ABSTRACT

BROWN, CAROLINE E. Effects of feeding pattern on plasma ghrelin concentrations in pigs. (Under direction of Dr. Scott C. Whisnant)

The independent role of ghrelin regulation continues to be controversial. Ghrelin, a 28 amino acid peptide identified as the endogenous ligand for growth hormone (GH) secretoagogue receptor, is found in the gastrointestinal tract, predominantly the stomach. Ghrelin stimulates GH secretion, increases feed intake, adipose tissue, and decreases gastric acid. The aim of this study was to determine if a change in meal patterns might affect ghrelin levels in barrows. Twelve crossbred barrows (67.1 $\pm$ 4.5 kg BW) were used. The pigs were placed on their corresponding diets on day 0. Six pigs were placed on continuous access to feed using a typical finishing diet and the treatment group was fed 2.73 kg of feed at 1200 and the remaining feed was removed at 1600. Catheters were placed in the jugular vein on day 7 and samples were taken on day 8, 9, and 11. Plasma ghrelin concentrations were measured every 15 minutes for 4 hours and then every 30 minutes the remaining 2 hours on days 8 and 9 using a commercially available RIA for active ghrelin. A glucose challenge (500 mg/kg BW) was administered on day 11 and a sample was taken before the infusion and then every 15 minutes for 3 hours after the infusion. Average daily gain during the experiment was 0.43 kg and 0.87 kg for the limited compared to continuous access to feed groups. Plasma ghrelin concentrations increased (20%) ($P<.01$) prior to feeding and decreased (20%) after feeding ($P<.01$) relative to baseline in the meal fed pigs. Ghrelin concentrations were decreased after glucose infusion ($P<.01$). Concentrations decreased by 40% after the initial infusion and then remained steady for approximately 2 hours post-infusion. In agreement with reports

from other species, ghrelin increased before and decreased after feeding in meal-fed animals. Ghrelin may be an important regulator of feed intake in swine.

EFFECTS OF FEEDING PATTERN ON PLASMA GHRELIN CONCENTRATIONS IN PIGS

by
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Chair of Advisory Committee

Dedication

I dedicate my M.S. thesis to my horse Daisy's Sensation (Dixie). She was my companion for nearly 15 years and passed away on May 18th, 2005. I spent most of my childhood with Dixie and will miss her dearly. Rest in peace Dixie Bell.

Biography

Caroline Elizabeth Brown was born in Sumter, SC on February 20th, 1981 to Mr. and Mrs. James Willis Brown. Caroline has one sister, Laura Brown who is thirteen months younger. Caroline grew up in Shelby, NC and graduated from Shelby High School in 1999. Following high school graduation, Caroline attended Clemson University for 1 year and then transferred to North Carolina State University to finish up her Bachelor's of Science degree in Animal Science. Caroline graduated Magna Cum Laude in May 2003. Immediately after graduating from undergraduate study, she continued on in the animal science department for my Master's of Science degree with a Physiology concentration. Caroline graduated with her Master's degree on August 9th, 2005. After graduation, Caroline will be a professor in the natural sciences department at Gardner-Webb University in Boiling Springs, NC teaching Anatomy and Physiology. On September 17th, 2005 Caroline will marry Joseph William Reynolds III, whom she met at North Carolina State University. Joe is originally from Statesville, NC and graduated in May 2003 with a Mechanical Engineer degree, Magna Cum Laude. Joe works at Milliken Chemical Company in Blacksburg, SC. After the wedding in September, Caroline and Joe will live in Cherryville, NC.

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INTRODUCTION

Ghrelin is a recently discovered hormone that is secreted primarily by the stomach/oxyntic cells and duodenum (Overduin et. al., 2004). Ghrelin is an acylated 28 amino acid peptide that is the natural ligand of the growth hormone (GH) secretagogue receptor (GHS-R) (Espelund et. al., 2004). Ghrelin stimulates GH secretion, increases feed intake, and increases adipose tissue. Upon administration of this orexigenic substance, there is a strong stimulation of endogenous GH release through binding to hypothalamic and pituitary GHS-R (Espelund et. al., 2004). Concentrations are altered during aberrations in nutritional status (Espelund et. al., 2004). Ghrelin levels tend to increase before a meal followed by low ghrelin levels after meal time which suggests that ghrelin could play a role in appetite regulation (Espelund et. al., 2004); therefore, meal pattern influences ghrelin secretion. This same hypothesis has been substantiated by studies in animal models as well as in human subjects (Espelund et. al., 2004).

In relation to GI tract hormones, insulin is an important regulator and has become of interest in studying the regulation of ghrelin. Previous evidence suggests that, in lean subjects, ghrelin levels increase before meals and fall within one hour of eating, which mirrors insulin patterns and shows less of an effect in obese subjects (Cummings et. al., 2001). The inverse relationship between circulating levels of ghrelin and insulin may suggest that post-meal hyperinsulinemia might inhibit ghrelin secretion during the absorptive state of digestion.

The current experiment was conducted in order to determine the effects of meal pattern combined with a glucose challenge on serum ghrelin and insulin levels in pigs.

REVIEW OF LITERATURE

Ghrelin

The hormone—ghrelin

Ghrelin is a 28 amino acid peptide identified as the endogenous ligand for the pituitary growth hormone (GH) secretagogue receptor (Briatore et al., 2003). Ghrelin has an octanoyl group on the serine at the 3rd position in the amino acid chain which gives the peptide hormone biological activity (Rosicka et. al., 2002). The name ghrelin comes from a Proto-Indo-European origin, *ghre*, which means growth (Kojima et al., 1999). The peptide differs by two amino acids in the rat and human. Ghrelin is synthesized and released primarily in the endocrine cells of the stomach and also synthesized in the neurons in the arcuate nucleus of the hypothalamus (Geary et. al., 2004). Ghrelin, like many substances, is found in the placenta as well. The ghrelin cells, originally named the X/A-like cells, now ghrelin cells, represent a large population (20%) of the cells in the gastric mucosa (Rosicka et. al., 2002). Other than the arcuate nucleus, there are ghrelin receptors in the brainstem, pituitary, as well as other brain areas (Geary 2004). Ghrelin robustly stimulates eating, suggesting the hypothesis that ghrelin is a coupled signal for hunger and meal initiation (Geary et. al 2004). In addition to stimulating GH secretion, ghrelin increases deposition of adipose tissue, and decreases gastric acid production (Rosicka et al., 2002). Ghrelin causes a decrease in the oxidation of fats and therefore causes more fat accumulation. Furthermore, ghrelin secretion is affected by the amount of

adipose tissue, which may indicate a relation to feed intake as well (Geary et al., 2004). The blood concentration of ghrelin is decreased by satiation or feeding and increased by fasting or hunger (Ueta et. al., 2003).

Ghrelin activates growth hormone secretagogue receptors (GHS-R) in the arcuate nucleus of the hypothalamus and stimulates the release of growth hormone (GH) and also in the vagal afferents to promote the release and expression of neuropeptide Y (NPY) and Agouti-related peptide (AgRP) further stimulating the paraventricular nucleus (PVN) and increasing ingestive behavior (Konturek et al., 2004). Gastric levels of ghrelin and its secretion may be regulated by central or local stimuli such as hunger (Rosicka et al., 2002). These stimuli could be substances within systemic circulation or signals from the central nervous system (CNS), yet the exact mechanism is unknown. It has also been found that expansion of the stomach does not cause ghrelin secretion, while glucose tends to be inhibitory to ghrelin. The regulation of ghrelin continues to be largely unknown. Broglio and colleagues (2002) reported that ghrelin levels are increased by fasting and decreased by food intake and glucose administration. Circulating levels of ghrelin are increased by anorexia but reduced in obese subjects but further research needs to be conducted to explain the relationship with adipose tissue.

Regulation of Leptin

Leptin is a 16kD single chain protein discovered in 1994 that decreases feed intake. Leptin is missing in ob/ob mice, which are obese, hyperphagic, sterile and have lower metabolic rate. Leptin acts through receptors (Ob-R) that are present in the afferent

visceral nerves and the arcuate nucleus of the hypothalamus. Leptin, the product of the 'ob' gene, is thought to play a role in the regulation of body weight (Korbonits et al., 2001). Leptin is produced by the differentiated adipocytes, although production has been demonstrated in other tissues. Again the neurons of the arcuate nucleus are capable of producing NPY and AgRP that tend to activate the feeding center located in the PVN (Konturek et al., 2002). The anorexigenic effect of leptin is mediated by inhibition of the synthesis and secretion of NPY. Leptin secretion shows a prominent circadian rhythm and tends not to be affected by individual meals (Geary 2004). Although leptin receptors are widespread throughout the body, there is some indication (from local infusions), that the populations of leptin receptors that mediate its effects on eating are in the arcuate nucleus, the brain, and in the dorsal vagal complex (Geary 2004). The normal endocrine function of leptin is complicated because the single chain peptide must pass through the blood-brain barrier to reach the receptors in these specified locations. Serum leptin levels are correlated with percentage body fat. Its serum concentrations are dependant on the amount of subcutaneous fat (Geary 2004). Leptin is not thought to be involved in meal feeding because its levels don not change acutely in response to meals. Also, human females tend to have more leptin for their particular body fat percentage (Anderwald et al., 2001 and Konturek et al., 2004). There is some evidence for an inhibitory effect of androgens, such as testosterone, on leptin secretion. Lean humans have a greater percentage of leptin bound than obese humans. Leptin has effects on food intake and the fat cells themselves in order that an increase in the level of leptin occurs with the increase in adipose tissue (Anderwald et al., 2002)

Hormonal Interactions

Interaction between leptin and ghrelin

Leptin acts at the central nervous system (CNS) in suppressing food intake and stimulating energy expenditure (Cummings et al., 2001). Recent studies point to adipose tissue as an active endocrine organ that secretes a range of hormones such as leptin and adiponectin. Leptin and ghrelin are part of the regulation of energy metabolism. The neurons of the lateral hypothalamus are involved in the release of NPY and AgRP that then activate the PVN or the "feeding center" (Cummings et al., 2001). Inhibition of the PVN induces satiety. Fasting stimulates the release of ghrelin and orexins (A and B) from the oxyntic mucosa. In the arcuate nucleus of the hypothalamus, ghrelin activated the GHS-R to stimulate GH release through stimulation of growth hormone releasing hormone (GHRH) (Murdolo et al., 2003). This also occurs in pituitary cultures in vitro. The stimulation of GH release then promotes the release of NPY and AgRP to stimulate the PVN and increase feeding behavior (Murdolo et al., 2003). Disruption in the balance between the anorexigenic and orexigenic factors can lead to disorders such as obesity or cachexia (weight loss) (Murdolo et al., 2003). Studies report that as weight loss occurs, ghrelin levels rise and leptin levels decrease. Ghrelin and leptin have diurnal rhythms, which further suggest, a relationship (a counterbalance between the two) (Cummings et al., 2001). In view of these diurnal rhythms, it is possible that leptin can affect ghrelin secretion by creating an inhibition in ghrelin's pathway. There are conflicting data

regarding this idea that show positive (Toshinaik et al., 2001) and negative (Asakawa et al., 2001) regulation of ghrelin by leptin. Ghrelin might stimulate leptin due to the observation that ghrelin receptors are found in adipose tissue (Kojima M. et al., 1999) but this warrants further investigation. Obesity is seen as a chronic adaptation to the hormonal changes that occur due to the increase in adipose tissue. With weight gain, there are increased leptin and decreased ghrelin levels but there may also be an up or down regulation of receptors in the hypothalamus, which in turn will cause sensitization or desensitization to the effects of the hormones.

Insulin and ghrelin interaction

In 1954, Sanger discovered the amino acid sequence of insulin, which is used for treatment of diseases like diabetes. Insulin is produced by the beta cells of the endocrine pancreas (~60-80% of pancreatic production). The primary function of the pancreas is to regulate glucose; insulin lowers glucose and inhibits glucagon. Within the body, a glucose sensing mechanism allows the beta cells to respond to a rise in glucose. Insulin is required for glucose uptake and utilization by most tissues (Gorbman et al., 1983). The brain, liver, and working skeletal muscles do not depend on insulin for uptake. Insulin increases lipogenesis by increasing glucose uptake to the fat-producing adipose tissue (Gorbman et al., 1983). Acetyl-Co-A Carboxylase is an enzyme that is lipogenic and increased by insulin as well. Insulin also acts on the muscle by stimulating amino acid uptake by the muscles. Studies have shown that, in normal subjects, ghrelin concentrations decrease after a meal and tend to increase just prior to a meal (Cummings

et al., 2001). Also, in lean subjects, plasma ghrelin levels increase before meals and fall within 1 hour of eating; this pattern is opposite to that of insulin (Cummings et al., 2001). The inverse relationship between circulating levels of ghrelin and insulin suggests that post-meal hyperinsulinemia might inhibit ghrelin secretion during the absorptive state of digestion. A recent study, which used Type II diabetic subjects, showed that insulin is a signal to suppress postprandial ghrelin levels, but not as much suppression in the normal patients before insulin therapy (Anderwald et al., 2003). The complete mechanism as to how insulin affects ghrelin has yet to be determined. Insulin could be utilizing indirect or direct paths in order to inhibit ghrelin synthesis or secretion from the oxyntic cells (X-A like cells). Interestingly, studies in rats showed that hypoglycemia (insulin-induced) increases ghrelin mRNA levels; however in humans, ghrelin concentrations are decreased during the hypoglycemic states [also insulin-induced] (Meier et al., 2004).

Glucose and ghrelin

Glucose concentrations are tightly regulated within the body. Glucose rises shortly after meal consumption and insulin functions to regulate high blood sugar levels. Recently, glucose has also been found to rapidly decrease ghrelin concentrations (Briatore et al., 2003). A recent clinical study compared the effect of glucose increase and early insulin response on ghrelin levels after i.v. glucose administration in Type II diabetic subjects (T2DM) and healthy subjects. T2DM patients have decreased sensitivity to insulin at the level of the pancreas from secondary characteristics such as obesity (Briatore et al., 2003). The conclusion was that hyperglycemia could directly inhibit

ghrelin. Administering glucose to diabetic patients reduced ghrelin even though there was no rise in insulin (Briatore et al., 2003). Non-diabetic subjects may also be used to study the relationship between insulin and ghrelin. Administration of insulin (a short acting analogue) did not affect ghrelin in non-diabetics (Poykko S et al., 2003). There are factors that lead to contradictions in research such as rate of administration, methods, timing (especially when considering pulsatile hormones), variation among subjects, and others. Insulin has opposite effects on plasma ghrelin and leptin and therefore could play an important role in regulating body weight.

Modulation of ghrelin and leptin

Insulin acts directly and indirectly with the CNS; directly by decreasing the hunger sensations and indirectly by modulation of leptin and ghrelin secretion (Anderwald et al., 2003). Insulin reduces ghrelin in healthy subjects and to a lesser extent in Type II patients prior to insulin therapy (Anderwald et al., 2003). The indirect effects of insulin can lead to suppression of hunger by modulating actions of ghrelin and leptin (Anderwald et al., 2003). Although insulin is the most important product from the beta cells within the islets of the pancreas, these cells are also involved in signal transduction pathways with leptin via leptin receptors that allow response to the hormone (Seufert et al., 2002). Insulin stimulates leptin from the adipose tissue and is called the "adipo-insular axis" (Seufert et al., 2002). Actions involving leptin and insulin occur both on the cellular level as well as at the level of the tissue. The molecular effects of leptin culminate to restrict insulin secretion by the pancreatic cells and biosynthesis to adapt

glucose homeostasis to body fat levels (Seufert et al., 2004). Leptin acts directly on beta cells in addition to hypothalamic action. At the cellular level, leptin is inhibitory toward insulin synthesis and secretion (Schofl C et al., 2002). Since insulin can stimulate leptin secretion and leptin can also inhibit insulin release, the two hormones have a classic negative feedback relationship. Further research needs to be conducted on the molecular aspects of leptin resistance in beta cells to possibly prevent the early stages or causes of Type 2 diabetes in obese patients. Ghrelin is suggested to function as an antagonist of leptin on hypothalamic neurons (Sun et al., 2004). Ueta and colleagues (2003) report that orexins-producing neurons express leptin receptors and that leptin may regulate the activity of orexin-producing neurons.

Metabolic disorders and other effects involving ghrelin

Disorders and ghrelin

Obesity is a common symptom/cause of many diseases, including diabetes. Reproductive status can also be affected by obesity. Women with polycystic ovary syndrome (PCOS) commonly suffer with obesity as well as hirsutism, irregular menstrual cycles, and the inability to get pregnant, due to follicular cysts (Neary et al., 2003). PCOS is also characterized by insulin-resistance and this furthers the clinical presentation of PCOS (Neary et al., 2003). Obesity begins the chain of events in PCOS women, which can be followed by insulin resistance. The fact that pregnancy causes an increase of hormones in circulation (such as progesterone, hCG, and others) would be a problem in this instance. Since ghrelin is involved in energy homeostasis and food intake, studying the relationship between ghrelin concentrations and the hormonal and metabolic features

of PCOS is important. There has not been extensive research with PCOS and ghrelin but it has been demonstrated that fasting plasma ghrelin levels in PCOS patents are comparable to controls (Neary et al., 2003). As previously seen, ghrelin concentration is inversely correlated with body mass index (BMI) further indicating that the excess fat mass in PCOS women reduces ghrelin levels as already seen in obese patients (Saad et al., 2002). Ghrelin seems to have several metabolic effects and can then have secondary effects that influence diabetes and PCOS.

Obesity

Again, obesity continues to be studied in the scientific community with its relationship to ghrelin. Many hormones, such as insulin, and environmental effects may alter metabolism. Along with ghrelin, leptin, and insulin; adiponectin also has important effects on metabolism. Adiponectin is secreted exclusively by adipose tissue and blood or tissue concentrations are found to be decreased in obesity and type 2 diabetes (Yildiz et al., 2004). Insulin resistance is also related to decreased adiponectin concentrations and seems to be more related to the degree of hypoadiponectinemia than the degree of adiposity (Yildiz et al., 2004). Glucose metabolism is also affected by adiponectin because this hormone is a potent insulin enhancer that links adipose tissue to the entire body's metabolism of glucose (Yildiz et al., 2004). Energy homeostasis is also linked to adiponectin in combination with leptin. Ghrelin has recently been attributed as a key regulator of body weight (Yildiz et al., 2004). Ghrelin levels are reported to be decreased in obesity and increased after diet-induced weight loss (Yildiz et al., 2004). Changes in

energy balance can be influenced by ghrelin as well (Yildiz et al., 2004). In a few studies that have been conducted, insulin may play a role in decreasing ghrelin levels after meals. After a meal, the increase in glucose and insulin could explain the decrease in ghrelin levels and the increase in plasma leptin in lean humans (Anderwald et al., 2003). However, Briatore and colleagues (2003) found that the administration of glucose to diabetic patients reduced ghrelin levels but with no rise in insulin. This observation could mean that glucose may have a direct effect on ghrelin levels, rather than modulation by insulin. Ghrelin concentrations are decreased in human obesity, whereas leptin levels are elevated, and the effects of ghrelin on energy homeostasis are the opposite of leptin's (Yildiz et al., 2004). Ghrelin and leptin have antagonistic effects via their specific receptors in the central nervous system (CNS) and in peripheral tissues (Anderwald et al., 2003). In the hepatocytes of the liver, ghrelin reduces and leptin stimulates insulin signal transduction, which then results in increasing and decreasing, respectively, glucose production (Anderwald et al., 2003). Ghrelin and leptin can indirectly enhance insulin's central action (having opposite mechanisms) on the sensation of hunger and appetite regulation. This being speculated, a study was performed that aimed to investigate the neuroendocrine regulation of appetite modulation by analyzing short-term changes and the relationship with ghrelin and leptin in healthy and diabetic subjects with and without prolonged insulin therapy (Anderwald et al., 2003). Their study found that, in healthy subjects, insulin causes a decrease in ghrelin dose-dependently and stimulates leptin secretion (Anderwald et al., 2003). A link between ghrelin and obesity has been observed from obese humans who underwent gastric bypass surgery and ghrelin production (in

ghrelin producing areas such as the fundus) decreased in parallel with a sustained weight loss and reduction in their appetite (Cummings and Shannon, 2003).

Although ghrelin has been noted for its regulation of food intake and energy homeostasis, it has also been suggested that it may play a role in reproductive physiology with its action on LH pulsatility by suppressing GnRH (De Souza et al., 2004). The known metabolic actions of ghrelin and now the potential reproductive role suggest that ghrelin could suppress the release of GnRH in women with exercise-related menstrual disturbances (De Souza et al., 2004). Ghrelin concentrations were approximately 100% higher in the group of exercising women that were considered amennorrheic because of excessive exercise (De Souza et al., 2004). In this same group, serum levels of estradiol remained constantly low, consistent with suppression of follicular development and the LH levels also remained low, which could account for inhibited follicular development (De Souza et al., 2004). There seems to be an impaired ghrelin response to feeding in amennorrheic patients due to plasma ghrelin levels remaining elevated after a meal (De Souza et al., 2004). Theoretically, a single meal would not be sufficient enough to restore energy homeostasis so ghrelin remains high in order that a signal remains to persuade energy intake. Ghrelin therapy could act as a compensatory mechanism to return subjects to a body weight that is seen as a set point.

Sleep effects

Many factors affect metabolism, body weight, and overall homeostasis. Sleep duration is one of the many factors that can alter the body's metabolism. It has previously

been reported that there is an association between short sleep times and an increase in BMI in many population samples (Taheri et al., 2004). It is of increasing interest whether or not metabolic hormones are involved in this relationship between body weights and sleep habits. In a Wisconsin sleep study, ~ 1000 participants took part in a study due to the observation that they had sleep disorders. Subjects underwent nocturnal polysomnography and also kept sleep diaries as well as completing questionnaires to determine that they were qualified for the experiment. Fasting blood samples were taken for plasma ghrelin and leptin measurement. The study found that in persons sleeping less than 8 h (74.4% of the sample), increased BMI was proportional to decreased sleep. Short sleep was associated with low leptin (p= 0.01), with a predicted 15.5% lower leptin for habitual sleep of 5 h versus 8 h, and high ghrelin (p= 0.008), with a predicted 14.9% higher ghrelin for nocturnal sleep of 5 h versus 8 h, independent of BMI (Taheri et al., 2004). They found that subjects with short sleep patterns had reduced leptin and elevated ghrelin (Taheri et al., 2004). This observation could explain an increase in appetite and further the urge to overeat. In American society, the vast selection and availability of food and lack of good sleeping habits could also explain the alterations in metabolic hormones, further increasing the risk for obesity and related disorders such as cardiovascular disease and diabetes.

Peptide Regulation

Neuropeptide regulation of feeding and NPY effects

Feeding regulation by neuropeptides involves the hypothalamic paraventricular nucleus (PVN), which contains magnocellular and parvocellular neurons. The magnocellular (major) neurons produce vasopressin (AVP) and oxytocin (OXT) and project their axons into the posterior pituitary for secretion into circulation. The parvocellular neurons in the PVN produce CRH and thyrotropin-releasing hormone (TRH) and also vasopressin and oxytocin, project their axons into the median eminence to secrete hormone into the bloodstream in order to control secretion of anterior pituitary hormones (Ueta et al., 2003). Neuropeptide Y (NPY) is produced by the neurons of the arcuate nucleus of the hypothalamus. These same neurons have growth hormone secretagogues (GHS) receptors on their surface. The effect of orexins on feeding can be attributed to stimulation by orexin-induced release of NPY in the arcuate nucleus (Ueta et al., 2003). Administration of ghrelin antibody has been shown to have no influence on the effect of NPY in the regulation of food intake and energy homeostasis (Rosicka et al., 2002). NPY alters the effects of ghrelin but ghrelin does not seem to alter NPY effects. Neuropeptide Y (NPY) has been shown to be the most potent orexigenic peptide and ghrelin has been noted to be the second most potent (Rosicka et al., 2002). NPY stimulates feeding and is increased in underfed animals. Leptin acts to decrease feed

intake by decreasing NPY and Agouti-related peptide (AgRP) while activating α-MSH. α-MSH inhibits feed intake via CRH; ghrelin stimulates NPY and AgRP. It has also been reported that α-MSH is released by the POMC neurons and activates OXT neurons (Ueta et al., 2003). Therefore, it is possible that OXT contributes to feed inhibition by α-MSH. AgRP is a protein that was first isolated in agouti mice and binds melanocortin (MC) receptors and antagonizes MSH. AgRP is also found in all mice and other mammals that have been studied. MSH inhibits food intake when it binds to the MC-4 receptor, which is found throughout the brain. Mutations in the MC-3 and MC-4 receptors produce obesity in mice and humans. Rosicka et. al. explains that the observation that ghrelin has an almost comparable effect to NPY has lead to the hypothesis that ghrelin's action could be mediated by NPY. NPY is an important factor for energy homeostasis and the peptide stimulates food intake and decreases energy output (Rosicka et al., 2002). Recent evidence also shows that ghrelin binds to the terminals of the neuropeptide Y (NPY) and AgRP neurons and that a portion of hypothalamic ghrelin-synthesizing neurons project to those nerve terminals and mediate ?-aminobutyric acid (GABA) currents that are involved in the stimulation of appetite and CRH release (Sun et al., 2004).

Neuromedin U (NMU) is a 23 amino acid neuropeptide that was discovered from porcine spinal cord tissue and functions to cause an elevation of blood pressure and regulation of the adrenocortical function (Ueta et al., 2003). Administration of the peptide indicates that NMU suppresses food intake and heat production (Ueta et al., 2003). Just as the Orexins A and B that were discovered in 1998, NMU is an endogenous ligand of G protein-coupled receptors, NMU1R and NMU2R. It has also been demonstrated that

NMU may be involved in stress responses (Ueta et al., 2003). There has been no evidence that NMU and ghrelin have a connection but NMU is another feed intake regulator that could interact with ghrelin.

Anorexigenic vs. Orexigenic

Anorexigenic substances

Anorexigenic substances are hormones, neuropeptides, and/or neurotransmitters that inhibit appetite. Anorexigenic substances include leptin, corticotropin-releasing hormone (CRH), pro-opiomelanocortin (POMC)/a-melanocyte-stimulating hormone (a-MSH), cocaine-amphetamine-related transcript (CART), cholecystokinin (CCK), peptide YY (PYY), insulin, and seratonin.

CCK is found in many forms (different lengths) within the body. The most common form in the brain is eight amino acids. CCK was originally found to regulate gall bladder function and can stimulate lutenizing hormone (LH) in rats and monkeys (Geary 2004). During the 1960s, CCK was the first hormone found to inhibit feed intake. CCK also stimulates pancreatic enzyme secretion and augments the effect of secretin on bicarbonate release. After eating, CCK transmits neural information regarding satiety to the hypothalamus. The signal travels via vagus nerves and is then distributed to the stomach and duodenum of the small intestine (Ueta et al., 2003). The substance also inhibits gastric emptying and stimulates intestinal motility. The release of CCK is stimulated by fat, amino acids, and by Ca^{2+} and Mg^{2+}. Injection of CCK antiserum increases feeding. CCK injections seem to reduce meal size but usually either fail to

affect the intermeal interval or shorten it (Geary 2004). Prior to a meal, CCK secretion has been reported to be greater in women than in men. CCK receptors are found on the cell membrane of its target cells. These receptors are found to be widespread in the gastrointestinal tract (GI tract) and also in the pancreas, gall bladder, and brain. On a meal-to-meal basis (short-term regulation), CCK and peptide YY (PYY) are released from the endocrine intestinal cells and by acting through G-protein coupled receptors have an effect on afferent nerves or directly on the arcuate nucleus (Konturek et al., 2004). This action inhibits food intake behavior, which stimulates NPY and AgRP and then inducing satiety through the inhibition of the PVN (Konturek et al., 2004).

Signals that are generated by the GI tract are able to regulate appetite. PYY 3-36 is produced in the small intestine and increases after a meal in proportion to calories and the composition of food consumed (Wynne et al., 2004). Higher levels of the peptide indicate consumption of fatty meals compared with that of protein and/or carbohydrates. PYY 3-36 is a satiety signal that is derived from the intestine as well as the pancreas (Wynne et al., 2004).

Proopiomelanocortin (POMC) is found in the arcuate nucleus but has fibers elsewhere. POMC produces ACTH and also β-endorphins (an opiod). When ACTH is released, the β-endorphins portion of the POMC molecule is released (a-MSH is part of POMC also) (Gorbman et al., 1983). The lateral portion of the arcuate nucleus includes neurons containing POMC and functions as a feeding inhibition center.

Orexins/Hypocretins

Orexins or hypocretins (stimulate secretions) were discovered in 1998. Orexins A and B were discovered as endogenous ligands of G protein-coupled receptors. Orexins are found in the lateral hypothalamus and resemble secretin in structure (a 27 amino acid peptide that is made in the duodenum and jejunum that stimulates water and bicarbonate secretion). These substances increase feeding and are seen to be elevated in fasted rats. The lateral hypothalamic area, known as the feeding center, includes separate neurons that contain orexins and melanin-concentrating hormone and functions as a feeding stimulation system (Ueta et al., 2003). Ghrelin is a significant orexigenic peptide by causing an increase in food intake and decrease in energy expenditure (Rosicka et al., 2002). Ueta and colleagues (2003) report that ghrelin acts directly on the hypothalamus to promote feeding activity. Ghrelin receptors are widely dispersed within in the brain including: the hypothalamus, hippocampus, substantia nigra, ventral tegmental area, and the raphe nuclei suggesting further physiological action by ghrelin other than feeding responsibilities (Ueta et al., 2003).

NPY stimulates feed intake through the Y1 or Y5 receptor. The hormone slows down the body's metabolic rate by decreasing energy expenditures so it slows weight loss or weight gain. Hypothalamic levels of NPY are increased in fasted animals, which indicate involvement with feed intake. If a substance stimulates feed intake, fasting should increase the levels of the hormone. Intracerebroventricular administration of orexins in the rat and mouse show that there is a increase in feeding behavior and fasting also increases the levels of orexins mRNA (Ueta et al., 2003). This observation is noted

to be short-term and no change was noticed in a 24-hr dietary intake or overall body weight.

Some evidence shows that orexin mRNA increases in response to restraint stress and cold stress. These results show that the central orexin system is related to physiologically related stress responses, such as CRH release (Ueta et al., 2003).

Growth Hormone

Growth Hormone—protein hormone

Growth hormone (GH), also known as somatotropin, is a protein hormone with approximately 190 amino acids as its structure. Somatotrophs are the cells that synthesize and secrete the hormone from the anterior pituitary of the brain. Growth hormone has two distinctively different effects: direct effects and indirect effects. GH has direct effects on fat tissue by stimulating triglyceride breakdown and suppressing uptake of circulating lipids. The indirect effects of GH are mediated by insulin-like growth factor-I (IGF-I) (Gorbman et al., 1983). IGF-I can also be produced locally in many tissues. IGF-1 is secreted from the liver and acts on target cells. The major role of GH is to stimulate whole body growth via IGF-1. The metabolic effects of GH include effects on protein, fat, and carbohydrate metabolism.

The production of GH is mediated by many factors including sleep, exercise, nutrition, and others. Growth hormone-releasing hormone (GHRH) is the primary stimulator of the peptide hormone. GHRH is a hypothalamic peptide that stimulates both the synthesis and secretion of GH (Neary et al., 2003). Another regulator of GH is

somatostatin and this inhibits GH release in response to GHRH and other factors such as low blood sugar. Ghrelin is a peptide hormone that binds to the receptors on somatotrophs to stimulate the secretion of somatostatin from the hypothalamus; if somatostatin is stimulated then GH will decrease (Neary et al., 2003). Ghrelin has now been identified as the growth hormone secretoagogue receptor ligand. Besides the pituitary and the hypothalamic areas of the brain that regulate GH release, the growth hormone secretoagogue receptor (GHSR) is expressed in the centers of the brain that control appetite, pleasure, mood, cognition, and biological rhythms (Sun et al., 2004). Ghrelin, as well as adenosine, have been identified as agonists for GHSR and administration of ghrelin and adenosine to rats stimulates feeding but only ghrelin stimulates the release of GH (Sun et al., 2004). It was assumed for a period of time that the GHSR was the only receptor for ghrelin but more evidence now shows that there is existence of receptor subtypes.

The generation of a *Ghsr*-null (-/-) mouse was done to understand more about the relationship between ghrelin and GHSR. By doing this, Sun and colleagues (2004) found that the well-characterized properties of acute ghrelin administration are its stimulatory type effects on GH release, adipose deposition, and appetite. Further, if GHSR is a relevant ghrelin receptor, it could be anticipated that *Ghsr*-null mice would show an anorexic dwarf phenotype, however they found that the appearance of the null mice could not be distinguished from that of the wild-type mice. The acute administration of ghrelin to normal (wild-type) animals stimulates the release of GH and to see if the effects of ghrelin on GH are mediated by GHSR, Sun and colleagues (2004) compared the effects of exogenous administration of ghrelin in normal vs. *Ghsr*-null mice. Their study found

that GH release was only detected in the normal mice as opposed to the *Ghsr*-null mice indicating that the biological effects of ghrelin on GH release are mediated by GHSR (Sun et al., 2004). Without GHSR, there was no GH release observed but the *Ghsr*-null mice grew comparative to that of the wild-type mice.

It is unknown if the GHSR agonists act through GHSR to stimulate GH release. Data shows that the activity of GHSR agonists does not depend on the expression of *Ghsr* by testing the stimulatory effect in *Ghsr*-null mice (Sun et al., 2004). The fact that ghrelin administration causes an acute increase in appetite and serum ghrelin is up-regulated during fasting suggests that ghrelin could be involved in fasting-induced hyperphagia in normal mice (Sun et al., 2004). Interestingly, their data show that serum ghrelin levels were elevated in *Ghsr* null mice also.

Stress

Stress and related peptides

Stress is defined as the sum of biological reactions to any adverse stimulus, physical, mental, or emotional that tends to disturb homeostasis. It has been known that various types of stress or stressors (causing stress) can cause appetite loss and further eating and behavioral disorders such as anorexia nervosa and bulimia. What is not known is the neural connection that is involved in the stress-induced changes that affect feeding behavior within the central nervous system (CNS). Accumulating evidence supports the notion that orexins and ghrelin may be involved in the stress responses via the CNS and

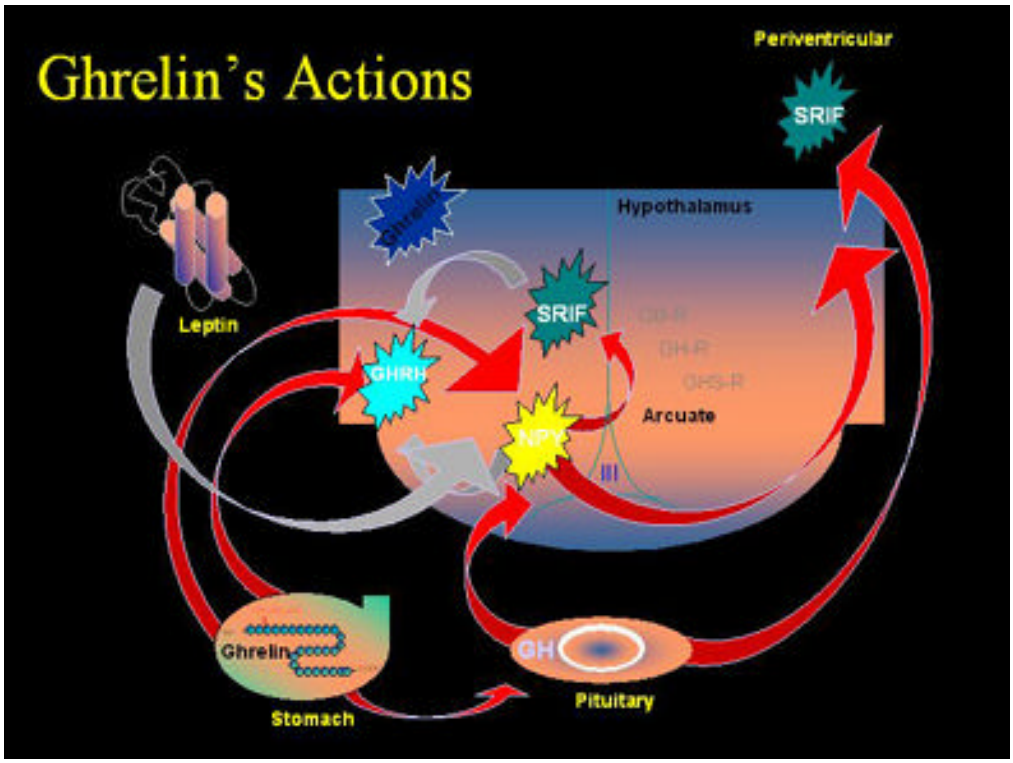
therefore affect feeding behavior (Ueta et al., 2003). Many studies have shown that neurotransmitters such as noradrenalin (NA), dopamine and serotonin, and stress-related hormones corticotrophin-releasing hormone (CRH) contribute to stress responses (Ueta et al., 2003). These neurotransmitters and/or stress-related hormones play a role at the level of the feeding and satiety center of the brain and can therefore stimulate or inhibit appetite.

The center of physiological response to stress is located on the hypothalamopituitary adrenal (HPA) axis. Production of CRH from the PVN causes the secretion of adrenocorticotropic hormone (ACTH) thereby causing the release of adrenal hormones such as glucocorticoids from the adrenal cortex. Urocortin, a stress related substance, also has feeding effects. Urocortin II (stresscopin-related peptide) and Urocortin III (stresscopin) have been proven to have a feeding inhibition effect (Ueta et al., 2003). Urocortin and CRH signal through the same receptors. CRH has been postulated to mediate both hormonal and behavioral responses to stressors as well (Ueta et al., 2003).

Oxytocin (OXT) administration inhibits feeding. Treatments have been done in order to promote OXT secretion (stress, peripheral administration, etc.) also tend be inhibitory towards feeding (Ueta et al., 2003).

The function of ghrelin during times of stress remains to be determined but a recent study has evidence to show that ghrelin augments ACTH release in response to stress secretion-inhibiting effect of leptin (Ueta et al., 2003). Since little is known about ghrelin, we studied its regulation in the pig relative to meal pattern.

Schematic representation of Ghrelin's actions



Imperial College London, School of Medicine 2004

MATERIALS AND METHODS

Animals

Twelve crossbred barrows (67.1 ± 4.5 kg BW) were used in our trial. Pigs were given water ad libitum and pelleted corn rations. Six pigs were randomly assigned to have continuous access to feed using a typical finishing diet and the remaining six barrows were allowed access to 2.73 kg of feed at 1200 each day and any remaining feed was removed at 1600. A typical finishing diet consists of crude protein, min-13.8%, crude fat, min- 5.8%, fiber, min- 5.0%, and salt, min-0.2% and max- 0.7%. All subjects were housed individually during the experiments. All procedures were done in accordance with North Carolina State University Animal Care and Use Committee approved research protocols. Weight was recorded on day zero, as well as on day 14 at the end of the trial.

Study Design

Beginning on day 0 of the experiment, the pigs were placed in individual pens and placed on their meal patterns. Catheters were threaded into the jugular vein to secure it into place and flushed with 5 mL of 3% sodium citrate to enable blood collection on day 7. Blood collection began on day 8 at 1000. Samples were taken every 15 minutes for 4 hours and every 30 minutes for the following 2 hours. Samples were placed in 6 mL vacutainer tubes containing sodium fluoride potassium oxalate. After every collection the catheters were flushed with 5 mL of the sodium citrate solution. These collections were done on day 8 and 9 of the trial to give the pigs a week to get accustomed to their diet and a day to rest from cannulation. Samples were centrifuged immediately and plasma was

collected and placed on ice until stored at -20°C for later assays. On day 11, glucose was administered intravenously at a dosage of 500 mg/kg BW to one half of the pigs and an equivalent volume of saline to the other half of the pigs, and the dosage was based on previous research done. One sample was collected prior to the glucose infusion and every 15 minutes until 1300. Samples were centrifuged and plasma was collected as stated above. Collection began at 1000 and ended 6 hours later. The pigs' meal schedule remained the same and no meal was omitted after the infusion. A circadian rhythm study was also performed beginning on day 11 after completing the glucose infusion collections. One sample per pig was taken every hour for 24 hours. Samples were centrifuged and plasma was collected after every sample just as the other days.

Radioimmunoassays

Ghrelin

Ghrelin was measured by RIA (Linco Research, Inc, St. Louis, MO), according to the manufacturer's directions. Active ghrelin standard preparation was done so by using serial dilutions into eight tubes on day one. This is a three day assay that requires incubation at 4°C overnight (22-24 hours) on day one and two of the assay. On day one of the assay the plasma from collected samples was added and on day two, 100 µL of trace (I^{125}) was added. On day three of the assay, the second antibody was added and then the tubes were centrifuged. A gamma counter was used to count the pellet. This RIA utilizes a double antibody, which is specific for the biologically active portion of ghrelin, which is the octanoyl group on the 3rd Serine. A standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated. The standard points covered from 7.81 pg/ml to

1000 pg/ml and the sensitivity of this assay shows that the lowest level of ghrelin that can be detected is 7.8 pg/ml when using a 100 µL sample size. This kit uses radiolabeled human ghrelin as the standard and tracers, which can be used for pig samples. The specificity of this test is <90% homology. Pig and human ghrelin differ by three amino acids at the 11, 22, and 26th position. Precision within and between the assays was found to be 8.6% (intraassay) and 9.8% (interassay)

Insulin

Insulin was measured using Coat-A-Count solid-phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA) designed to measure insulin in serum or plasma for multiple species. This RIA is a procedure where the labeled insulin competes for a fixed time with insulin in the sample for sites on insulin-specific antibody. The total incubation time for the assay is overnight (18-24 hours) using all the standards (A-G). The collected serum was placed in the coated tubes from the kit along with all the calibrators. Tubes were incubated at room temperature (15-28°C) for the allotted time of 18-24 hours. The standard curve points of the insulin assay measure from 5.0 µIU to 350 µIU. The sensitivity of the assays shows that 5 µIU is the lowest detectable level of insulin. The specificity finds that pro-insulin has 32% cross reactivity and C-peptide and glucagons are not detectable. Precision for the assay shows that the intraassay coefficient of variation is 5.8% and the interassay to be 9.2%. Tubes were decanted thoroughly to remove all visible moisture and counted in the gamma counter.

Statistical Analysis

Treatment effects were assessed using PROC GLM, PROC MIXED, and PROC TTEST using SAS statistical software (8.2) (SAS, Cary, NC 2000). The experimental unit for all measures was the individual pig as each pig was housed separately throughout the duration of the experiment. The fixed effects were time and treatment, while response was a random effect. Time by treatment interactions was also analyzed. The model statement used in SAS (8.2) was [response = treatment, time, day, and time by treatment interaction]. Least squares means were used to determine significance between treatment and control groups at specific time points. Asterisks in the tables and figures indicate statistical significance with a P value < 0.01 except Figure 2 where an asterisk represents a P value of < 0.001.

RESULTS

The initial stratification ensured that there was little variation between initial weights of the treatment and control groups (within treatment or control; Table 1). There was no difference in the initial weights of the barrows ($P<0.4885$). Over the 14-day study, ADG (average daily gain) was lower for the treatment group ($P<0.001$) (Table 1). The pigs in the treatment group had no feed remaining after the first day assigned to the treatment of restricting feed.

Ghrelin

Consistent with previous reports ghrelin was high before mealtime and leveled off afterwards. Plasma ghrelin concentrations increased (20%) ($P<0.01$) prior to feeding and decreased (20%) after feeding ($P<0.01$) relative to baseline (50 pg/ml) in the meal fed pigs (Figure 1, 2).

Day effect

Ghrelin levels were higher in the meal fed pigs in comparison to the ad libitum animals. This effect was more pronounced in the day nine samples vs. the day eight samples. The treatment effect was significant for day nine ($P<0.0001$) and slightly significant for the day eight experiment ($P<0.04$). Time 9 was the feeding point in our experimentation for the day samples. Ghrelin concentrations were higher in meal fed pigs before feeding and lower for most samples afterward. In the ad libitum group no such pattern was observed because we did not observe the ad libitum pigs at another time point.

Day 9 results show that treatment and time by treatment effect were both significant for the model ($P<0.0001$) on day 9 of sampling. Time by treatment effect shows significance from that of feeding (time 9) including: time 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20. This is shown in Figure 2. The two groups were also found to be significantly different from one another (34.3 pg/mL $\pm$ 12.3 vs. 77.0 pg/mL $\pm$ 19.6—meal fed vs. ad libitum respectively; $P<0.0001$). Ghrelin was higher in meal-fed on both days 8 and 9 although there was a day difference. Day 9 results show more of a marked response to the experimentation [meal pattern] (Figure 2) when compared to Day 8 (Figure 1).

Glucose challenge

Ghrelin concentrations were decreased after glucose infusion ($P<0.01$). Concentrations decreased by 40% after the initial infusion and then remained steady for approximately 2 hours post-infusion ($P<0.01$) (Figure 5).

The glucose challenge shows time explaining most of the variation ($P<0.0014$). There is no significant difference between the ad libitum vs. meal fed group ($P>0.1809$) for the glucose challenge results. There was an initial decrease of ghrelin ($P<0.01$) after the glucose response for a couple of hours with a rebound of ghrelin levels closer to the time of feeding at 1200.

Circadian Rhythm

The circadian rhythm samples show that time explained most of the variation ($P<0.0001$). The circadian data shows that the ad libitum vs. meal fed groups are not significantly different. Figure 3 shows these individual differences. In the circadian rhythm, peaks occurred at time points that are seen in Figure 3. Figure 3 also shows that times 0500, 0700, 1100 were significantly different when comparing ad libitum and meal

fed groups. Soon after feeding the circadian results do show a peak in ghrelin and then a decrease but no significant difference between the ad libitum and meal fed animals ($P<0.01$).

Insulin

Day effect

The GLM procedure shows that treatment, time, and/or treatment by time effect were not significant for the day 8-insulin concentrations. Ad libitum vs. meal fed animals were not significantly different ($P>0.1604$). For the day 8 samples of the insulin assay, time and response to treatment were both different ($P<0.0001$). Time 1 vs. time 20 (1000 vs. 1530 sample) tended to be different ($P<0.053$) for the effect of time. The treatment by time effect for the control and treatment was different ($P<0.01$) at specific points for the day 8 samples (Figure 4). In the treatment group, time 1 and time 2 were significantly different ($P<0.0009$). For the day 9 sampling, treatment and time were significantly different from one another. The time of sampling had influence over the study ($P<0.01$) and the treatment that the pig was placed on had significance as well ($P<0.01$) (Figure 8). This caused variable insulin concentrations where insulin did increase after feeding in the meal-fed group more so than the ad libitum animals (Figure 4). Day 8 was not different from day 9 in insulin concentration ($P>0.58$). Analogous to the ghrelin data, the day by treatment effect is significant ($P<0.0148$). Treatment by time also had an effect for the insulin data, but more so than the ghrelin data ($P<0.0009$). Results also show that the treatment (meal fed or ad libitum) was not significant ($P<0.8135$).

Glucose challenge

Time, treatment, and time by treatment interaction all showed significance for the glucose challenge ($P<0.0001$). Data for the effect of time shows significance between time 1 (pre-infusion; 0800), time 4 ($P<0.0048$; 0845), and time 13 (last sample; 1100), ($P<0.0096$). Glucose was higher in the ad libitum animals after the glucose infusion and also peaked after time 9, which was 1000 during the challenge. The effect of time also shows significance between the 2 groups ($P<0.0001$) for the glucose challenge. The sample taken before the infusion had low concentrations of insulin; after the glucose infusion, levels rose in both groups ($P<0.01$), but in ad libitum more so than in the meal fed animals. Also, after time 9 (1000) the ad libitum pigs had another rise in insulin that was different from that of the meal fed animals again ($P<0.01$).

Table 1				
Pig #	**Weight (before) kg**	**Weight (after) kg**	**Kg gained**	**ADG (kg)**
Treatment Average	*68.7*	*72.8*	*5.9*	*0.5*
28711	*73.1*	*77.3*	*4.2*	*0.3*
28208	*77.9*	*	*	*
28809	*65*	*68.6*	*3.6*	*0.3*
28512	*61.8*	*70*	*8.2*	*0.6*
27510	*67.7*	*75.5*	*7.7*	*0.6*
28712	*66.4*	*	*	*
Control Average	65.6	76.6	12.2	0.9
28408	76.4	82.1	12.7	0.9
28706	51.1	62.7	11.6	0.8
28407	70	80	10	0.7
28406	63.2	80	16.8	1.2
28710	65.9	80	14.1	1
28908	67.3	75	7.7	0.6

Table 1. Mean weight (kg) of pigs before and after the 14-d trial and average daily gain (ADG). * represents animals that did not complete the trial. Standard error (SE) was 4.9 for all treatments and 3.2 for controls. ADG was significantly different between treatment animals and controls ($P<0.001$).

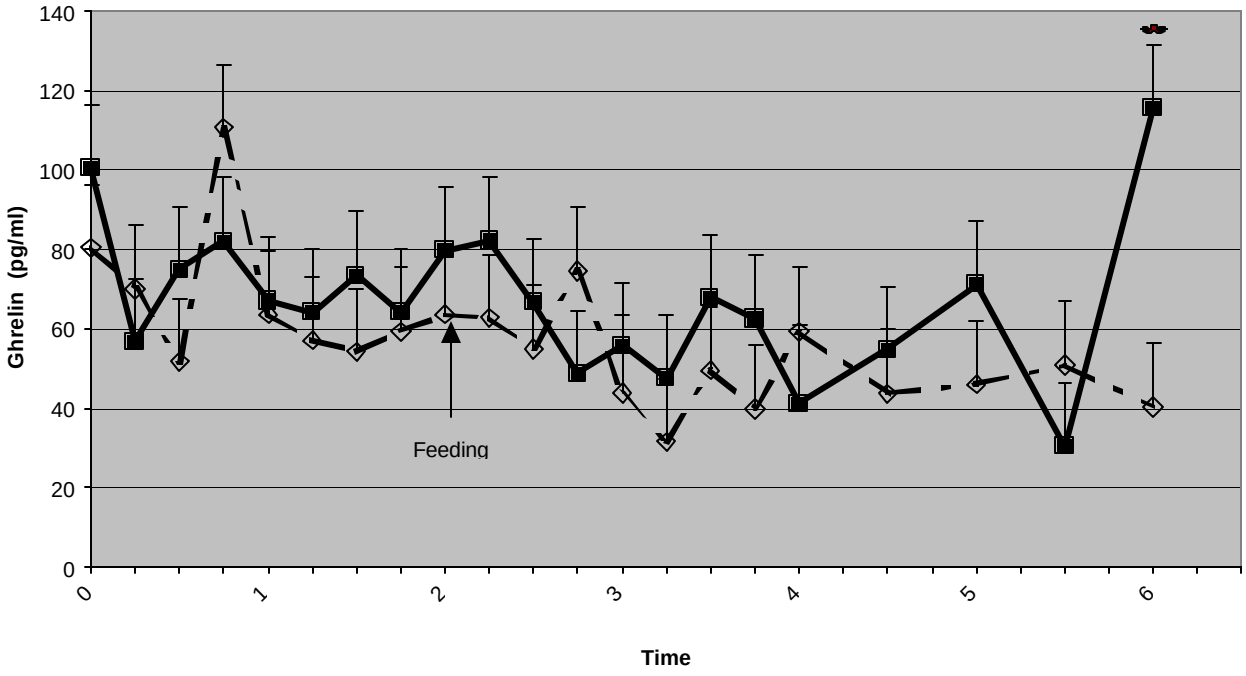


Figure 1. Day 8 sampling, ghrelin response (pg/ml) in either meal fed (T; closed squares) or ad libitum (C; open diamonds) groups Time is given in 15 minute increments and the last four samples were taken 30 minutes apart. Data represent mean + SEM; *, $P<0.01$, indicates where meal fed animals differ from the ad libitum animals

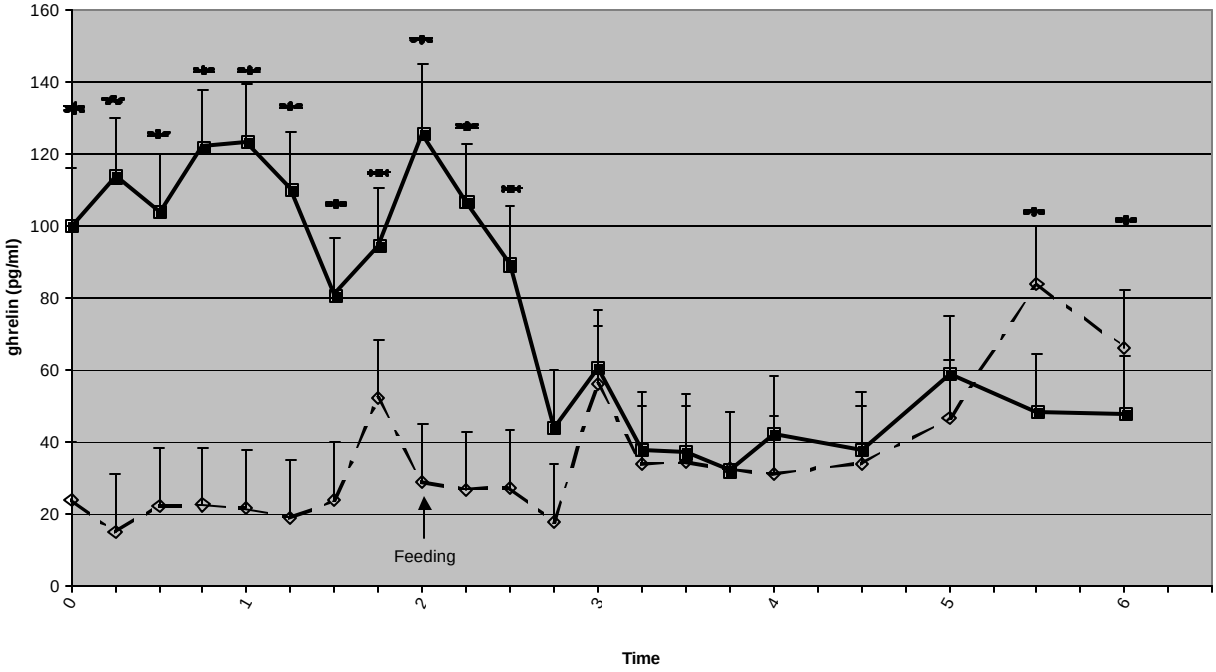


Figure 2. Day 9 sampling, ghrelin response (pg/ml) in either meal fed (T; closed squares) or ad libitum (C; open diamonds) groups Time is given in 15 minute increments and the last four samples were taken 30 minutes apart. Data represent mean + SEM; *, P<0.001, indicates where meal fed animals differ from the ad libitum animals

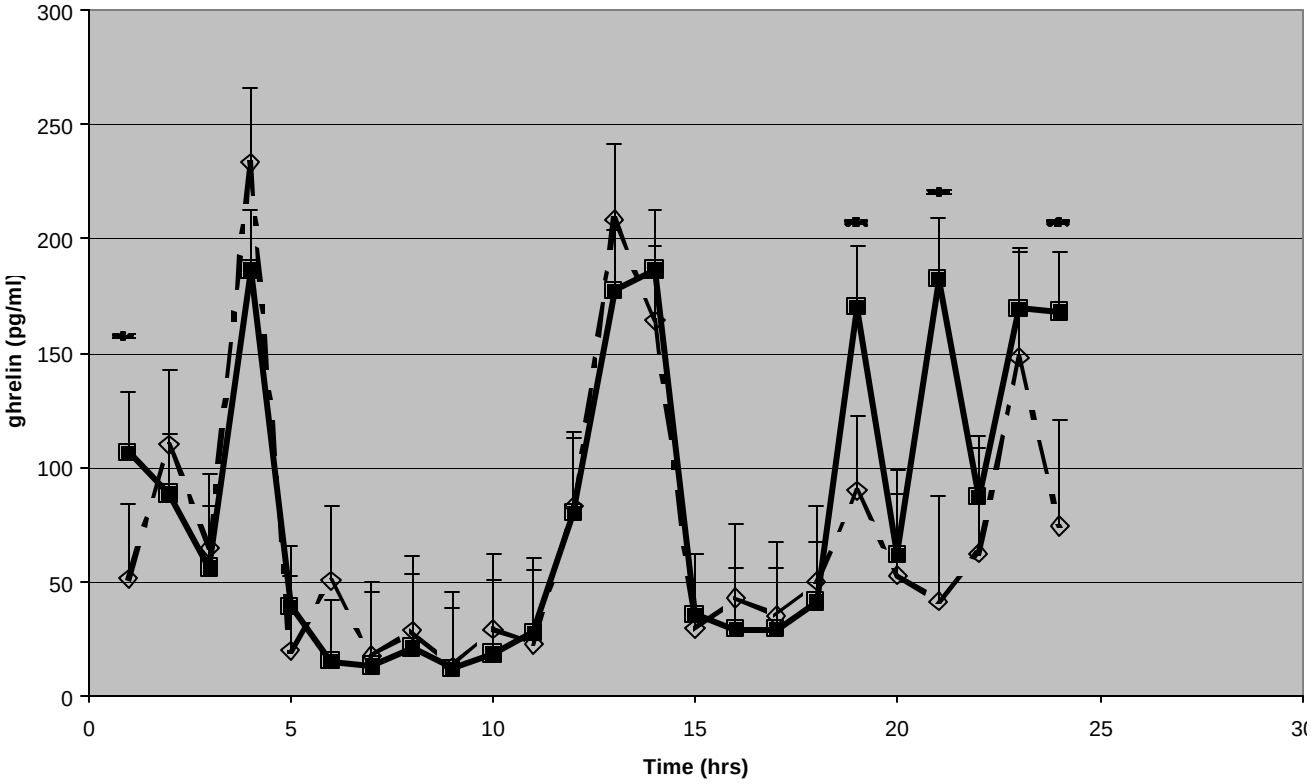


Figure 3. Ghrelin response from circadian rhythm samples taken once an hour for 24 hours beginning at 1100 in either meal fed (T; closed squares) or ad libitum (C; open diamonds) groups. Data represent mean + SEM; *, $P<0.01$, indicates where meal fed animals differ from the ad libitum animals

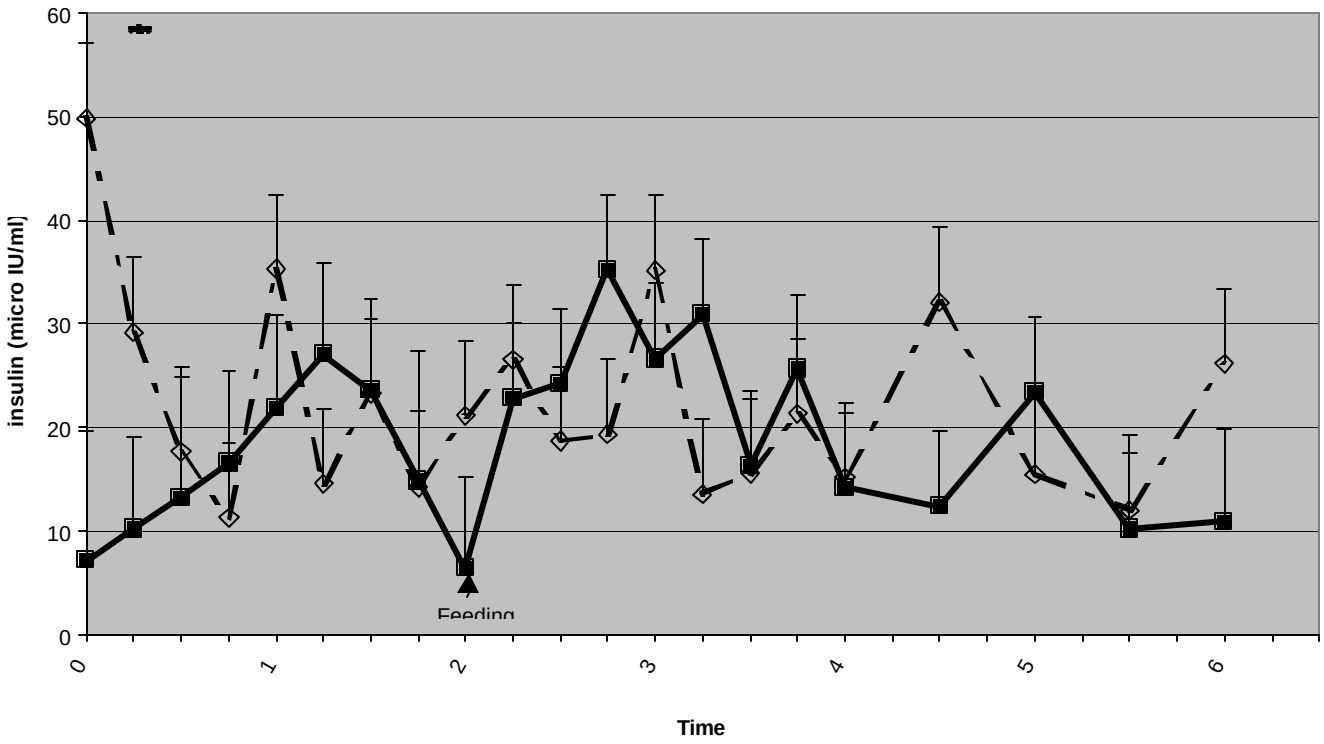


Figure 4. Day 8 sampling, insulin response (µIU) in either meal fed (T; closed squares) or ad libitum (C; open diamonds) groups. Data represent mean + SEM; *, P<0.01, indicates where meal fed animals differ from the ad libitum animals

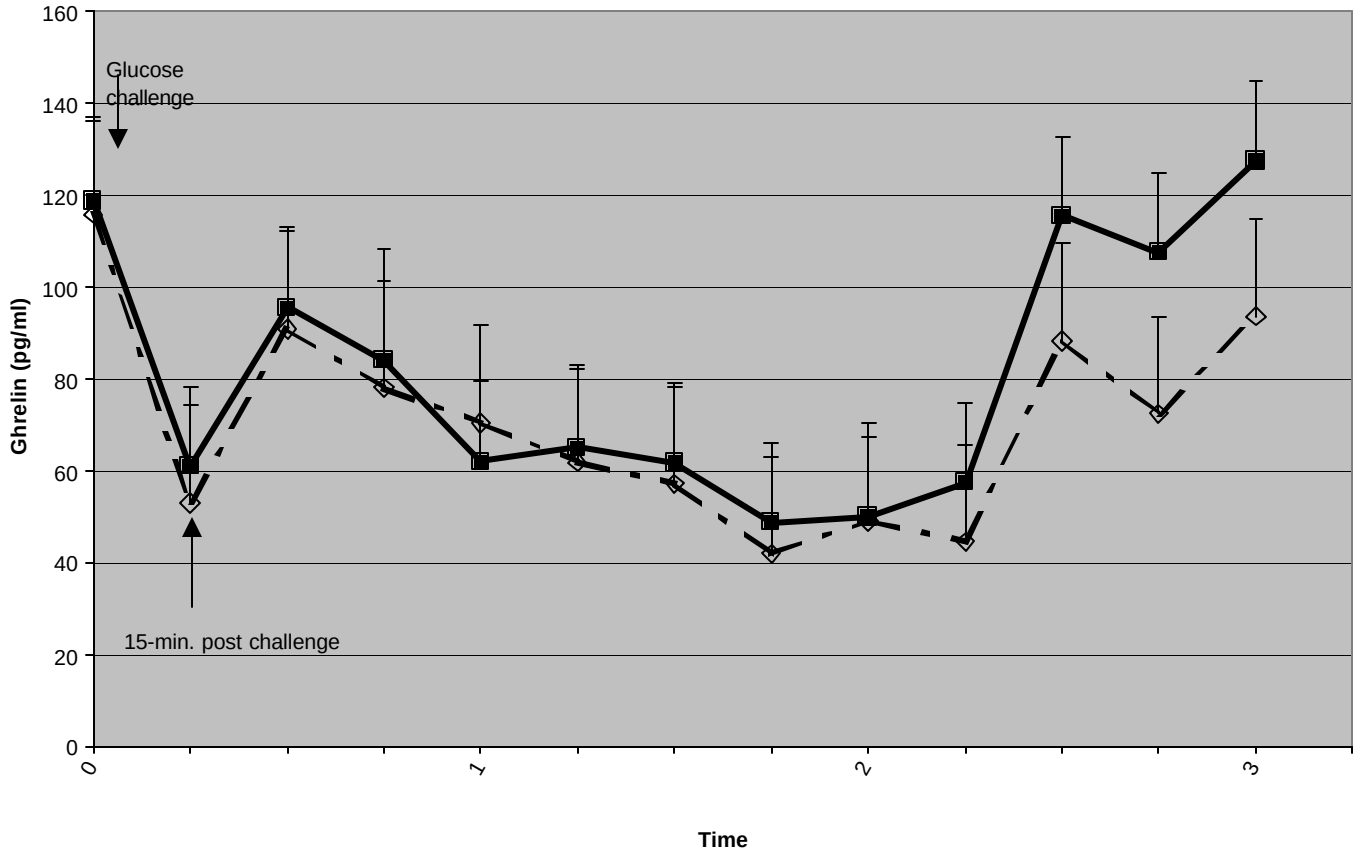


Figure 5. Glucose challenge and ghrelin response (pg/ml). Ghrelin response in either meal fed (T; closed squares) or ad libitum (open diamonds). Time increments are every 15 minutes with a glucose challenge given immediately following collection of the first sample. The challenge began at 0800 and ended at 1100.

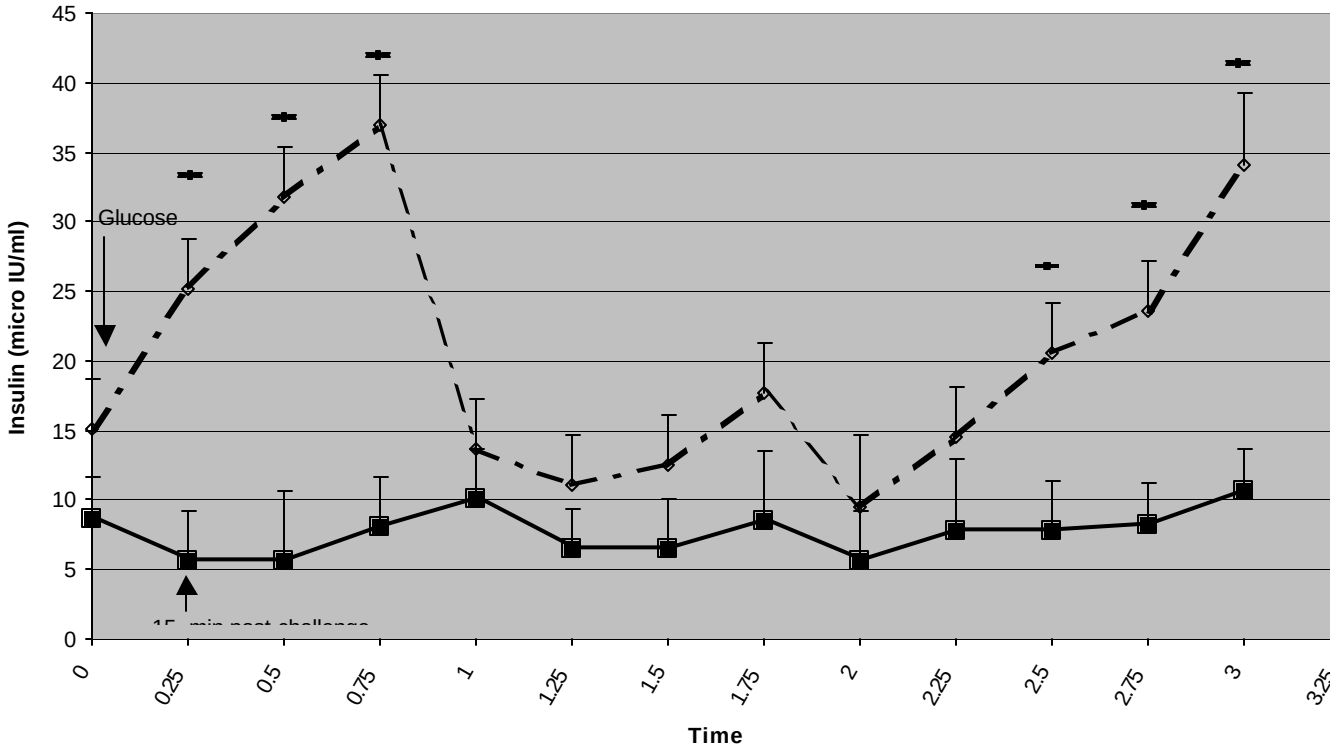


Figure 6. Glucose challenge and insulin response (µIU/ml). Insulin response in either meal fed (T; closed squares) or ad libitum (open diamonds). Time increments are every 15 minutes with a glucose challenge given immediately following collection of the first sample. The challenge began at 0800 and ended at 1100.

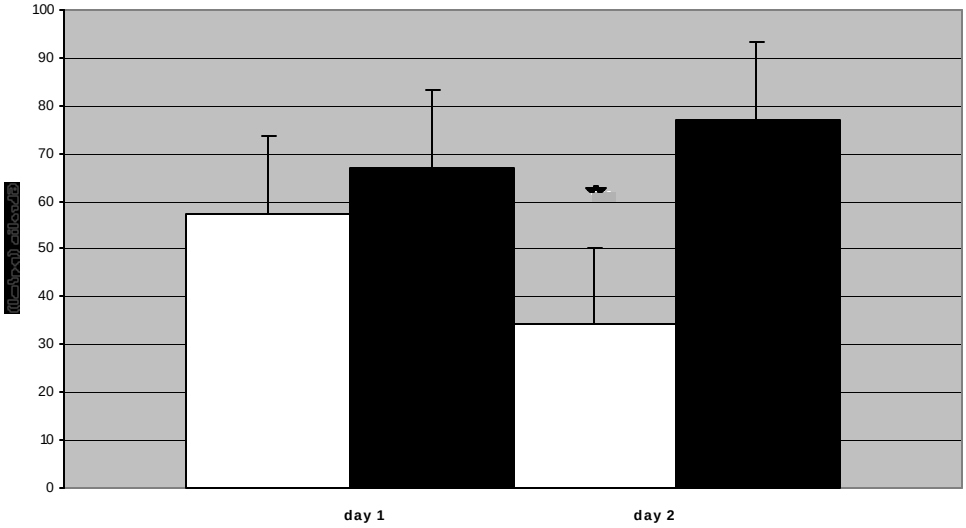


Figure 7. Ghrelin response (pg/ml) in day 8 and day 9 sampling. Day 8 and 9 values are an average of the ad libitum (white bars) or meal fed (black bars) animals. White bars represent ad libitum animals and black represents the meal-fed animals. *, ($P<0.01$) shows significance.

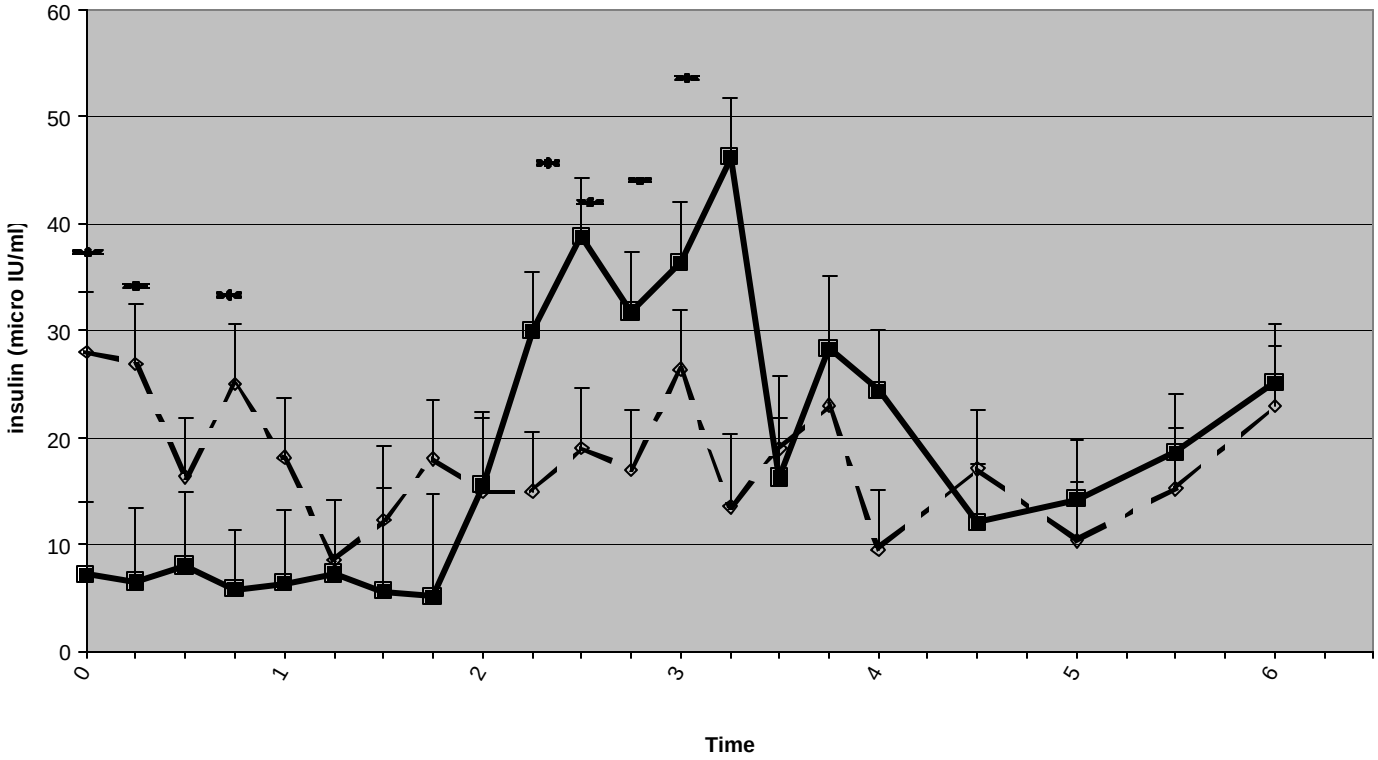


Figure 8. Day 9 sampling, insulin response (μIU/ml) in either meal fed (T; closed squares) or ad libitum (C; open diamonds) groups Time is given in 15 minute increments and the last four samples were taken 30 minutes apart. Data represent mean + SEM; *, P<0.01, indicates where meal fed animals differ from the ad libitum animals

DISCUSSION

Based on the weight data, the treatment pigs (restricted diet) could have possibly been fed more than 2.72 kg/day. The control animals gained double the amount of weight that the treatment group did and all pigs were close in initial weight before the study began. The control animals consumed ~ 4.5 kg/day eating ad libitum. It had been planned to feed the groups similar amounts but we underestimated feed consumption in the control pigs. The difference between groups was not only in the pattern of feeding but also in the amount.

Ghrelin

Day effect

We found that the samples from day 1 and day 2 samples were significantly different from one another, although, ideally, conditions would remain the same 24 hours after the day 1 sampling occurred. We conclude that variation could be due to changes in the eating, or because the day 2 samples were retrieved 24 hours after the first days samples. The variation could also be attributed to a disruption to their routine on the first day of sampling. At the end of the 14-day study, ghrelin assays were performed. As we would expect when comparing day 1 and 2, treatment explains most of the variation. Our results, conclusive with other data (Saad et al., 2004), show that ghrelin rises just prior to a meal and falls afterwards. Day 2 shows that ghrelin levels in the meal fed animals were markedly higher at many time points throughout the sampling. The controls were lower due to the fact that they were not eating and were supposedly full. This is consistent with the idea that ghrelin is high when the animals are hungry. We may have seen more

conclusive results with our day 2 sampling because the day 1 samples may have thrown the animals off of their routine

Glucose challenge

There remains to be a dispute as to the relationship between ghrelin and glucose (Briatore et al., 2003), so we studied ghrelin levels after glucose infusions and found that ghrelin levels decreased after infusion. During the experimental conditions (restricted feed), ghrelin levels tended to depend on food intake with preprandial elevations and suppressed postprandial levels. Based on the day one and day two samples, ghrelin had a markedly higher response in the treatment group further securing the notion that restricted feed causes a rise in ghrelin (Misra et al., 2004, Saad et al., 2004). Another observation with the glucose challenge data is that after the glucose infusion, ghrelin concentrations decreased with a rebound a couple of hours later, suggesting that ghrelin again rises as feeding time approaches.

Circadian Rhythm

One of the main observations is that there is a distinct circadian rhythm in circulating ghrelin where the peptide hormone is high during the evening hours and treatment subjects gave higher responses of ghrelin. The rise in the evening could be explained by the fact that the ad libitum animals' feeders were refilled and they were eating so the meal-fed animals observed this since they were housed together. The circadian rhythm might have correlation with other hormones, namely an inverse correlation with serum cortisol levels (Espelund et al., 2004). Stress hormones, such as cortisol, could have a contributory role in physiological reactions (i.e. appetite, blood pressure, and metabolism) [Ueta et al., 2004]. There is evidence that ghrelin promotes

feeding via hypothalamic activity and is conversely increased by hunger or anorexia nervosa (Ueta et al., 2004).

Insulin

Insulin has previously been reported to suppress ghrelin levels (Cummings et al., 2003, Konturek et al., 2004, Saad et al., 2004, Murdolo et al., 2003, Anderwald et al., 2003). Our insulin data show that the pancreatic hormone could be a physiological modulator of ghrelin levels. It is unclear whether insulin is acting directly or indirectly.

Day effect

Based on the insulin data collected from the day one samples, the ad libitum animals had random fluctuations of insulin in comparison to the treatment animals, which was expected. The day two results point out the same pattern with control animals' insulin levels continuing to fluctuate. The insulin response from the meal-fed group was more marked in the day two samples with more difference between the two groups. For example, at the time of feeding, the meal-fed animals are significantly different from the controls on day two, where there are not on day one. We show that restricting feed gives a more marked insulin response to feeding when compared to ad libitum feeding. When comparing day one and two, there was a day by treatment effect, which shows us that the day of sampling along with whether the animal is meal-fed or ad libitum is significant to our study.

Glucose challenge

Based on our findings, it is clear that insulin rises postprandially as ghrelin levels fall, yet it continues to be unclear whether it is the glucose increase or the rise in insulin in response to the glucose that could elicit the ghrelin response; ghrelin levels notably

suppressed. Saad and colleagues (2002) report that insulin injections suppress ghrelin secretion as long as the injections are continued but had a rapid rise to near-basal levels within 60 minutes of discontinuation. Our treatment was significant in that the control and treatment group differ from one another.

The mechanism by which insulin has an inhibitory effect on ghrelin cannot be explained by our study. We can only note that restricting feed gives less of a response to glucose and therefore less insulin response than ad libitum fed animals. Insulin could have a direct effect on the ghrelin-producing cells that results in lower ghrelin levels. Contradictory reports (Caixas et al., 2002) show that subcutaneous (s.c) administration of insulin analogues did not affect plasma ghrelin levels. Schaller and colleagues (2003) did not find a role of insulin at physiological concentrations in the regulation of plasma ghrelin, although they did not study periods shorter than 30 min. In contrast to Schaller's study (2003), other studies using hyperinsulinaemic clamp techniques reported that insulin infusion reduces ghrelin concentration and the inhibitory effects seem independent of glucose concentrations (Saad et al., 2002, Mohlig et al., 2002). Saad and colleagues (2002) reported that insulin is a physiological and dynamic modulator of ghrelin and that insulinemia possibly mediates the effect of nutritional status on its concentration. Our results suggest that glucose mediates the effect of nutrition on ghrelin levels directly and ghrelin levels are inversely proportional to insulin and not necessarily directly correlated to insulin. Briatore and colleagues (2003) state that it is possible that a more sustained or protracted hyperinsulinemia, rather than a short-lived burst like the early insulin response, is required for the inhibition of plasma ghrelin. In this study, it was possible for them to observe the effect of hyperglycemia alone on plasma ghrelin

because they used diabetic subjects as their treatment group. Type 2 diabetics were used in their study because of the early insulin response to i.v. glucose is abolished (allows the discrimination of glucose effects). Under these conditions, Briatore and colleagues (2003) observed that in Type 2 Diabetes Mellitus (T2DM) subjects a significant reduction in plasma ghrelin after i.v. glucose bolus suggests that hyperglycemia could possibly play a role in ghrelin regulation. Our findings tend to agree more with this conclusion than to say that insulin definitively has direct effects on ghrelin. Although parenteral nutrient and/or insulin infusions are able to suppress ghrelin levels when administered for prolonged periods or at supraphysiological doses in rats and humans (Saad et al., 2002, Flanagan et al., 2003), physiological doses that mimic post-meal fluctuations do not affect ghrelin in humans (Caixas et al., 2002). In contrast to this idea, enteral nutrition consistently suppresses ghrelin levels in humans and rodents, even at lower doses (Overduin et al., 2004, Caixas et al., 2002, Cummings et al., 2001). The decline in insulin in the meal-fed animals gives a rise in their respective ghrelin levels, which postprandially decrease due to the rise in insulin after a meal. Cummings and colleagues (2001) also observed the postprandial suppression of ghrelin and also reported that insulin suppresses ghrelin levels (Cummings et al., 2003, Konturek et al., 2004). Overduin and colleagues (2004) reported that it is unlikely that solely circulating glucose and insulin drives nutrient-related ghrelin suppression, although these could be factors to contribute to the response in ghrelin levels. These findings were based on the observation that, regardless of the macronutrient type infused into their subjects, ghrelin levels remained suppressed long after blood glucose and insulin levels had returned to baseline.

Some studies suggest the idea that insulin could play a role in regulating body weight through a direct effect, but less pronounced in Type II diabetics (Anderwald 2002). In addition to regulating ghrelin, insulin could modulate leptin effects to increase adiposity. Ghrelin stimulates appetite to increase weight gain (Nakazato et al., 2001) while leptin inhibits food intake.

Ghrelin levels have also been studied in other species, including ruminants. Sugino and colleagues (2002) report that there is a transient surge of ghrelin secretion before feeding is modified by different feeding regimens in sheep. The study was intended to modify ghrelin levels by affecting feed regimens in the ruminant species.

CONCLUSIONS

One of the goals of swine producers is to optimize feed efficiency in the herd. Many factors can compromise these goals such as undernutrition, stressors, and others. The pig is a complex mammal and feeding behavior is regulated by many integrated systems. In an attempt to understand the physiological mechanisms within the animal, our focus cannot be limited to just one system or another. Also, as these animals are used for experimental models for other species, such as the rodent and human, physiology becomes even more relevant. Although our study focused on the swine species, some of our findings could be relevant to the human community, such as diabetic patients.

In our current study, we examined the effects that meal pattern has on secretion of ghrelin and insulin in pigs. Although we did not study the direct route that the change in meal pattern has within the body, we studied the hormonal consequence to give us more understanding of internal effects. We hoped that level of feed intake would be more similar in the two groups. It is not possible to definitely state that differences between groups were due to meal pattern since overall intake was different. However results in the meal-fed group did show hormonal patterns similar to those reported in other species. We did find a relationship between ghrelin and hunger, in that ghrelin concentrations tended to decrease after meals, as previously documented but also that glucose tends to suppress ghrelin in restricted fed animals. We cannot conclude that insulin directly suppresses ghrelin but only that restricting feed causes less fluctuation in insulin levels and that there was less of an insulin response to feeding in meal fed animals. Our results show the

important relationship between the appropriate amount of feeding and subsequent hormonal concentrations.

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