLH
Human	GSSFLSPEHQRVQQRKESKKPPAKLQPR

As mentioned above ghrelin's biological actions are mediated by the ghrelin receptor (GHS-R). The ghrelin receptor gene codes for two separate transcripts, GHS-R1a and its splice variant, GHS-R1b (Kaiya et al., 2008). GHS-R1a mediates ghrelin's biological actions and while GHS-R1b has been identified in most tissues, its function remains unknown (Ghigo, 2004). We have recently identified the two GHS-R isoforms in the tilapia and determined their tissue distribution (Fox et al., 2007). Both GHS-R1a and GHS-R1b were found in all tissues assayed including the liver, muscle, and the proximal pars distalis of the pituitary, which contains GH cells. In all tissues, GHS-R1a was found in higher quantities.

Ghrelin was originally identified in rats as the first natural ligand for the orphan GHS-R (Kojima et al., 1999). In mammals, ghrelin has been shown to directly stimulate GH release from the pituitary; however, release of GH-releasing hormone (GHRH) from the hypothalamus may be involved in ghrelin achieving a maximal stimulation of GH release. While GHRH and ghrelin stimulate GH release through independent signaling pathways, they appear to act synergistically. Co-administration of GHRH and ghrelin stimulates a greater amount of GH release than administration of either hormone alone (Arvat et al., 2001; Hataya et al., 2001). Ghrelin has been shown to stimulate GH release *in vitro* in seabream (Chan et al., 2004), *in vivo* in rainbow trout (Shepherd et al., 2007) and channel catfish (Kaiya et al., 2005), and both *in vivo* and *in vitro* in tilapia (Riley et al., 2002; Kaiya et al., 2003; Fox et al., 2007), hybrid striped bass (Picha et al., 2009) and goldfish (Unniappan and Peter, 2004). Recently, much attention has been given to ghrelin's role in stimulating food intake and adipogenesis. In rats, ghrelin has been shown to rapidly and acutely increase food intake (Tschöp et al., 2000), with similar findings in goldfish (Unniappan et al., 2004). However, in rainbow trout, ghrelin treatment failed to stimulate appetite (Jönsson et al., 2007). In tilapia, chronic treatment with ghrelin-C10 significantly increased food intake as well as total fat content in the liver and muscle, whereas ghrelin-C8 had no effect (Riley et al., 2005).

More studies need to be conducted in fish to determine ghrelin's role in regulating appetite and metabolism. Ghrelin's orexigenic actions have been shown to be mediated through neuropeptide Y (NPY) (Theander-Carillo et al., 2006). NPY is known to be a very potent stimulator of appetite in vertebrates (Stanley et al., 1986). In goldfish, both central and peripheral ghrelin treatment

stimulate feeding through the NPY pathway (Miura et al., 2006). These findings provide evidence that ghrelin plays an important role in energy regulation.

Glucose Metabolism

Glucose metabolism is characterized by the absorption, peripheral tissue uptake, and hepatic production and storage of glucose. These processes are controlled by a variety of hormones. Insulin, IGF-I, and leptin are hypoglycemic hormones, while glucagon, GH, and cortisol increase blood glucose levels (Yeo and Sawdon, 2007). The ability to transport glucose into and out of cells is critical for maintaining a proper balance between energy storage and usage. Glucose cannot diffuse across the plasma membrane due to its polar nature. Glucose transport is mediated by several cell surface glucose transport proteins (GLUTs) (Yeo and Sawdon, 2007). Thirteen GLUTs have been identified in mammals, with GLUTs 1-4 being the most widely and abundantly expressed (Joost and Thorens, 2001). In mammals, GLUT4 is found primarily in skeletal muscle and adipose and is responsible for peripheral glucose uptake (Yeo and Sawdon, 2007). GLUT4 is an insulin-responsive GLUT and is expressed at low levels on the surface of the plasma membrane when blood glucose levels are low. An increase in blood glucose levels stimulates the release of insulin from the β -cells of the pancreas. As insulin binds to its receptor (INS-R), a cascade of cell-signaling events induce recruitment of additional GLUT4 proteins to the cell surface, increasing peripheral glucose uptake and returning blood glucose levels to normal (Yeo and Sawdon, 2007). The INS-R is composed of two α and two β subunits. When insulin binds to the α subunit, tyrosine kinase activity is stimulated at the β subunit. The phosphorylation cascade that follows is crucial for insulin to carry out its cellular effects (Leibush et al., 1996). This mechanism by which insulin

and its receptor (INS-R) interact is similar amongst mammals, birds, amphibians and fish (Navarro et al., 1999). Our lab has determined the tissue distribution for the INS-R in tilapia, with the highest expression seen in the adipose, similar to what is seen in mammals, with a relatively low expression in the muscle (unpublished data).

Circulating levels of ghrelin and glucose appear to form a feedback system. In humans, ghrelin has been shown to induce hyperglycemia; acute ghrelin administration resulted in an increase in serum glucose levels and a decrease in serum insulin levels (Broglia et al., 2001). These changes suggest a direct glycogenolytic effect of ghrelin. Ghrelin also stimulates the release of glucose from cultured pig and rat hepatocytes (Gauna et al., 2005). It has also been suggested that ghrelin may modulate the release of insulin from the pancreas. In rats, ghrelin stimulated insulin secretion from isolated pancreatic islets (Date et al., 2002). Conversely, infusion of ghrelin into the portal vein of Wistar rats inhibited glucose-stimulated insulin release (Cui et al., 2008). On the other hand, plasma ghrelin levels are reduced by oral and intravenous glucose administration in normal, obese, and diabetic humans (Shiyya et al., 2002; Soriano-Guillen et al., 2004) as well as in rats (McCowen et al., 2002). In addition, hyperinsulinemic/euglycemic studies have shown that insulin is a regulator of circulating ghrelin levels, causing a decrease in plasma ghrelin levels following insulin infusion (Saad et al., 2002; Ghigo, 2004).

While there is much evidence to support ghrelin's role in glucose metabolism in mammals, there is a lack of research demonstrating if ghrelin functions similarly in fish. Although many of the mechanisms regulating glucose metabolism are similar in fish and mammals, fish are considered glucose intolerant, unable to quickly clear glucose from the bloodstream. Understanding

ghrelin's effects on glucose metabolism in fish may shed light on why fish metabolize carbohydrates in this way.

Growth Hormone/Insulin-like Growth Factor-I (GH/IGF-I) Axis

The GH/IGF-I axis is the primary regulator of growth in vertebrates, including fish (Rousseau and Dufour, 2007). GH is involved in several physiological processes such as reproduction and metabolism (Rousseau and Dufour, 2007). In both fish and mammals, GH promotes protein synthesis while stimulating lipid breakdown and suppressing peripheral carbohydrate uptake (LeRoith et al., 2001).

The biological effects of GH are mediated by its receptor (GHR). In fish, two phylogenetic groups of the GH receptor have been identified, GHR1 and GHR2 (Saera-Vila et al., 2005; Jiao et al., 2006). GHR2 appears to be the functional GH receptor since it is more closely related to the GHR found in mammals (Jiao et al., 2006). GHR1 is suggested to be a receptor for somatolactin (SL). Somatolactin belongs to the same hormone family of GH and prolactin, but it has only been identified in fish (Jiao et al., 2006). Studies in fish have shown that SL plays a role in gonadal maturation, stress response, immune function, fat metabolism and skin color, however, its function is not fully characterized (Jiang et al., 2008). This theory has been supported in the tilapia where salinity and fasting experiments revealed the tissue distribution of GHR1 to be consistent with that of somatolactin activity (Pierce et al., 2007).

GH is secreted from the pituitary and stimulates secretion of IGF-I, a mitogenic factor (LeRoith et al., 2001). IGF-I is an important intermediate for many of the growth-promoting effects of GH and is produced in all tissues with the liver being the major source of circulating IGF-I (Rousseau and Dufour, 2007).

In fish, IGF-I has been shown to increase growth (McCormick et al., 1992) and circulating IGF-I levels have been correlated with growth rate (Pierce et al., 2001; Mingarro et al., 2002). IGF-I is also regulated by nutrition, as circulating IGF-I levels are reduced by food deprivation (Rousseau and Dufour, 2007). IGF-I also plays an important role in carbohydrate metabolism, balancing the effects of GH and insulin (Yakar et al., 2004). In liver IGF-I deficient mice, low circulating IGF-I levels were associated with hyperinsulinemia and muscle insulin insensitivity (Yakar et al., 2001).

Ghrelin has been shown to regulate the GH/IGF-I axis in all vertebrates, including fish (as mentioned above). In mammals, ghrelin is hypothesized to regulate the GH response to fasting and caloric restriction (Ghigo et al., 2005). GH response to ghrelin administration is reduced in both obesity and anorexia nervosa (Tessone et al., 2003; Alvarez-Castro et al., 2004; Broglio et al., 2004). The hyper- and hyposecretion of GH in conditions of anorexia and obesity, respectively, may reflect the hyper- and hyposecretion of ghrelin that occurs in these same conditions (Ghigo et al., 2005).

The effect of ghrelin on IGF-I is not as well characterized. In a study in children with GH-insensitivity syndrome, IGF-I treatment reduced plasma ghrelin levels (Uckun-Kitapci et al., 2008). In rainbow trout, both a low dose (1.0 pmol/g) and high dose (10 pmol/g) of ghrelin increased plasma IGF-I levels at 3 h post-injection, whereas, only the low dose caused a significant decrease in plasma IGF-I at 6 h post-injection (Shepherd et al., 2007). In tilapia, long-term ghrelin administration (3 weeks) has been shown to increase plasma GH levels, lower plasma IGF-I levels, and increase body weight and total fat content in the liver (Riley et al., 2005). This suggests that ghrelin may be inhibiting linear growth via suppressing IGF-I levels. Acute ghrelin treatment increased liver IGF-I mRNA

levels 5 h post-injection in the tilapia (Fox et al., 2007). These data suggest that ghrelin exhibits a temporal effect on IGF-I in tilapia.

Hypotheses

Hypothesis 1: Ghrelin will induce hyperglycemia by suppressing mRNA levels of the insulin receptor (INSR) and GLUT4 in muscle tissue.

Hypothesis 2: Ghrelin will up-regulate components of the GH/IGF-I axis.

Hypothesis 3: Ghrelin will increase NPY mRNA levels in the brain.

Objectives

1. To examine the effect of ghrelin on plasma glucose levels, and GLUT4 and INSR expression in muscle.

2. To examine the effect of ghrelin on circulating GH and IGF-I levels, and IGF-I, GHR1 and GHR2 expression in liver.

3. To examine the effect of ghrelin on NPY, GHSR-1a and GHSR-1b expression in brain.

MATERIALS AND METHODS

Experimental Design

Sexually mature male tilapia (30-150 g), *Oreochromis mossambicus*, were reared in fresh water under simulated natural photoperiod [14 h:10 h (light:dark)] and allowed to acclimate to the tanks for two weeks prior to experimentation. Fish were fasted for 24 h prior to the experiment to ensure similar metabolic states in all fish. Fish were given a single intraperitoneal injection of tilapia ghrelin-C8 or ghrelin-C10 at either 1 ng/g body weight (low dose) or 10 ng/g body weight (high dose). These doses have been shown to elicit a biological response in tilapia (Fox et al, 2007). Control animals received a saline injection (1 μ l/g body weight). Injections were given at 0900 h. This experiment was replicated with a sample size of five fish per treatment in each experiment. Tissue and blood samples were taken at 0, 2, and 4 h post-injection. Blood was collected from the caudal vasculature in heparin-coated (200U, Sigma, St. Louis, MO) syringes. Plasma was separated by centrifugation at 10,000 rpm for 10 min at 4 °C and stored at -20 °C until analysis for glucose, GH and IGF-I. Muscle, liver and brain samples, collected for various mRNA analyses, were placed in 500 μ l Tri-Reagent (Ambion, Austin, TX) and stored at -80°C for later analysis.

Radioimmunoassays

Radioimmunoassays (RIA) were performed to determine plasma levels of GH and IGF-I. Our colleague, Dr. E. Gordon Grau at the Hawaii Institute of Marine Biology, performed the RIA for GH following Yada et al. (1994). For plasma IGF-I in tilapia, we have validated the RIA kit from GroPep (Adelaide, Australia), as described by Shimizu et al. (1999) for tilapia. Twenty-five

microliters of sample plasma were extracted with 100 μ L of acid-ethanol (87.5% ethanol and 12.5% 2M HCl). Ten microliters of extracted plasma and standards were incubated with anti-barramundi IGF-I at a concentration of 1:10,000 for 24 h at 4 °C. Samples were precipitated with 100 μ L of anti-rabbit IgG goat serum (1:75) in EDTA-PBS + 10% polyethylene glycol then incubated for 2 h at room temperature. Free and bound tracers were separated by centrifugation at 3200 rpm for 1 h at 4 °C. The supernatant was aspirated and samples were counted using the 1470 Wizard gamma counter (Perkin Elmer, Waltham, MA).

Plasma Glucose

Plasma glucose levels were determined by using the glucose (HK) kit (Sigma) with some modifications. Five microliters of plasma and standards (10 mg/mL to 0 mg/mL) were added to a 96-well plate followed by 200 μ l of assay reagent in each well. The reaction proceeded for 15 min. The absorbance was determined at 340 nm using the ELX800 plate reader from BioTek (Winooski, VT).

Quantitative PCR

Total RNA was extracted from individual tissues using Tri-Reagent following the manufacturer's protocol (Ambion). RNA concentration was determined using a spectrophotometer and then diluted to either 100 ng/ μ l or 200 ng/ μ l, depending on the tissue. Ten microliters of diluted RNA was reverse transcribed to synthesize cDNA using the High-Capacity cDNA Reverse Transcription system (Applied Biosystems, Foster City, CA). All mRNA levels were determined by real-time quantitative RT-PCR (qPCR). This assay has been previously validated in the PI's laboratory for all the following tilapia mRNAs (INS-R, GH, GHR-I, GHR-II, GHSR-1a, GHSR-1b, NPY, IGF-I, ARP) (Table 2).

I have validated GLUT4 for this study. For GLUT4, degenerative primers were developed based on a sequence alignment from brown trout (GenBank #AF247395), Coho salmon (GenBank #AF502957) and Atlantic cod (GenBank #DQ109810). From these primers, a partial gene sequence was prepared by PCR and ligated into a pCR-II TOPO vector (Invitrogen, Carlsbad, CA). Plasmids were purified and sequenced to obtain specific tilapia GLUT4 primers. All target mRNA values were normalized by dividing the copy number value of each individual sample with each samples corresponding copy number of the house keeping gene, acidic ribosomal phosphoprotein P0 (ARP) (Pierce et al., 2007; Fox et al., 2009).

The assay was the same for all mRNAs and only that for GLUT4 mRNA will be described. Plasmid DNA containing the amplified fragment of tilapia GLUT4 mRNAs was serially diluted and prepared as standard samples. The PCR reaction contained 0.2 μ M of each primer, 0.6 μ M of probe (when used), and 7.5 μ l of Taqman Master Mix (or Power Sybr Green, Applied Biosystems). The cycling parameter was as follows: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 sec and 58 °C for 1 min.

Statistics

Differences among means were determined using a factorial analysis of variance (ANOVA) followed by Fisher's Least Significant Difference. Data are expressed as means \pm S.E.M., relative to the control group. Calculations were performed using STATISTICA (Statsoft, Tulsa, OK).

Table 2
Primer sequences

Target mRNA	Primer Sequence
ARP	F: 5'- TTT GAA AAT CAT CCA ACT TTT GGA T-3' R: 5'- GCA GGG ACA GAC GGA TGG T-3'
GH	F: 5'- TTA CAT CAT CAG CCC GAT CG- 3' R: 5'- AGA TCG ACA GCA GCT TCA GGA- 3' P: 5'- CAA ACA CGA GAC TCAGCG CAG CTC G- 3'
GHR1	F: 5'- CAC AGA CTT CTA CGC TCA GGT CA- 3' R: 5'- TGA GTT GCT GRC CAG GAG ACA- 3' P: 5'- CAA TGT TAT GCC AAC TGG TGG TGT GGT G- 3'
GHR2	F: 5'- CAC ACC TCG ATC TGG ACA TAT TAC A-3' R: 5'- CGG TTG GAC AAT GTC ATT AAC AA-3' P: 5'- CGT CCA GCT CCG CTC CAG GGA-3'
GHSR-1a	F: 5'- CTG GTG GTT GTT GTG CTA GCC-3' R: 5'- TAC TCG GAT AAC AAC GAC AGC AA-3' P: 5'- CCG CTC TCT GGA TGC TCC TTC ACC-3'
GHSR-1b	F: 5'- AGT GCT CTA CAG CCT GAT AGG-3' R: 5'- TTG CGC GTT CTG GAA ACT TAC-3' P: 5'- CTG TGG CAA AGG CAC CGA GAG ACG-3'
GLUT4	F: 5'-TGA CTG GAA CCC TCG CTC TG-3' R: 5'-TGT AGT CCC CCT CAA TGA TCT TCT-3' P: 5'- TGT CCT TGG ATC TTT GG-3'
IGF-I	F: 5'- CTG CTT CCA AAG CTG TGA GCT-3' R: 5'- GAT CGA GAA ATC TTG GGA GTC TTG-3' P: 5'- CAG CGC CTT GAG ATG TAC TGT GCA CCT-3'
INS-R	F: 5'- CAC GCT GGT GGT GAT GGA-3' R: 5'- GGC AGC GCA GGT AGC TCT C-3'

RESULTS

Plasma Levels of Glucose, GH, and IGF-I

Fish treated with the low dose of ghrelin-C8 exhibited plasma glucose levels significantly higher ($P < 0.01$) than control at 4 h post-injection (Fig. 1).

Neither dose of ghrelin-C8 had an effect on plasma glucose levels at 2 h. Ghrelin-C10 had no effect on plasma glucose levels at any time point.

There was no difference in plasma GH amongst the various treatments at any time point (Fig. 2).

Fish treated with ghrelin-C8 (low and high dose) as well as fish treated with the low dose of ghrelin-C10 exhibited plasma IGF-I levels significantly lower ($P < 0.05$) than control at 2 h post-injection. The high dose of ghrelin-C10 had no effect at 2 h. None of the treatments elicited an effect at 4 h post-injection (Fig. 3).

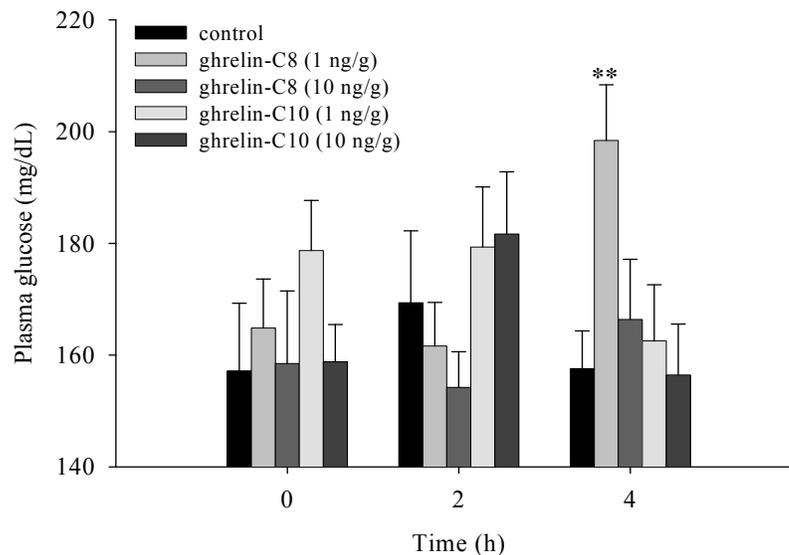


Fig.1. Effect of ghrelin on plasma glucose levels. The low dose of ghrelin-C8 significantly increased plasma glucose levels at 4 h post-injection relative to the control. Vertical bars represent mean \pm SEM (n=6-9). ** significantly different from control at $P < 0.01$.

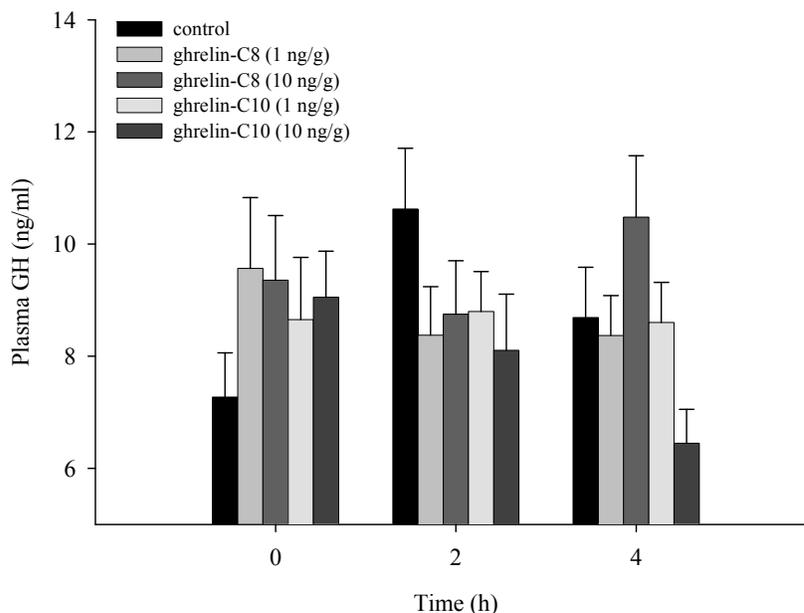


Fig. 2. Effect of ghrelin on plasma GH. All treatments failed to elicit an effect in plasma GH levels. n=6-10.

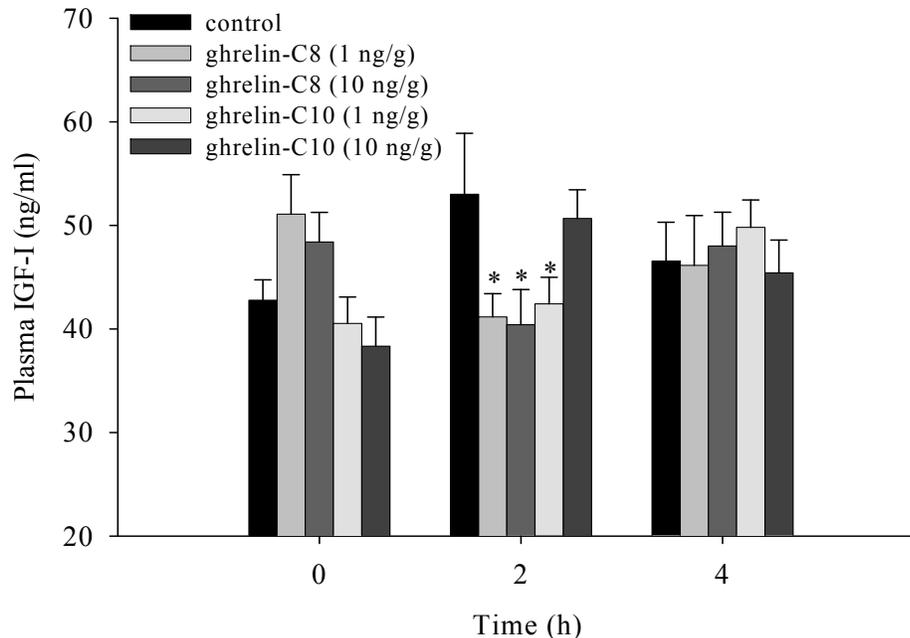


Fig. 3. Effect of ghrelin on plasma IGF-I levels. Both doses of ghrelin-C8 as well as the low dose of ghrelin-C10 significantly reduced plasma IGF-I levels at 2 h post-injection relative to the control. Vertical bars represent mean \pm SEM (n=7-10). * significantly different from control at $P < 0.05$

mRNA Levels in the Brain

Fish treated with the high dose of ghrelin-C10 exhibited significantly higher ($P < 0.001$) NPY mRNA levels than control at 4 h post-injection (Fig. 3). Ghrelin-C10 (low and high dose) had no effect on brain NPY mRNA levels at 2 h post-injection. At all time points tested, ghrelin-C8 failed to elicit an effect on brain NPY mRNA levels (Fig. 4).

Fish treated with the low dose of ghrelin-C8 and ghrelin-C10 exhibited significantly higher ($P < 0.01$, $P < 0.05$, respectively) GHS-R1a mRNA levels than the control at 4 h post-injection (Fig. 5). The high doses of ghrelin-C8 and ghrelin-C10 had no effect at 4 h. All treatments failed to elicit an effect at 2 h post-injection.

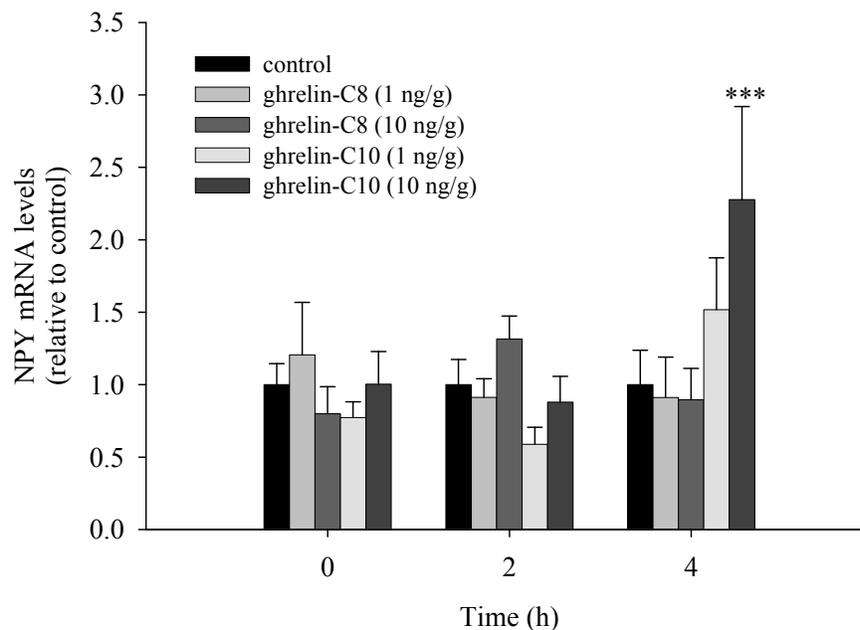


Fig. 4. Effect of ghrelin on NPY mRNA levels in the brain. The high dose of ghrelin-C10 significantly increased brain NPY mRNA levels at 4 h post-injection relative to the control. Vertical bars represent mean \pm SEM (n=6-10). *** significantly different from control at $P < 0.001$.

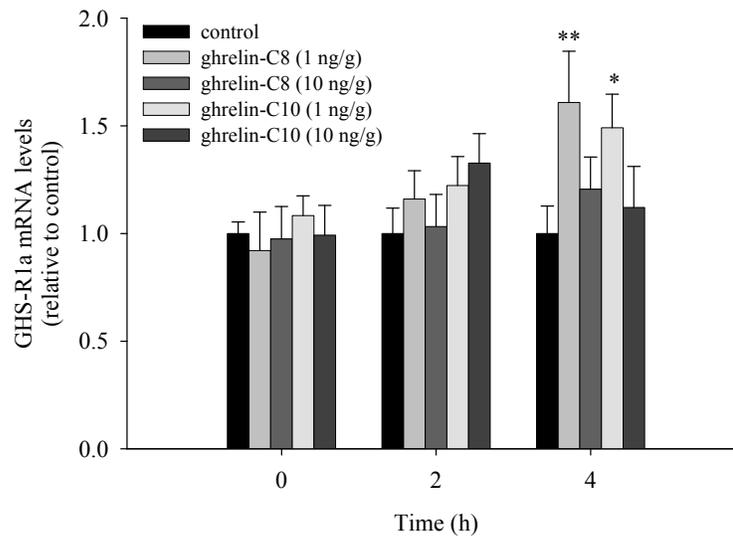


Fig. 5. Effect of ghrelin on GHS-R1a mRNA levels in the brain. The low doses of both ghrelin-C8 and C-10 significantly increased brain GHS-R1a mRNA levels at 4 h post-injection relative to the control. Vertical bars represent mean \pm SEM (n=6-10). *, ** significantly different from control at $P < 0.05$ and $P < 0.01$, respectively.

At 2 h post-injection, all treatment groups exhibited a significant increase in GHS-R1b mRNA levels compared to control (C8 low, $P < 0.05$; C8 high, $P < 0.01$; C10 low, $P < 0.001$; C10 high, $P < 0.001$) (Fig 6). All treatments failed to elicit an effect at 4 h post-injection.

mRNA Levels in Muscle

Fish treated with either dose of ghrelin-C8 and ghrelin-C10 exhibited GLUT4 mRNA levels significantly ($P < 0.01$) lower than control at 2 h post-injection (Fig. 7). All treatments failed to elicit an effect at 4 h post-injection.

At both time points, all ghrelin treatments failed to elicit an effect in INS-R mRNA levels (Fig. 8).

mRNA Levels in the Liver

Ghrelin treatment failed to exhibit an effect on GHR1 mRNA levels (Fig. 9).

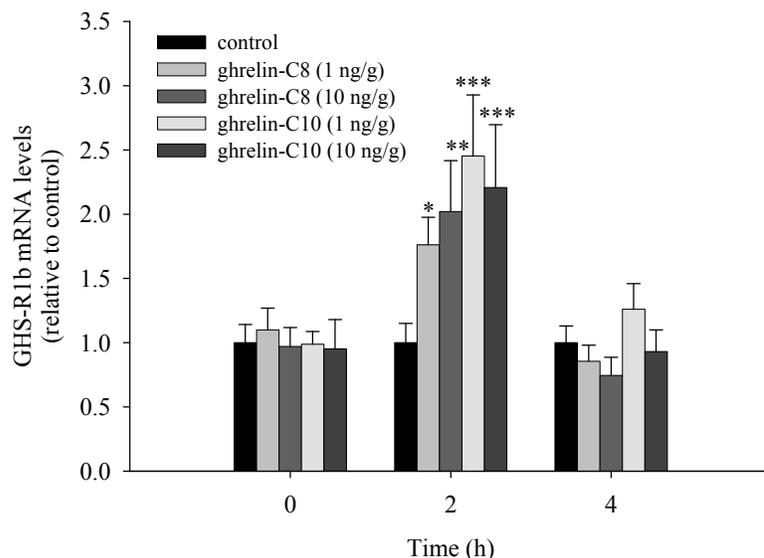


Fig. 6. Effect of ghrelin on GHS-R1b mRNA levels in the brain. Both low and high doses of ghrelin-C8 and ghrelin-C10 significantly increased brain GHS-R1b mRNA levels at 2 h post-injection relative to the control. Vertical bars represent mean \pm SEM (n=5-9). *, **, *** significantly different from control at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

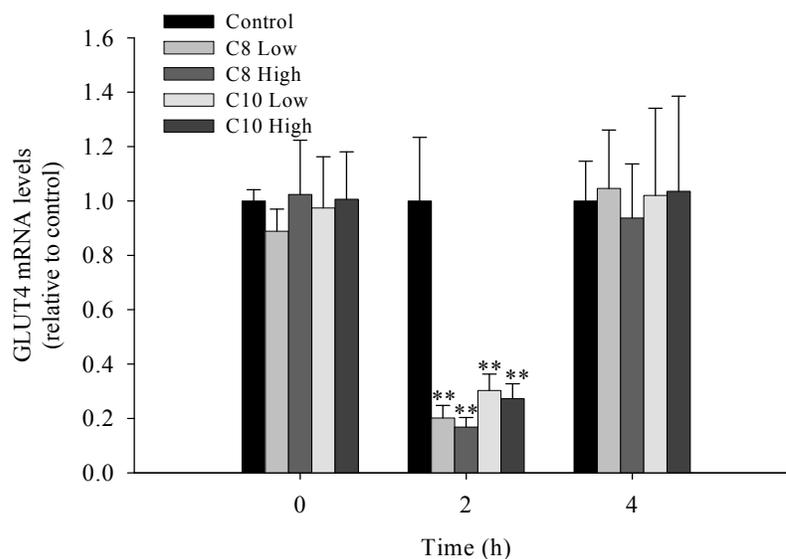


Fig. 7. Effect of ghrelin on GLUT4 mRNA levels in muscle. Both doses of ghrelin-C8 and ghrelin-C10 significantly reduced GLUT4 mRNA levels at 2 h post-injection relative to the control. Vertical bars represent mean \pm SEM (n=5-10). ** significantly different from control at $P < 0.01$.

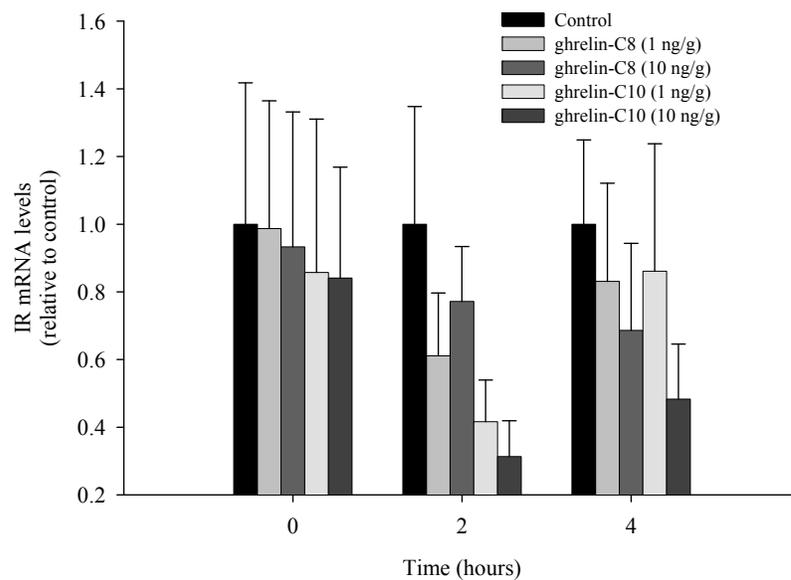


Fig. 8. Effect of ghrelin on INS-R mRNA levels in muscle. All ghrelin treatments failed to elicit an effect on INS-R mRNA levels in muscle (n= 4-8).

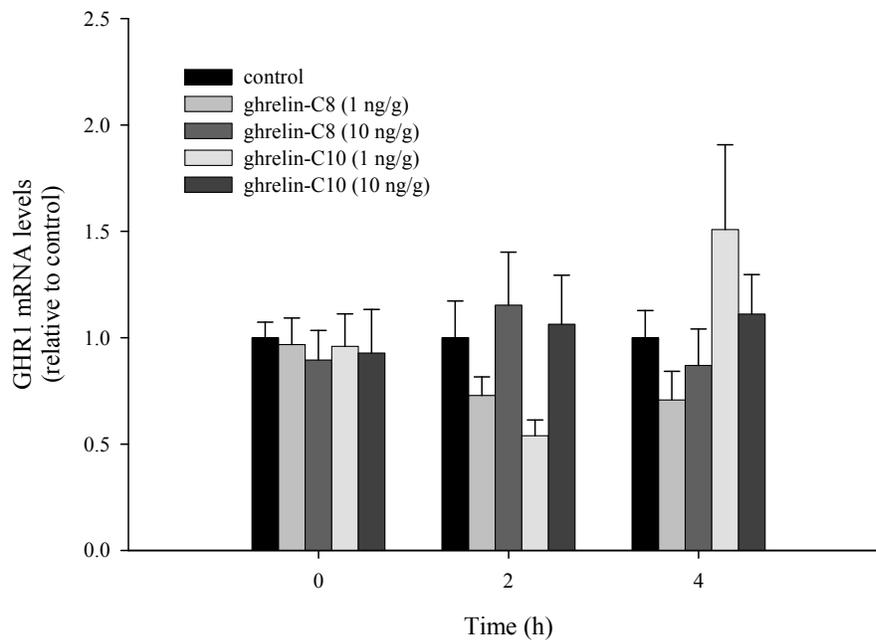


Fig. 9. Effect of ghrelin on GHR1 mRNA levels in liver. All ghrelin treatments failed to elicit an effect on liver GHR1 mRNA levels (n= 6-10).

Fish treated with ghrelin-C8 (low and high dose) and the low dose of ghrelin-C10 exhibited GHR2 mRNA levels significantly ($P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively) higher than control at 2 h post-injection (Fig. 10). The low dose of ghrelin-C10 had no effect at 2 h post-injection. At 4 h post-injection all treatments failed to elicit an effect on GHR2 mRNA levels.

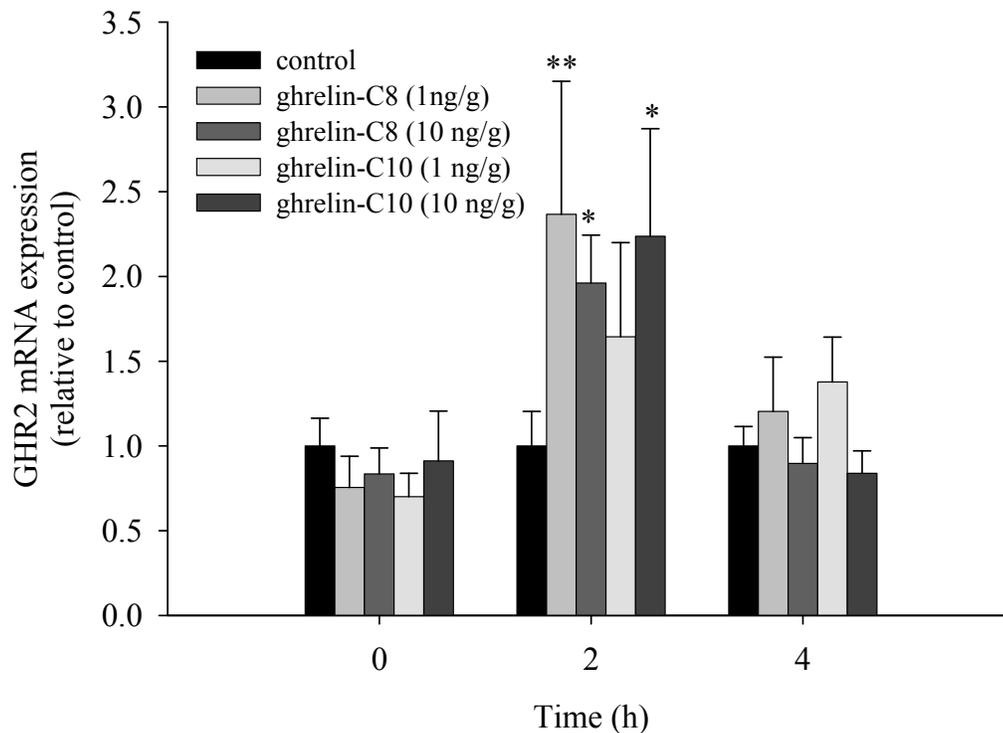


Fig. 10. Effect of ghrelin on GHR2 mRNA levels in the liver. Both doses of ghrelin-C8 and the high dose of ghrelin-C10 significantly increased liver GHR2 mRNA levels at 2 h post-injection relative to the control. Vertical bars represent mean \pm SEM (n=4-9). *, ** significantly different from control at $P < 0.05$ and $P < 0.01$, respectively.

There was a significant ($P < 0.01$) interaction of time and treatment on IGF-I mRNA levels. Fish treated with the high dose of ghrelin-C10 exhibited IGF-I mRNA levels significantly ($P < 0.05$) higher than control at 2 h post-injection (Fig. 11). The low dose of ghrelin-C10 had no effect at 2 h post-injection. At 4 h

post-injection, fish treated with the low dose of ghrelin-C10 exhibited IGF-I mRNA levels significantly ($P < 0.05$) higher than control. The high dose of ghrelin-C10 had no effect at 4 h post-injection. Both doses of ghrelin-C8 failed to elicit an effect at all time points tested.

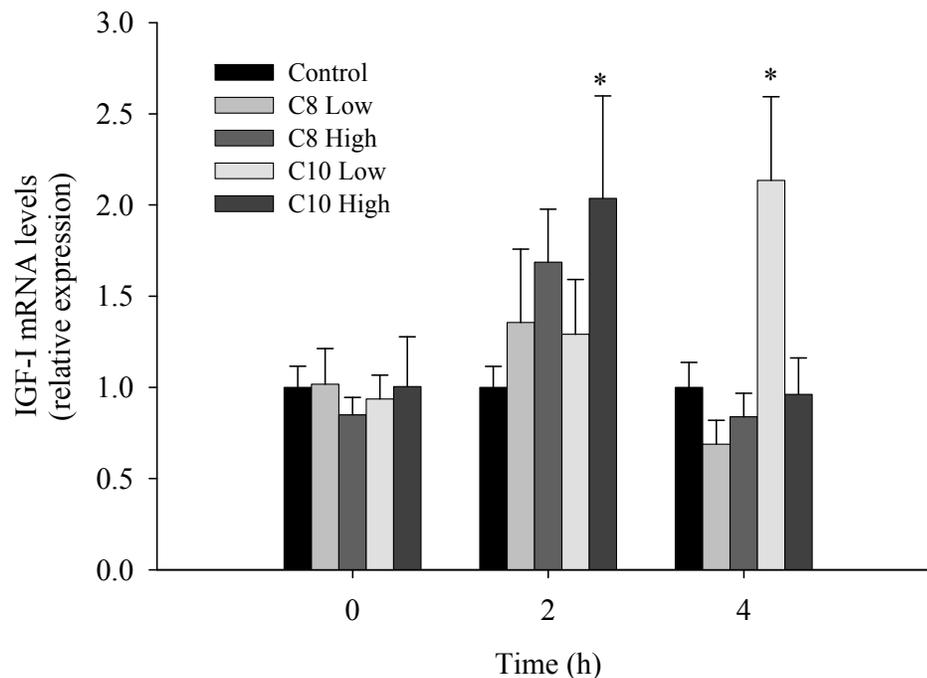


Fig. 11. The effect of ghrelin on IGF-I mRNA levels in the liver. The high dose of ghrelin-C10 significantly increased liver IGF-I mRNA levels at 2 h post-injection relative to the control. At 4 h post-injection the low dose of ghrelin-C10 significantly increased liver IGF-I mRNA levels relative to control. Vertical bars represent mean \pm SEM (n=6-10). * significantly different from control at $P < 0.05$.

DISCUSSION

The results of this study demonstrate the influence of ghrelin on glucose metabolism and the GH/IGF-I axis as well as its role in the endocrine control of food intake. Ghrelin treatment elevated plasma glucose levels, similar to that in mammals, likely through its suppression of muscle GLUT4 expression. While plasma GH levels were unaffected by ghrelin treatment, liver mRNA levels of the functional GH receptor, GHR2, were significantly elevated. Although ghrelin treatment suppressed plasma IGF-I levels, treatment with ghrelin-C10 elevated liver IGF-I mRNA levels. These data confirm ghrelin's influence on the GH/IGF-I axis, but a clear cause-and-effect relationship between ghrelin and the GH/IGF-I axis in tilapia remains to be clearly established. This study also reinforces the role of ghrelin in appetite control. Ghrelin treatment elevated brain mRNA levels of NPY, a potent appetite stimulator.

Ghrelin treatment caused an up-regulation of GHS-R expression in the brain. The low dose (1 ng/g) of both ghrelin-C8 and ghrelin-C10 significantly increased GHS-R1a mRNA levels at 4 h post-injection. At 2 h post-injection, all ghrelin treatments significantly increased brain GHS-R1b mRNA levels. These findings are unique in that GHS-R1a is thought to be responsible for mediating ghrelin's biological effects with GHS-R1b having no known biological effects (Ghigo, 2004). GHS-R1a is also expressed in higher quantities in tilapia brain than GHS-R1b (Fox et al., 2007). In HEK 293 cells, GHS-R1a signaling was reduced in the presence of GHS-R1b; elevated GHS-R1b expression caused translocation of GHS-R1a from the cell surface to the nucleus (Leung et al., 2007). The increased levels of GHS-R1b mRNA seen at 2 h could be responsible for a

down-regulation in GHS-R1a mRNA. At 4 h, GHS-R1b mRNA levels were not elevated, but GHS-R1a mRNA levels were. The absence of GHS-R1b's inhibitory effect at 4 h may have allowed the elevated GHS-R1a levels to be observed.

At 4 h post-injection, fish receiving the low dose of ghrelin-C8 exhibited plasma glucose levels significantly higher than that of the control group. This is consistent with research showing ghrelin to have a hyperglycemic effect in pigs, rats, and human (Broglia et al., 2001; Gauna et al., 2005). At 2 h post-injection, muscle GLUT4 mRNA levels were significantly reduced by all treatments. A reduced number of GLUT4 molecules being recruited to the plasma membrane would decrease glucose uptake and may provide a possible explanation for the elevated plasma glucose levels seen at 4 h. Muscle INS-R mRNA levels were unaffected by ghrelin treatment. Although GLUT4 is insulin-responsive, insulin is not the only hormone that is capable of activating GLUT4. IGF-I may stimulate GLUT4 recruitment as well. The IGF-I receptors and the insulin receptor have a high homology (Federici et al., 1997), IGF-I and insulin can bind to each other's receptor with a lower affinity, and the signaling pathways of IGF-I and insulin have a large amount of overlap (Navarro et al., 1999). In fish, insulin appears to be more involved in growth-regulation than in carbohydrate metabolism (Plisetskaya, 1998; Wright et al., 1998) and IGF-I appears to be closely tied to glucose metabolism (Yakar et al., 2001). Both doses of ghrelin-C8 and the low dose of ghrelin-C10 significantly reduced plasma IGF-I levels at 2 h post-injection. If IGF-I is playing a larger role in glucose metabolism than insulin, suppressed GLUT4 mRNA levels at 2 h and elevated plasma glucose levels at 4 h may be due to a reduction in circulating IGF-I, or ghrelin may exhibit a direct effect on reduced GLUT4 mRNA levels.

In this study, ghrelin treatment influenced the GH/IGF-I axis in a tissue-specific manner. Only liver mRNA values were up-regulated, while values of circulating hormone were unaffected or even suppressed. There was no change in plasma GH levels. This finding is similar to that in fish exposed to ghrelin for 21 days (Riley et al., 2005). However, tilapia given intraperitoneal injections of ghrelin-C8 (1 ng/g body weight) and ghrelin-C10 (0.1 and 1 ng/g body weight) showed increased levels of plasma GH (Fox et al., 2007). The animals used in that study weighed 30-50 g, while the fish in this study were much larger, 30-150 g. Considering that smaller tilapia exhibit faster growth rates (Kuwaye et al., 1993) this may account for the different responses seen.

Liver GHR1 mRNA expression remained unchanged, however, liver GHR2 mRNA expression increased at 2 h in fish receiving both ghrelin treatments. These results coincide with the idea that GHR2 is the actual GH receptor, while GHR1 remains the SL receptor and is not involved in GH binding (Jiao et al., 2006; Pierce et al., 2007). Plasma IGF-I levels were suppressed in fish treated with the low and high doses of ghrelin-C8 and the low dose of ghrelin-C10 at 2 h post-injection. In a study by Riley et al. (2005) tilapia exposed to ghrelin-C10 for 21 days also exhibited a suppression in plasma IGF-I levels while no change was seen in a similar experiment in which tilapia were injected with both doses of ghrelin-C8 or ghrelin-C10 (Fox et al., 2007). Liver IGF-I mRNA expression was increased at 2 h by the high dose of ghrelin-C10 and at 4 h by the low dose of ghrelin-C10. These results are similar to the findings of Fox et al. (2007) in which intraperitoneal injection of both ghrelin-C8 and ghrelin-C10 resulted in increased IGF-I mRNA expression in the liver. Conversely, Riley et al. (2005) found no change in hepatic IGF-I mRNA expression following chronic ghrelin exposure. It is surprising to find a concurrent elevation of liver IGF-I expression and

suppression of circulating IGF-I levels, however other studies have yielded similar results. In hybrid striped bass undergoing compensatory growth, a decrease in plasma IGF-I was reported along with an increase in liver IGF-I mRNA levels (Picha et al., 2006). In the tilapia, fasting resulted in significantly decreased plasma IGF-I levels with an increase, although not significant, in liver IGF-I mRNA levels (Pierce et al., 2007). It is also interesting to note that while the high dose of ghrelin-C10 did not suppress plasma IGF-I levels, it was the only treatment that elevated liver IGF-I mRNA levels. The exact relationship between ghrelin and IGF-I remains unclear. As the mechanisms regulating the GH/IGF-I axis are numerous, the unexpected data reported in these experiments are likely a result of this inherent complexity. Only more detailed studies will be able to further elucidate the effects of ghrelin on the GH/IGF-I axis in tilapia.

In the brain, fish given the high dose of ghrelin-C10 displayed a significant increase in NPY mRNA levels at 4 h post-injection. In goldfish, ghrelin-C8 stimulated feeding has been shown to be mediated by NPY and intracerebroventricular administration of ghrelin also resulted in an increase in NPY mRNA in the brain (Miura et al., 2006). Earlier findings in tilapia show that fish receiving treatments of ghrelin-C10 showed an increase in appetite and weight as well as an increase in fat content in the liver, while ghrelin-C8 had no effect (Riley et al., 2005). In the Nile tilapia, ghrelin mRNA increases prior to periods of rapid growth (Parhar et al., 2003). Also in the tilapia, intraperitoneal injection of NPY stimulated food intake and increase in body weight (Carpio et al., 2006). NPY levels have also been shown to increase pre-prandially and decrease post-prandially in tilapia (Peddu et al., 2009). Taken altogether, these findings indicate NPY as a mediator for the growth-promoting effects of ghrelin, via an increase in energy intake (appetite).

CONCLUSIONS

This study has shown that ghrelin influences glucose metabolism and the GH/IGF-I axis in tilapia and affirms its role in appetite control. Consistent with what has been shown in pigs, rats, and humans, ghrelin treatment resulted in increased plasma glucose levels, confirming the hyperglycemic activity of ghrelin. Ghrelin treatment also resulted in suppressed GLUT4 mRNA levels in the muscle. Reduced GLUT4 expression may be a contributing factor in ghrelin's hyperglycemic effect. Ghrelin treatment resulted in increased NPY mRNA expression in the brain. Along with what is known about NPY's role in appetite and growth, these results show NPY may be responsible for mediating ghrelin's growth-promoting actions. Ghrelin treatment did not affect plasma GH levels, but did suppress plasma IGF-I levels at 2 h post-injection. In the liver, ghrelin treatment increased both GHR2 and IGF-I mRNA levels. While these results show that ghrelin does up-regulate some components of the GH/IGF-I axis, its complex involvement in a network of physiological events make it difficult to clearly isolate ghrelin's precise actions. More studies must be done to understand the mechanisms by which ghrelin affects glucose metabolism and to further elucidate its effects on the GH/IGF-I axis.

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