

**UNRAVELLING THE MECHANISM OF GHRELIN SECRETION AND THE
EFFECTS OF GHRELIN REDUCTION USING A RECEPTOR DECOY
APPROACH**

by

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DALHOUSIE UNIVERSITY
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ABSTRACT

The incidence of obesity, and the associated morbidities and mortality are increasing. Strategies to manage this disease hinge on the balance of caloric intake and energy expenditure. This regulation depends largely on endocrine input from the periphery. The recently discovered stomach derived hormone, ghrelin, has emerged as a key player in the regulation of appetite and energy storage. Ghrelin achieves these functions through binding the ghrelin receptor in appetite regulating neurons and in peripheral metabolic organs including the pancreas and adipose tissue. Since ghrelin acts on energy regulating metabolic organs, its secretion from the stomach is tightly coupled to energy availability. Ghrelin levels increase during periods of fasting and decrease after a meal is consumed. Under chronic energy surplus (such as obesity) ghrelin levels decrease while in chronic energy deficit (anorexia nervosa, weight loss) ghrelin levels increase.

While major advances have been made in understanding both the function of ghrelin and its dysregulation in disease, little is known about the cellular regulation of ghrelin secretion. This is due to the lack of cellular models of ghrelin secretion. In this thesis, I describe the development of a novel ghrelin secreting primary rat stomach cell culture. Using this system I elucidated the roles and mechanisms of neurotransmitters, hormones (insulin and glucagon), nutrients (glucose) and anti-diabetics (metformin) in the regulation of ghrelin secretion. These findings have clearly demonstrated the ability of ghrelin cells to sense energy availability and provide important insights for ghrelin altering therapies. To evaluate both the function of ghrelin and the feasibility of reducing circulating ghrelin, I developed a novel *in vivo* ghrelin-reducing strategy. *In vivo* expression of a decoy protein based on the ligand binding domains of the ghrelin receptor was expressed in mice. Mice treated with this plasmid construct had reduced circulating levels of ghrelin. Interestingly, reduced circulating ghrelin was protective from high fat diet-induced obesity and resulted in improved glucose metabolism. This work demonstrates both the importance of ghrelin in peripheral energy storage and the feasibility of this novel ghrelin reducing approach for the treatment of obesity and insulin resistance.

LIST OF ABBREVIATIONS USED

ACTH	Adrenocorticotropin hormone
AG	Acylated ghrelin
AgRP	Agouti related protein
AMPK	Adenosine mono-phosphate kinase
AN	Anorexia nervosa
ARC	Arcuate nucleus
BBB	Blood brain barrier
BMI	Body mass index
CART	Cocaine amphetamine regulated transcript
CCK	Cholecystokinin
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
DIO	Diet-induced obesity
fMRI	Functional magnetic resonance imaging
FSH	Follicle stimulating hormone
GH	Growth Hormone
GHRH	Growth hormone releasing hormone
GHSR	Growth hormone secretagogue receptor (Ghrelin receptor)
GI	Gastrointestinal
GLP-1	Glucagon like peptide-1
GLP-2	Glucagon like peptide-2
GnRH	Gonadotropin releasing hormone
GLUT2	Glucose transporter 2

GLUT4	Glucose transporter 4
GOAT	Ghrelin octanoyl acyl transferase
HFD	High fat diet
ICV	Intracerebroventricular
IGF-1	Insulin-like growth factor-1
IRec	Insulin receptor
IRS	Insulin receptor substrate
KO	Knockout
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
MCH	Melanocortin hormone
α MSH	α -Melanocyte stimulating hormone
NPY	Neuropeptide Y (tyrosine)
NTS	Nucleus tractus solitarius
NE	Norepinephrine
PC1	Proprotein convertase 1
PI3K	Phosphoinositide 3-kinase
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus
PYY	Peptide YY (tyrosine tyrosine)
TRH	Thyroid releasing hormone
TSH	Thyroid stimulating hormone
UAG	Un-acylated ghrelin
VTA	Ventral tegmental area

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CHAPTER 1: INTRODUCTION

1.1 Obesity, Co-morbidities and Energy Balance

According to the World Health Organization (WHO), obesity is responsible for at least 2.8 million deaths worldwide due to linked diseases including diabetes, cardiovascular disease and cancer (WHO 2012). The WHO defines obesity as having a body mass index (BMI; height in cm/weight in kg²) of over 30 and overweight as having a BMI over 25 (WHO 2012). In Canada, the most recent measured data indicates that over 60% of the adult population is overweight or obese (Canada 2012). Furthermore, a recent study conducted by Statistics Canada indicated that over 30% of Canadian children are now overweight or obese (Karen Robert 2012). While the development of obesity is complex and has many contributing factors (psychological, social and genetic), in the simplest sense weight gain occurs when the amount of calories ingested exceeds the amount of calories used (largely through basal cellular metabolism, physical activity, and brown adipose tissue thermogenesis) (Figure 1). Therefore, current non-surgical strategies aimed at reducing weight target both food intake and energy expenditure. An understanding of the physiological mechanisms responsible for coordinating energy balance is vital in developing treatments for obesity and its co-morbidities.

Since the mid 1990's, with the discovery of the anorexic hormone leptin (Zhang, Proenca et al. 1994), a major focus on the molecular mechanisms regulating energy balance has taken place (reviewed in (Flier 2004)). The consensus is whole body

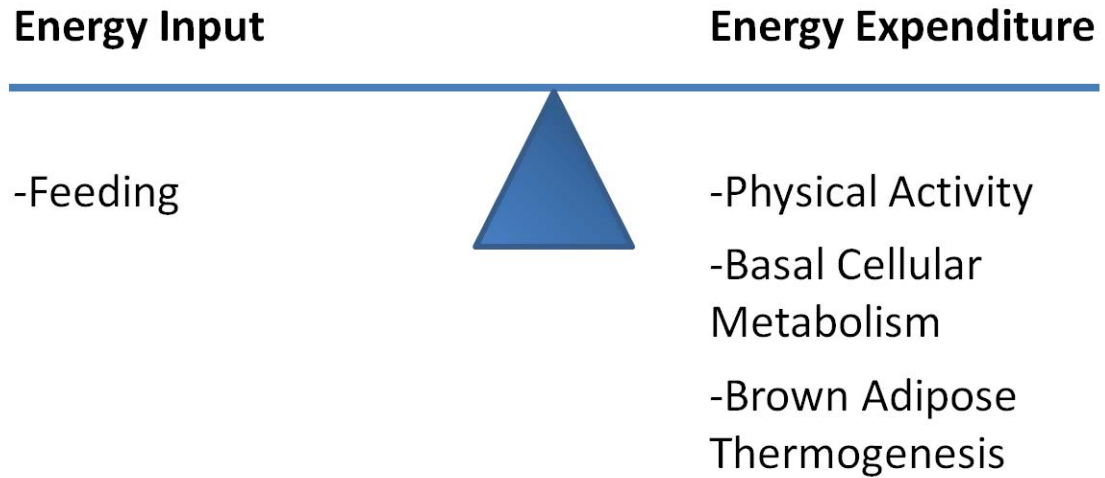


Figure 1 Energy balance schematic. Sources of energy input are balanced by sources of energy expenditure

energy status is communicated from peripheral metabolic organs (including the stomach, intestine, pancreas and adipose) to energy-regulating regions in the hypothalamus (Flier 2004). The importance of peripheral energy sensing on body weight are exemplified by the development of obesity in individuals that lack the anorexic hormone leptin (Farooqi, Jebb et al. 1999), or in individuals with Prader-Willi Syndrome who have elevated levels of the orexigenic hormone ghrelin (Cummings, Clement et al. 2002). Furthermore, dysregulation in either the production, or the targeted signalling of energy regulating factors during obesity, prevents the normal balance in the regulation of appetite and metabolism (Levin 2006). To fully understand the importance of peripheral and central energy regulation, a detailed description of these systems is required.

1.2 Central Energy Regulation

The brain coordinates energy regulation by both hedonic and homeostatic means. The former occurs through the cortical-limbic area containing the reward circuitry associated with eating. The latter occurs mainly through the hypothalamus and brain stem to interpret signals from the periphery on energy availability.

1.2.1 Hedonic/Reward Based Eating

If humans only consumed food based on homeostatic needs, the development of obesity would be unlikely. The drive to consume calorie rich palatable foods, while known to increase under energy deficit, often still persists after caloric requirements have been met (reviewed in (Erlanson-Albertsson 2005)). A study comparing various high fat

and sugar diets in rats demonstrated that even in the presence of the post-meal homeostatic cues, animals still continue to consume high fat and sugar food (la Fleur, van Rozen et al. 2010). This suggests that the hedonic aspects of consuming palatable foods play an important role in food intake. The pathways associated with food rewards are now beginning to be understood. Many of the same neurotransmitters that are associated with pleasure and addiction, such as dopamine (Radhakishun, van Ree et al. 1988) and opioids (Gosnell and Majchrzak 1989), are implicated in food reward. Supporting this, antagonists of opioids have been demonstrated to reduce palatable food choices in humans (Yeomans, Wright et al. 1990). However, while opioid knockout mice (β -endorphin and enkephalin) have a reduced drive to acquire palatable food in a food reward challenge setting, this difference is lost when the animals are fasted (Hayward, Pintar et al. 2002). This finding suggests that hedonic appetite regulation may not be as relevant in a deprived state.

One of the key regions of the brain involved in reward based eating appears to be the nucleus accumbens. Injection of opioid agonists in this region causes rats to increase consumption of high fat diet (Zhang and Kelley 2000). In contrast, injections of opioid antagonists in this region reduces the consumption of high sucrose solution without affecting normal chow consumption in rats (Kelley, Bless et al. 1996). While the downstream signalling from this region are not fully understood, some evidence suggests that GABAergic neuron projections from the nucleus accumbens to the lateral hypothalamus may be responsible for mediating the effects on appetite and food preference (Stratford and Kelley 1999).

1.2.2 Homeostatic Eating

The central nervous system (CNS) has several mechanisms and locations by which it can sense energy availability and respond with either a stimulation or inhibition of appetite. The communication between the periphery and the homeostatic appetite regulating brain regions, including the hypothalamus and brainstem, ensure energy levels are maintained at an appropriate level.

1.2.3 The Role of the Hypothalamus

The hypothalamus is structurally small (roughly the size of an almond in humans), but it secretes a large amount and variety of neuropeptides. The axis between the hypothalamus, the pituitary gland and the target organs has been thoroughly studied and plays a vital role in coordinating growth, reproduction and whole body metabolic function. This is achieved through the release of thyrotropin releasing hormone (TRH), growth hormone releasing hormone (GHRH), corticotropin-releasing hormone (CRH), and gonadotropin releasing hormone (GnRH) from the hypothalamus. Subsequently, TRH stimulates thyroid stimulating hormone (TSH) release from the anterior pituitary, which then activates thyroid hormone production (T4 and T3) from the follicular cells of the thyroid gland. Thyroid hormone activates many cellular pathways associated with energy consumption and catabolism. GHRH stimulates growth hormone (GH) release by the somatotroph cells of the pituitary. GH then stimulates the production of insulin-like growth factor-1 (IGF-1) from hepatocytes, which activates anabolic pathways like muscle and bone development. CRH stimulates the release of adrenocorticotropic hormone

(ACTH) from the corticotrophs of the anterior pituitary. ACTH then stimulates the production of glucocorticoids from the adrenal cortex, which will promote the production and availability of glucose. GnRH stimulates the production of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the gonadotrophs in the pituitary. These will then target sex organs in both men and women and are required for reproduction and sex hormone production.

From the above description of the hypothalamic-pituitary-target organ axis, it is clear that the hypothalamus plays a critical role in the maintenance of growth, reproduction, and energy metabolism through activation of secondary pathways. Direct evidence for an energy regulatory component within the hypothalamus came over 80 years ago when Hetherington *et al.* discovered that inducing lesions in the hypothalamus of rats led to obesity (Hetherington and Ranson 1939). Later research showed that the hypothalamus contains several distinct regions (nuclei) that are involved in energy balance. In addition, the hypothalamus is well suited to interpret circulating indicators of energy status due to its proximity to the median eminence (a region devoid of the blood brain barrier (BBB)). As such, the hypothalamus is considered a circumventricular organ and receives direct input from circulating hormones and nutrients (reviewed (Cone, Cowley et al. 2001)).

The arcuate nucleus (ARC) is the primary region of the hypothalamus involved in energy regulation. It contains two distinct populations of neurons that have opposing functions on appetite. One group expresses neuropeptide-Y (NPY) and Agouti-related protein (AgRP), which are known to stimulate appetite (Stanley, Kyrkouli et al. 1986) and reduce energy expenditure (Billington, Briggs et al. 1991). The other group of

neurons produces α -melanocyte stimulating hormone (α -MSH) and cocaine and amphetamine regulated transcript (CART), which are known to inhibit appetite (Fan, Boston et al. 1997; Lambert, Couceyro et al. 1998) and stimulate energy expenditure (Pierroz, Ziotopoulou et al. 2002). α -MSH is derived from the precursor proopiomelanocortin (POMC); therefore, these neurons are often referred to as POMC neurons. The contrasting roles of NPY and POMC neurons serve to maintain energy levels in both situations of low and high energy availability. Cross-talk is also present between both the orexigenic and anorexigenic neurons in the ARC, as NPY administration has been demonstrated to cause hyperpolarisation (inactivation) in POMC neurons (Roseberry, Liu et al. 2004). Once activated, projections from neurons within the ARC can activate other “second order” hypothalamic neurons in the paraventricular nucleus (PVN) and lateral hypothalamus (LH). In support of this, injection of NPY or α -MSH into the PVN of rats was demonstrated to stimulate or block feeding respectively (Cowley, Pronchuk et al. 1999). Furthermore, neural projections from the ARC were also found to innervate the lateral hypothalamus (LH), which contains appetite-stimulating orexin and melanin-concentrating hormone (MCH) containing neurons (Elias, Saper et al. 1998). These neurons send projections throughout the brain including autonomic pre-ganglionic nerves, which may be a key region in mediating the output of energy regulation from the brain to the periphery (Elias, Saper et al. 1998; Peyron, Tighe et al. 1998). A summary of hypothalamic feeding neurons is provided in Figure 2.

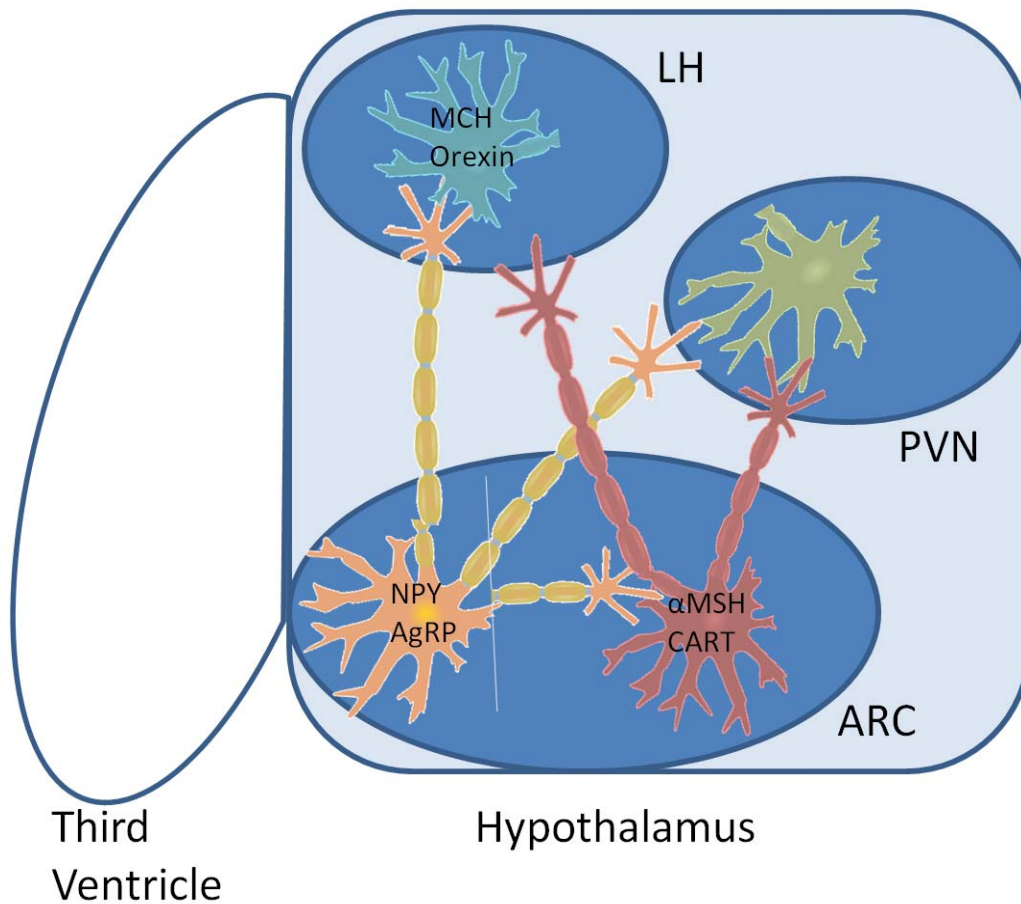


Figure 2 Hypothalamic nuclei involved with central energy regulation. The main hypothalamic nuclei involved in homeostatic appetite regulation are the arcuate nucleus (ARC), paraventricular nucleus (PVN) and the lateral hypothalamus (LH). Within the ARC are two populations of neurons that either stimulate (through neuropeptide Y (NPY) and Agouti related protein (AgRP)) or inhibit (through α melanocyte stimulating hormone (α MSH) and cocaine amphetamine regulated transcript (CART)) appetite and energy utilization. Activation of these neurons leads to activation or inhibition of second order neurons in the LH and PVN.

1.2.4 The Role of the Brainstem

The brainstem (in particular the nucleus tractus solitarius (NTS)) has extensive connections with the hypothalamus (ter Horst, Luiten et al. 1984), other brain regions including the cortex and amygdala (van der Kooy, Koda et al. 1984), and the periphery including the gastrointestinal (GI) tract (Buyse, Ovesjo et al. 2001; Date, Murakami et al. 2002). Afferent signals from the peripheral metabolic organs transmit energy availability information to the brainstem in part through the vagus nerve (reviewed (Berthoud 2008)). These vagal afferents send input from chemoreceptors both in taste buds and along the gastrointestinal (GI) tract (Eram and Michel 2006) and from mechanoreceptors along the GI tract (Berthoud, Lynn et al. 2001). Furthermore, information from metabolic hormones is also transmitted through receptors contained within the terminals of vagal afferents (Buyse, Ovesjo et al. 2001; Date, Murakami et al. 2002). Indeed, severing the afferent vagal nerve fibres in rats prevents the appetite satiating effects of the gut hormone cholecystokinin (CCK) (Smith, Jerome et al. 1985).

1.3 Peripheral Energy Regulation

As described in the previous section, a major component of how homeostatic energy regulation is achieved in the hypothalamus depends on the input from signals in the periphery. Interestingly, many of the same organs that have important roles in absorbing, metabolising, and storing food-derived energy, also release factors that communicate both the absence and presence of energy. This section will describe key hormones released from these organs and their role in energy metabolism.

1.3.1 The Endocrine Pancreas

During situations of energy deficit and surplus, the endocrine pancreas serves to regulate circulating glucose. This is achieved by the release and action of two glucose regulating hormones, insulin and glucagon, from the islets of Langerhans.

Insulin is a 5.8 kD peptide hormone produced from the β cells within the islets of the pancreas. The β cells sense the levels of glucose by first allowing glucose to enter through the glucose transporter 2 (GLUT2) expressed in the plasma membrane. The glucose is then metabolized through glycolysis leading to increased levels of ATP. The increased ATP:ADP ratio closes voltage gated K^+ channels leading to a build-up in positive charge, cell depolarization, and Ca^{++} influx. The increase in intracellular Ca^{++} causes the release of secretory vesicles containing insulin (Matschinsky, Magnuson et al. 2006). In the periphery, insulin acts on several metabolic targets including skeletal muscle (myocytes), liver (hepatocytes), and adipose tissue (adipocytes). Insulin binds to the insulin receptor (IRec), which is a tyrosine kinase heterodimer (2 α and 2 β subunits). Binding of insulin leads to a conformational change moving the allosteric α subunits away and allowing the β subunits to autophosphorylate the tyrosine kinase domains. The activated receptor then phosphorylates the insulin receptor substrate 1 (IRS-1) protein. Phosphorylated IRS-1 will then activate phosphoinositide 3-kinase (PI3K), which in turn adds another phosphate group to phospho- inositol-2 (PIP_2), resulting in PIP_3 (Watson, Kanzaki et al. 2004). Protein kinase B (AKT) is then phosphorylated which stimulates the activity of Rab proteins and translocation of the glucose transporter 4 (GLUT4) to the cell membrane, as demonstrated in adipocytes (Miinea, Sano et al. 2005). The increased level of GLUT4 at the cell membrane allows for increased glucose influx. In hepatocytes, the

phosphorylation of AKT leads to the inactivation of glycogen synthase kinase, which enables glycogen synthase to function to produce glycogen polymers from the excess glucose.

In the brain, insulin does not appear to regulate glucose uptake as was demonstrated by the lack of cerebral uptake of radiolabeled glucose in insulin infused humans (Cranston, Marsden et al. 1998). However, the IRec is widely expressed in the brain, specifically at high levels in the hypothalamus (Havrankova, Roth et al. 1978). There is now evidence that insulin can enter the brain by a saturable IRec mechanism present in the endothelial barrier or through circumventricular organs devoid of the BBB (reviewed in (Woods, Seeley et al. 2003)). Other than peripheral insulin, there is also some evidence from rat neuronal cultures that insulin can be produced centrally (Clarke, Mudd et al. 1986). In the CNS, insulin acts to reduce appetite and promote energy expenditure. Indeed, intracerebroventricular (ICV) injection of insulin was demonstrated to reduce appetite and weight gain in rats (Ikeda, West et al. 1986). Interestingly, the ability of insulin to reduce appetite in obese/diabetic rats was lost (Ikeda, West et al. 1986) suggesting central insulin resistance can occur. The effects of insulin on appetite are believed to occur through reduced NPY production and release within the hypothalamus (Schwartz, Sipols et al. 1992). Insulin may also regulate appetite through effects on other peripheral satiety factors. Acute insulin infusions in humans cause a rapid drop in the orexigenic hormone ghrelin (Saad, Bernaba et al. 2002). In addition, insulin stimulates the release of the anorexigenic hormone glucagon like peptide-1 (GLP-1) (Lim, Huang et al. 2009). Overall, insulin is released in a state of energy surplus and acts to promote the storage and reduce the intake of excess calories.

In contrast to insulin, the pancreatic hormone glucagon is released from the α -cells of the pancreatic islets in situations of low circulating glucose. Glucagon acts to restore normoglycemia through activating hepatic gluconeogenesis and glycogenolysis (Pilkis and Granner 1992). As such, the circulating level of glucagon fluctuates in the opposite manner as insulin, with peaks during fasting state and troughs postprandially. Somewhat paradoxically, glucagon has been demonstrated to also reduce appetite. Early studies with glucagon administration in men showed that it caused a reduction in caloric intake with concomitant weight loss (Schulman, Carleton et al. 1957). While it appears that the appetite suppressing effects of glucagon occur through hepatic afferents (Geary and Smith 1983), direct ICV administration of glucagon also reduces appetite in rats (Inokuchi, Oomura et al. 1984).

1.3.2 Adipose Tissue

While adipose tissue plays an important role in long-term energy storage, it also plays several key roles in signalling energy availability. Adipose tissue produces several hormones (called adipokines) that are released into circulation based on energy status in the tissue. Leptin is an example of an adipokine that acts centrally to reduce appetite (Halaas, Gajiwala et al. 1995). Indeed, humans that have larger fat stores have higher levels of leptin expression (Lonnqvist, Arner et al. 1995). Leptin receptor mRNA was initially described to colocalize with NPY/AgRP neurons in mice (Mercer, Hoggard et al. 1996) and POMC/CART neurons in rats (Cheung, Clifton et al. 1997). Later work demonstrated that leptin treatment increased hypothalamic mRNA expression of POMC in mice (Thornton, Cheung et al. 1997). Mice that are leptin deficient (*ob/ob*) are

hyperphagic and obese; however, this can be reversed by leptin administration (Pelleymounter, Cullen et al. 1995). A similar reversal of obesity and hyperphagia in humans with a congenital leptin mutation can be achieved with leptin administration (Farooqi, Jebb et al. 1999). While, much promise was held for leptin in the treatment of obesity, later evidence demonstrated that the chronic hyper-leptinemia state occurring in obesity leads to leptin resistance in the intracellular signalling of hypothalamic neurons (Bjorbaek, Elmquist et al. 1998).

1.3.3 The Gastrointestinal Tract

The GI tract consists of hollow organs stretching from the mouth to the anus and several accessory glands. Each region of the GI tract is specialized for aspects of mechanical or chemical/enzymatic breakdown of food and nutrient absorption. The stomach is primarily involved in mechanical digestion through peristalsis, chemical digestion through release of hydrochloric acid from parietal cells, and enzymatic digestion through pepsinogen release from chief cells. The majority of nutrient absorption takes place in the small intestine comprised of the duodenum, jejunum and ileum, while the large intestine absorbs water from digested material. The presence of nutrients in the GI tract gives this set of organs the unique ability to be one of the early sensors of energy availability. As will be described, the GI tract, through both the enteric nervous system and GI secreted hormones, plays a critical role in energy metabolism.

The enteric nervous system is a branch of the autonomic nervous system that coordinates all aspects of digestion, but also communicates nutrition status to the CNS

(reviewed in (Konturek, Konturek et al. 2004)). Fluorescent dye injection into the brainstem of rats retrogradely labelled afferent nerves throughout the GI (Berthoud, Jedrzejewska et al. 1990). These afferent nerve fibres are activated by both mechanical (stretch during stomach distention) and by chemical (presence of nutrients, hormones and neurotransmitters) receptors on the nerve terminals (reviewed (Konturek, Konturek et al. 2004)).

GI hormones play a major role in regulating whole body energy metabolism. Indeed the GI tract, both in terms of the number of cells and hormones produced, is the largest endocrine organ in the body (Gagnon, Mayne et al. 2009). Several GI hormones are regulated in response to nutrient ingestion including cholecystokinin (CCK), peptide YY (PYY), glucagon like peptide-1 (GLP-1), and ghrelin.

Cholecystokinin is primarily produced in the I-cells of the small intestine and induces gall bladder contraction, releasing bile to aid in the digestion of lipids (reviewed in (Liddle 2000)). Therefore, the levels of CCK production are highly stimulated by the presence of lipids in the GI tract (reviewed in (Beglinger and Degen 2004)). In a seminal paper by Gibbs *et al.*, the appetite reducing effects of CCK were described by injecting purified CCK into rats (Gibbs, Young et al. 1973). This study was the first evidence indicating that GI hormones could affect appetite, and became a foundation for the discovery of several other appetite regulating GI hormones. The appetite regulating effects of CCK are mediated by binding to the CCK_A receptor present in NPY neurons within the dorsal medial hypothalamus (DMH) (Bi, Ladenheim et al. 2001) and by vagal afferents innervating the brainstem (NTS) (Day, McKnight et al. 1994). Interestingly, daily administration of CCK in rats, while decreasing meal size, leads to increased meal

frequency with an overall stabilization of daily energy intake (West, Fey et al. 1984). As such, CCK is likely more relevant in the regulation of short term energy intake.

Peptide tyrosine tyrosine (PYY) is produced by enteroendocrine L-cells located in the distal ileum and colon (Lundberg, Tatemoto et al. 1982). Concentrations of PYY increase in circulation after a meal (Pedersen-Bjergaard, Host et al. 1996). Peripheral injection of PYY in rodents leads to decreased hypothalamic NPY activity and in humans causes a decrease in feeding over 24 hours (Batterham, Cowley et al. 2002). However, Tschop *et al.* were unable to replicate these experimental findings, calling into question the effects of PYY on appetite (Tschop, Castaneda et al. 2004).

The proglucagon protein is also produced in enteroendocrine L-cells (which colocalize with PYY). The posttranslational processing of proglucagon in the L-cells releases several peptides that are involved in energy metabolism including GLP-1, GLP-2 and oxyntomodulin (Herrmann, Goke et al. 1995). GLP-1 is an important regulatory hormone that stimulates glucose-dependent insulin secretion. In humans, injection of GLP-1 potentiates the postprandial increase of insulin (and therefore is referred to as an incretin) (Kreymann, Williams et al. 1987). This has been the basis of GLP-1 targeted therapies for the treatment of type 2 diabetes mellitus (T2DM). GLP-1 potentiates insulin secretion by acting on the GLP-1 receptor, which is present on the β cells of the pancreas (Thorens 1992). Several long-lasting GLP-1 analogues and medications that increase GLP-1 half-life are now approved for the treatment of T2DM (reviewed in (Fineman, Cirincione et al. 2012)). GLP-1 also has extra-pancreatic effects, as it has been demonstrated that GLP-1 or analogues increase hepatic glycogen synthase activity and glucose disposal in both the liver and muscle of rats (Alcantara, Morales et al. 1997).

Both these extra-pancreatic effects are of additional benefit in the normalization of elevated blood sugar. In addition to the glucose regulating aspects of GLP-1, there is evidence supporting its role in regulating appetite. Indeed, ICV injection of GLP-1 into rats caused a reduction in appetite (Turton, O'Shea et al. 1996). This group also demonstrated that the GLP-1 administration activated feeding neurons within the hypothalamus (and these actions could be blocked using the GLP-1 receptor antagonist Exendin 9-39) (Turton, O'Shea et al. 1996). Similarly, IV administration of GLP-1 in humans causes enhanced satiety and reduced *ad libitum* food consumption (Flint, Raben et al. 1998).

Overall, it is clear that peripheral hormones from metabolic tissues have important roles in maintaining energy homeostasis. This occurs from both the direct effects on peripheral tissues as well as action in the CNS. The GI hormones mentioned so far all cause a satiating effect on appetite. The only peripheral hormone known to stimulate appetite is the stomach derived ghrelin. Ghrelin has many unique effects on both peripheral and central energy regulation and will be the focus of the remaining introduction. A summary of the GI hormones and their actions is presented in Figure 3.

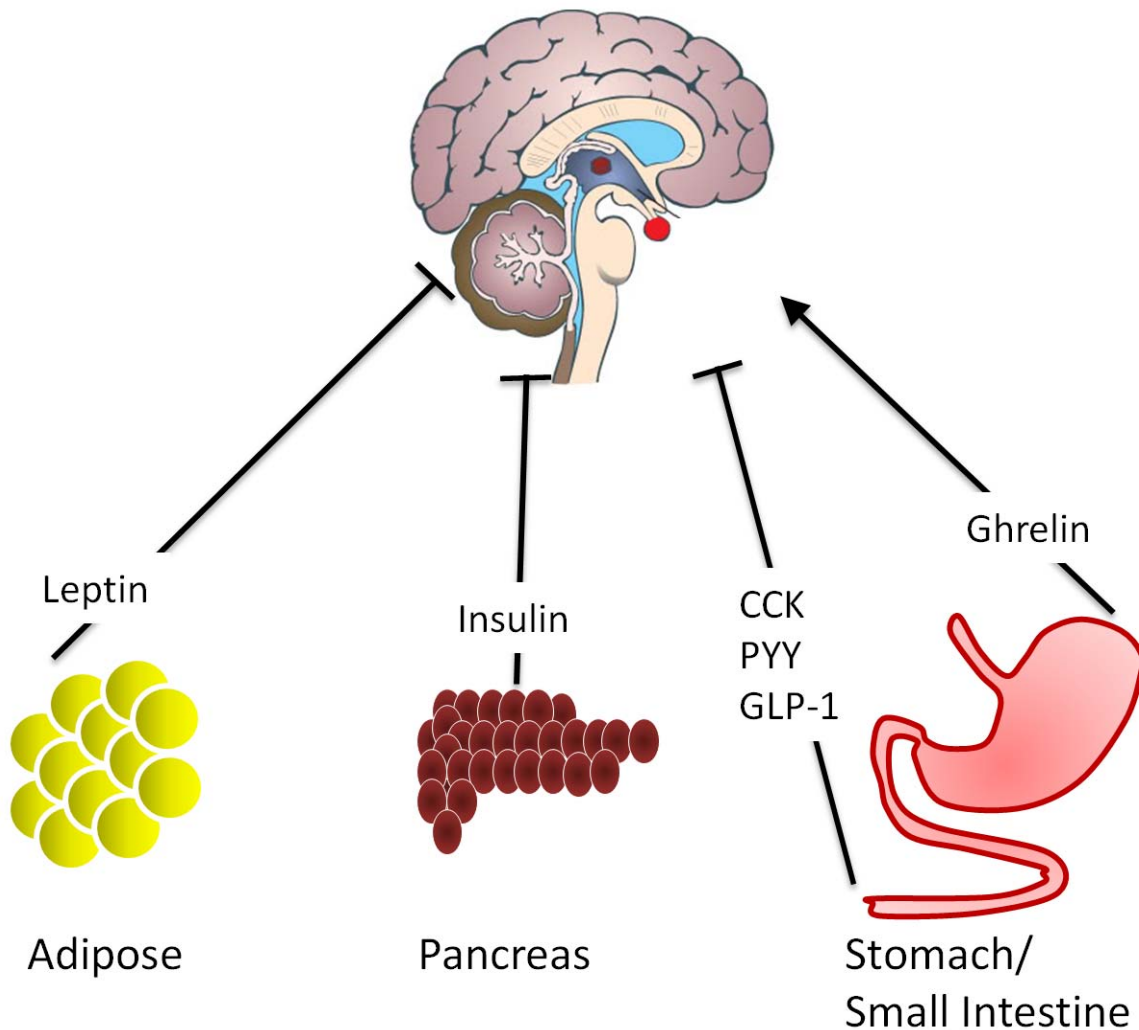


Figure 3 Peripheral hormones involved in appetite regulation. Leptin is released from adipose tissue in response to long term energy surplus and inhibits appetite. Insulin is released from pancreatic β cells in response to increased glucose and inhibits appetite. Several satiety hormones are released from the small intestine including cholecystokinin (CCK), peptide YY (PYY) and glucagon like peptide-1 (GLP-1). Ghrelin is released from the oxyntic glands in the gastric fundus and stimulates appetite.

1.4 Ghrelin

Ghrelin is a unique hormone both in terms of its structure, function, and its regulation of secretion. As ghrelin is the central focus of this thesis, a detailed background on the discovery, structure, and regulation of its secretion and signalling will follow.

1.4.1 Ghrelin Discovery and Proghrelin-Derived Peptides

Ghrelin was first discovered in 1999 as the ligand to the (then orphan) growth hormone secretagogue receptor (GHSR) (Kojima, Hosoda et al. 1999) and as such, was initially shown to stimulate growth hormone secretion. This was a case of reverse pharmacology, as the GHSR had been previously identified (Pong, Chaung et al. 1996), but no endogenous ligand had been known until ghrelin was discovered. As activation of the GHSR (with synthetic agonists) was previously known to stimulate increases in intracellular Ca^{++} , the authors used GHSR transfected Chinese hamster ovary cells and a fluorescent Ca^{++} assay to screen for the GHSR ligand. Various tissue extracts were examined in this assay (brain, lung, heart, kidney, stomach and small intestine), and surprisingly the stomach extract yielded the greatest Ca^{++} response. Purification and sequencing later determined that a 28 amino acid (aa) peptide was responsible for these effects and was given the name ghrelin (Proto-Indo-European root “ghre”, meaning “to grow”). Mass spectrometry experiments identified a post-translational modification on the 3rd serine residue corresponding to an octanoic acid moiety. The researchers also confirmed that this octanoylation was required for the activation of the GHSR (Kojima, Hosoda et al. 1999).

Ghrelin is derived from a 114 aa preprohormone precursor which requires both signal peptidase cleavage and endoproteolytic cleavage by proprotein convertase-1 (PC1/3) to produce the 28 aa peptide (Zhu, Cao et al. 2006). In addition to this processing, ghrelin is also modified by the addition of an oxygen-linked 8-carbon fatty acid (octanoic acid) on its 3rd serine residue (acylated-ghrelin or AG) (Kojima, Hosoda et al. 1999). This unique acylation is produced by the ghrelin O-acyl transferase (GOAT) and was discovered simultaneously by two different groups (Gutierrez, Solenberg et al. 2008; Yang, Brown et al. 2008). The exact timing/location of when ghrelin acquires this acylation is still under investigation. Zhu *et al.* demonstrated that AG is still produced in the PC1/3 knockout mouse, indicating that octanoylation is not dependent on post-translational processing (Zhu, Cao et al. 2006). In addition, GOAT is localized in the ER membrane, which supports its ability to modify proghrelin as it is being produced (Yang, Brown et al. 2008). While only AG is able to bind the ghrelin receptor, another unacylated (or deacylated) ghrelin (UAG) form exists in circulation at >10-fold higher concentration (Hosoda, Kojima et al. 2000; Foster-Schubert, Overduin et al. 2008; Morash, Gagnon et al. 2010). Despite UAG's inability to interact with the ghrelin receptor, some GHSR independent actions have been documented (reviewed in (Baragli, Lanfranco et al. 2011)).

In addition to these two forms of ghrelin, there may also be a third peptide that is produced from the C-terminal end of the proghrelin prohormone called obestatin (Figure 4). Initially, this hormone was believed to have an opposite effect on appetite to that of ghrelin (Zhang, Ren et al. 2005). However, several other groups were not able to confirm these findings (Seoane, Al-Massadi et al. 2006; Nogueiras, Pfluger et al. 2007). Indeed,

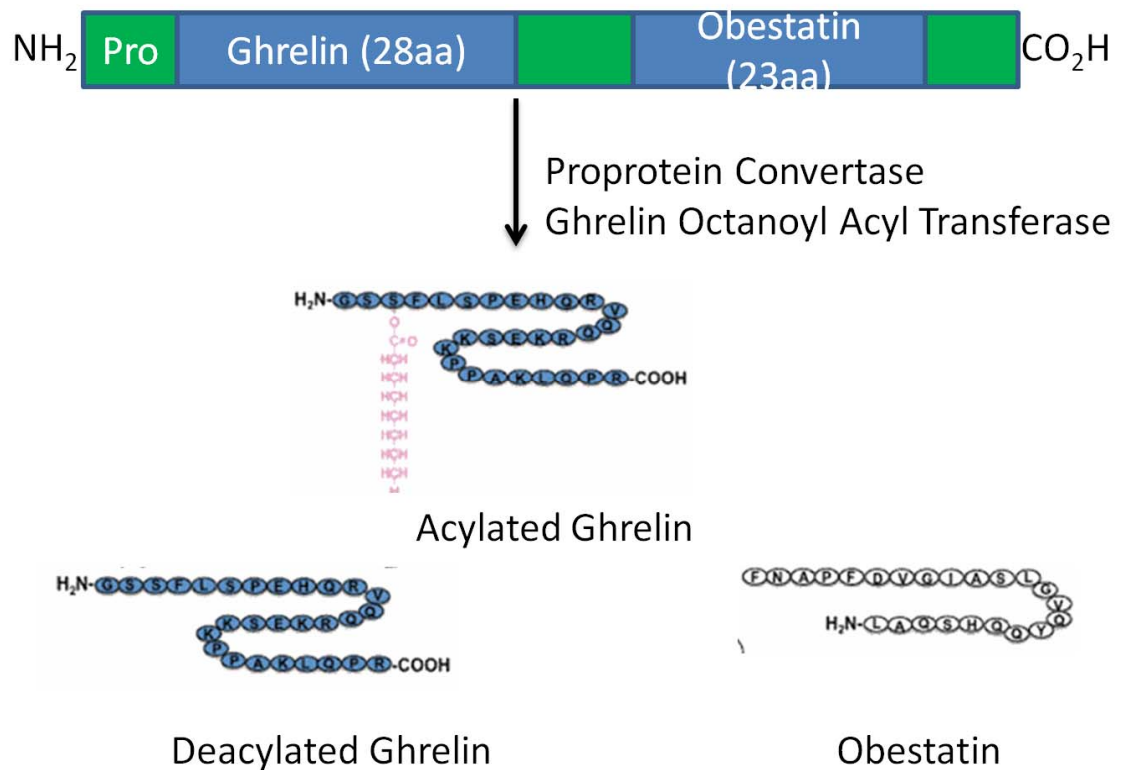


Figure 4 Proghrelin structure and derived peptides. Preproghrelin is first produced as a 117aa precursor. During its production or post-translationally, an octanoic acid moiety is added to the 3rd serine of the ghrelin peptide and the precursor is processed by PCSK1 to release the 28aa ghrelin peptide. Acylated ghrelin can become deacylated. A third putative hormone, obestatin, may also be produced from the proghrelin precursor.

the original authors were unable to reproduce their findings on the binding between obestatin and the orphan GPR39 (Chartrel, Alvear-Perez et al. 2007) (and in response (Zhang, Klein et al. 2007)). There is also some debate to whether this putative 23 aa hormone actually exists in circulation (Bang, Soule et al. 2007).

As indicated above, ghrelin exists in two forms in circulation (acylated and deacylated). The levels of ghrelin reported in circulation vary considerably and appear to depend on the method of plasma preparation and the type of assay used. Early reports on ghrelin were largely done with assays that detected total ghrelin (acyl + deacyl). In humans, fasting levels of total ghrelin were found in the low nanomolar range (Wren, Seal et al. 2001) or high picomolar range (Ikezaki, Hosoda et al. 2002). It was later determined that acidification of plasma was an important step in retaining the acylated ghrelin in a sample (Hosoda, Doi et al. 2004). While reports vary on the concentrations detected, it is generally observed that levels of deacylated ghrelin are 5-10-fold higher (fasting) than acylated ghrelin in circulation (Harada, Nakahara et al. 2008). In that report, healthy fasting females had 22 pg/ml (6.5 pM) of acylated and 230 pg/ml (68 pM) of deacylated ghrelin in circulation.

The half-life of ghrelin in circulation was characterized by pharmacokinetics where both forms of ghrelin were infused in humans and plasma was continually assayed by specific radioimmunoassays. According to these studies, AG has a half-life of ~10 minutes while UAG is ~30 minutes (Akamizu, Takaya et al. 2004). *In vitro* studies examining ghrelin degradation in plasma found that butylcholinesterase plays a key role in the degradation of AG to UAG (De Vriese, Gregoire et al. 2004). To determine how ghrelin is cleared from circulation Wu *et al.* tracked the clearance and tissue localization

of radiolabeled ghrelin injected into rats. They found that the majority of labeled ghrelin was localized to the kidney, and in support, inducing renal failure by sepsis led to greater half-life of ghrelin in circulation (Wu, Zhou et al. 2003).

1.4.2 Regions of Ghrelin Production

As described earlier, ghrelin was first discovered in the stomach, but further work identified ghrelin-producing cells in other tissues. A discussion on the various locations of ghrelin production follows.

Soon after the discovery of ghrelin, the same group conducted *in situ* hybridization and immunohistochemistry experiments to map out ghrelin expression within the stomach and GI tract. They found that ghrelin was primarily localized in the gastric fundus (upper region) within the parietal cell rich oxyntic gland (Date, Kojima et al. 2000) (Figure 5). They also determined that ghrelin signal was not colocalized with other gastric cell types (enterochromaffin-like cells, enterochromaffin cells, and somatostatin cells) and was localized in the previously uncharacterized endocrine X/A cells (reviewed in (Stengel and Tache 2012)). Ghrelin cells represent ~20% of the endocrine cells present in the gastric fundus and are also sparsely found in the upper regions of the small intestine (Date, Kojima et al. 2000). The finding that ghrelin is primarily produced in the stomach is supported by a study examining the post-operative levels in patients receiving varying degrees of gastrectomy. In this study removal of the proximal stomach (fundus) caused a sustained (7 days post-operative) 70% drop

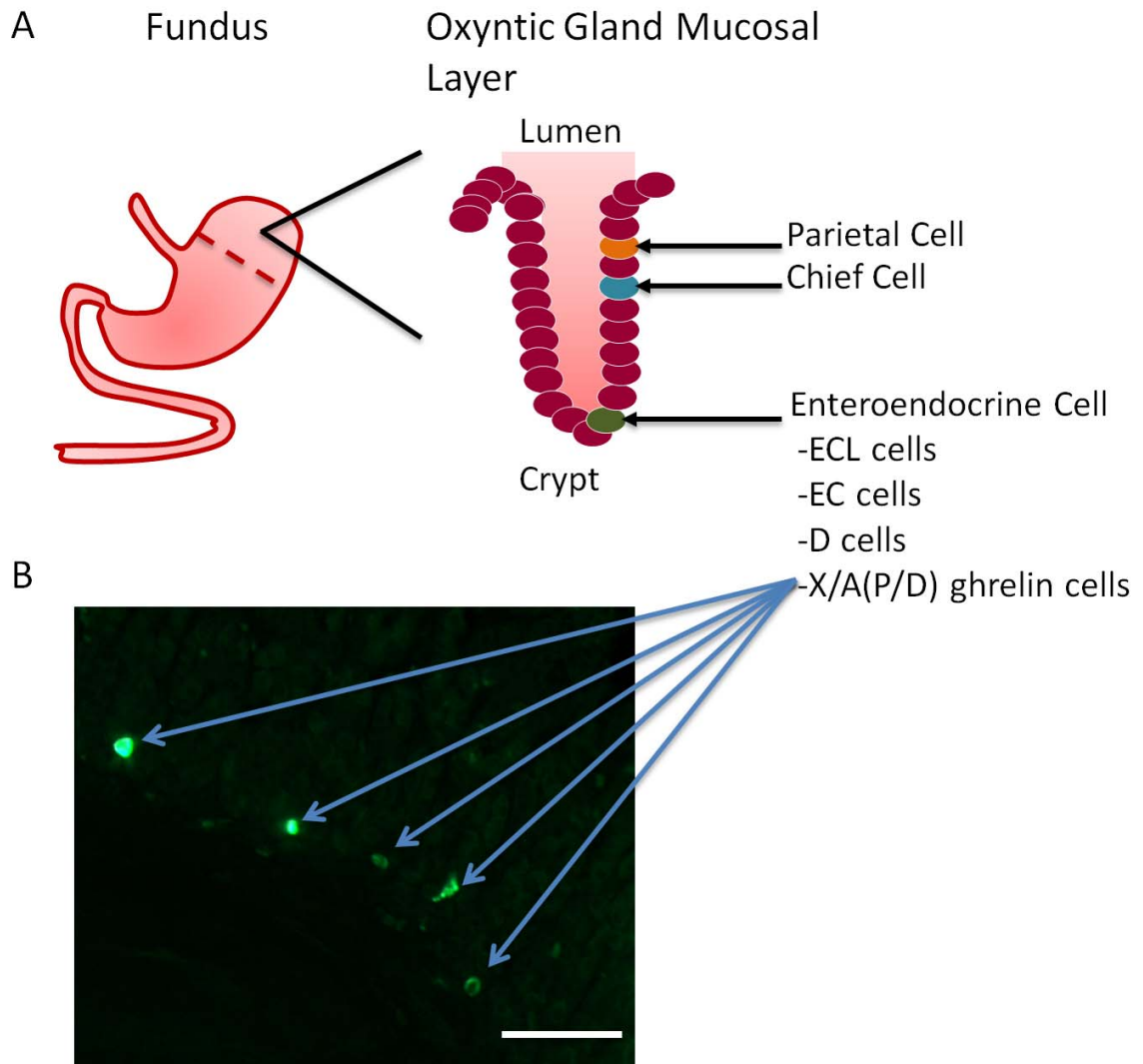


Figure 5 Primary location of ghrelin production. The majority of ghrelin produced comes from the oxyntic mucosal layer in the gastric fundus region of the stomach. Ghrelin producing (X/A or P/D) enteroendocrine cells are primarily present in the crypts of the mucosal layer. Immunofluorescence of ghrelin protein from 8 day old rat stomach tissue (scale bar = 100 μ m).

in circulating ghrelin, while more distal gastrectomies caused an initial decrease with eventual recovery in ghrelin levels (Jeon, Lee et al. 2004). These results further indicate that the proximal stomach is the primary source of ghrelin in the body.

While the stomach may be the major source of ghrelin in the circulation, ghrelin is also produced in other tissues where it may have a paracrine effect. Such production has been observed within the endocrine pancreas. Ghrelin expression was shown to localize within pancreatic α cells (major glucagon secreting cell) as well as alone in a distinct population of cell referred to as the epsilon cells (ϵ -cells) (Heller, Jenny et al. 2005). Interestingly, during development pancreatic ghrelin production precedes stomach ghrelin production; however, pancreatic ghrelin cells decrease in number into adulthood (Wierup, Svensson et al. 2002). This high level of ghrelin expression in the early pancreas may indicate a role for ghrelin in the development of the endocrine pancreas.

Ghrelin is also expressed in several brain regions, primarily in the hypothalamus (Cowley, Smith et al. 2003), but also in the cerebral cortex and brainstem (Hou, Miao et al. 2006). Axons from these ghrelin-expressing neurons were shown to make synapses with neurons expressing NPY and AgRP (Cowley, Smith et al. 2003). In addition, using retrograde labelling from the hypothalamus, Hou *et al.* determined that the majority of ghrelin producing neurons within the hypothalamus project to the dorsal ventral complex (Hou, Miao et al. 2006). The presence of ghrelin producing neurons was also confirmed in transgenic mice that expressed enhanced green fluorescent protein under the ghrelin promoter (Kageyama, Kitamura et al. 2008).

1.4.3 The Growth Hormone Secretagogue Receptor and Ghrelin Signalling

Growth hormone release is stimulated by GHRH and GH secretagogues through distinct receptors. GH-secretagogues act on the GHSR while GHRH acts on the GHRH receptor (Howard, Feighner et al. 1996; Pong, Chaung et al. 1996). Correspondingly, the highest levels of GHSR expression are largely found within the anterior pituitary. However, mRNA for GHSR is ubiquitously expressed in several regions including the brain, pancreas, liver, ovaries and adipose tissue (Guan, Yu et al. 1997). Interestingly, the gene encoding for the GHSR produces two alternatively spliced proteins - GHSR1a is the full length receptor while GHSR1b is a truncated splice variant that is not localized to the cell membrane (Howard, Feighner et al. 1996). The GHSR1a will be the subject of future discussion and will be referred to as GHSR or ghrelin receptor.

The GHSR is a G-protein coupled receptor with seven trans-membrane domains and three extracellular loops (McKee, Palyha et al. 1997). Pedretti *et al.* found that the GHSR had nearly 50% of maximal signalling in the absence of its ligand and using mutational analysis they further demonstrated that this high constitutive activity occurs via interaction between the second, sixth, and seventh trans-membrane domains (Pedretti, Villa et al. 2006). They also found that the second extracellular loop is critical for the interaction with hydrophobic AG (Pedretti, Villa et al. 2006). Indeed, synthetic forms of ghrelin as short as four aa's can activate the receptor, providing the hydrophobic octanoyl moiety is present on the peptide (Bednarek, Feighner et al. 2000). The receptor structure and its important regions are illustrated in Figure 6. The downstream signalling of GHSR after ligand binding has been well characterized in pituitary somatotrophs but also in hypothalamic neurons

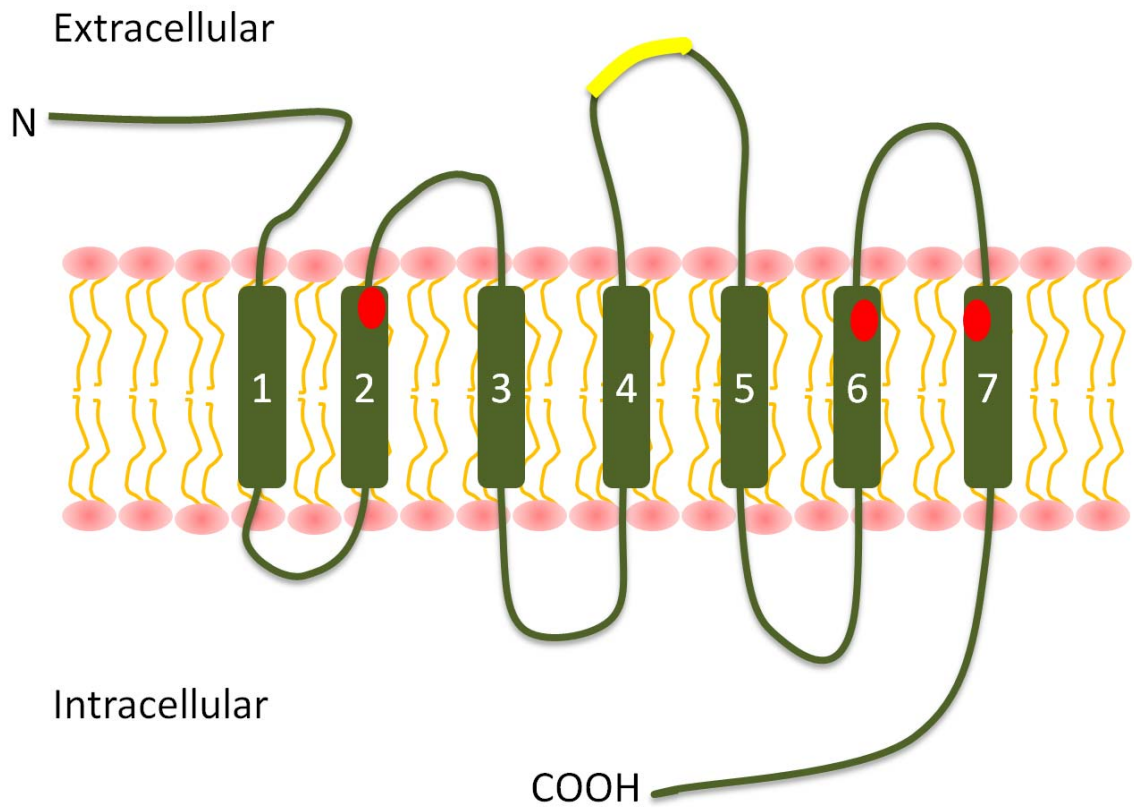


Figure 6 Illustration of the growth hormone secretagogue receptor (ghrelin receptor) with important regions identified. The ghrelin receptor maintains ~50% signalling in the absence of a ligand through the interaction of the second, sixth and seventh trans-membrane domains (red). The second extracellular loop is important in ligand interaction (yellow).

(Kohno, Gao et al. 2003). Once activated by ghrelin or another GH secretagogue, the GHSR causes an increase in intracellular Ca^{++} . Indeed, the group that discovered ghrelin used intracellular Ca^{++} as their screening assay (Kojima, Hosoda et al. 1999). In dispersed primary NPY expressing neurons, ghrelin treatment caused increases in Ca^{++} that are dependent on protein kinase A (PKA) (but not PKC) and on N-type Ca^{++} channels (and not L-type). Interestingly, co-administration with the appetite lowering hormone leptin attenuated the Ca^{++} response to ghrelin in NPY neurons (Kohno, Gao et al. 2003). In adipocytes, ghrelin treatment has anti-apoptotic effects, which occur through activation of both the mitogen activated protein kinase (MAPK) and PI3K pathways (Kim, Yoon et al. 2004).

1.4.4 The Functions of Ghrelin

1.4.4.1 Ghrelin Stimulates GH Release

As described earlier in the context of its discovery, ghrelin was initially shown to stimulate GH release from the anterior pituitary in rats. This was later confirmed in humans receiving various doses of ghrelin intravenously (Peino, Baldelli et al. 2000). A major component of GH stimulation occurs through the action of the hypothalamic release of GHRH. Interestingly, ghrelin has been shown to have a greater stimulatory effect on GH release than GHRH (Arvat, Maccario et al. 2001). Low dose ghrelin, when administered with GHRH, causes a synergistic effect on GH release when compared to the effect of either hormone alone (Hataya, Akamizu et al. 2001). GH has important roles in promoting general anabolism in the body, many of which occur through the production

of IGF-1. In terms of glucose homeostasis, GH is known to stimulate glucose production and inhibit glucose uptake in peripheral tissues (Altzuler, Rathgeb et al. 1968). This is especially vital in situations of prolonged fasting in order to mobilize energy. Evidence of ghrelin's importance in this context comes from GOAT knockout animals (which do not produce AG). When these animals are placed on a low calorie diet, they develop severe hypoglycaemia and become moribund as result of lower GH levels (Zhao, Liang et al. 2010). Treating animals with either ghrelin or GH can reverse this state (Zhao, Liang et al. 2010).

1.4.4.2 Appetite Stimulation

One of the well characterized functions of ghrelin is appetite stimulation. Initial studies determined that injecting ghrelin into the ICV space of rats caused a dose-dependent stimulation in feeding (Tschop, Smiley et al. 2000). This hyperphagia was later confirmed with peripheral injections of ghrelin (Wren, Small et al. 2001). Using c-Fos immunolabeling, IV administration of ghrelin in mice was shown to stimulate hypothalamic activity in the arcuate nucleus that was largely (90%) localized to the appetite stimulating NPY producing neurons (Wang, Saint-Pierre et al. 2002). Indeed, ghrelin receptor mRNA had been previously shown in rats to be expressed in the majority of the NPY neurons while it was largely absent in POMC neurons (Willesen, Kristensen et al. 1999). In addition to stimulating appetite through the hypothalamus, ghrelin has a similar effect when injected into the dorsal ventral complex (DVC) of the brainstem in rats (Faulconbridge, Cummings et al. 2003). In support of this, it was later shown via *in*

situ hybridization that ghrelin receptor is widely expressed throughout the DVC of rats and mice (Zigman, Jones et al. 2006).

Ghrelin may also stimulate appetite through the vagus nerve. Ghrelin receptor expression and radiolabeled ghrelin binding has been demonstrated on afferent vagal nerve terminals in rats (Date, Murakami et al. 2002). The same study showed that severing the vagus nerve by subdiaphragmatic vagotomy, or administering the vagal blocker capsaicin, prevents the appetite stimulatory effects of peripheral ghrelin administration (Date, Murakami et al. 2002). However, another study that investigated the effect of peripheral ghrelin administration in rats using two forms of vagotomy, found that appetite was stimulated similar to controls (Arnold, Mura et al. 2006). It is also possible that peripheral ghrelin may stimulate appetite by crossing the blood brain barrier (BBB). Studies injecting various forms of radiolabeled ghrelin (mouse/human AG/UAG) found that human AG could cross the BBB by a saturable mechanism. The same group later determined that energy state plays a role in the transport of ghrelin across the BBB. They found that obese mice had reduced transport of ghrelin into the brain compared to that of normal weight animals (Banks, Burney et al. 2008). Interestingly, mice perfused with triglycerides along with ghrelin had increased transport across the BBB (Banks, Burney et al. 2008), possibly indicating that certain macronutrients promote a greater ability for ghrelin to act in appetite regulation.

Ghrelin is also implicated in hedonic appetite regulation. Ghrelin receptors were identified in the ventral tegmental area (VTA) which projects to several nuclei involved in the food reward pathway (Guan, Yu et al. 1997; Zigman, Jones et al. 2006). Supporting a role for ghrelin in this region, mice that had ghrelin injected into the VTA observed an

increase in appetite (Naleid, Grace et al. 2005). A role for ghrelin in food reward is further supported by studies that block ghrelin signalling. Ghrelin receptor knockout (KO) mice or rats treated with ghrelin receptor antagonists show reduced motivation to acquire palatable food (Egecioglu, Jerlhag et al. 2010). Recently, hedonic feeding was examined in GOAT KO mice (which do not produce AG). These animals had an attenuated effort to pursue HFD food after a fast and reduced effort to pursue HFD (dessert) after being calorically-sated (Davis, Perello et al. 2012). The hedonic feeding effects of ghrelin were also demonstrated in humans; using functional magnetic resonance imaging (fMRI), intra-venous ghrelin administration was shown to increase the brain activity in regions associated with food reward (Malik, McGlone et al. 2008). Overall, these studies demonstrate that ghrelin, through several pathways, stimulates appetite.

1.4.4.3 Glucose Metabolism

In addition to the effects on GH release and appetite, ghrelin is involved in regulating several aspects of glucose metabolism. Early after its discovery, ghrelin was shown to cause a drop in insulin levels when injected in humans (Broglia, Arvat et al. 2001). Later, it was demonstrated in humans that IV ghrelin, while not having a major effect on fasting insulin, reduced glucose stimulated insulin secretion (Tong, Prigeon et al. 2010). The effects of ghrelin on insulin secretion are likely direct as ghrelin receptor expression is found in the islets of the pancreas (Guan, Yu et al. 1997). The majority of studies have demonstrated that ghrelin inhibits glucose stimulated insulin secretion from the pancreas (reviewed (Dezaki, Sone et al. 2008)). However, it has also been

demonstrated that IV injection of ghrelin in rats can stimulate insulin secretion (Lee, Wang et al. 2002).

As described earlier, the pancreas is also a site of ghrelin production (from the ϵ -cells). To elucidate the role of endogenous pancreatic ghrelin, Dezaki *et al.* used ghrelin receptor antagonists and antiserum against ghrelin in mice and isolated rat islets. They determined that ghrelin blocked glucose stimulated Ca^{++} levels in part by increasing outward K^+ currents (hyperpolarizing) (Dezaki, Hosoda et al. 2004). The ghrelin-induced reduction in insulin secretion (as determined using siRNA knockdown experiments in isolated β -cells) occurs through the G-protein α_i , which is known to reduce intracellular cAMP and Ca^{++} (Dezaki, Kakei et al. 2007). This is in contrast to the ghrelin receptors G-protein α_q signalling that occurs in pituitary GH secretion (Howard, Feighner et al. 1996). Very recently, Park *et al.* demonstrated the interaction between GHSR and somatostatin receptor in β -cells determined whether ghrelin activated the G-protein α_i and inhibited insulin release, or activated the G-protein α_q to stimulate insulin release (Park, Jiang et al. 2012).

Ghrelin also affects hepatic glucose regulation. In cultured hepatocytes, AG was demonstrated to stimulate glucose production while UAG had the opposite effect (Gauna, Delhanty et al. 2005). In rats, chronic administration of a non-appetite stimulating low dose of ghrelin led to reduced glycogen synthase kinase and increased PGC1 α (activator of gluconeogenesis) protein expression in livers (Barazzoni, Zanetti et al. 2007). Despite the increase in proteins associated with hepatic glucose production, an increase in glucose uptake machinery (GLUT4) was observed in muscle tissue (Barazzoni, Zanetti et al. 2007). In support of this, studies measuring hepatic glucose production and glucose

disposal using radiolabeled glucose tracer method in mice, found that ghrelin increased glucose disposal (in muscle and fat) and partially blocked the insulin-induced suppression of gluconeogenesis (Heijboer, van den Hoek et al. 2006). So while ghrelin appears to cause elevated glucose production from the liver, it also promotes glucose uptake into peripheral tissues.

Interestingly, a recent study has also demonstrated a role for ghrelin in CNS-regulated glucose homeostasis. Ghrelin receptor knockout mice have low fasting blood glucose compared to controls (Sun, Butte et al. 2008). To determine if this was partially due to central ghrelin receptor signalling, Scott *et al.* designed a ghrelin receptor knockout animal with selective expression restored in the brainstem. They found that selective ghrelin receptor expression in the brainstem restored the ability to maintain fasting blood glucose (Scott, Perello et al. 2012). Overall, these studies clearly indicate that ghrelin can regulate glucose metabolism through both peripheral and central mechanisms.

1.4.4.4 Adipose Tissue Regulation

The initial study that described the appetite stimulating effects of central ghrelin administration also observed a dose-dependent increase in body weight. Specifically, peripheral ghrelin administration led to an increase in fat mass without affecting appetite, which was likely due to increased storage (increased respiratory quotient independent of GH effects) of calories in adipose tissue (Tschop, Smiley et al. 2000). To further separate the appetite effects of ghrelin administration from adipose tissue storage effects, Perez-

Tilve *et al.* used a high fat diet, which allowed for a similar hyperphagia between control and ghrelin-injected animals. In their study, ghrelin caused a significant increase in adipose tissue mass and lipogenic gene transcripts in white adipose tissue without any additional hyperphagia (Perez-Tilve, Heppner *et al.* 2011). Interestingly, while both ghrelin and ghrelin receptor knockout mice have normal feeding and body weight on a chow diet, they are protected from high fat diet-induced obesity (DIO) (Wortley, del Rincon *et al.* 2005; Zigman, Nakano *et al.* 2005). These studies indicate that while the role for ghrelin in appetite may be compensated for in its absence, such compensation does not occur in terms of fat regulation.

At the cellular level, ghrelin promotes differentiation in adipocyte cell lines (Kim, Yoon *et al.* 2004) and in primary cultured adipocytes (Choi, Roh *et al.* 2003). However, similar increases in differentiation were also observed using UAG and the putative pro-ghrelin derived hormone obestatin (Miegyeu, St Pierre *et al.* 2011). Choi *et al.* also observed that isoproterenol-induced lipolysis was significantly reduced by ghrelin treatment in cultured primary adipocytes (Choi, Roh *et al.* 2003). The increases in fat mass observed with ghrelin treatment may also be due to reduced lipid export from adipocytes. Rats treated with ghrelin have reduced mRNA levels of the lipid exporting ATP binding cassette G1 (Davies, Kotokorpi *et al.* 2009). Furthermore, this study demonstrated by MRI that ghrelin treatment resulted in increased adiposity in the retroperitoneal rather than subcutaneous cavities (Davies, Kotokorpi *et al.* 2009). This region of adipose tissue is of particular concern as it is largely responsible for the metabolic complications that occur in obesity (reviewed in (Wajchenberg 2000)).

1.4.4.5 Reproduction

Ghrelin has been established to have several important functions in reproduction. These effects are mediated by action on both the hypothalamic-pituitary-gonadal axis and directly on the gonads in both males and females (reviewed by (Garcia, Lopez et al. 2007)). Ghrelin ICV injections caused a reduction in LH and FSH release in ovariectomized and normal rats (Fernandez-Fernandez, Tena-Sempere et al. 2005). In agreement, ghrelin treated excised hypothalamus have reduced gonadotropin releasing hormone (GnRH) secretion (Fernandez-Fernandez, Tena-Sempere et al. 2005). In men, peripheral ghrelin injections also caused a delay and reduction of LH release leading to reduced levels of testosterone (Kluge, Schussler et al. 2007).

Gonadal expression of both ghrelin and the ghrelin receptor has been demonstrated in the human ovaries (Gaytan, Barreiro et al. 2003) and testis (Gaytan, Barreiro et al. 2004). *In vitro*, ghrelin administration prevents the development of pre-implantation embryos (Kawamura, Sato et al. 2003) and reduces the litter size of rats treated daily for the first half of pregnancy (Fernandez-Fernandez, Navarro et al. 2005). In addition, low ghrelin may also be detrimental in reproduction as embryos transferred to ghrelin-deficient mice have reduced embryo implantation (Martin, Lieber et al. 2011). These studies suggest that ghrelin has an important role as an energy sensor in reproduction, and levels on either extreme can lead to impaired reproductive function.

1.4.4.6 Cardiac Functions

In addition to the above mentioned and well-studied aspects of ghrelin function, ghrelin may also have beneficial roles in cardiac health. In support of this, low levels of ghrelin are associated with hypertension (Poykko, Kellokoski et al. 2003). Ghrelin and the GHSR are both present in the cardiovascular system as the mRNA for both are expressed throughout the myocardium in humans (Gnanapavan, Kola et al. 2002). Several studies have demonstrated that ghrelin is a potent vasodilator, an effect mediated by nitric oxide production in rats (Shimizu, Nagaya et al. 2003; Tesauro, Schinzari et al. 2005) and humans (Tesauro, Schinzari et al. 2005). Ghrelin may also be beneficial in preventing myocyte apoptosis during cardiomyopathies (Baldanzi, Filigheddu et al. 2002; Marleau, Mulumba et al. 2006). A summary of the functions of ghrelin is provided in Table 1.

Table 1 Summary table on the functions of ghrelin.

Function	Effect	Target	Mechanism
GH Release	Increase	Pituitary	GHSR dependent cAMP increase
Appetite	Increase	Hypothalamus, VTA, Brainstem	Increased NPY Release
Insulin Secretion	Decrease	Pancreatic β Cells	Decreased Intracellular Ca^{++}
Adipose Growth	Increase	White Adipose Tissue	Reduced Lipolysis
Reproductive Outcome	Decrease	Pituitary, Embryo	Reduced LH/FSH and Implantation
Cardiovascular Health	Increase	Endothelial and Myocytes	Vasodilatation and Reduced Apoptosis

1.4.5 The Mechanism of Ghrelin Secretion

From the above description of the many functions of ghrelin, one concept emerges. A key role for ghrelin is to signal the availability of energy and in turn, maintain the body at sufficient energy levels. This is achieved through stimulating GH to mobilize energy stores during periods of fasting, acting on the hypothalamus to increase appetite, and promoting the storage of calories once ingested. Furthermore, ghrelin also regulates how high-energy demand events, such as reproduction, occur. Accordingly, it is vital that X/A cells can tightly regulate their secretion of ghrelin by sensing energy status. Therefore, how ghrelin levels fluctuate and the mechanisms of ghrelin secretion are an important aspect of ghrelin physiology.

1.4.5.1 Fasting Induced Ghrelin Production

The initial paper that demonstrated ghrelin to be an appetite stimulating and adiposity promoting peptide also found that ghrelin levels were elevated in rat serum during the fasting state and decreased after nutrients were consumed (Tschop, Smiley et al. 2000). Shortly after, the same group demonstrated a similar variation in humans (Tschop, Wawarta et al. 2001). By sampling ghrelin over a 24-hour period, Cummings *et al.* identified the peaks and troughs of circulating ghrelin that occurred pre-meal and post-meal, respectively (Figure 7 (Cummings, Purnell et al. 2001)). Initial investigation on how ghrelin secretion was regulated compared several factors including stomach distention and meal type. A water meal was unable to cause the drop in ghrelin secretion as observed with carbohydrate, ruling out the effect of stretch signalling

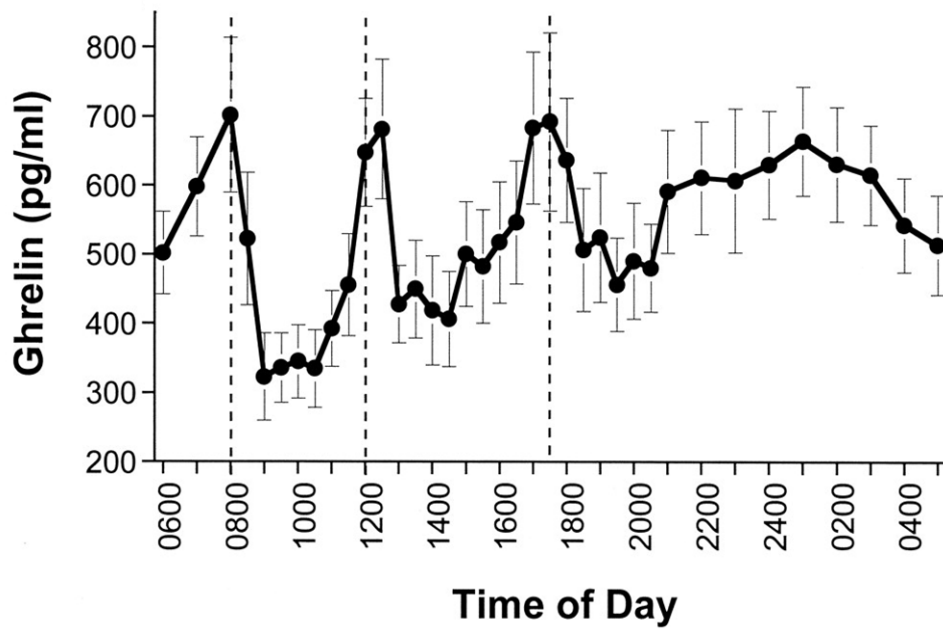


Figure 7 Diurnal variation in ghrelin levels. 38 plasma collections were completed over a 24 hour period with scheduled meal times at 8, 12 and 17.5 hours in healthy individuals. Adapted from Cummings *et al.* 2001 Diabetes vol 50 n8 pg 1714-19.

(Tschop, Smiley et al. 2000). Furthermore, blocking gastric emptying by occluding the pyloric sphincter prevented the effect of carbohydrate on reducing ghrelin levels (Williams, Cummings et al. 2003). This suggested that stomach luminal sensing might not play an important role compared to post gastric nutrient sensing. Interestingly, during a 24 hour fast, ghrelin levels still rise and fall before and after expected meal times (Natalucci, Riedl et al. 2005). Although actual meal consumption has been demonstrated to be an important regulator of ghrelin secretion, this work suggests that diurnal patterning of ghrelin secretion, based on expected meal times, is occurring.

1.4.5.2 Nutrients and Ghrelin Secretion

The amount and the type of nutrients consumed affect both the degree and the length of the postprandial drop in ghrelin. In humans, Callahan *et al.* examined the effect of caloric content in liquid meals, and found meals with higher calories caused a greater drop in ghrelin and prolonged satiety (Callahan, Cummings et al. 2004). With regards to the type of macronutrient, several groups have determined their effect on ghrelin secretion. In normal weight women, a meal comprised mainly of carbohydrate caused a greater drop in ghrelin compared to an iso-caloric meal comprised primarily of fat (Monteleone, Bencivenga et al. 2003). A similar finding was later reported in rodents (Sanchez, Oliver et al. 2004). As previous reports had indicated that ghrelin is not regulated by stomach nutrient sensing, Overduin *et al.* perfused nutrients further down the GI tract into the duodenum and jejunum of the small intestine of rats. They determined that glucose infusion caused the most rapid onset and longest duration of

ghrelin suppression, while lipid infusions caused the smallest effect on plasma ghrelin (Overduin, Frayo et al. 2005). These studies have indicated that carbohydrates may cause a more rapid and longer suppression of ghrelin levels, however there may also be a rebound effect. Comparing the levels of ghrelin after different iso-caloric meals made from carbohydrate, fat, or protein, it was shown that carbohydrates cause a large biphasic (drop then rise) in ghrelin postprandially (Foster-Schubert, Overduin et al. 2008). This greater rise in ghrelin may have implications in rebound hunger after a primarily carbohydrate meal, although this was not investigated in the mentioned study.

The perception of calories and “richness” in a meal may also be involved in ghrelin secretion. In a study where individuals were given two identical milkshakes, one on the pretence of being a “rich” high calorie and the other being healthy and low calorie, individuals told they were consuming the high calorie milkshake had a greater drop in post-meal ghrelin levels (Crum, Corbin et al. 2011). This supports that cortical limbic brain regions (hedonic) play a role in the regulation of ghrelin secretion.

1.4.5.3 Hormones Regulating Ghrelin Secretion

Since circulating hormones have key roles in regulating energy homeostasis, and ghrelin producing cells have access to circulation on the basolateral side, hormones are likely candidates in the regulation of ghrelin secretion. Pancreatic hormones like insulin and glucagon, as well as GI hormones like GLP-1, are tightly coupled to glucose homeostasis and appetite (as described earlier). Their potential role in ghrelin secretion is now beginning to be examined.

Many of the initial works investigating the levels of ghrelin under different energy states also examined the levels of insulin. These studies demonstrated that ghrelin levels fluctuated in reverse to insulin throughout the day (Cummings, Purnell et al. 2001). Furthermore, obese individuals who are hyperinsulinemic have lower levels of ghrelin (Tschop, Weyer et al. 2001). As insulin increases after a meal is consumed, and insulin is known to have satiating effects, these findings were not surprising. A more direct link between the effects of insulin on ghrelin was obtained using hyperinsulinemic euglycemic pancreatic clamp experiments in humans. Using this strategy, insulin infusion (while maintaining constant glucose) was demonstrated to cause a rapid and reversible drop in circulating ghrelin (Saad, Bernaba et al. 2002). These data support the notion that insulin is able to regulate ghrelin secretion; however, they do not provide direct evidence at the cellular level.

Glucagon fluctuates in the same manner as ghrelin, that is, both hormones increase during periods of fasting and are suppressed postprandially. As a principle function of glucagon is to restore energy availability (as described earlier) in periods of fasting, one might expect glucagon to stimulate ghrelin release. However, the effects of glucagon on ghrelin secretion have been conflicting. Two studies examined the effects of glucagon injection in humans and found glucagon reduced circulating ghrelin levels (Arafat, Otto et al. 2005; Soule, Pemberton et al. 2005). Both studies also observed an increase in insulin and glucose upon injection of glucagon, which is acknowledged as being a potential contributor to the inhibition of ghrelin release. In contrast, studies that examined the effects of glucagon directly on ghrelin release from the stomach in rodents had opposite results. In isolated whole rat stomachs, injection of glucagon into the gastric

artery stimulated ghrelin release (Kamegai, Tamura et al. 2004). Furthermore, glucagon injection into the femoral vein of rats was shown to increase ghrelin release into the gastric vein after 10 minutes (Katayama, Shimamoto et al. 2007). Additionally, this study also demonstrated the colocalization of glucagon receptor and ghrelin in stomach tissue using immunohistochemistry (Katayama, Shimamoto et al. 2007). The discrepancy between the human injection studies and the rodent injection/perfused-stomach studies may arise from both the timing of when ghrelin levels were sampled as well as the non-specific effects of injecting exogenous glucagon in an *in vivo* setting.

GLP-1 may also be involved in regulating ghrelin secretion. GLP-1 levels increase after a meal (opposite of ghrelin) to promote insulin secretion (as described above). The effects of GLP-1 on the postprandial drop of ghrelin levels were examined in humans. While an initial delay in the drop was observed (likely due to the delayed gastric emptying effect of GLP-1), the degree of ghrelin suppression was greater in GLP-1 treated individuals (Hagemann, Holst et al. 2007). Insulin, as expected, was significantly increased in this group and may be the mechanism by which GLP-1 can suppress ghrelin secretion. Exendin-4 is a degradation resistant GLP-1-like peptide that was originally extracted from the saliva of the lizard *Heloderma Suspectum* and is currently used in the treatment of T2DM (DeFronzo, Ratner et al. 2005). Injection of exendin-4 in rats caused a sustained (8 hr) reduction on circulating ghrelin (Perez-Tilve, Gonzalez-Matias et al. 2007). Importantly, the effect of exendin-4 on ghrelin levels was independent of increases in insulin (Perez-Tilve, Gonzalez-Matias et al. 2007). Surprisingly, injection of GLP-1 had no effect on ghrelin secretion (Perez-Tilve, Gonzalez-Matias et al. 2007). Further *in*

in vitro studies are needed to elucidate the mechanism of how GLP-1 or analogues may regulate ghrelin secretion.

1.4.5.4 Nervous System Regulation of Ghrelin Secretion

The vagus nerve (part of the parasympathetic nervous system) extends afferent fibres throughout the viscera to sense and relay information to the central nervous system, which then communicates with organs via its efferent fibres. A role for the vagus nerve in the regulation of ghrelin secretion was established soon after ghrelin's discovery. Studies conducting subdiaphragmatic vagotomy (severing of the vagus nerve) were used to establish the role of the vagus nerve in ghrelin secretion. Rats that had undergone vagotomy no longer exhibited the fasting induced increases in ghrelin (Williams, Grill et al. 2003). Interestingly, these animals still had the postprandial suppression in ghrelin secretion, indicating that the vagus nerve may have a more critical role in stimulating fasting ghrelin secretion rather than suppression after a meal.

There is also evidence for the involvement of the sympathetic nervous system in ghrelin secretion. The ability of norepinephrine (NE) to alter energy regulating hormone secretion has precedent since Hansen *et al.* demonstrated (using a porcine ileum preparation) that NE treatment inhibited GLP-1 secretion (Hansen, Lampert et al. 2004). How sympathetic tone is affected by fasting is relevant to its ability to regulate ghrelin. In support of fasting induced sympathetic activity, increased sympathetic turnover was found in the intraperitoneal fat stores of fasted rats (Migliorini, Garofalo et al. 1997). This indicates that sympathetic tone may be important in mobilizing fat stores in periods of

fasting to restore energy availability. Some evidence has also indicated the effects of sympathetic activity on ghrelin secretion. Activation of sympathetic nerves in rats by chemical (using the sympathomimetic tyramine) or electrical stimulation led to a stimulation in ghrelin secretion (Munding, Cummings et al. 2006). Interestingly, no effect of epinephrine on ghrelin secretion was observed, indicating that only neurotransmitter and not neuro-humoral effects were responsible (Munding, Cummings et al. 2006). Overall, it appears that the increased sympathetic activity during periods of fasting may contribute to the increases in ghrelin.

1.4.5.5 Cellular Mechanism of Ghrelin Release

Examining the effects of how the nervous system, nutrients and hormones alter ghrelin secretion, and investigating how ghrelin levels change in response to varying metabolic milieus, provides important information towards understanding the mechanism of ghrelin secretion. However, the information provided by these studies is indirect, as only certain aspects can be controlled when investigating ghrelin secretion in a living system. To clearly understand how ghrelin secretion is regulated, and by which intracellular pathways within the ghrelin producing cells, an *in vitro* cellular model of ghrelin secretion is required. At the time this question was investigated during my thesis, no other groups had published findings of such nature. During the course of my graduate degree several groups have published works alongside my own. These studies are described in relation to my works in the discussion.

1.4.6 Ghrelin Levels in Metabolic Disease States

As is clear from the previous sections, ghrelin secretion is tightly regulated based on energy availability. This regulation is important as ghrelin, through its various targets, will alter energy intake and metabolism. The current understanding of how the regulation of ghrelin levels is dysregulated in metabolic diseases and at extremes in body weight is discussed below.

1.4.6.1 Ghrelin in Obesity

As described earlier, ghrelin administration has been shown to cause obesity (Tschop, Smiley et al. 2000). In addition, individuals with Prader-Willi Syndrome (the most common form of syndromic obesity) have elevated plasma ghrelin levels (Cummings, Clement et al. 2002). So it was surprising when ghrelin levels were found to be negatively correlated with BMI in normal weight and obese Caucasians and Pima Indians (Tschop, Weyer et al. 2001), a finding that has been confirmed by other groups (Shiia, Nakazato et al. 2002). This is paradoxical since lower ghrelin levels should lead to reduced appetite and reduced storage of calories, yet this agrees with the concept that ghrelin levels are expected to decrease in response to an energy surplus.

Despite the lower fasting levels in obese individuals, there appears to be a dysfunction in their postprandial drop in ghrelin. Le Roux *et al.* compared the levels of ghrelin before and after meals (of varying calories) between obese and normal weight individuals. They found that obese individuals (while having lower baseline ghrelin) had

a significantly smaller percent drop in their circulating ghrelin (le Roux, Patterson et al. 2005). Recently, a study examining the postprandial drop in ghrelin in Hispanic adolescents also found the postprandial response to be blunted in obese individuals (Mittelman, Klier et al. 2010). However, another study in female adolescents observed a similar postprandial percent reduction in ghrelin for obese, control, and anorexic children (Stock, Leichner et al. 2005). It should be noted that in this study, the drop in absolute ghrelin levels was smaller for obese adolescents (100pg/ml vs 200pg/ml in control) (Stock, Leichner et al. 2005). It may be that the smaller drop in ghrelin after a meal is contributing to the reduced satiation that is observed in obese individuals. Indeed, time spent feeding and calories required to satiate are known to be directly associated with BMI (Delgado-Aros, Cremonini et al. 2004).

1.4.6.2 Ghrelin in Insulin Resistance

Several of the above studies examining the levels of ghrelin in obesity have also examined the correlation between ghrelin and insulin resistance. Indeed, obesity is often associated with T2DM and a compensatory hyperinsulinemia. As described above, insulin has been demonstrated to reduce ghrelin secretion in both rodents and humans. Efforts have been made to separate the effects of obesity and hyperinsulinemia when examining ghrelin levels in humans. Cummings *et al.* examined a group of 40 obese individuals separated into insulin sensitive and insulin resistant groups. The insulin resistant group had elevated fasting insulin and reduced total ghrelin compared to the insulin sensitive group (with no confounding effect from BMI) (McLaughlin, Abbasi et

al. 2004). There is also some evidence indicating that effects of insulin resistance on ghrelin secretion may only be relevant in the scenario of increased weight. A study examining the correlation between insulin resistance (measured through a euglycemic hyperinsulinemic clamp) in normal weight middle aged (58) men found no interaction between insulin resistance and ghrelin levels when adjusting for body fat (Fagerberg, Hulten et al. 2003). It appears that the hyperinsulemia associated with insulin resistance at least in the context of obesity is an important factor leading to the decreased levels of ghrelin.

1.4.6.3 Ghrelin in Anorexia Nervosa

Anorexia Nervosa (AN) is an eating disorder that is characterized by a refusal to maintain normal body weight, manifested through a fear of gaining weight and disturbance in one's view of their body weight or shape (Diagnostic and Statistical Manual for Mental Disorders IV). In contrast to the lower levels of ghrelin in obesity, individuals who are underweight with AN have elevated fasting ghrelin levels (Shiyya, Nakazato et al. 2002). Interestingly, a study examining the ghrelin response to an oral glucose tolerance test in females found that even after the postprandial drop, AN women had levels above that of fasting controls (Nakai, Hosoda et al. 2003). Since these studies indicated that ghrelin levels are elevated in both fasting and postprandial AN individuals, the concept that "ghrelin resistance" is occurring was tested. Broglio *et al.* examined GH responses to a bolus ghrelin injection in AN and control women. They found AN women, despite having a hyper-responsiveness to GHRH injection, had a reduced GH response to

ghrelin (Broglia, Gianotti et al. 2004). They concluded that at least in the pituitary, chronic elevated levels of ghrelin can lead to a loss of ghrelin sensitivity. Indeed, a similar study that examined the hunger response to an injection of ghrelin found that AN women (in addition to having lower GH response) had no ghrelin mediated increase in hunger after one hour, and only partially increased hunger after five hours (Miljic, Pekic et al. 2006). These studies indicate that ghrelin levels do increase in the situation of extremely low energy availability of AN, however it appears that those with AN are resistant to the appetite inducing effects of ghrelin.

1.4.6.4 Ghrelin After Medical and Surgical Weight Loss

It is well established that weight loss (through non-surgical methods) in overweight and obese individuals is very difficult to maintain as documented in long term weight loss studies (reviewed in (Safer 1991)). There is an interesting connection between the drive toward rebound weight gain after weight loss and the levels of ghrelin. In a study with overweight women, levels of ghrelin were investigated before and after weight loss (through diet and exercise programs) and the authors found that fasting ghrelin levels were directly correlated with percentage weight loss (Hansen, Dall et al. 2002). Therefore it is possible that the elevated levels of circulating ghrelin after weight loss are contributing to the difficulty associated with maintaining weight loss. The role that ghrelin plays in weight loss intervention is perhaps best understood through the success of sustained weight loss in bariatric surgery. Indeed, bariatric surgeries have much higher levels of sustained weight loss (25% weight loss after 10 years) (2005)

compared with diet and exercise alone (5% after 2 years) (O'Brien, Dixon et al. 2006). Cummings *et al.* compared the daily ghrelin levels in individuals that had received Roux en Y gastric bypass surgery, caloric restriction weight loss, or in normal weight no weight loss individuals. In agreement with previous work, they found ghrelin levels were elevated in the diet weight loss group; however, they saw significantly reduced levels and no diurnal rhythm of ghrelin release in the bypass group (Cummings, Weigle et al. 2002). This (and other groups findings) is in contrast to some studies that did not observe any effect on post-operative ghrelin levels or even increased ghrelin levels (reviewed in (Tymitz, Engel et al. 2011)). These discrepancies may be explained on the weight loss state at the time of ghrelin measurement, as individuals that are actively losing weight have elevated post operative ghrelin compared to those that had reached a stable weight at the time measurement (Faraj, Havel et al. 2003). Nevertheless, it appears that reduced ghrelin levels may play a role in the success of maintained weight loss in bariatric surgeries.

1.4.7. Strategies to Modulate Ghrelin Levels

As described, ghrelin has important roles in promoting energy intake and promoting adiposity. In metabolic diseases, the regulation of ghrelin secretion is perturbed. These aspects make ghrelin an important target for therapy in metabolic diseases. As such, several studies have examined the feasibility of altering ghrelin levels through different strategies. These strategies and the state of ghrelin related clinical trials are discussed below.

1.4.7.1 Ghrelin Receptor Antagonism

Early on, before the discovery of ghrelin, GHSR antagonists had been developed (Smith, Cheng et al. 1993). Early studies demonstrated that peripheral and ICV administration of the GHSR antagonist [D-Lys]-GHRP-6, reduced feeding in obese and normal weight mice (Asakawa, Inui et al. 2003). Since this discovery, novel small molecule ghrelin receptor antagonists have been developed (such as YIL-781) that confirmed the reduced feeding and weight gain results previously shown, but also demonstrated an additional improvement in glucose tolerance tests (Esler, Rudolph et al. 2007). While research continues to be published on the development and use of ghrelin receptor antagonists, these compounds are still in the pre-clinical stage of drug development (reviewed in (Delporte 2012)). It is possible that due to broad spectrum of ghrelin targets, including GH release and the reproductive axis, that non-specific blocking of the ghrelin receptor may have negative side effects. Nevertheless, animal work and eventually clinical trials may shed light on the feasibility of this approach.

1.4.7.2 GOAT Antagonism

As described earlier, GOAT is the acyl transferase responsible for adding the octanoyl moiety to ghrelin. This modification is unique as no other GOAT substrates have been identified. Since the appetite and adipogenic effects are primarily mediated by the acylated form of ghrelin, preventing this modification is an attractive approach to ghrelin reducing therapies. Using GOAT-transfected insect membranes, Yang *et al.* designed an octanoylated peptide (based on ghrelin) with a modified diaminopropionic

ester (DAP³) at the 3rd position instead of serine that was able to inhibit the octanoylation of ghrelin (Yang, Zhao et al. 2008). Subsequently, Barnett *et al.* designed a similar compound (GO-CoA-Tat) and tested its effects in mice. Daily administration of GO-CoA-Tat reduced circulating AG and prevented weight gain after eight days of treatment (Barnett, Hwang et al. 2010). Additionally, they demonstrated pre-treatment of GO-CoA-Tat in both human islet cells and mice leads to increased insulin production and improved glucose stimulated insulin release respectively (Barnett, Hwang et al. 2010). As this is a peptide-based drug, there may be some limitations for its use in therapy; however, future stable or synthetic GOAT inhibitors may hold promise for a ghrelin altering therapy.

1.4.7.3 Ghrelin Neutralization

Several strategies have been developed to reduce the levels of ghrelin in circulation. Zorrilla *et al.* immunized rats using short, ghrelin hapten peptides based on octanoylated ghrelin, and observed a reduction in feeding and weight gain (Zorrilla, Iwasaki et al. 2006). Despite this promising finding in animals, phase 2 clinical trials from Cytos Biotech using a ghrelin vaccine did not cause weight loss in obese patients (Delporte 2012). Using a different approach, Mayorov *et al.* designed catalytic antibodies which specifically bound to ghrelin and hydrolysed the octanoyl moiety. Mice treated with this catalytic antibody had a reduced metabolic rate and reduced re-feeding after a fast (Mayorov, Amara et al. 2008). In addition to these antibody-based approaches to reduce ghrelin, novel ribonucleic spiegelmers (L-RNA instead of D-RNA) have been designed to bind to AG. This novel ghrelin binding spiegelmer (L-NOX-B11) prevented the activation of the GHSR *in vitro* and reduced the stimulatory effect of ghrelin on GH

release in rats (Helmling, Maasch et al. 2004). Later work demonstrated that the L-NOX-B11-2 spiegelmer was also able to block ghrelin induced feeding (Shearman, Wang et al. 2006). In addition, they demonstrated that chronic (13 day) daily administration of the spiegelmer caused reduced feeding and increased fat weight loss in high fat diet-induced obesity (DIO) mice (Shearman, Wang et al. 2006). To verify that these effects were occurring through interaction with ghrelin, the treatments were examined in ghrelin knockout animals and had no effect on feeding (Shearman, Wang et al. 2006). Development of the NOX compounds is currently underway at Pfizer Pharmaceuticals (Delporte 2012). The various pharmacological tools described are presented in Figure 8.

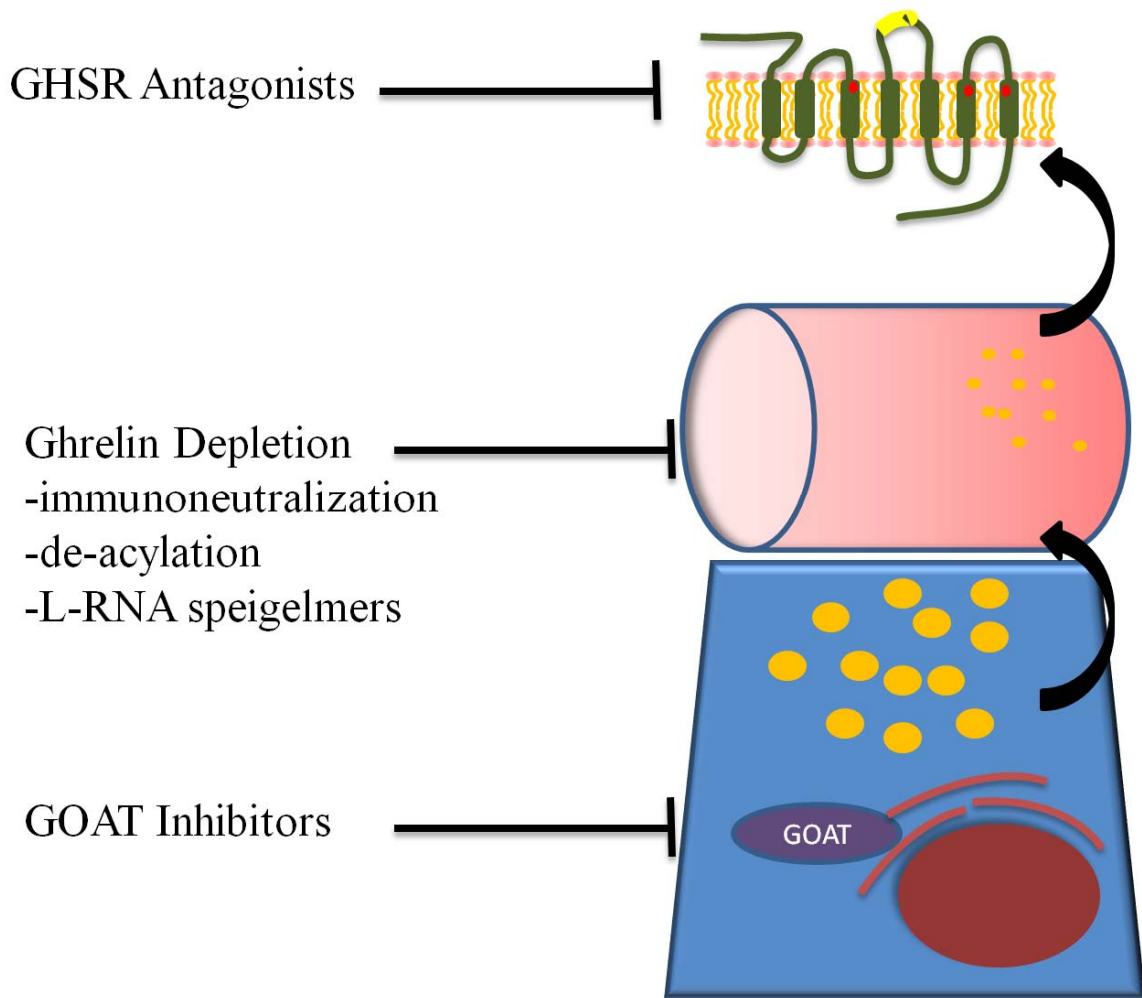


Figure 8 Strategies to reduce ghrelin action. Ghrelin receptor antagonism using small peptide antagonists can reduce weight gain and improve glucose tolerance in animals. Depleting circulating acylated ghrelin by administering anti-ghrelin or catalytic antibodies that de-acylate ghrelin can block diet induced obesity. Inhibitors of GOAT prevent the acylation of ghrelin, and have shown some therapeutic promise in animals.

1.5 Preface to Manuscripts

Ghrelin has key roles in regulating whole body energy metabolism. Therefore, the ability of ghrelin cells to sense and respond to the energy milieu is important. However, the cellular mechanism(s) of ghrelin secretion are sparsely understood, due in part to the lack of a readily available ghrelin secreting cell lines. Chapters 2-5 describe the development of a primary stomach cell culture from young rats and its use in elucidating the roles of neurotransmitters, hormones, nutrients, and antidiabetics in the cellular mechanism of ghrelin secretion.

Ghrelin administration has been demonstrated to increase feeding and adiposity as well as lead to impaired glucose homeostasis. These findings have led to the investigation of strategies that reduce active ghrelin or inhibit the activation of the ghrelin receptor. In the final manuscript (Chapter 6), a novel strategy, using a circulating ghrelin receptor based decoy protein to reduce circulating ghrelin is presented.

Chapters 2, 3 and 5 are published manuscripts (Chapter 6 under minor revisions) and are presented with full discussions. The discussion for chapter 4 occurs in the overall discussion section. Permission has been obtained for the use of these publications and letters from the publishers are appended at the back of this thesis.

CHAPTER 2: INSULIN AND NOREPINEPHRINE REGULATE GHRELIN SECRETION FROM A RAT PRIMARY STOMACH CELL CULTURE

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2.1 Abstract

Ghrelin is a peptide hormone primarily produced in the previously unidentified X/A endocrine cells of the stomach. Extensive studies have focused on the effects of ghrelin on growth hormone release and appetite regulation; however, the mechanisms regulating ghrelin secretion are less understood. In the present study, we developed a primary culture of newborn rat stomach cells to investigate the mechanisms regulating ghrelin synthesis and secretion. We demonstrated that this cell preparation secretes ghrelin in a regulated manner through the increase of cAMP, intracellular calcium and activation of protein kinase C. Norepinephrine (0.1-10 μ M) stimulated ghrelin secretion through the β 1 adrenergic receptor via increased cAMP and protein kinase A activity, while acetylcholine had no effect. Since circulating ghrelin levels were previously shown to be inversely correlated with insulin levels, we investigated the effect of insulin on ghrelin secretion. We first demonstrated that ghrelin cells express the insulin receptor α and β subunits. Next, we determined that insulin (1-10nM) inhibited both basal and norepinephrine-stimulated ghrelin secretion, caused an increase in phosphorylated AKT and a reduction in intracellular cAMP, but did not alter proghrelin mRNA levels. The inhibitory effect of insulin was blocked by inhibiting phospho-inositol-3-kinase and AKT but not MAPK. Higher dose insulin (100 nM) did not suppress ghrelin secretion which prompted the investigation of cellular insulin resistance by pretreating the cells with 100nM insulin for 24 hours. This caused a reduction in insulin receptor expression and prevented the insulin mediated AKT activation and the suppression of ghrelin secretion with no impact on norepinephrine-stimulated ghrelin secretion. Our findings highlight the

role of the sympathetic nervous system, insulin and insulin resistance in the regulation of ghrelin secretion.

2.2 Introduction

Ghrelin is a 28 amino acid peptide that is primarily produced in the previously uncharacterized X/A (as their product was unknown at the time) enteroendocrine cells of the gastric mucosa (Kojima, Hosoda et al. 1999). Ghrelin stimulates growth hormone release and appetite, and alters energy metabolism (Wang, Lee et al. 2002; Higgins, Gueorguiev et al. 2007). Ghrelin levels in circulation fluctuate in response to energy availability, with higher levels in the fasting state and lower at the fed state (Tschop, Smiley et al. 2000; Gomez, Englander et al. 2004). The elevated ghrelin present in the fasted state promotes an orexigenic response from the appetite neurons within the arcuate nucleus of the hypothalamus (Tschop, Smiley et al. 2000). Under chronic energy surplus or deficit, ghrelin levels are also altered. In obese patients, circulating ghrelin levels are reduced (Tschop, Weyer et al. 2001). In contrast, chronic energy deficit, such as anorexia nervosa, is associated with increased circulating ghrelin (Shiia, Nakazato et al. 2002). These studies illustrate that ghrelin secreting cells have the ability to sense and respond to energy status by increasing or decreasing ghrelin synthesis and secretion. However, the mechanisms by which ghrelin cells modulate secretion during these energy states are not fully understood.

A likely candidate in the regulation of ghrelin secretion is the autonomic nervous system. The gastrointestinal tract is heavily innervated by the intrinsic (enteric) and extrinsic (parasympathetic and sympathetic) nervous systems which control many aspects of gastro-intestinal physiology including motility and secretion (Holmgren and Olsson 2011; Olsson and Holmgren 2011). There is some evidence that sympathetic nerve

stimulation can stimulate ghrelin secretion (Mundinger, Cummings et al. 2006), however the cellular mechanism is not known.

The endocrine system responds actively to energy state with changes in circulating hormones like insulin and glucagon. Insulin levels are inversely related to ghrelin during both fasted and fed states making insulin a strong candidate for modulating ghrelin secretion (Cummings, Purnell et al. 2001). In humans, acute insulin infusion causes a rapid and reversible drop in ghrelin levels (Saad, Bernaba et al. 2002). Interestingly, hyperinsulinemic obese individuals have lower circulating ghrelin compared to normal insulin obese individuals (McLaughlin, Abbasi et al. 2004). Even in anorexia, where the levels of ghrelin are elevated, an infusion of insulin causes a marked reduction in ghrelin levels (Karczewska-Kupczewska, Strackowski et al. 2010). These results suggest that insulin is an important hormone regulating ghrelin secretion; however, the cellular evidence and the downstream intracellular pathway of insulin on ghrelin secretion is lacking. Recently, two research groups have developed ghrelin secreting cell lines derived from mouse ghrelinomas (Iwakura, Li et al. 2010; Zhao, Sakata et al. 2010). The effect of insulin on ghrelin secretion in these cell lines is conflicting. Iwakura et al. showed that insulin was able to significantly reduce ghrelin secretion and proghrelin mRNA expression (Iwakura, Li et al. 2010) while Zhao et al. reported no effect of insulin on ghrelin secretion (Zhao, Sakata et al. 2010). Although these ghrelin cell lines are important models for investigating the mechanisms of ghrelin secretion, they may not be under normal metabolic regulation due to their tumor origin. Indeed, recent studies comparing tumor derived with primary cultured hepatocytes found considerable differences in their protein profiles (Pan, Kumar et al. 2009). Furthermore,

problems in gene transcription of hormones has been observed in tumor derived intestinal cell lines (Cao, Flock et al. 2003).

Insulin activates the insulin receptor tyrosine kinase domain, which phosphorylates and recruits different substrate adaptors such as the insulin receptor substrate (IRS) family of proteins (Bevan 2001). Tyrosine phosphorylated IRS then displays binding sites for numerous signaling partners (Bevan 2001). Among them, phospho-inositol-3 kinase (PI3K) has a major role in insulin function, mainly via the activation of AKT/PKB (Watson, Kanzaki et al. 2004). Insulin stimulates glucose uptake in muscle and adipocytes via translocation of glucose transporter 4 vesicles to the plasma membrane, through the PI3K/AKT pathway (Miinea, Sano et al. 2005). Insulin signaling also has growth and mitogenic effects, which are mediated by the activation of the mitogen-activated protein kinase (MAPK) pathway (Bevan 2001). The specific pathway by which insulin regulates ghrelin secretion is unknown. Interestingly, insulin was shown to stimulate glucagon-like peptide-1 secretion from the enteroendocrine “L” cells through the activation of the PI3K and MAPK (Lim, Huang et al. 2009) suggesting this pathway may be relevant in ghrelin secretion.

In this study, we established a primary culture of gastric ghrelin secreting cells from postnatal day 8 rats. We characterized the expression and secretion of ghrelin and the presence of its post-translational modifying enzymes. We then demonstrated that norepinephrine stimulated ghrelin secretion through the β 1 adrenergic receptor, increased intracellular cAMP and PKA activity, while insulin reduced ghrelin secretion through the PI3K/AKT pathway. Interestingly, the inhibitory effect of insulin on ghrelin secretion

was lost when cells were preincubated in high concentration insulin for 24h, suggesting that insulin resistance can occur at the level of stomach ghrelin cells.

2.3 Materials and Methods

Animals

All animal studies were approved by the Dalhousie University animal care committee and strictly adhere to the policies of the Canadian Council for Animal Care. Untimed pregnant Sprague Dawley rats were purchased from Charles River (Wilmington, MA). The mother and the pups were housed in a large rat cage with free access to food and water. The average litter size was 13 pups. Litters were sacrificed at postnatal day 8 (PND8).

Primary cell culture preparation

All cell culture media and reagents, unless otherwise stated, were obtained from Sigma Aldrich (Oakville, ON, Canada). Primary cell culture was prepared from mixed pups (male and female) as there was no difference between genders for basal ghrelin percentage secretion ($11.9 \pm 1.37\%$ male and $12.38 \pm 1.24\%$ female). Mixed pups were anaesthetized with isoflurane and sacrificed by decapitation. From each litter, 3 separate cellular preparations comprised of 3 animals each were prepared. The stomach endocrine cell extraction protocol was adapted from Buchan and colleagues (Buchan 1996) with some modifications. Briefly, the stomachs were immediately removed, cut longitudinally and contents were gently removed with care not to destroy the mucosal cell layer. The stomachs were placed in wash buffer (Hanks buffered saline salts supplemented with 0.1% BSA and 10 mM HEPES). Media was then aspirated and stomachs were minced

into small pieces using surgical scissors. Stomachs were resuspended in 5ml of wash buffer and incubated at room temperature for 5 minutes. The stomachs were then subjected to enzymatic digestion in 5ml of wash buffer supplemented with type 1A collagenase (6mg/ml) with orbital shaking at 37 C for 20 minutes. Five ml of wash buffer was added and digestion mixture was incubated at room temperature for 5 minutes. The supernatant was collected, centrifuged at 200 g for 10 minutes and set aside as digestion fraction #1. The remaining undigested material was treated to a second 20 minute digestion in collagenase (3mg/ml) followed by an additional 5 minute incubation with 100ul of 5mM EDTA. Cells were rinsed with 5ml of DMEM 10% fetal bovine serum (FBS) and passed through a 200uM nylon cell strainer (VWR, ON, Canada) into a 50ml tube along with digestion fraction #1. The filtrate was spun down at 200 g for 10 minutes. The cell pellet was washed with culture media (DMEM (high glucose) supplemented with 10% FBS and 1% penicillin/streptomycin) and spun down at 200 g for another 10 minutes. The final cell pellet was then resuspended in 10 mL of culture media. Cell count was determined using a hemocytometer. Cells were plated in 6 well or 10 cm cell culture plates for ghrelin secretion or proghrelin mRNA expression experiments, respectively.

Ghrelin secretion experiments

Secretion experiments were completed in 6-well cell culture plates (BD Falcon, Mississauga, ON Canada). Cells were plated at 1×10^6 cells per well in 2ml of culture

media. For total ghrelin secretion experiments, treatments were started 48 hours after cells were put in culture. For acyl-ghrelin secretion experiments, after the first 24 hours, cells were cultured for 24 hours in medium containing 50uM octanoic acid-BSA conjugate prepared as per Zhao et al (Zhao, Sakata et al. 2010). For insulin resistance experiments, after the first day in culture, cells were incubated in fresh media (10% FBS DMEM) supplemented with 100nM insulin for 24 hours. On the day of secretion experiment, cells were washed with PBS and incubated with different treatments dissolved in 2mL of low glucose (1000mg/L), phenol red free DMEM and 0.5% FBS for 4 hours. After incubation, culture media was collected and spun at 1000 x g for 5 minutes to remove any floating cell debris. The supernatant was acidified with 200 uL of 1% trifluoroacetic acid (TFA) and kept on ice to prevent protease activity and loss of the acylated ghrelin. Cells were scraped in 500ul of an acidic lysis buffer (1M HCl, 1% TFA and 50mM NaCl), and were sonicated for 5 seconds on ice. The lysate was spun down for 5 minutes at 11,000 x g at 4C. The supernatants from the culture media and cell extract were then subjected to hydrophobic reverse phase resin chromatography (C-18 SepPak cartridges, Waters) according to the manufacturer's instructions, eluted in 4 ml of 80% isopropanol in water containing 0.1% TFA, and dried completely in a vacuum concentrator. Recovery of both ghrelin forms from the Sep-Pak columns (as determined from pure ghrelin) was >85%. Pellets were kept at -80C and resuspended in immunoassay buffer immediately before assay. To control for the variable number of ghrelin expressing cells in each well, ghrelin was measured in the culture media and the cell extract. Therefore ghrelin secretion was always normalized to total ghrelin content of each well (media/(media+lysate)). This “percentage secretion” controls for changes in the

level of secreted ghrelin that may occur due to the proportion of ghrelin cells in each experiment and has been previously used successfully in other primary cell preparations (Brubaker 1988).

Ghrelin assays

Total ghrelin (acyl and non-acyl) level was determined using total ghrelin RIA (Phoenix Pharmaceuticals, Burlingame CA) as per the manufacturer's guidelines. This assay recognizes both acylated and non-acylated ghrelin with a minimum detection limit of 10 pg/ml. Acylated ghrelin was determined using active ghrelin enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor MI) as per the manufacturer's guidelines. This assay recognizes only the acylated form of ghrelin (cross reactivity with non-acylated ghrelin <0.001%) with a detection limit of 1 pg/ml. Accuracy of assay was confirmed using provided quality controls. Ghrelin levels were determined for entire media and cell lysate from each sample. Results for various treatments were presented as percentage secretion relative to untreated control.

Immunocytochemistry

Cells were plated on glass coverslips in 12 well plates at 1×10^5 cells per well. Cells were then rinsed with TRIS buffered saline (TBS) and fixed in 4% paraformaldehyde for 20 minutes at room temperature. Cells were rinsed 3 x 5 minutes in TBS before being permeabilized in 0.1% triton X -100 for 15 minutes. Cells were

blocked for 20 minutes in a TBS blocking buffer containing 0.1% Triton X and 5% normal donkey serum (Vector Labs, Burlington ON Canada). Antibodies for proprotein convertase 1 (PC1) (Abcam, Cambridge MA), ghrelin-o- acyl transferase (GOAT), insulin receptor α and β subunits and proghrelin (Santa Cruz biotechnology, Santa Cruz CA) were used at a 1:100 dilution in blocking buffer overnight at 4C. Fluorescent secondary antibodies were obtained from Invitrogen (Carlsbad CA) and incubated at 1:150 for 45 minutes at room temperature. Coverslips were then mounted on slides in Vectashield mounting medium containing DAPI (Vector Labs, Burlington ON Canada) and visualized using a Zeiss Axioplan 2 fluorescent microscope.

Quantitative RT-PCR

Cells were plated into 10 cm plates at 4×10^6 cells per plate and grown for 24 hours. Cells were rinsed with PBS, and then treated in DMEM/0.5% FBS with \pm 10nM insulin for 4, 8 or 16 hours. Media was then aspirated and cells were rinsed with PBS before RNA extraction was performed using Bio-Rad Aurum total RNA extraction kit (Bio-Rad labs, ON, Canada). One μ g of RNA (quantified using absorbance at 260nm) was converted to cDNA using Bio-Rad iScript cDNA synthesis kit. Realtime PCR was completed using SYBR green master mix (Bio-Rad) with primers for proghrelin (Forward: TGG CAT CAA GCT GTC AGG AGC, Reverse: AGC TGG CGC CTC TTT GAC CT), and for reference genes, 18S (Forward: TCA ACT TTC GAT GGT AGT CGC CGT, Reverse: TCC TTG GAT GTG GTA GCC GTT TCT) and Rpl13a (Forward: ATG GCG GAG GGG CAG GTT CT, Reverse: CCA CCA CCT TTC GGC CCA GC).

Primers were designed based on rat intron-spanning oligos for proghrelin and rpl13a using primer quest software (Integrated DNA Technologies, Coralville IA).18S. Data was analyzed using Bio-Rad CFX Manager software version 1.6 using the relative quantification method (standard curve). A single PCR product was confirmed by agarose gel electrophoresis and products were verified by sequencing.

Western blotting

Cells were plated into 10 cm plates at 4×10^6 cells per plate and allowed to grow for 24 hours at 37C, 5% CO₂. Insulin treatments (10nM) were prepared in Krebs Ringer Buffer with 10mM HEPES (pH 7.4) and given to cells for 5, 10, 15, 20 30 and 60 minutes at 37C. Cells were then scraped on ice in Cytobuster cell lysis buffer (EMD Biosciences, Gibbstown NJ) supplemented with protease and phosphatase inhibitor cocktails (Complete Mini/Phospho-Stop, Roche applied science, ON Canada). Cells were sonicated and protein was quantified using the Bradford method. Protein extracts from mouse 3T3-L1 differentiated adipocytes were used as a positive control for insulin receptor western blots. Forty ug of protein extract was denatured and separated in 8% acrylamide SDS Tris tricine gels at 100V. Gels were transferred for 1 hour at 100V to PVDF membranes. Western blots were blocked in 5% milk 0.1% Tween-20. Primary antibodies for AKT, pAKT and insulin receptor (β -subunit) were obtained from Cell Signaling (Danvers, MA), and actin from Abcam (Cambridge MA). Secondary HRP conjugated antibodies were obtained from GE Healthcare (Piscataway NJ).

Chemiluminescence was measured using the Bio-Rad Chemidoc XRS and analyzed using the 1-D software from Bio-Rad.

cAMP assays

Cells were plated in 6 well plates at 1×10^6 cells per well and grown for 24 hours in standard culture media. Cells were then placed in serum free media for 2 hours followed by serum free media with 10uM IBMX for 30 minutes. Treatments were then given in fresh serum free media with 10uM IBMX for 30 minutes. Media was then discarded and cells were collected and assayed with the cAMP EIA kit (Cayman Chemical, Ann Arbor MI) following the manufacturer's guidelines. To control for possible variations in total cell number after treatments, cells were counted after 30 minute treatment period. No significant difference was observed in the number of cells present after treatments ($1.62 \times 10^6 \pm 0.03 \times 10^6$ for control, $1.60 \times 10^6 \pm 0.16 \times 10^6$ for insulin and $1.53 \times 10^6 \pm 0.05 \times 10^6$ for norepinephrine).

Data analysis

All data are expressed as mean \pm SEM. Data was analyzed by Student's *t* test or one/two factor ANOVA with appropriate post hoc testing. Comparison of stomach and intestine ghrelin from embryonic and postnatal animals was completed with two factor ANOVA with Bonferroni post hoc test. Dose response experiments were analyzed using one factor ANOVA with Dunnett's post hoc test (p values < 0.05 were considered statistically significant).

2.4 Results

Establishment of rat stomach primary culture

The first objective was to determine a source of ghrelin cells for the production of primary culture that was capable of being cultured and had high levels of ghrelin producing cells. Embryonic day 18 (E18) and PND8 cells extracted from both stomach and intestine attached to culture plates while adult cells were largely unable to adhere and survive. The levels of total ghrelin were examined in whole tissue homogenates of stomach and intestine from E18 and PND8. The stomachs of PND8 pups had the highest levels of ghrelin (1163 ± 98.4 pg ghrelin/ug in PND8 and 61.2 ± 12.1 pg ghrelin/ug tissue in E18, $p < 0.001$, (Figure 9A, B). Cells extracted from digested stomachs contained a heterogenous population of stomach cells including ghrelin positive endocrine cells (Figure 9C). To quantify the percentage of ghrelin cells present in the preparation, 3 fields of view (at 400x magnification) from 3 distinct cell preparations were counted for ghrelin immuno-positive cells and total cells (using DAPI nuclear staining). Ghrelin cells represented 4.54 ± 0.31 out of 100 cells (Figure 9C). To determine if ghrelin positive cells express PC1 and GOAT (enzymes necessary for the processing of proghrelin (Zhu, Cao et al. 2006) and the octanoylation of ghrelin (Yang, Brown et al. 2008), respectively) double immunofluorescence with anti-PC1 or anti-GOAT with anti-ghrelin antibodies was completed. Ghrelin positive cells (green) were colocalized with PC1 (in red, upper panel) and GOAT (in red, lower panel) in fluorescent double immunocytochemistry (Figure 9D). The basal rate of ghrelin percentage secretion was $7.80 \pm 0.70\%$ at 1hr, $13.1 \pm 2.0\%$ at 2hrs and $19.7 \pm 3.2\%$ at 4 hours (Figure 9E). After 4 hours, total ghrelin (deacyl + acyl) content per well was 964 ± 58.3 pg in media and 5395 ± 775 pg in cells

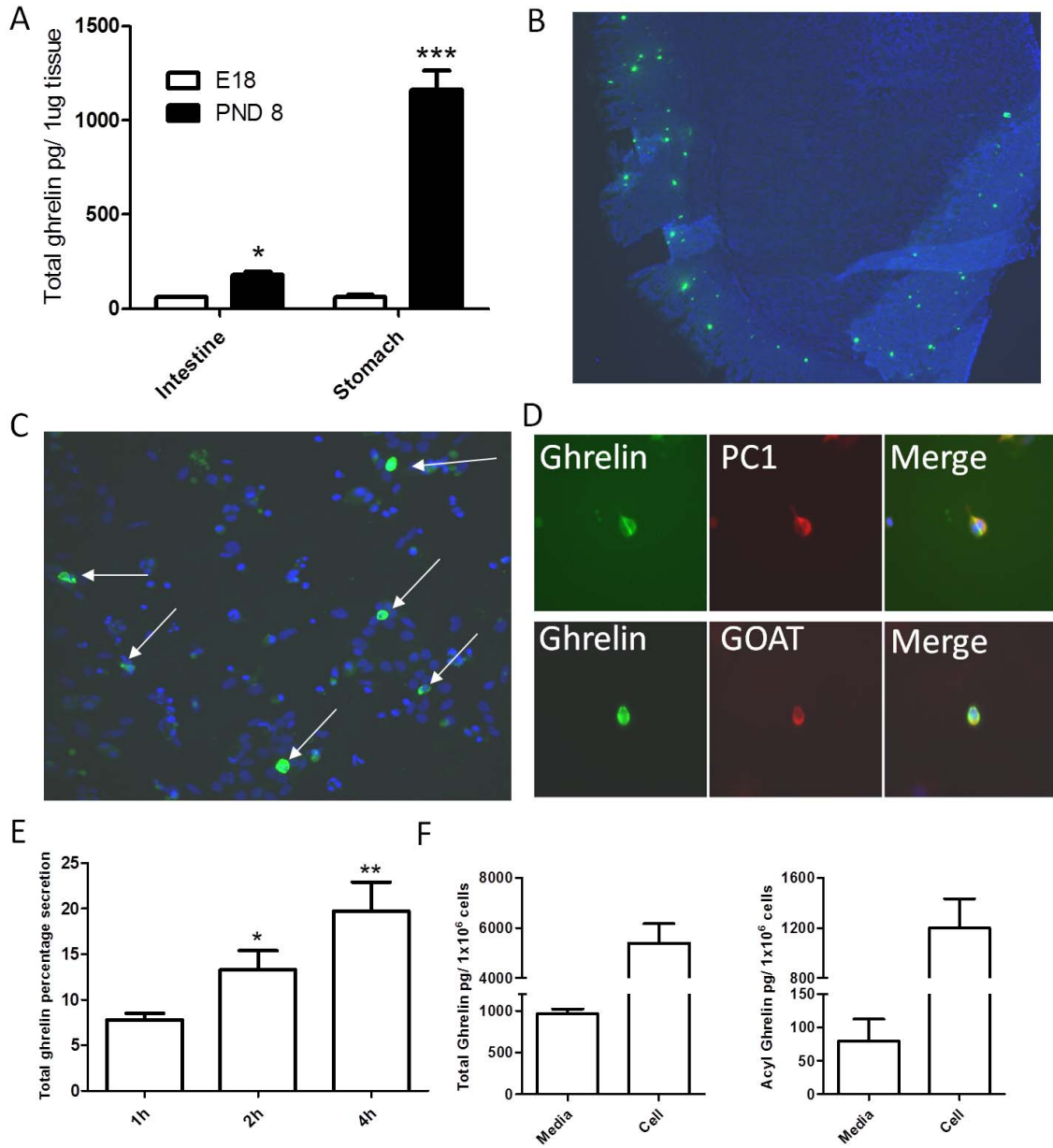


Figure 9 Development and characterization of rat stomach primary culture. Total ghrelin levels from 1ug of tissue were examined in stomach and small intestine from embryonic (white) and postnatal day 8 (PND8) (black) rats (A). Ghrelin positive cells (in green) were examined in stomachs of PND8 pups by fluorescent immunohistochemistry with DAPI nuclear stain (blue) (B). After extraction, ghrelin positive cells (green) were detected in the cell preparation by fluorescent immunocytochemistry of primary culture (C). Ghrelin positive cells (green) colocalized with proprotein convertase 1 (PC1, in red, upper panel) and ghrelin o acyl transferase (GOAT, in red, lower panel) in fluorescent double immunocytochemistry (D). Percentage secretion for total ghrelin (acyl and de-acyl) was examined after 1, 2 and 4 hours in fresh media (comparison between 1hr with 2 and 4hrs (E). Total (acyl and de-acyl, on left) and acyl (right) ghrelin levels in media and cell lysate from 1×10^6 cells were examined after 4 hours (F). ***, $p < 0.001$, **, $p < 0.01$ and *, $p < 0.05$ vs. control (n=6).

, while acyl ghrelin was 79.9 ± 35 pg in media and 1200 ± 233 pg in cells (Figure 9F).

Intracellular pathways regulating ghrelin secretion

To evaluate the intracellular pathways involved in ghrelin secretion, cells were pharmacologically treated to increase PKC, cAMP/PKA and intracellular calcium. Incubating the cells with the phorbol ester PMA (10uM), which activates PKC, stimulated acyl ghrelin secretion (2.83 ± 0.492 fold of control, $p < 0.05$, Figure 10A). Activating adenylyl cyclase with forskolin (10uM), which increases cAMP production and PKA activation, significantly increased acyl ghrelin secretion (3.56 ± 0.61 fold of control, $p < 0.01$, Figure 10A). IBMX (10uM), a phosphodiesterase inhibitor that increases cAMP levels, significantly stimulated acyl ghrelin secretion (2.81 ± 0.672 fold of control, $p < 0.05$, Figure 10A). Next, ghrelin cells were treated with compounds known to increase intracellular calcium including the calcium ionophore ionomycin and the calcium store depleting thapsigargin. Both compounds led to a robust increase in acyl ghrelin secretion (20.3 ± 6.28 , $p < 0.05$ and 16.1 ± 7.31 , $p < 0.05$, fold of control, respectively, Figure 10B).

Ghrelin secretion is regulated through the β -adrenergic system

To determine the role of the nervous system, cholinergic and adrenergic neurotransmitters were tested on ghrelin secretion. Acetylcholine (10uM, n=6) had no effect on ghrelin secretion (data not shown). However, norepinephrine (0.1, 1 and 10uM)

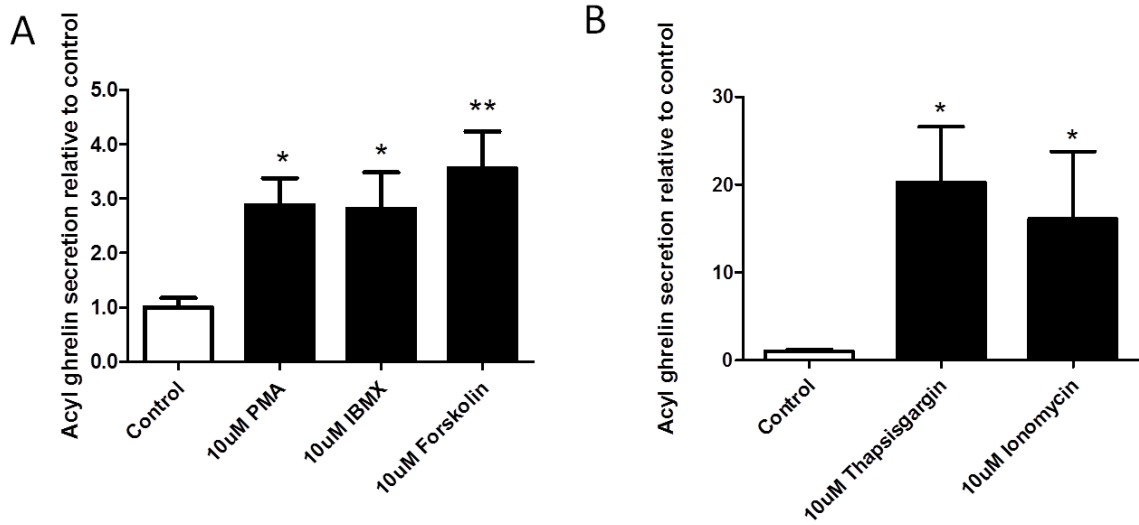


Figure 10 Intracellular regulation of ghrelin secretion. Acyl ghrelin secretion was determined after 4 hour treatments with PMA (10uM), IBMX (10uM) or forskolin (10uM) (A). Acyl ghrelin secretion was determined after 4 hour treatments with thapsigargin (10uM) and ionomycin (10uM) (B). Data is presented as mean relative to control \pm SEM with *, $p < 0.05$ and **, $p < 0.01$ vs. control (n=6).

significantly stimulated acyl ghrelin secretion (4.30 ± 1.02 $p < 0.05$, 9.87 ± 1.27 $p < 0.01$ and 8.61 ± 1.52 $p < 0.01$ respectively, Figure 11A). The stimulatory effect of norepinephrine was blocked by pre-incubating the cells with the β_1 adrenergic receptor antagonist atenolol (10 μ M) (Figure 11B). As the β_1 adrenergic receptor was shown in previous studies to activate the G-protein α_s subunit and subsequently adenyl cyclase (Bahouth and Lopez 1992), cAMP levels were examined next. Norepinephrine (10 μ M) significantly increased cAMP production (1.87 ± 0.37 fold of control, $p < 0.05$, Figure 11C). Finally, confirming that the effect of norepinephrine was mediated through PKA, co-incubation of norepinephrine with the PKA inhibitor H89 (10 μ M) completely blocked the stimulation of ghrelin secretion (Figure 11D).

Insulin reduces ghrelin secretion

Since circulating ghrelin levels are inversely correlated to the levels of insulin, and several in vivo studies have indicated that insulin injection leads to reduced ghrelin levels, we investigated the role of insulin on ghrelin secretion. First, ghrelin cells were examined for expression of the insulin receptor. As shown in Figure 12A, using double immunofluorescence staining (panel i), ghrelin (green) was colocalized with the insulin receptor α (red) and β (red) subunits. Insulin receptor was also detected in cell lysates of primary stomach culture via western blot against insulin receptor β subunit, with differentiated adipocyte (3T3-L1) lysate used as a positive control (panel ii, Figure 12A). The effect of insulin on basal ghrelin secretion was examined in a dose range of 0.01 to 100nM. Insulin significantly reduced acyl ghrelin secretion at 1nM (0.661 ± 0.108 fold,

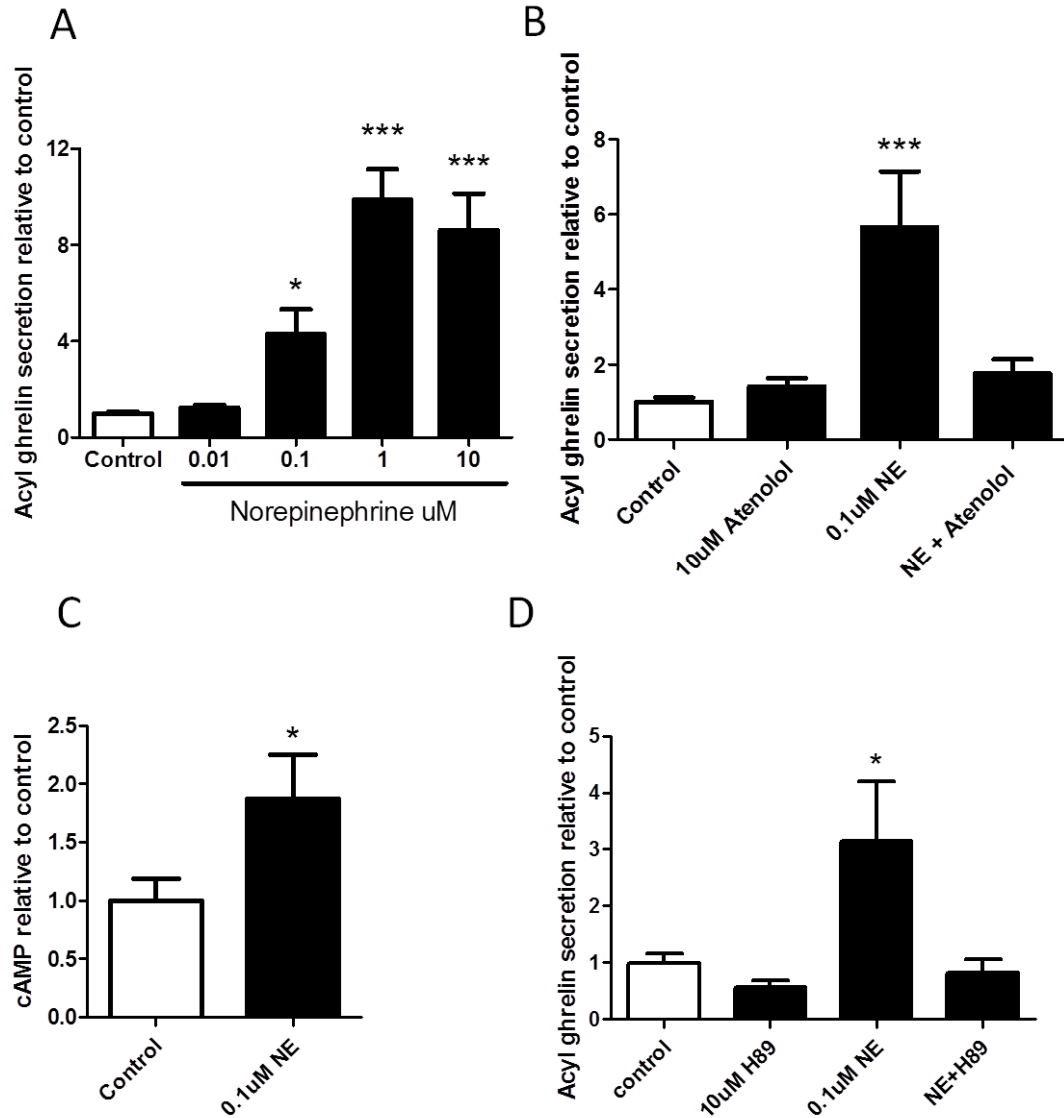


Figure 11 β adrenergic regulation of ghrelin secretion through PKA and cAMP. Acyl ghrelin secretion was examined after 4 hour treatments with 0.01, 0.1, 1 and 10uM norepinephrine (A, n=9). Norepinephrine (NE) treatment (0.1uM) was tested in combination with the β 1 receptor antagonist atenolol (10uM) (B, n=6) and PKA inhibitor H89 (10uM) (D, n=6). Intracellular cAMP level was measured after 30 minute treatment with norepinephrine (0.1uM) (C, n=6). Data is presented as mean relative to control \pm SEM with ***, $p < 0.001$, **, $p < 0.01$ and *, $p < 0.05$ vs. control.

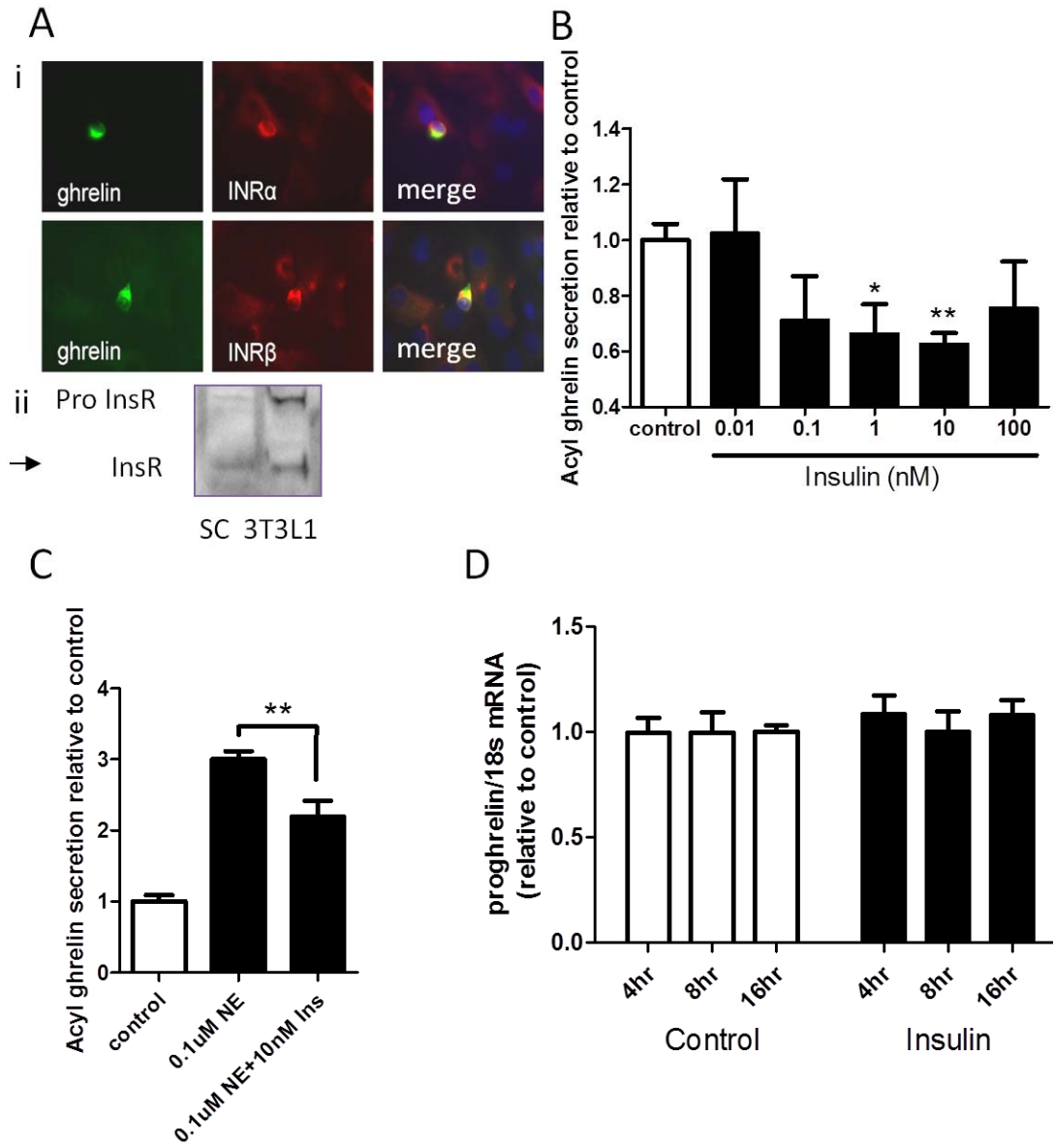
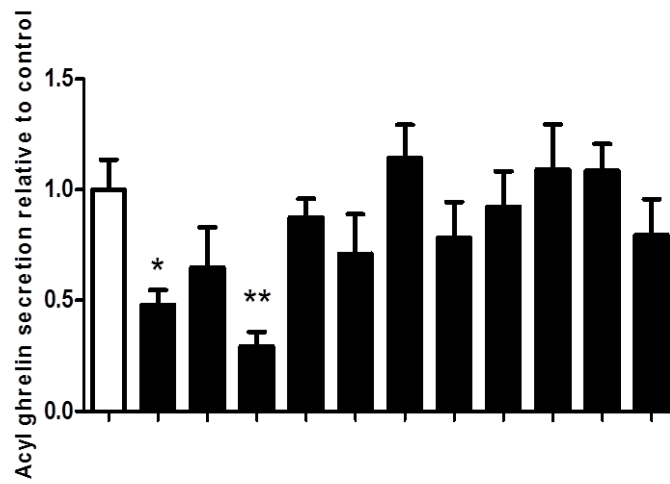


Figure 12 The effect of insulin on ghrelin secretion. Colocalization of insulin receptor α and β subunits (red) with ghrelin (green) was examined using double immunofluorescence (Ai). The protein expression of insulin receptor (β -subunit, pro-form upper 220kd band and mature-form lower 100kd band) was confirmed by western blotting of total cell lysates from stomach culture (SC) and differentiated 3T3L1 mouse adipocytes (used as positive control Aii). Acyl ghrelin secretion was examined after 4 hour treatment of insulin at various doses (0.01, 0.1, 1, 10 and 100nM) (B, n=9). To test the effect of insulin on NE stimulated ghrelin secretion, insulin (10nM) was co-incubated with norepinephrine (NE) (0.1uM) (C, n=6). The effect of insulin (10nM) on Proghrelin/18S mRNA expression was examined by quantitative RT-PCR after 4, 8 and 16 hour treatment with 10nM insulin (D, n=6). Data presented as mean relative to control \pm SEM with ** p<0.01 and *P<0.05 vs. control.

p<0.05) and 10nM (0.624 ± 0.04 fold, p<0.01, Figure 12B). The effect of insulin was in a U-shape dose effect, with low (0.01 and 0.1 nM) and very high (100 nM) insulin concentrations having no effect on ghrelin secretion. To determine if insulin could reduce stimulated ghrelin secretion, insulin was given in combination with NE. Insulin (10nM) in combination with NE (0.1uM) was able to significantly reduce the elevated secretion levels of NE alone (2.19 ± 0.227 vs 3.00 ± 0.117 for NE alone, p<0.05, Figure 12C). The role of insulin on proghrelin mRNA expression was examined after 4, 8 and 16 hours of incubation with insulin (10nM). Quantitative real time PCR of proghrelin relative to the reference genes 18s and Rpl13a (Rpl13a not shown) showed that proghrelin mRNA levels were not altered by insulin treatment (Figure 12D).

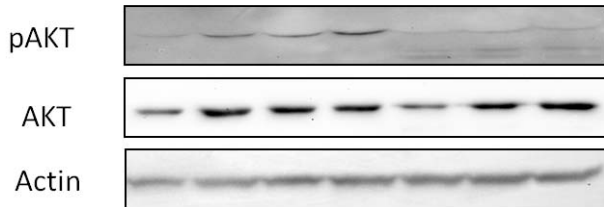
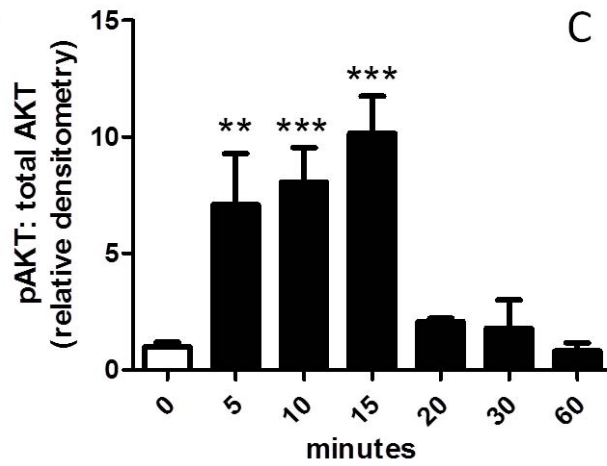
To determine the intracellular pathway of insulin action, intracellular signaling inhibitors were tested for their ability to block insulin-mediated inhibition of acyl ghrelin secretion. Co-incubation of insulin (10nM) with the MAPK inhibitor PD98059 (1uM) did not block the insulin-mediated reduction in ghrelin secretion (0.294 ± 0.06 fold of control, p<0.05, Figure 13A). However, the PI3K inhibitors LY294002 (0.1uM) and Wortmannin (0.1uM) as well as the AKT phosphorylation inhibitors triciribine (10uM) and AKT inhibitor VIII (20nM) prevented the inhibitory effect of insulin on ghrelin secretion (Figure 13A). Inhibitors alone did not have a significant effect on ghrelin secretion (Figure 13A). To demonstrate that insulin can activate the AKT pathway in the primary stomach culture, cells were incubated with insulin (10 nM) for various time points and pAKT was measured via western blot. Insulin treatments for 5, 10 and 15 minutes significantly increased pAKT levels relative to total AKT (7.1 ± 2.2 , 8.1 ± 2.6 and

A



Insulin	-	+	-	+	-	+	-	+	-	+	-	+
PD98059	-	-	+	+	-	-	-	-	-	-	-	-
LY294002	-	-	-	-	+	+	-	-	-	-	-	-
Wortmannin	-	-	-	-	-	-	+	+	-	-	-	-
Triciribine	-	-	-	-	-	-	-	-	+	+	-	-
AKT 1/2	-	-	-	-	-	-	-	-	-	-	+	+

B



C

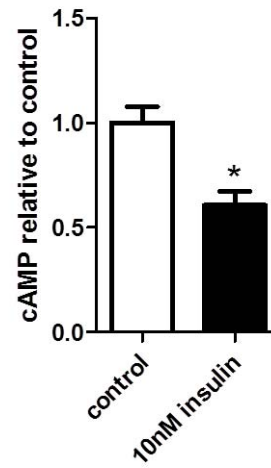


Figure 13 The mechanism of insulin action on ghrelin secretion. The effect of insulin (10nM) on ghrelin secretion was examined in the presence of MAPK inhibitor PD98059 (10uM), AKT inhibitors triciribine (10uM) and AKT inhibitor VIII (60nM), and PI3K inhibitors LY294002 (0.1uM) and Wortmannin (10uM) (n=6, A). Western blot densitometry of pAkt/Akt was examined after treatment with 10nM insulin for 5-60 minutes (a representative western blot is shown in B, n=6). Intracellular cAMP was measured in culture after 30 minute treatment with 10nM insulin (C, n=6). Data is presented as mean relative to control \pm SEM with ***, p<0.001, **, p<0.01 and *, p<0.05, compared to control.

10.2±3.2 fold of control, $p<0.01$,) while beyond 15 minutes this increase was lost (Fig. 13B). Finally, to examine the potential role of cAMP in reduction of ghrelin secretion, total cellular cAMP was assayed after insulin treatment. Insulin (10 nM) significantly reduced the levels of intracellular cAMP (0.61 ± 0.066 fold of control, $p<0.05$ Figure 13C).

High concentration insulin (100nM) had no effect on ghrelin secretion. This prompted the investigation into why the ghrelin cell preparation no longer responded to insulin using a model of cellular insulin resistance. Insulin resistance (IR) was induced by 24 h pretreatment with media containing 100 nM insulin. Previous studies demonstrated that similar conditions are sufficient to decrease insulin action in adipocytes (Thomson, Williams et al. 1997) and primary intestinal endocrine cells (Lim, Huang et al. 2009). Cells preincubated with insulin (100nM) for 24 hours no longer responded to insulin treatment (10nM) (Figure 14A). To examine if cellular insulin resistance affected NE-stimulated ghrelin secretion, preconditioned cells (100nM insulin, 24 hours) were treated with NE (0.1uM). Norepinephrine stimulated ghrelin secretion was comparable in IR and non IR cells (6.22 ± 1.7 vs 6.69 ± 1.63 , Figure 14B), indicating that cellular insulin resistance did not affect the stimulatory effect of NE on ghrelin secretion. To determine the possible mechanism of this loss in insulin action, the levels of insulin receptor expression were examined in IR cells. Total receptor expression was significantly reduced in IR cells by 25% ($75 \pm 7.5\%$ of control, $p<0.05$, Figure 14C). In addition, we examined the effect of IR on the downstream insulin signaling pathway in rat stomach primary culture. Insulin treatment (10nM) was no longer able to activate AKT phosphorylation in IR cells (Figure 14D). These results indicated that cellular insulin

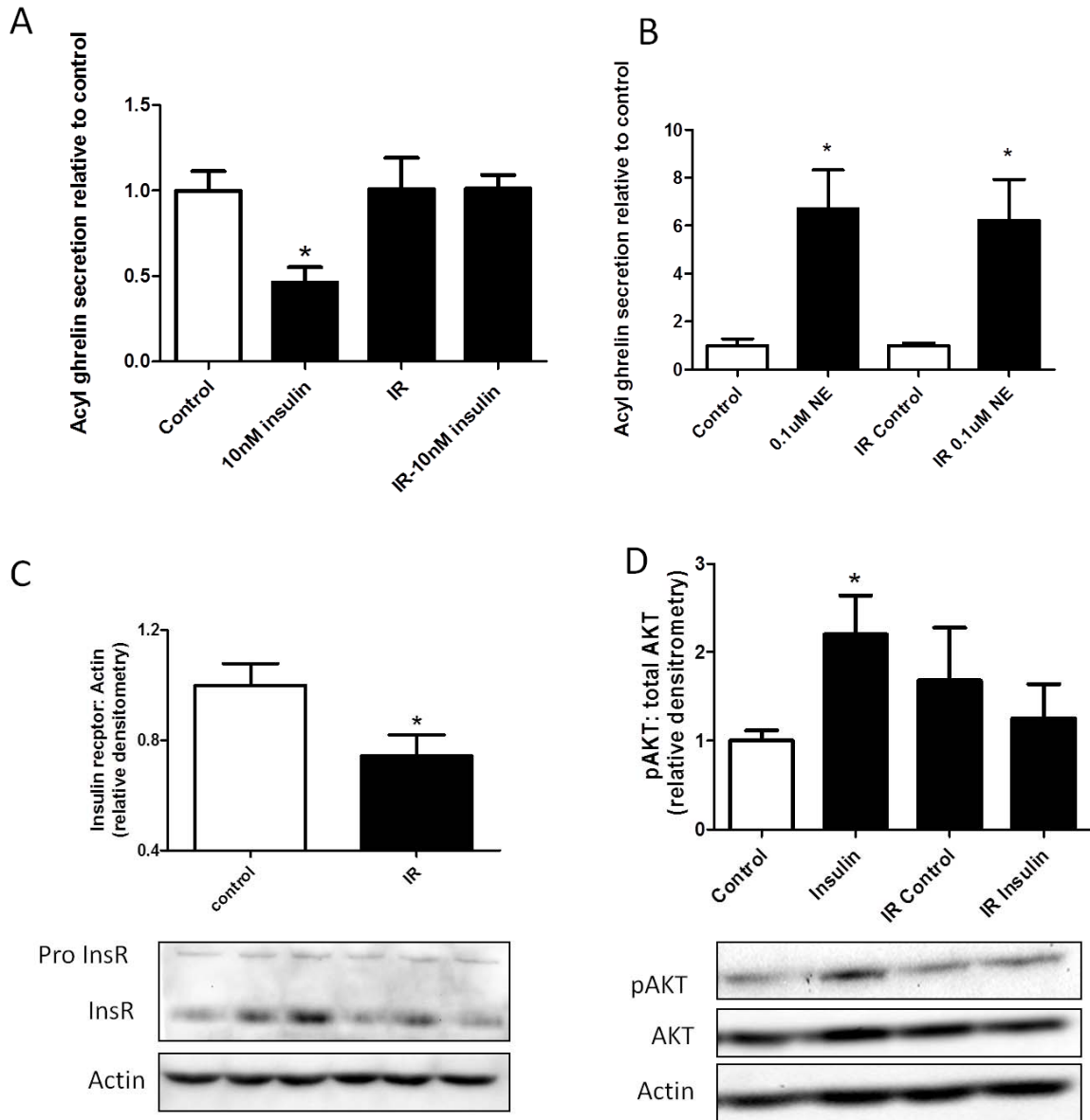


Figure 14 Effect of cellular insulin resistance on ghrelin secretion. Acyl ghrelin secretion was examined after 4 hours insulin (10nM) treatment in cells pre-treated with and without 100nM insulin for 24 hours (insulin resistant = IR) (A, n=9). Norepinephrine (0.1uM) stimulated ghrelin secretion was examined in control and IR cells (B, n=6). Western blot relative densitometry of insulin receptor/actin was completed in control and IR cells (C, n=3 shown, n=6 total). Western blot relative densitometry of pAKT/AKT was examined after 5 minute treatment of 10nM insulin in control and IR cells (representative western blot shown in D, n=6). Data presented as mean relative to control \pm SEM with **, $p < 0.01$ and *, $p < 0.05$ vs. non IR control.

resistance in the rat stomach primary culture resulted in reduced insulin receptor expression, and the loss of the insulin ability to induce the phosphorylation of AKT and the reduction of ghrelin secretion.

2.5 Discussion

Ghrelin is a hormone that regulates key metabolic actions ranging from the stimulation of appetite to the promotion of adipogenesis (Higgins, Gueorguiev et al. 2007). These actions ultimately lead to a shift from a whole body energy deficit to a surplus. As such, it is critical that ghrelin producing cells are able to sense energy status and respond with an appropriate increase or decrease in ghrelin secretion. Although there are many literature reports on ghrelin action (see reviews (Nogueiras, Tschop et al. 2008; Castaneda, Tong et al. 2010)), there is a lack of understanding of the cellular mechanisms controlling ghrelin secretion. In the present study we developed a primary stomach cell culture with ghrelin producing enteroendocrine cells to examine the mechanism of ghrelin secretion.

Similar to other groups, we found stomach ghrelin levels to be very low during the embryonic stage and much higher levels in neonates (Liu, Yakar et al. 2002). Therefore, we used 8-day-old rat pups for the production of the stomach primary culture. We demonstrated the presence of PC1 and GOAT in all ghrelin cells, confirming their ability to produce the mature acyl-ghrelin hormone. A comparison between total and acyl ghrelin secretion showed that total ghrelin levels were approximately 10 fold higher than acylated, which is in agreement with previous findings in rodents and humans (Foster-Schubert, Overduin et al. 2008; Morash, Gagnon et al. 2010). To further validate our cell system's ability to secrete ghrelin in a regulated manner, we tested several compounds known to increase cAMP, activate PKC and increase intracellular calcium. Forskolin and IBMX, which both lead to increases in cAMP, increased ghrelin secretion. Our results are in agreement with Zhao et al. who showed that increasing cAMP through forskolin

treatment led to an increase in ghrelin secretion in their tumor derived ghrelinoma cell line (Zhao, Sakata et al. 2010). Not surprisingly, treating the cell system with compounds known to increase the levels of intracellular calcium (thapsigargin and ionomycin) also led to a significant increase in ghrelin secretion.

The autonomic nervous system has extensive connections with the stomach and plays an essential role in motility (Olsson and Holmgren 2011) and secretion (Holmgren and Olsson 2011). We found that norepinephrine stimulated ghrelin secretion through the β 1 adrenergic receptor; however, acetylcholine did not affect ghrelin secretion. Previous studies that artificially stimulated sympathetic neurons *in vivo* using electrical stimulation showed an increase in ghrelin release (Munding, Cummings et al. 2006). Additionally, Zhao and colleagues showed that blocking catecholamine release with reserpine prevented the fasting induced increase in ghrelin secretion (Zhao, Sakata et al. 2010). We further demonstrated that norepinephrine treatment led to increased intracellular cAMP in the primary culture. We acknowledge that the changes in cAMP may be in part from the non-ghrelin cells in the preparation. However, since compounds that increase cAMP (IBMX and forskolin) stimulated ghrelin secretion, and blocking cAMP dependent kinase (PKA) with H89 prevented NE stimulated secretion, it is likely that ghrelin secretion is regulated by intracellular cAMP levels. Taken together, these findings further support the role of the sympathetic nervous system in the stimulation of ghrelin secretion.

To further explore how ghrelin secretion is affected by energy state, we investigated the role of insulin. An inverse relationship between the levels of insulin and ghrelin exists; ghrelin levels are highest in the fasted state and lowest in the post prandial state (Tschop, Smiley et al. 2000; Cummings, Purnell et al. 2001). Studies have

correlated low ghrelin levels with elevated insulin in obese individuals (Tschop, Weyer et al. 2001). In addition, euglycemic insulin infusions led to a reversible reduction in ghrelin levels (Saad, Bernaba et al. 2002). These studies implicate insulin in the regulation of ghrelin secretion; however, they were not designed to determine if insulin's effects on ghrelin levels occur directly on stomach ghrelin production. We demonstrated that ghrelin cells express insulin receptor α and β subunits. Although insulin receptor positive immunofluorescence was found in other non ghrelin cells, the signal was strongest in the double labeled ghrelin cells. Furthermore, when cells were treated with insulin in the physiological (1nM) and high physiological (10nM) range, we observed a 34% and 38% reduction in ghrelin secretion, respectively. This finding is in line with human studies showing a 31% reduction in ghrelin levels following insulin infusion using euglycemic hyperinsulinemic clamp technique (Saad, Bernaba et al. 2002). Insulin was also able to partially reduce norepinephrine-stimulated ghrelin secretion. This partial reduction suggests that the actions of insulin and norepinephrine are mediated by independent pathways. We then investigated if insulin regulated proghrelin mRNA expression. Insulin treatment did not affect proghrelin gene expression at the 4, 8 and 16 hour time points examined. Our findings were in contrasts to those found by Iwakura et al. who showed that insulin at 10 and 100nM was able to reduce proghrelin expression (Iwakura, Li et al. 2010). These differences may be due to differing mechanisms of insulin action within the primary and the tumor derived cell lines. In agreement with our findings, the tumor derived ghrelin secreting cell line produced by Iwakura et al. had a reduction in ghrelin secretion with insulin treatment (Iwakura, Li et al. 2010). This is in contrast to the ghrelinoma cell line developed by Zhao et al. in which no effect of insulin

treatment on ghrelin secretion was observed (Zhao, Sakata et al. 2010). The difference between the results reported by these two groups may stem from the origin of the ghrelin producing cells. The cells used by Iwakura and colleagues were derived from stomach tumors while those used by Zhao and colleagues in the insulin treatment experiments were from pancreatic tumors (Zhao, Sakata et al. 2010). It is possible that ghrelin cells within the pancreas possess signaling machinery distinct from those in the stomach. In addition, Zhao and colleagues only tested insulin at 100nM, a dose we also found to have no effect on ghrelin secretion.

To further explore the mechanism of the insulin mediated suppression of ghrelin secretion, we investigated the intracellular insulin signaling pathways. We found that PI3K and AKT were essential, while the ERK/MAPK pathway was not essential, in mediating insulin's effect on ghrelin secretion. While western blot experiments would be analyzing protein (pAKT) from both ghrelin and non-ghrelin cells, our finding that the AKT inhibitors tricibirine and AKT inhibitor VIII prevented ghrelin secretion suggests the importance of this pathway in the ghrelin cells. Other work examining primary culture of stomach cells has also shown that gastric acid secretion is regulated through the PI3K/AKT and not the MAPK pathway (Todisco, Pausawasdi et al. 2001). We found AKT phosphorylation occurred within the first 5 to 15 minutes after insulin treatment but dropped at the later time points. This rapid activation and subsequent deactivation of AKT has been demonstrated in other cell culture models (Mariappan, Feliars et al. 2007).

The lack of inhibitory effect with 100nM insulin prompted the investigation of how signaling was modified during cellular insulin resistance. We examined cells subjected to a high concentration of insulin (100 nM) for 24 hrs. Several groups have

used this technique to model the hyperinsulinemia that causes insulin resistance in both immortalized (Thomson, Williams et al. 1997) and primary (Lim, Huang et al. 2009) cell cultures. Interestingly, this pretreatment prevented the insulin-induced reduction in ghrelin secretion. Insulin resistance treated cells were still able to secrete ghrelin to the same extent as control cells when stimulated by NE. This confirms that the insulin signaling pathway is not critical for adrenergic stimulated ghrelin secretion. To further understand the mechanism of this cellular insulin resistance, we examined the level of insulin receptor expression and the downstream activation of AKT. We observed reduced insulin receptor expression in insulin resistant cells and further downstream, insulin induced AKT phosphorylation was also lost. The loss of insulin response and signaling found in high insulin pretreated cells only considers the role of elevated insulin and not other hormones (leptin, adiponectin etc) or proinflammatory cytokines (TNF- α , IL-6) known to be important in insulin resistance. Nevertheless, our findings support the notion that ghrelin cells can become insulin resistant *in vitro*.

In summary, we have developed a primary stomach cell system that enables the examination of the mechanism of ghrelin secretion. We have shown the importance of intracellular cAMP/calcium, the β 1 adrenergic system and the role of insulin in the regulation of ghrelin secretion. We have shown that insulin's action on ghrelin secretion is mediated through the activation of PI3K and AKT, but not MAPK (summarized in figure 15). The primary rat stomach cell culture is an important tool for examining physiologically relevant information on the mechanisms regulating ghrelin secretion.

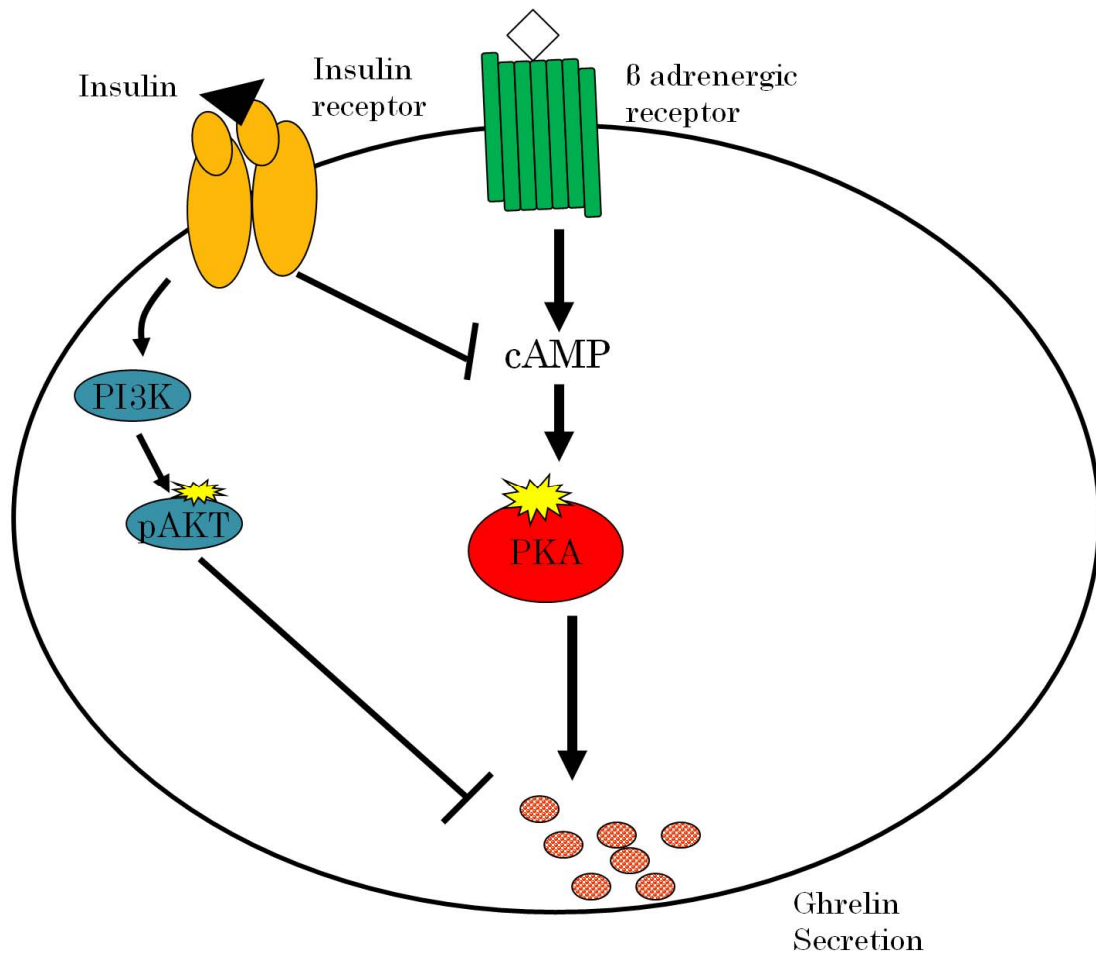


Figure 15 Summary of Chapter 2 results.

**CHAPTER 3: GLUCAGON STIMULATES GHRELIN SECRETION
THROUGH THE ACTIVATION OF MAPK AND EPAC AND
POTENTIATES THE EFFECT OF NOREPINEPHRINE**

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3.1 Abstract

Ghrelin is a stomach derived orexigenic hormone whose levels in circulation are altered by energy availability. Like ghrelin, the glucotropic hormone glucagon increases in the fasting state and serves to normalize energy levels. We hypothesized that glucagon can directly stimulate stomach ghrelin production. To verify this hypothesis, we used a primary culture of dispersed rat stomach cells. We first demonstrated that stomach ghrelin cells express the glucagon receptor (GlucR). Glucagon (1-100nM) significantly stimulated ghrelin secretion and proghrelin mRNA expression and co-incubation with a GlucR inhibitor prevented glucagon's action. The MEK inhibitor (PD98058) reduced the glucagon-stimulated ghrelin secretion and proghrelin mRNA expression. Furthermore, glucagon treatment increased the phosphorylation of ERK1/2. Glucagon also increased intracellular cAMP levels, and inhibition of adenylate cyclase reduced glucagon's effect on ghrelin secretion. Surprisingly, inhibiting PKA (using H89 and RP-cAMP) did not prevent glucagon stimulated ghrelin secretion. Instead, inhibiting the exchange protein activated by cAMP (EPAC) with Brefeldin-A was able to significantly reduce glucagon stimulated ghrelin secretion. Furthermore, the EPAC agonist (8-pCPT) significantly stimulated ghrelin secretion. Depleting endoplasmic reticulum calcium stores or blocking voltage dependent calcium channels prevented glucagon stimulated ghrelin secretion. Finally, co-incubation with the sympathetic neurotransmitter norepinephrine potentiated the glucagon stimulation of ghrelin secretion. Our findings are the first to show a direct link between glucagon and stomach ghrelin production and secretion and highlight the role of MAPK, the PKA independent EPAC pathway and the synergy between norepinephrine and glucagon in ghrelin release.

3.2 Introduction

Ghrelin is a 28-amino acid peptide that is secreted from the X/A like endocrine cells of the stomach in response to low energy or fasting conditions (Kojima, Hosoda et al. 1999; Cummings, Purnell et al. 2001). Ghrelin acts to restore energy balance by stimulating food intake through its action on the appetite regulating neurons within the hypothalamus (Morton, Cummings et al. 2006). In the periphery, ghrelin appears to be involved in the storage of ingested calories. This is supported by the observations that ghrelin (Wortley, Anderson et al. 2004) and ghrelin receptor (Zigman, Nakano et al. 2005) deficient mouse models are resistant to high fat diet induced weight gain. Furthermore, during a prolonged calorie restriction, acylated ghrelin is essential for survival through its growth hormone releasing effects (Zhao, Liang et al. 2010).

As ghrelin plays a key role in maintaining normal energy balance, ghrelin secretion is sensitive to energy state. Indeed ghrelin levels are highest in fasting conditions and in individuals with anorexia nervosa and lower postprandially and in obese individuals (Shiyya, Nakazato et al. 2002). The actual cellular mechanisms of ghrelin regulation are beginning to emerge thanks to the development of ghrelin secreting cell lines (Iwakura, Li et al. 2010; Zhao, Sakata et al. 2010; Sakata, Park et al. 2012) and primary culture of rodent gastric cells (Gagnon and Anini 2012; Sakata, Park et al. 2012). The increase in ghrelin secretion during energy insufficiency was shown to be, in part, due to the direct action of norepinephrine (NE) released from the sympathetic nervous system on β_1 -adrenergic receptors expressed by the ghrelin cells (Zhao, Sakata et al. 2010; Gagnon and Anini 2012). In contrast, insulin was shown to act directly on ghrelin

cells to inhibit ghrelin secretion (Iwakura, Li et al. 2010; Gagnon and Anini 2012; Sakata, Park et al. 2012).

Like ghrelin, the pancreatic hormone glucagon plays a vital role in restoring energy balance under fasting conditions. Low circulating glucose stimulates glucagon release from α cells of the pancreatic islets. Glucagon then restores normoglycemia through activation of hepatic gluconeogenesis and glycogenolysis (Pilkis and Granner 1992). The circulating level of glucagon fluctuates in the same manner as ghrelin, with peaks during fasting state and troughs postprandially. Several studies have examined the possible role of glucagon in ghrelin secretion. Two studies in humans, examined the effect of glucagon injection on circulating ghrelin levels (Arafat, Otto et al. 2005; Soule, Pemberton et al. 2005). In both studies, glucagon injection caused a drop in ghrelin levels. It must be noted that in these studies, glucagon injection also caused an increase in blood glucose and plasma insulin levels. Since both glucose (Sakata, Park et al. 2012) and insulin (Gagnon and Anini 2012) were shown to inhibit ghrelin secretion, it is hard to conclude from these studies if the drop in circulating ghrelin was due to the direct effect of glucagon on ghrelin cells or through the action of glucagon on blood glucose and insulin secretion. Interestingly, when glucagon was perfused directly into the gastric vein (Katayama, Shimamoto et al. 2007) or delivered in an isolated stomach model (Kamegai, Tamura et al. 2004), it significantly stimulated ghrelin secretion. The discrepancy in these studies likely results from the complexity of injecting glucagon in vivo where many other factors that influence ghrelin release are being altered. In the present study, we used a primary culture of rat gastric cells to understand the direct effect of glucagon on ghrelin

secretion and proghrelin expression and elucidate the intracellular pathways involved in glucagon action.

3.3 Materials and Methods

Dissociated stomach culture

All animal studies were approved by the Dalhousie University animal care committee and strictly adhere to the policies of the Canadian Council for Animal Care. Pregnant Sprague Dawley rats were purchased from Charles River (Wilmington, MA). Primary rat stomach culture was prepared as previously described in detail (Gagnon and Anini 2012). Briefly, 8 day old mixed pups (male and female), were anaesthetized with isoflurane. Stomachs were dispersed using type 1A collagenase and seeded in 25mM glucose, 10% fetal bovine serum (FBS) DMEM into 6 well plates (1×10^6 cells/well) and 10 cm plates (5×10^6 cells) for secretion or RNA/protein experiments, respectively. All media and compounds unless otherwise stated were purchased from Sigma Aldrich (Oakville, ON, Canada).

Ghrelin secretion experiments

Secretion experiments were completed in 6-well cell culture plates (BD Falcon, Mississauga, ON Canada). Cells were plated at 1×10^6 cells per well in 2ml of culture media. After 24 hours, cells received medium containing 50uM octanoic acid for an additional 24 hours. Cells were then washed with PBS and incubated with different treatments dissolved in 2mL of low glucose (1000mg/L), phenol red free DMEM and 0.5% FBS for 4 hours. Media and cell lysates were collected and acidified then extracted using SEP-Pak reverse phase chromatography. All ghrelin assays were completed for

acylated ghrelin and were determined using an active ghrelin enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor MI) as per the manufacturer's guidelines. This assay recognizes only the acyl form of ghrelin (cross reactivity with non-acylated ghrelin <0.001%) with a detection limit of 1 pg/ml. Ghrelin levels were determined for entire media and cell lysate from each sample. Results for various treatments were presented as percentage secretion relative to untreated control.

Immunocytochemistry

Cells were plated for 24 hours on glass coverslips in 12 well plates at 1×10^5 cells per well. Cells were then rinsed with TRIS buffered saline (TBS) and fixed in 4% paraformaldehyde for 20 minutes at room temperature. Cells were rinsed 3 x 5 minutes in TBS before being permeabilized in 0.1% Triton X -100 for 15 minutes. Cells were blocked for 20 minutes in a TBS blocking buffer containing 0.1% Triton X and 5% normal donkey serum (Vector Labs, Burlington ON Canada). Antibodies for glucagon receptor and ghrelin (Santa Cruz biotechnology, Santa Cruz CA) were used at a 1:100 dilution in blocking buffer overnight at 4C. Fluorescent secondary antibodies were obtained from Invitrogen (Carlsbad CA) and incubated at 1:150 for 45 minutes at room temperature. Coverslips were then mounted on slides in Vectashield mounting medium containing DAPI (Vector Labs, Burlington ON Canada) and visualized using a Zeiss Axioplan 2 fluorescent microscope. The absence of primary antisera was used as a negative control.

Quantitative RT-PCR

Cells were plated into 10 cm plates at 5×10^6 cells per plate and grown for 24 hours. Cells were rinsed with PBS, and then incubated in low glucose DMEM/0.5% FBS with indicated treatments for 24 hours. Media was then aspirated and cells were rinsed with PBS before RNA extraction was performed using Bio-Rad Aurum total RNA extraction kit (Bio-Rad labs, ON, Canada). One μg of RNA (quantified using absorbance at 260nm) was converted to cDNA using Bio-Rad iScript cDNA synthesis kit. Realtime PCR was completed using SYBR green master mix (Bio-Rad) with primers for proghrelin (Forward: TGG CAT CAA GCT GTC AGG AGC, Reverse: AGC TGG CGC CTC TTT GAC CT), and for Rpl13a (Forward: ATG GCG GAG GGG CAG GTT CT, Reverse: CCA CCA CCT TTC GGC CCA GC) as the reference gene. Data was analyzed using Bio-Rad CFX Manager software version 1.6 using the relative quantification method (standard curve).

Western blotting

Cells were plated into 10 cm plates at 4×10^6 cells per plate and allowed to grow for 24 hours at 37C, 5% CO₂. Glucagon treatments (10nM) were prepared in phenol red free, low glucose DMEM/0.5% FBS (pH 7.4) and given to cells for 5, 10, 15 minutes at 37C. Cell lysate from the human hepatocyte cell line HEP-G2 was used as a positive control for GluR (a generous gift from Dr Roger McLeod, Dalhousie University). Cells were then scraped on ice in Cytobuster cell lysis buffer (EMD Biosciences, Gibbstown NJ) supplemented with protease and phosphatase inhibitor cocktails (Complete

Mini/Phospho-Stop, Roche applied science, ON Canada). Cell extracts were separated on SDS-PAGE 8% poly-acrylamide gels and transferred to PVDF membranes. Primary antibodies for total ERK 1/2 and pERK were obtained from Cell Signalling and GluR from Santa Cruz Biotechnology. Secondary HRP conjugated antibodies were obtained from GE Healthcare (Piscataway NJ). Chemiluminescence was measured using the Bio-Rad Chemidoc XRS and analyzed using the 1-D software from Bio-Rad.

cAMP assays

Cells were plated in 6 well plates at 1×10^6 cells per well and grown for 24 hours in standard culture media. Cells were then placed in serum free media for 2 hours followed by serum free media with 100uM IBMX for 30 minutes. Treatments were then given in fresh serum free media with 10uM IBMX for 30 minutes. Media was then discarded and cells were collected and assayed with the cAMP EIA kit (Cayman Chemical, Ann Arbor MI) following the manufacturer's guidelines.

Data analysis

All data are expressed as mean \pm SEM. Data was analyzed by Student's *t* test or one factor ANOVA with appropriate post hoc testing. Comparisons between control and treatment experiments were analyzed using one factor ANOVA with Dunnett's post hoc test (p values <0.05 were considered statistically significant). Comparisons between treatments were analyzed using one factor ANOVA with Bonferroni post hoc test.

3.4 Results

Glucagon stimulates ghrelin secretion and mRNA production

Ghrelin expressing cells in the rat stomach culture were first examined for the expression of the glucagon receptor (GluR) using double-immunofluorescence. As shown in Figure 16A, ghrelin immunostained cells (in green) were colocalized with glucagon receptor immunostained cells (in red). GluR expressing cell were only found in ghrelin positive cells. To further examine the expression of glucagon receptor in ghrelin cells we performed western blotting experiments using protein extracted from primary culture (P.Cult) of ghrelin cells and hepatic HEPG-2 (HG2) cells used as positive control. Next, the effect of glucagon treatment directly on ghrelin secretion and proghrelin mRNA expression was examined. Glucagon at 1nM ($p < 0.01$), 10 nM ($p < 0.01$) and 100nM ($p < 0.05$), caused a significant increase in ghrelin secretion compared to baseline after 4 hours of incubation (2.46 ± 0.48 fold of control for 1nM, 2.56 ± 0.60 fold of control for 10nM and 2.36 ± 0.37 fold of control for 100nM) (Figure 16B). Glucagon (10nM) also caused a significant increase in proghrelin mRNA expression after a 24 hour incubation (1.37 ± 0.10 fold of control, $p < 0.05$) (Fig. 16C). To further confirm the action of glucagon on ghrelin cells was occurring through the GluR, primary stomach cultures were incubated for 4 hours with glucagon in the presence or absence of the GluR antagonist (Glucagon receptor antagonist 1). The GluR antagonist completely blocked the stimulatory effect of glucagon on ghrelin secretion (Figure 16D). These results indicate that glucagon stimulates ghrelin secretion and proghrelin mRNA expression via the glucagon receptor expressed by ghrelin cells.

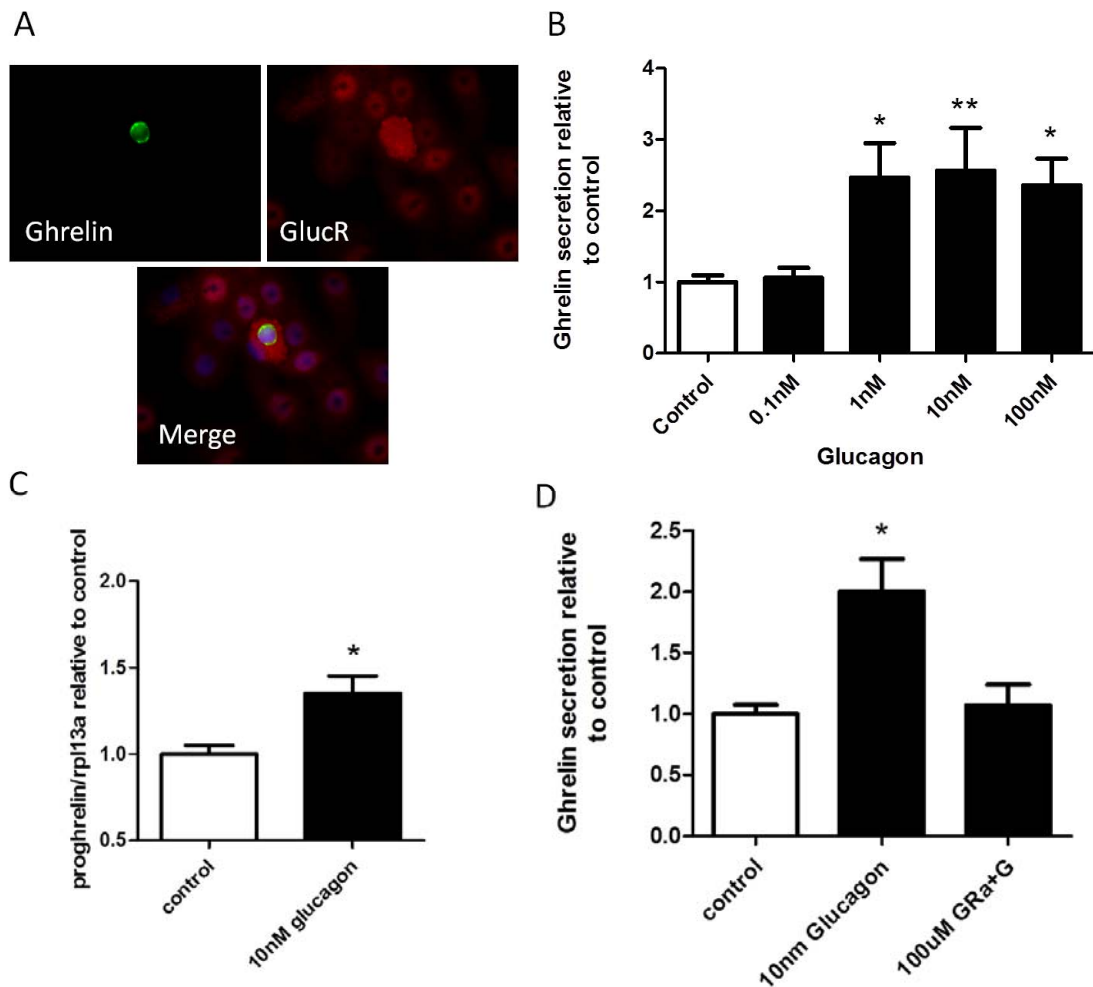


Figure 16 Glucagon inhibits ghrelin secretion. Ghrelin (green) and glucagon receptor (red, GlucR) expression was examined by double immunofluorescence microscopy in primary culture and a Western blot was completed against GlucR with cell lysates from HEP-G2 hepatocytes (HG2) and primary culture (P.Cult) (A). Ghrelin secretion was examined in cells treated with glucagon (0.1-100nM) for 4 hours (B). Proghrelin gene expression relative to ribosomal protein 13a was examined in the cells after 24hour treatment with 10nM glucagon (C). Ghrelin secretion was examined in cells treated with 10nM glucagon alone or in combination with 100uM of the glucagon receptor antagonist 1 for 4 hours (D). Data is presented as mean (n=6) \pm standard error of mean with * = $p < 0.05$ and ** = $p < 0.01$.

Glucagon activates EPAC but not PKA

As glucagon's mechanism of action has been shown to be mainly through the activation of adenylate cyclase and protein kinase A (PKA) in hepatocytes (Pilkis and Granner 1992), this pathway was investigated in ghrelin cells. First, glucagon treatment was found to increase the levels of intracellular cAMP (1.48 ± 0.21 fold of control, $p < 0.05$) (Figure 17A). Next, the role of adenylate cyclase (AC) activation in glucagon stimulated ghrelin secretion was determined. Glucagon treatment in combination with 100uM of the AC inhibitor SQ22536 (SQ) reduced the stimulatory effect of glucagon on ghrelin secretion (Figure 17B). To determine if PKA activation was required for glucagon-stimulated ghrelin secretion, two different inhibitors of PKA (1uM H89 and 50uM RP-cAMP) were tested. Surprisingly, neither compound was able to block glucagon stimulated ghrelin secretion (Figure 17C) indicating that the effect of glucagon on ghrelin secretion is PKA independent. As the Exchange Protein Activated by cAMP (EPAC) is a cAMP dependent signalling protein, ghrelin secretion was examined with the EPAC specific agonist 8-CPT-2Me-cAMP (CPT). CPT treatment (10uM) caused a significant increase in ghrelin secretion (1.37 ± 0.136 fold of control, $p < 0.05$) (Figure 17D). To determine if EPAC activation is involved in glucagon-stimulated ghrelin secretion, cells were treated with glucagon alone or in combination with an inhibitor of EPAC, brefeldin-A (BFA). Co-incubating 100uM BFA with glucagon reduced the significant stimulation of ghrelin secretion seen with glucagon alone (Figure 17E).

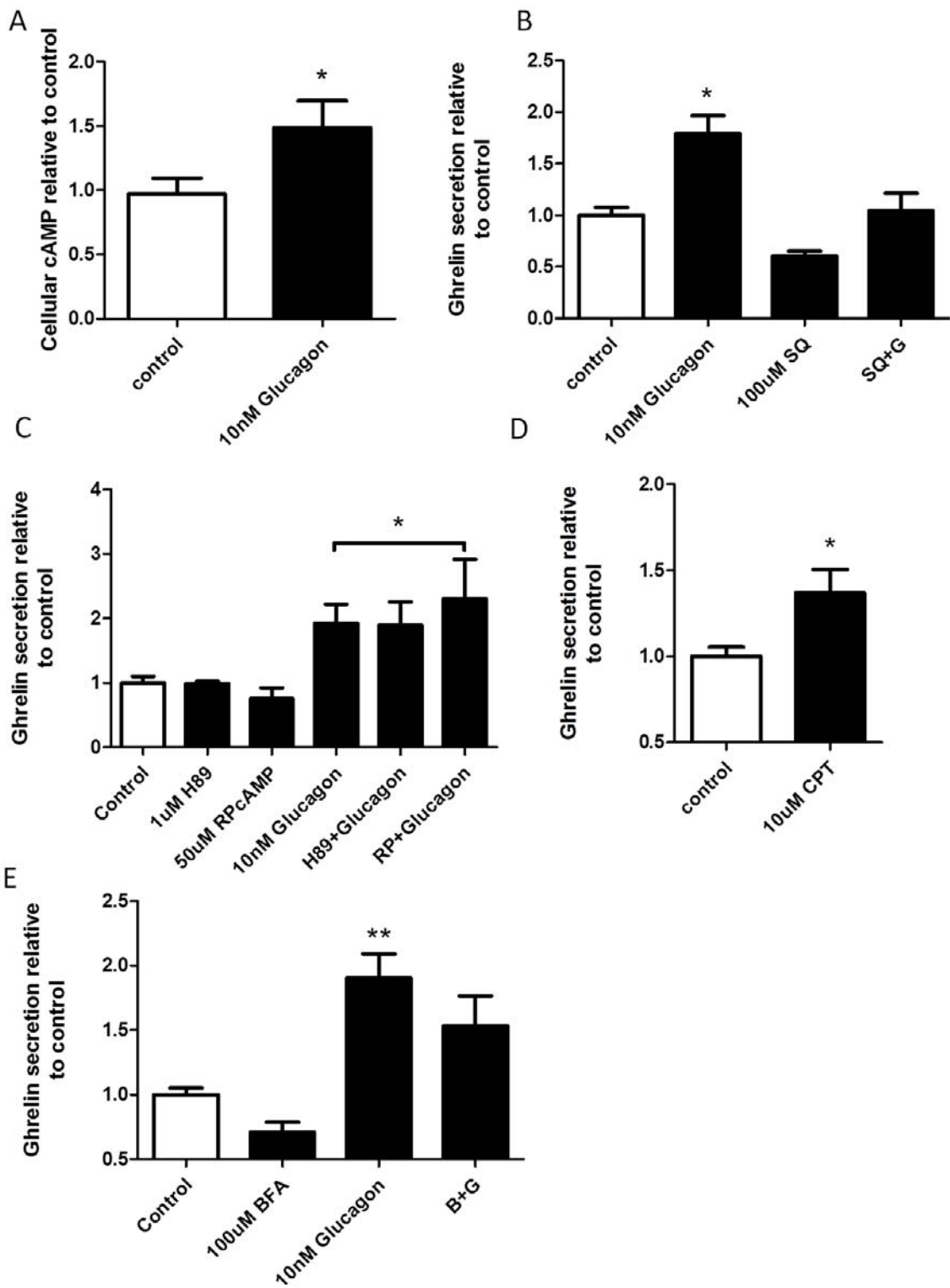


Figure 17 Glucagon stimulates cAMP production and activates the EPAC pathway. Intracellular cyclic AMP (cAMP) was examined in cells treated with 10nM glucagon for 30 minutes (A). Ghrelin secretion was examined in cells treated with 10nM glucagon alone or in combination with 100uM of the adenylate cyclase inhibitor SQ 22536 (SQ) for 4 hours (B). Glucagon stimulated ghrelin secretion was not affected by co-incubation with the protein kinase A inhibitors Rp-cAMP (50uM) or H89 (1uM) for 4 hours (C). Ghrelin secretion was examined in cells treated with 10uM of the EPAC agonist 8-CPT-2Me-cAMP (CPT) for 4 hours (D). Ghrelin secretion was examined in cells treated with 10nM glucagon alone or in combination with 100uM of the EPAC antagonist Brefeldin-A (BFA) for 4 hours (E). Data is presented as mean (n=6) \pm standard error of mean with * = p<0.05 and ** = p<0.01.

Role of MAPK in glucagon stimulated ghrelin secretion and expression

Since previous studies have demonstrated glucagon signalling through the MAPK pathway (Pedretti, Villa et al. 2006), the role of MAPK in glucagon stimulated ghrelin secretion and proghrelin mRNA expression was investigated. Cells were incubated with glucagon alone or in combination with the MAPK kinase inhibitor PD98059 (20uM). Co-incubating PD98059 with glucagon reduced the glucagon-stimulated ghrelin secretion (Figure 18A). To determine if MAPK is also involved in glucagon stimulated proghrelin mRNA expression, cells were incubated with glucagon alone or in combination with PD98059 for 24 hours and mRNA expression was measured by quantitative RT-PCR. Similarly, inhibition of MAPK with PD98059 blocked glucagon-stimulated proghrelin mRNA expression (Figure 18B). Furthermore, to verify that glucagon treatment led to MAPK activation in ghrelin cells, phosphorylated ERK (pERK) was examined by western blot in cells treated with 10nM glucagon for 5, 10 and 15 minutes. Glucagon treatment for 10 minutes caused a significant increase in relative phosphorylation of ERK (3.07 ± 0.91 fold of control, $p < 0.05$) (Figure 18C).

The role of calcium in glucagon stimulated ghrelin secretion.

Since we have previously demonstrated that increased intracellular calcium is linked with increased ghrelin secretion (Gagnon and Anini 2012), the roles of both voltage-dependent calcium channels (VDCC) and intracellular calcium stores in glucagon stimulated ghrelin secretion were examined. To determine the importance of VDCC's in glucagon stimulated ghrelin secretion, cells were incubated with glucagon alone or in the

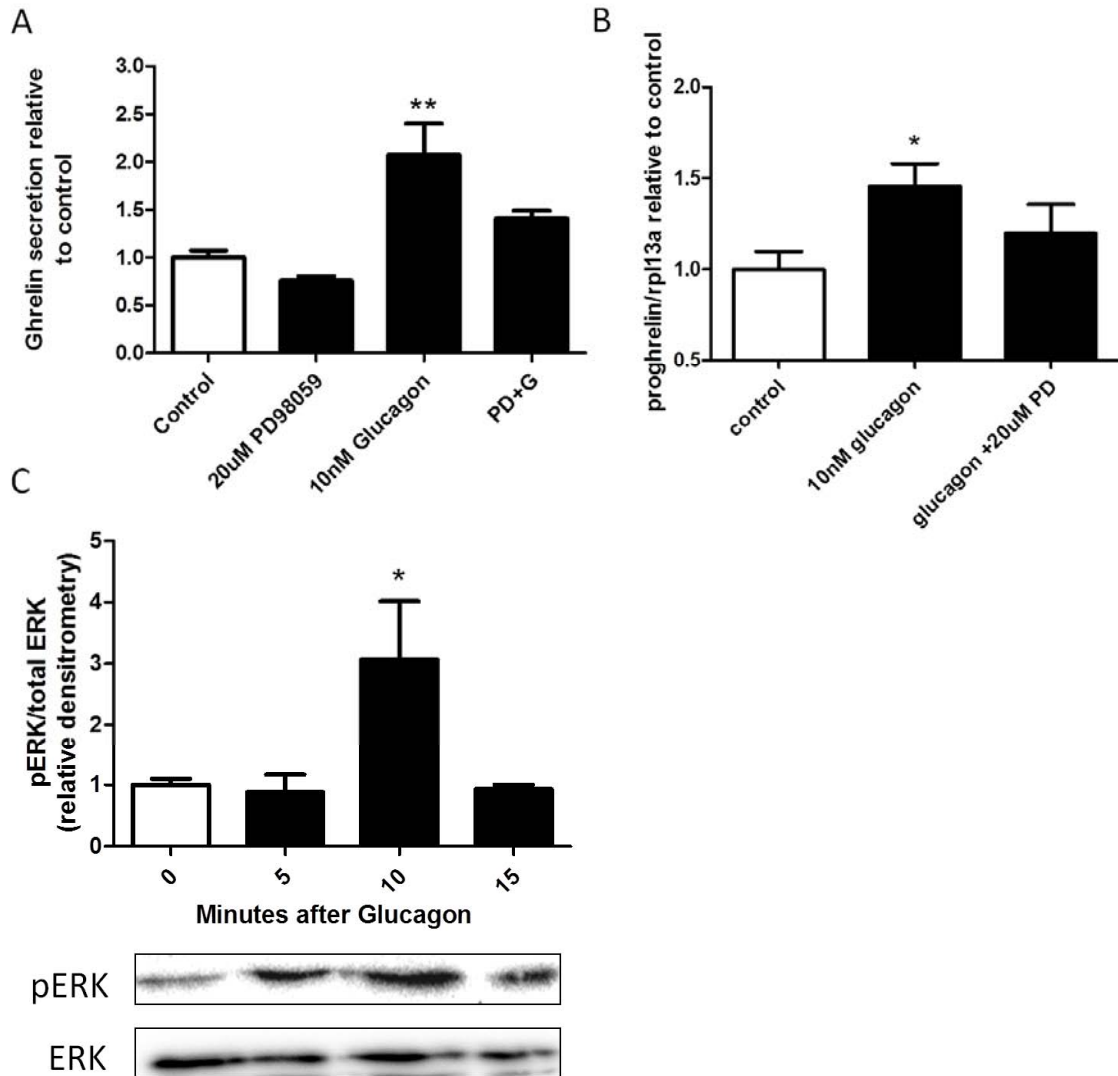


Figure 18 Glucagon stimulates ghrelin secretion through MAPK. Ghrelin secretion was examined in cells treated with 10nM glucagon alone or in combination with the 20uM of MAPK kinase inhibitor PD98059 for 4 hours (A). Proghrelin gene expression relative to ribosomal protein 13a was examined in the cells after 24 hour treatment with 10nM glucagon alone or in combination with 20uM PD98059 (B). Relative densitometry of phosphorylated ERK (pERK) over total ERK was completed by western blot on cells treated with glucagon for 0, 5, 10 and 15 minutes (representative blot shown in C). Data is presented as mean (n=6) \pm standard error of mean with * = $p < 0.05$ and ** = $p < 0.01$.

presence of the L-type VDCC blocker Nitrendipine. Nitrendipine completely blocked the glucagon-stimulated ghrelin secretion (Figure 19A). To determine the role of endoplasmic reticulum (ER) calcium store release in glucagon stimulated ghrelin secretion, cells were preincubated with the calcium ATPase inhibitor thapsigargin (1 μ M) for 30 minutes to deplete the ER calcium stores, then the cells were rinsed and incubated with glucagon for 4 hours. Cells pretreated with 1 μ M thapsigargin had significantly reduced glucagon stimulated ghrelin secretion (1.38 ± 0.177 vs 1.81 ± 0.25 for glucagon alone, Figure 19B).

Norepinephrine potentiates glucagon stimulated ghrelin secretion.

As previous studies demonstrated the role of sympathetic tone in the stimulation of ghrelin secretion (Mundinger, Cummings et al. 2006), we hypothesized that glucagon and NE act synergistically to stimulate ghrelin secretion during energy insufficient states. To verify this hypothesis, cells were incubated with a sub-activating concentration of NE (0.01 μ M) alone or in combination with glucagon (10 nM). While 0.01 μ M NE had no effect on ghrelin secretion, when combined with glucagon, it caused an increase in glucagon stimulated ghrelin secretion that was significantly greater than glucagon alone (2.09 ± 0.162 in combination vs 1.532 ± 0.147 glucagon alone, $p < 0.05$ Figure 20). These results indicate that NE and glucagon can act synergistically to stimulate ghrelin secretion.

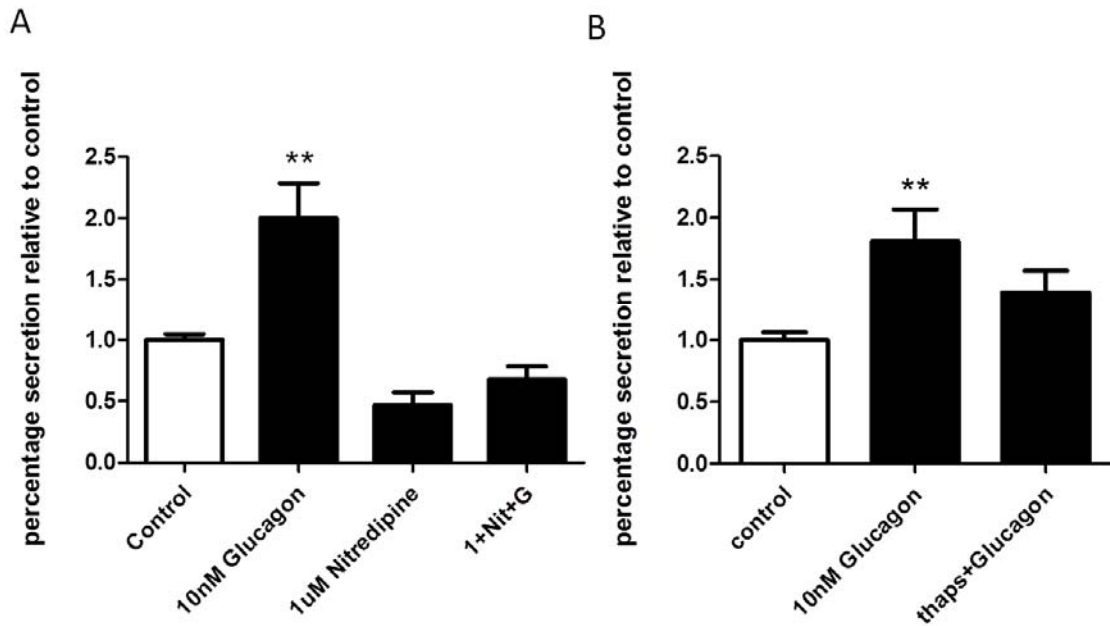


Figure 19 Glucagon stimulates ghrelin release through ER calcium and voltage gated calcium channels. Ghrelin secretion was examined in cells treated with 10nM glucagon alone or in combination with 1uM of the L-type voltage gated calcium channel inhibitor Nitrendipine for 4 hours (n=6, A). Ghrelin secretion was examined in cells with or without pre-treated with 1uM thapsigargin for 30 minutes then washed and treated with 10nM glucagon (n=9, B). Data is presented as mean (n as indicated) \pm standard error of mean with ** = $p < 0.01$.

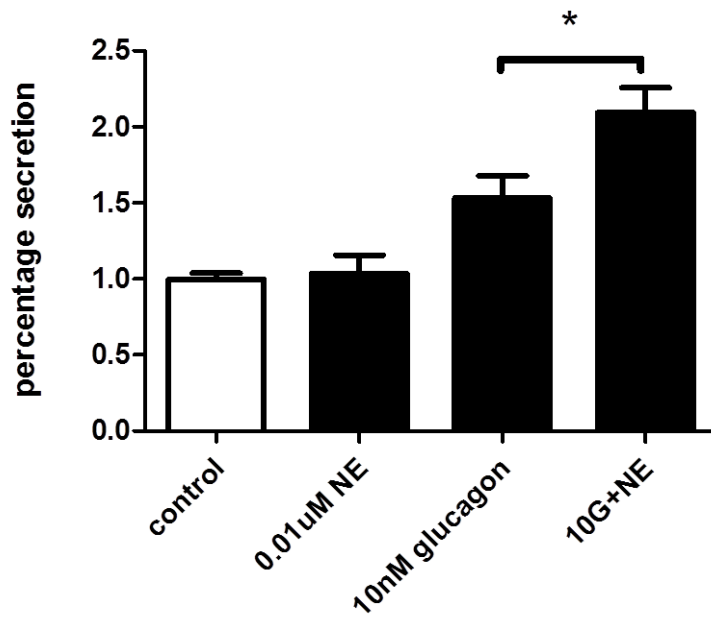


Figure 20 Norepinephrine potentiates glucagon's effect on ghrelin secretion. Ghrelin secretion was examined in the cells treated with 0.01uM of norepinephrine (NE), 10nM glucagon or a combination of NE and glucagon. Data is presented as mean (n=9) \pm standard error of mean with * = $p < 0.05$.

3.5 Discussion

The secretion of ghrelin in response to prolonged energy deficit is a vital process in restoring energy balance. As such, other energy regulating pathways, including the glucagon signalling pathway, are likely to play a role in ghrelin regulation. The findings of the present study demonstrate that glucagon is able to directly stimulate ghrelin mRNA production and hormone secretion through the MAPK and EPAC pathway in a dispersed stomach cell culture model. Furthermore, glucagon potentiates the stimulatory action of the sympathetic nervous system on ghrelin release.

To establish a direct link between glucagon treatment and the effect on ghrelin cells, we first showed that ghrelin expressing stomach cells co-expressed the glucagon receptor. Our report is in agreement with other studies showing the presence of glucagon receptor in stomach ghrelin cells (Katayama, Shimamoto et al. 2007). In our immunohistochemistry experiments, glucagon receptor was only found colocalized with ghrelin positive cells. This is in agreement with a recently published study showing significantly higher glucagon receptor mRNA expression in a pure population of isolated green fluorescent protein expressing ghrelin cells (Lu, Zhao et al. 2012). Next we found glucagon stimulated ghrelin secretion and mRNA production. Our use of glucagon in the low nanomolar range is representative of the fasting levels of circulating glucagon in humans (Ortega, Moreno-Navarrete et al. 2011) and has been used by other groups to study the effects of glucagon in vitro (Kachra, Barash et al. 1991). The stimulatory effect of glucagon on ghrelin secretion we observed was also demonstrated in models where glucagon was directly perfused into the femoral vein (Katayama, Shimamoto et al. 2007) or in isolated stomachs (Kamegai, Tamura et al. 2004). These findings and the findings of

this report are in contrast to the human studies where glucagon injection caused a reduction in circulating ghrelin. This discrepancy can be explained by the fact that exogenous injection of glucagon results in increased blood glucose and plasma insulin levels. Both glucose (Sakata, Park et al. 2012) and insulin (Gagnon and Anini 2012) were shown to inhibit ghrelin secretion and therefore the direct effect of glucagon on ghrelin cells is masked by the effect on glucose and insulin in these studies.

Glucagon action in hepatocytes has been thoroughly studied and shown to signal through the activation of AC, increases in intracellular cAMP and activation of PKA (Jiang and Zhang 2003). Indeed, we found that incubation of rat stomach cultures with glucagon resulted in a significant increase in intracellular cAMP and that blocking AC reduced glucagon stimulated ghrelin secretion. It should be noted that the stimulation of cAMP was modest compared to that seen with NE in previous work (Gagnon and Anini 2012). Surprisingly, inhibition of PKA did not prevent glucagon's action on ghrelin secretion in primary stomach culture. Previous studies had shown EPAC activation by glucagon in hepatocytes (Khouri, Dittrich et al. 2011). Indeed, the EPAC specific agonist, CPT, was able to significantly stimulate ghrelin secretion (although not at the same extent as glucagon). Blocking EPAC activity using BFA reduced the stimulatory effect of glucagon. It must be acknowledged that while BFA is not an EPAC specific antagonist, it has been widely used by other investigators to inhibit EPAC (Wang, Gu et al. 2007; Mironov, Skorova et al. 2011). Although PKA was not found to be involved in glucagon-induced ghrelin secretion, our previous study using the same cell model, demonstrated that PKA is required for norepinephrine-stimulated ghrelin secretion (Gagnon and Anini 2012). These results indicate that ghrelin secretion can be stimulated through PKA-

dependent or EPAC-dependent pathways depending whether the glucagon receptor or the β_1 adrenergic receptor is activated.

Glucagon treatment led to increased phosphorylation of ERK1 in rat stomach cultures. Since the primary stomach culture contains both ghrelin cells and non ghrelin cells, we cannot rule out the possibility that the phosphorylated-ERK observed in the Western blot is derived from non-ghrelin cells. However, the demonstration that inhibition of the upstream MAPK kinase prevented glucagon's effect on both ghrelin secretion and mRNA production supports a direct role of MAPK in ghrelin secretion.

Since we have previously shown that increases in intracellular calcium are linked with the stimulation of ghrelin secretion, we investigated the importance of intracellular calcium in glucagon stimulated ghrelin secretion. Blocking VDCC using the L-type calcium channel blocker nitrendipine not only prevented glucagon stimulated ghrelin secretion, but also reduced basal ghrelin secretion. This suggests that an influx of extracellular calcium is required for ghrelin secretion. We found cells with thapsigargin-induced depletion of ER calcium no longer had a significant response to glucagon. Interestingly, glucagon was previously found to activate small inwardly rectifying Ca^{2+} current through activation of AC and EPAC but not PKA (Aromataris, Roberts et al. 2006). It is also possible that calcium could be mobilized as a consequence of Ca^{2+} -induced Ca^{2+} release from caffeine and ryanodine-sensitive Ca^{2+} stores in ghrelin cells. To what extent Ca^{2+} derived from various subcellular compartments plays in the stimulation of ghrelin secretion needs further investigation.

During the fasting stage, the body attempts to restore energy balance by coordinating hormones that mobilize energy stores and increase appetite. We have demonstrated that glucagon, a hormone which increases during fasting, stimulates the synthesis and secretion of ghrelin. This increased production of ghrelin will then stimulate a feeding response to restore energy levels. This concept of fasting induced changes and ghrelin secretion is reinforced by recent work by Sakata *et al.* that demonstrated ghrelin secretion was stimulated in a low glucose environment (Sakata, Park et al. 2012). In contrast, high glucose (when glucagon levels would be suppressed) ghrelin secretion is suppressed (Nakagawa, Nagaya et al. 2002).

Previously our group (Gagnon and Anini 2012) and others (Mundinger, Cummings et al. 2006; Zhao, Sakata et al. 2010) have shown that ghrelin secretion is stimulated by sympathetic nervous system activation. It appears that the pre-meal spikes in ghrelin are in part regulated by sympathetic tone. In other systems, increased sympathetic activity under fasting conditions has been shown to stimulate hepatic glucose production (Edwards and Silver 1970) and adipose tissue lipolysis (Patel, Coppack et al. 2002). We sought to determine if the sympathetic neurotransmitter NE could potentiate the effects of glucagon on ghrelin secretion. We found that 0.01 μ M NE, which had no effect on its own, significantly increased glucagon-stimulated ghrelin secretion. This potentiation suggests that sympathetic activity may have an important role in priming ghrelin cells for maximal response to glucagon on fasting conditions. Indeed, this would be a physiologically important scenario where both stimulatory factors would act together to promote appetite and restore energy levels.

These findings demonstrate that the X/A cells secrete ghrelin in response to glucagon. Interestingly, ghrelin was shown to stimulate glucagon secretion (Chuang, Sakata et al. 2011), suggesting the existence of a positive feedback loop. Such interactions are not common in physiology but are well established to occur during the potentiation of luteinizing hormone release during the menstrual cycle. Moreover, a positive feedback loop between the intestinal hormone glucagon-like peptide 1 and insulin have been reported (Lim, Huang et al. 2009). It is possible that the ghrelin-glucagon positive loop exists to ensure energy availability during extended periods of fasting.

Our studies were all completed using a dispersed primary stomach cell culture. While we observed a high level of ghrelin expressing cells in this preparation, it should be noted that other non-ghrelin cells were present in culture. Other groups have recently developed tumor derived ghrelin cell lines (Iwakura, Li et al. 2010; Zhao, Sakata et al. 2010). However recently, one of these groups investigated the effects of glucose in ghrelin secretion using a similar dispersed primary cell model (Sakata, Park et al. 2012). While the tumor derived cell lines are important tools in understanding the mechanism of ghrelin secretion, the authors found several of the glucose metabolising enzymes were altered in the cell lines, suggesting potential limitations of that model. In addition conflicting results regarding the effect of insulin on ghrelin secretion have been published between each cell line (Iwakura, Li et al. 2010; Zhao, Sakata et al. 2010).

In summary, our results demonstrate the role of glucagon in stimulating ghrelin secretion. We have confirmed the findings of other groups that glucagon stimulates ghrelin secretion but have shown this with a more direct approach and elucidated for the

first time the intracellular pathways regulating this response (Figure 21). Furthermore we have shown that sympathetic neurotransmitter can potentiate the response to glucagon which is likely an important component of fasting induced increases in ghrelin.

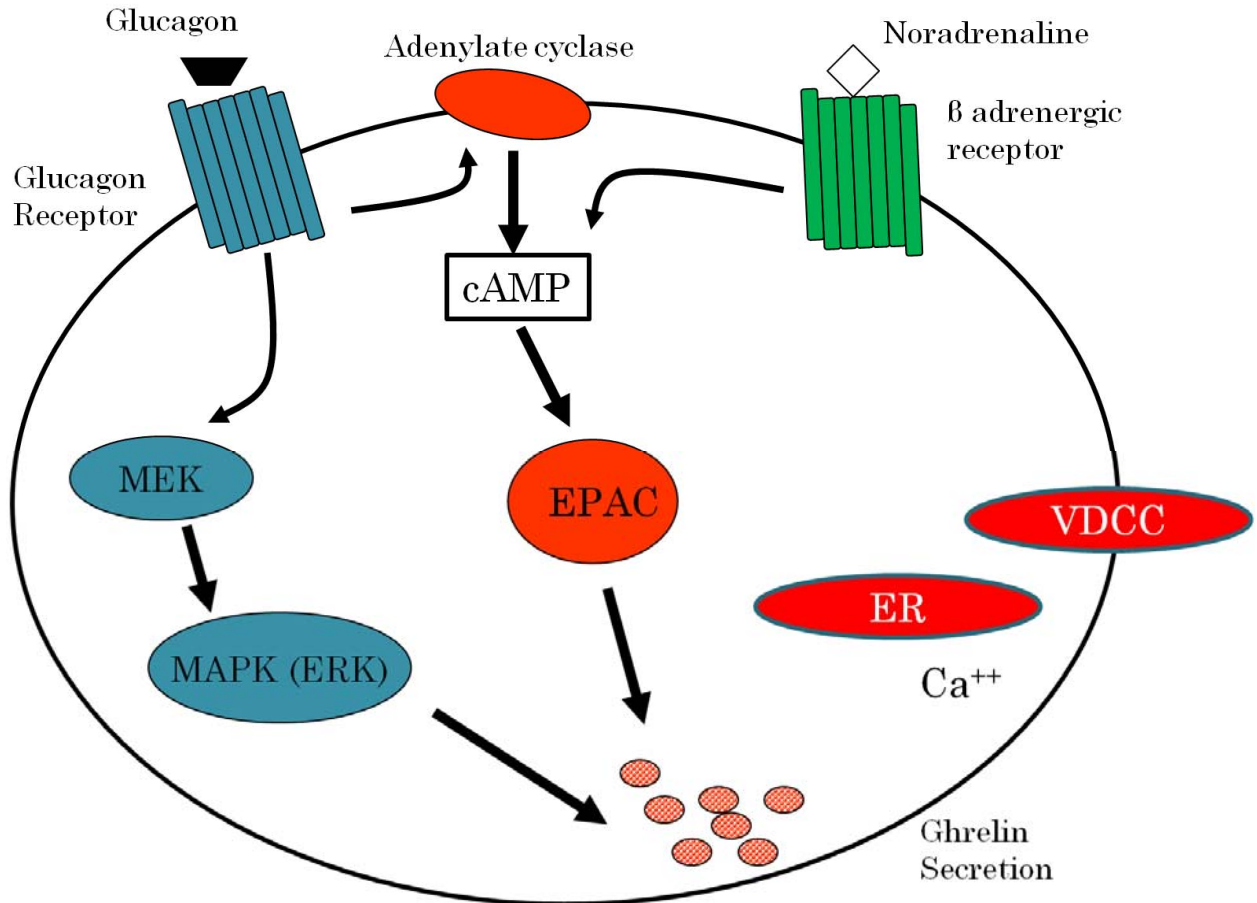


Figure 21 Summary of Chapter 3 results

CHAPTER 4: THE ROLE OF GLUCOSE IN GHRELIN SECRETION

Jeffrey Gagnon, Younes Anini

4.1 Introduction

Glucose has been shown *in vivo* to cause a drop in circulating ghrelin levels. Since glucose administration in a living system causes changes in several other regulatory peptides (including insulin and glucagon), it is unclear if and how ghrelin cells can directly sense glucose concentration. Additionally, the glucose dependency of metabolic hormone effects on ghrelin secretion is unknown. Insulin secretion is stimulated in high glucose conditions. Furthermore, we have demonstrated that insulin can inhibit ghrelin secretion from rat primary stomach culture (Gagnon and Anini 2012). Therefore, it is plausible that the concentration of glucose to which ghrelin secreting cells are exposed plays a regulatory role in their response to insulin. To answer these questions, the primary culture of rat stomach cells as described in detail in the above studies was used. Ghrelin secretion from cells was measured in response to varying concentration of glucose alone and in combination of insulin.

4.2 Materials and Methods

Cell preparations for ghrelin secretion experiments were completed as previously indicated in the above manuscripts with the following changes. After the first 24 hours in 25 mM glucose DMEM, media was replaced with 5 mM glucose DMEM containing 50 uM octanoic acid for 24 hours. Cells were then rinsed with PBS and treatments of varying glucose concentrations were prepared in glucose free, phenol red free, 0.5% FBS DMEM supplemented with 1 M sterile filtered D-glucose to the required concentrations. In the hormone incubation experiments, recombinant insulin (Humulin) was given at 10

nM final concentration. Ghrelin secretion data is presented as percent secretion (ghrelin in media/(ghrelin in media + ghrelin in cell lysate) x100) relative to 5mM glucose alone.

4.3 Results

To test the effects of glucose on ghrelin secretion, media supplemented with varying concentrations of glucose (1, 5, and 15mM) was given to ghrelin cells. Ghrelin secretion was altered by glucose concentration with 1mM glucose at $1.23 \pm 0.158\%$, 5mM at $1.00 \pm 0.071\%$ and 15mM at $0.774 \pm 0.065\%$ (Figure 22). There was a significant difference in the levels of secretion between the 1mM and 15mM glucose groups.

To determine if the varying concentrations of glucose had any impact on hormone-regulated ghrelin secretion, insulin was co-incubated with the varying concentrations of glucose. Insulin treatment (10nM) was able to reduce ghrelin secretion at all concentrations of glucose tested, with $0.525 \pm 0.034\%$ at 1mM, $0.467 \pm 0.021\%$ at 5mM, and $0.481 \pm 0.118\%$ at 15mM glucose; however, there was no significant interaction between glucose concentration and insulin treatment on ghrelin secretion (Figure 22). Results will be discussed in the overall thesis discussion.

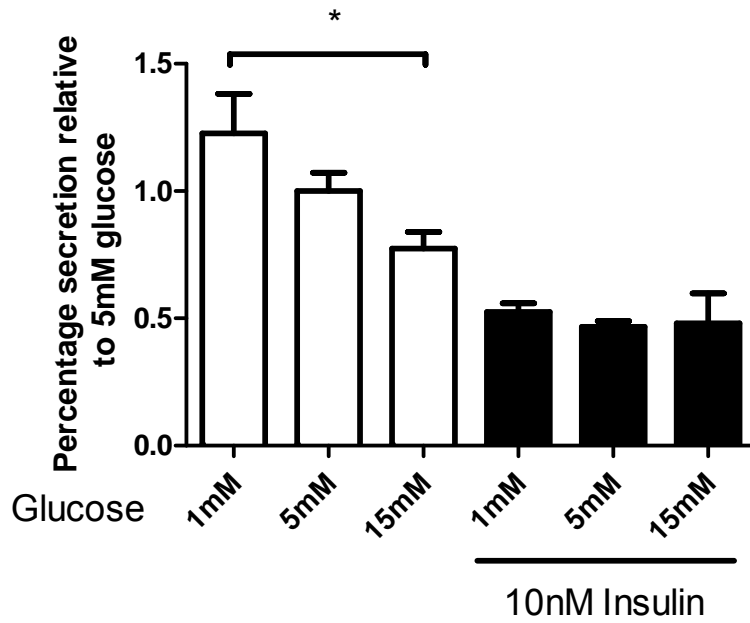


Figure 22 Percentage ghrelin secretion in response to glucose and insulin. Ghrelin secretion was examined after 4 hour incubation in the presence of 1, 5 and 15mM D-glucose alone or in combination with 10nM insulin (black bars). n=6 for glucose and n=3 for insulin treatments.

CHAPTER 5: METFORMIN DIRECTLY INHIBITS GHRELIN SECRETION THROUGH AMP-ACTIVATED PROTEIN KINASE IN RAT PRIMARY GASTRIC CELLS

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The authors have no conflict to declare.

5.1 Abstract

The anti diabetic drug metformin causes weight loss in both diabetic and non diabetic individuals. Metformin treatment is also associated with lower circulating levels of the orexigenic hormone ghrelin. To test whether metformin directly effects stomach ghrelin production, we treated ghrelin producing rat primary stomach cells with metformin and examined the levels of ghrelin secretion, proghrelin gene expression, and activation of AMP-activated protein kinase (AMPK). Metformin significantly reduced ghrelin secretion and proghrelin mRNA production and both these effects were blocked by co-incubation with the AMPK inhibitor Compound C. Additionally, ghrelin cells were shown express AMPK using immunocytochemistry. Finally, metformin treatment caused a significant increase in the level of phosphorylated (active) AMPK. Our results demonstrate that metformin directly inhibits stomach ghrelin production and secretion through AMPK. This reduction in ghrelin secretion may be one of the key components in metformin's mechanism of weight loss.

5.2 Introduction

The antidiabetic biguanide metformin is one of the most prescribed, first line medications in the treatment of T2DM. In contrast to some antidiabetics (sulphonoureas and insulin), metformin does not cause weight gain and can lead to significant weight loss(1998). One of the known targets of metformin action is the intracellular signalling enzyme, AMP-activated protein kinase (AMPK). In the liver and muscle, AMPK activation reduces hepatic gluconeogenesis and promotes fatty acid oxidation, respectively (see review (Viollet, Guigas et al. 2012)). In addition to these effects, metformin treatment also appears to affect the levels of the orexigenic hormone, ghrelin. Ghrelin is produced primarily in the X/A like endocrine cells of the stomach and acts to increase appetite and promote energy storage (Horvath, Diano et al. 2001). Accordingly, ghrelin levels rise before a meals and drop postprandial (Cummings, Purnell et al. 2001). Studies in humans have shown that metformin treatment prolongs the postprandial or post-glucose drop in circulating ghrelin levels (English, Ashcroft et al. 2007; Kusaka, Nagasaka et al. 2008). However, other studies found no difference in the postprandial drop in ghrelin in metformin treated individuals (Kiyici, Ersoy et al. 2009). As ghrelin secretion is known to be altered by hormones and nutrients including insulin (Gagnon and Anini 2012) and glucose (Sakata, Park et al. 2012), it is difficult to conclude from in vivo studies if metformin has a direct role on ghrelin secretion. In the present study, we used primary culture of rat gastric cells to demonstrate a direct effect of metformin on ghrelin secretion.

5.3 Materials and Methods

Animals

All animal studies were approved by the Dalhousie University animal care committee and strictly adhere to the policies of the Canadian Council for Animal Care. Pregnant Sprague Dawley rats were purchased from Charles River (Wilmington, MA).

Primary Culture

Primary rat stomach culture was prepared as previously described in detail (Gagnon and Anini 2012). Briefly, 8 day old rat pup stomachs were dispersed using type 1A collagenase and seeded in 25mM glucose, 10% fetal bovine serum (FBS) DMEM into 6 well plates (1×10^6 cells/well) and 10 cm plates (5×10^6 cells) for secretion or RNA/protein experiments, respectively. All media and compounds unless otherwise stated were purchased from Sigma Aldrich (Oakville, ON, Canada).

Ghrelin Secretion

After 24 hours in culture, cells were incubated in media supplemented with 10 μ M octanoic acid for an additional 24 hours. Cells were rinsed with phosphate buffered saline and given fresh DMEM with 5mM glucose and 0.5% FBS containing treatments for 4 hours. Both media and cell lysates were acidified and concentrated using reverse phase SepPak column (C-18 SepPak cartridges, Waters) according to the manufacturer's

instructions. Acylated ghrelin levels were determined using active ghrelin enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor MI) as per the manufacturer's guidelines. Ghrelin secretion was normalized to total ghrelin content of each well (media/(media+lysate)) to control for changes in the proportion of ghrelin cells in each experiment.

Proghrelin mRNA Expression

After 48 hours in culture, cells were incubated in DMEM with 5mM glucose and 0.5% FBS containing treatments for 24 hours. Relative proghrelin/RPL13a mRNA expression was determined using standard curve method with the following primers: proghrelin (Forward: TGG CAT CAA GCT GTC AGG AGC, Reverse: AGC TGG CGC CTC TTT GAC CT), and Rpl13a (Forward: ATG GCG GAG GGG CAG GTT CT, Reverse: CCA CCA CCT TTC GGC CCA GC).

AMPK phosphorylation

After 48 hours in culture, cells were stabilized in 0.5% FBS DMEM with 5mM glucose for 2 hours then given 10mM Metformin in fresh media for 4 hours. Lysates were separated in 8% acrylamide SDS Tris tricine gels and transferred to PVDF membranes. Primary blotting antibodies for phosphorylated Thr172 -AMPK and AMPK were obtained from Millipore (Billerica, MA). Immunocytochemistry was completed on 4% paraformaldehyde fixed cells on coverslips with the same AMPK antibody.

Statistics

Experiments were analyzed using either Student's t-test or one factor ANOVA with Dunnett's post hoc test to untreated control (p values <0.05 were considered statistically significant).

5.4 Results

To determine the direct effect of metformin on stomach ghrelin secretion, cells were incubated with metformin (0.1-100mM) for four hours. Metformin caused a significant reduction in ghrelin secretion at 1mM (0.632 ± 0.071 fold of control, $p > 0.05$), 10mM (0.617 ± 0.060 fold of control, $p < 0.01$) and 100mM (0.365 ± 0.042 fold of control, $p < 0.01$) (Figure 23A). To examine if metformin treatment could alter proghrelin gene expression, quantitative RT-PCR was completed. Metformin treatment (10mM) caused a strong reduction in proghrelin gene expression (0.106 ± 0.019 fold of control, $p < 0.001$, Figure 23B).

As metformin has been shown to activate AMPK, we tested if the AMPK agonist, AICAR could also reduce ghrelin secretion. Cells treated with 0.5mM of AICAR had a significant reduction in ghrelin secretion (0.453 ± 0.063 fold of control, $p < 0.01$, Figure 24A). To further investigate the role of AMPK, cells were incubated with metformin (10 mM) in the presence of the AMPK inhibitor, compound C (10uM). Inhibition of AMPK completely blocked the effect of Metformin on ghrelin secretion (Figure 24B). Similarly, the inhibitory effect of metformin on proghrelin mRNA expression was also blocked by co-incubation with the AMPK inhibitor (Figure 24C). To confirm that ghrelin cells express the AMPK protein, double immunofluorescence using anti-ghrelin and anti-AMPK antibodies was completed. As shown in Figure 24D, ghrelin cells co-expressed AMPK. To further demonstrate AMPK activation, phosphorylated AMPK was examined after 4 hours of metformin treatment. Metformin (10mM) significantly increased levels of phosphorylated AMPK (2.20 ± 0.232 fold of control, $p > 0.01$, figure 24E).

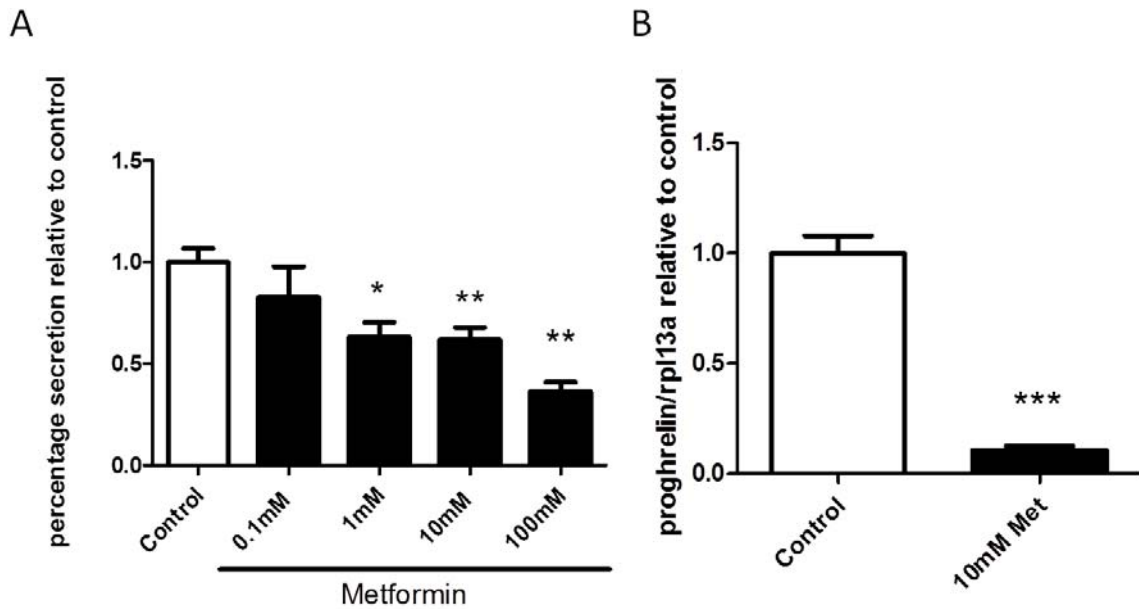


Figure 23 Metformin inhibits ghrelin secretion and mRNA expression. Acyl ghrelin secretion was determined after 4 hour incubation of the rat gastric primary culture with Metformin (0.1 – 100 mM) (A). Proghrelin/Rpl13a mRNA expression was examined by quantitative RT-PCR after 24 h incubation of the rat gastric primary culture with Metformin (10mM) (B). Results are expressed as mean \pm SEM (n = 6). * $p < 0.05$ and ** $p < 0.01$ versus control.

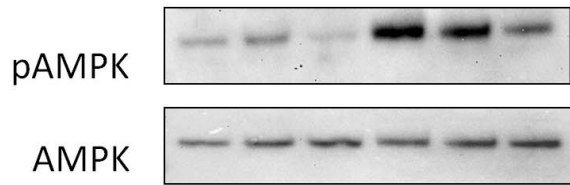
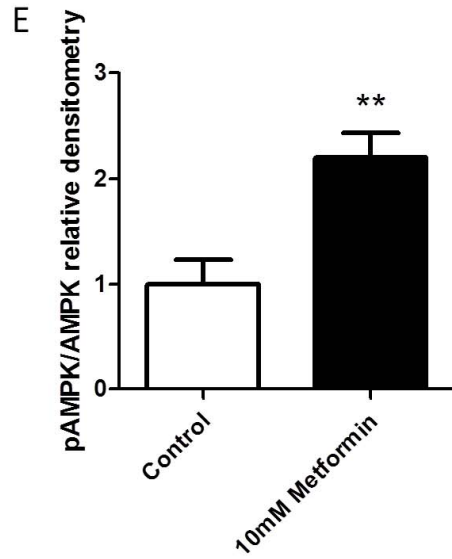
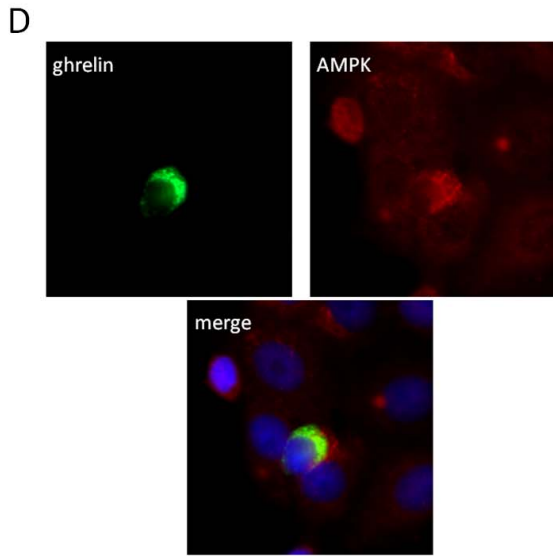
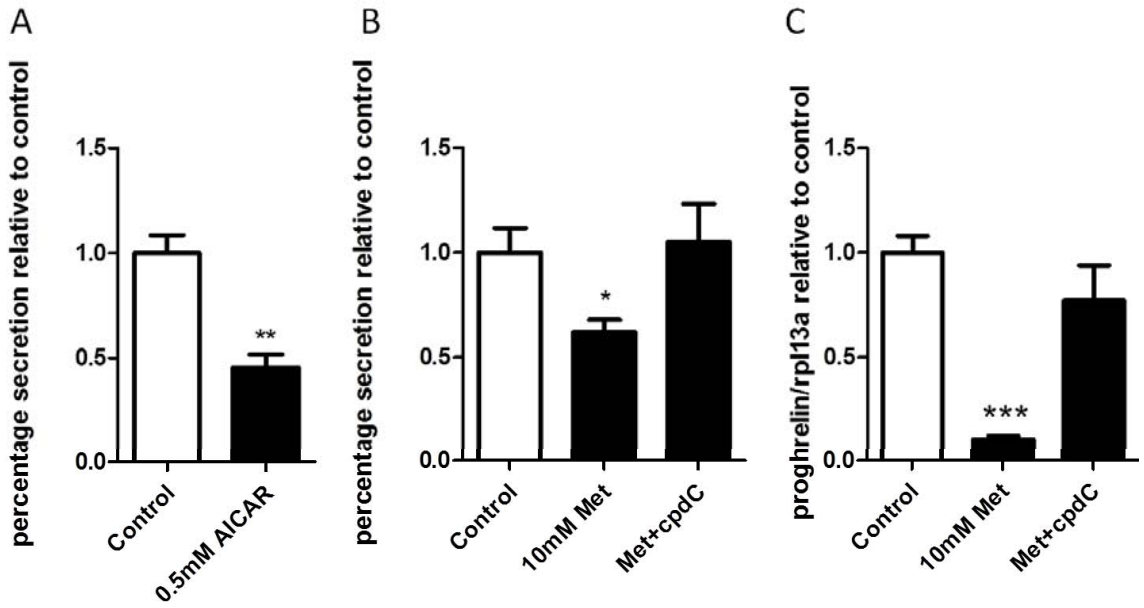


Figure 24 Metformin acts through AMPK to inhibit ghrelin secretion. Acyl ghrelin secretion was determined after 4 hour incubation with 0.5mM of the AMPK agonist AICAR (A). Acyl ghrelin secretion was determined in cells treated with 10mM Metformin alone or in the presence of 10uM of the AMPK inhibitor, compound C (cpdC) (B). Quantitative RT-PCR was completed for proghrelin/Rpl13a after 24 hour treatment with media alone (control), metformin (10mM) and metformin (10 mM) in the presence of cpdC (10uM) (C). Double immunofluorescence for ghrelin (green) and AMPK (red) was completed in rat primary culture (D). Western blotting of primary stomach culture treated with metformin (10mM) for 4 hours was completed with phospho-specific AMPK and total AMPK antibodies (representative blot of 3 repeats, E). Results are expressed as mean \pm SEM (n = 6). * p<0.05, ** p<0.01 and ***p<0.001 versus control.

5.5 Discussion

Our findings indicate that metformin inhibits stomach proghrelin mRNA production and ghrelin secretion, ghrelin cells co-express AMPK, and metformin treatment leads to increased AMPK phosphorylation. To our knowledge, our study is the first to show the direct effect of metformin on ghrelin secretion and proghrelin mRNA expression. We found the 1-100 mM dose range to be effective at reducing ghrelin secretion. The 10 mM dose used in subsequent experiments provided a strong effect without causing any cell death during our experiments and has been used by other groups studying the effects of metformin in vitro (Mueller, Stanhope et al. 2000). This is also the first report linking AMPK activation and reduced ghrelin secretion. Although it is possible that the AMPK phosphorylation in western blots was from non ghrelin stomach cells in the preparation, our findings that the AMPK agonist AICAR strongly reduced ghrelin secretion and that the AMPK inhibitor blocked metformin's effect on ghrelin mRNA production and secretion supports its direct mechanism of action. As the effects on mRNA production may not be directly associated with secretion, we hypothesize that they may be occurring through AMPK activation of the mammalian target of Rapamycin (Viollet, Guigas et al. 2012) pathway, however this requires further investigation. The secretion dependent effects of metformin may be occurring through AKT as AMPK activation has been shown to increase AKT activity (Leclerc, Leclerc et al. 2010). This is supported by our previous studies showing that increases in AKT phosphorylation by insulin led to reduced ghrelin secretion (Gagnon and Anini 2012).

Most interesting is the possible link between metformin's known effects on weight loss and ghrelin's actions on appetite and energy storage. Indeed, metformin

therapy has been shown to reduce daily caloric intake (Makimattila, Nikkila et al. 1999) and prolong the feeling of fullness after a meal (English, Ashcroft et al. 2007). This combined with metformin's ability to lengthen the postprandial drop in circulating ghrelin further supports a role for ghrelin in mediating metformin's effects on weight loss (English, Ashcroft et al. 2007; Kusaka, Nagasaka et al. 2008). In summary, our results using the ghrelin secreting primary stomach culture are the first to show the direct effect of metformin on ghrelin production.

A final mechanistic summary of Chapters 2-5 is presented in Figure 25

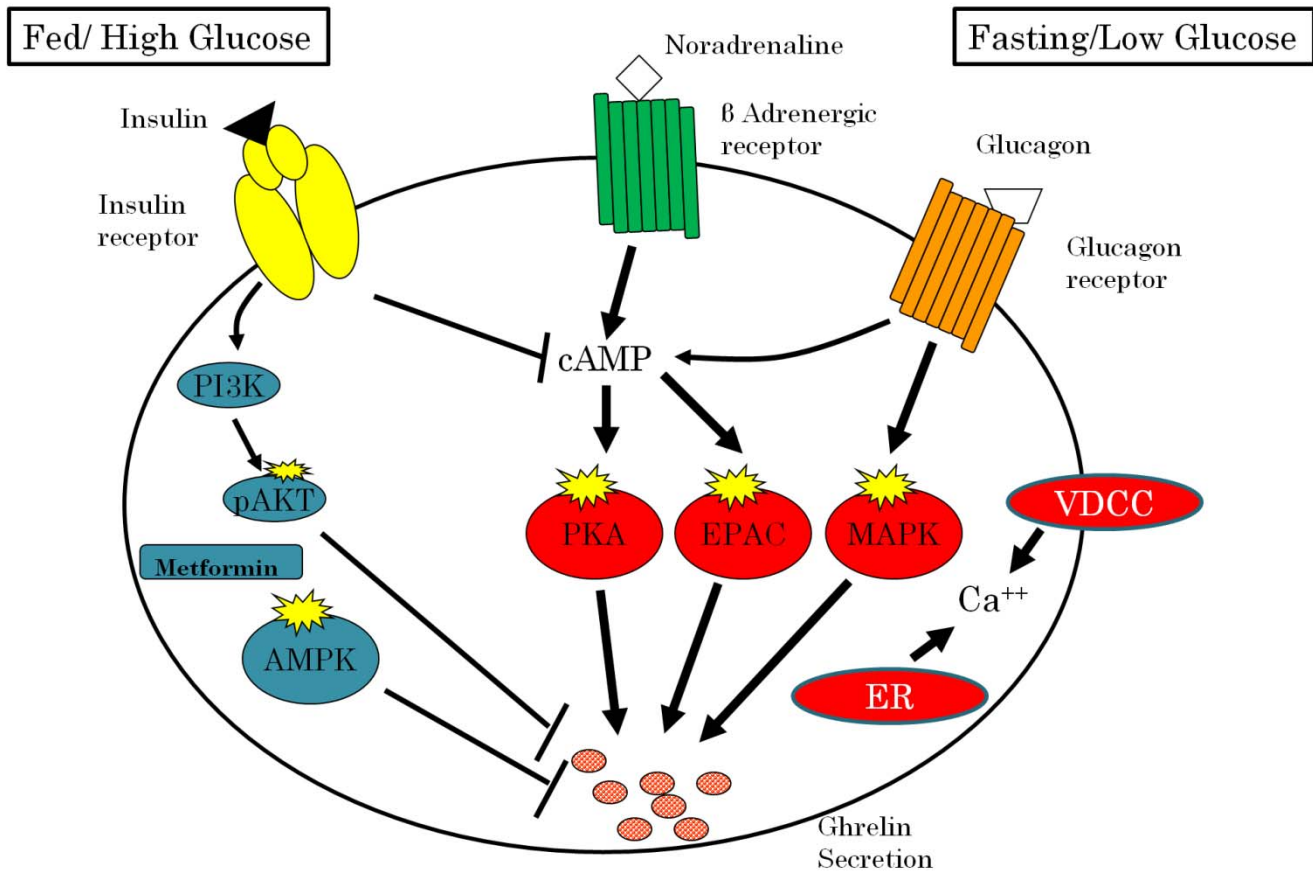


Figure 25 Final summary of ghrelin's mechanism of secretion

**CHAPTER 6: LOWERING CIRCULATING GHRELIN BY THE
EXPRESSION OF A GHRELIN RECEPTOR BASED DECOY
PROTEIN PROTECTS AGAINST HIGH FAT DIET-INDUCED
OBESITY IN MICE**

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6.1 Abstract

Ghrelin is a stomach-derived peptide hormone that stimulates appetite and promotes adiposity through binding to the growth hormone secretagogue receptor (GHS-R1a). Reducing the levels of circulating ghrelin holds the potential to reduce appetite and promote energy utilization. We designed and injected a GHS-R1a based DNA vector in mice that lowered the levels of circulating acylated-ghrelin (AG). When placed on a high fat diet, treated mice had reduced weight gain compared to controls, caused by reduced fat accumulation in the peritoneum with no significant effect on lean mass. White adipose tissue gene expression was examined by quantitative RT-PCR and showed increased PPAR γ and hormone sensitive lipase levels in treated animals, indicating a preference for increased fat utilization, while pro-inflammatory IL-1 and TNF α levels were reduced. Intraperitoneal glucose tolerance and insulin tolerance tests showed improved glucose clearance and insulin sensitivity in treated animals. Our findings show that ghrelin neutralization using *in vivo* expression of the GHS-R1a-based fusion protein was able to prevent diet-induced weight gain, alter adipose gene expression, and improve glucose clearance. These findings, while confirming the role of ghrelin in peripheral energy metabolism, suggest that plasmid-based gene delivery of a GHS-R1a-based protein may be a useful tool in the treatment of obesity.

6.2 Introduction

Excessive adiposity is a risk factor for many chronic diseases including T2DM, cardiovascular disease, and cancer (Wang, McPherson et al. 2011). Several studies have shown that even a modest reduction in weight has a positive impact on cardiovascular risk factors (Blackburn 1995; Pi-Sunyer 1996) and is associated with a reduced risk for developing both T2DM and diabetes-associated complications (Bosello, Armellini et al. 1997). Lifestyle interventions aimed at reducing caloric intake and increasing physical activity through behavioural changes are currently recommended as the first-line approach for weight management. However, these strategies alone are less successful when compared to pharmacological interventions for maintained weight loss (6 to 12 months) (Gray, Cooper et al. 2012). Unfortunately, most of the drugs approved for the treatment of obesity have been withdrawn from use due to their side effects (Gray, Cooper et al. 2012). Recently, targeting of gut hormones for the treatment of obesity has garnered interest (Neary and Batterham 2009). Indeed, infusions of glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) in humans are able to reduce appetite by acting on feeding regions in the brain (De Silva, Salem et al. 2011). Another promising candidate is the stomach derived peptide hormone ghrelin. Ghrelin levels peak in circulation during energy depleted states leading to activation of the appetite stimulating neuropeptide Y (NPY)/ Agouti gene related peptide (AgRP) neurons within the arcuate nucleus of the hypothalamus (Nakazato, Murakami et al. 2001). This action occurs via ghrelin binding to the growth hormone secretagogue receptor (GHS-R1a) (Kamegai, Tamura et al. 2000). In addition to appetite, ghrelin promotes the differentiation of adipocytes and the

preference for storage of calories in adipose tissue (Tschop, Smiley et al. 2000; Rodriguez, Gomez-Ambrosi et al. 2009).

The ghrelin peptide is derived from proghrelin, which is a precursor peptide that is proteolytically cleaved and acylated to produce acylated ghrelin (AG) (Zhu, Cao et al. 2006), un-acylated ghrelin (UAG) (Zhu, Cao et al. 2006) and obestatin (Zhang, Ren et al. 2005); however, *in vitro* studies have shown that both UAG (Kojima, Hosoda et al. 1999) and obestatin (Zhang, Ren et al. 2005) are unable to bind to GHS-R1a. The GHS-R1a is a G-protein-coupled receptor (Howard, Feighner et al. 1996), and its activation stems from the binding of its only known endogenous ligand, AG (Kojima, Hosoda et al. 1999). Specifically, it is hypothesized that AG binds to GHS-R1a in the second extracellular loop (EC2), which forms a hydrophobic pocket that allows the lipophilic acylated side chain of ghrelin to be stabilized during binding (Pedretti, Villa et al. 2006).

Depleting circulating AG holds the potential to reduce caloric intake and promote fat energy utilization. As such, we constructed mammalian expression plasmid vectors encoding the ligand binding domains of the GHS-R1a, specifically the N-terminal (Nt) and/or the first and second extracellular domains (EC1, EC2) then fused this with the mouse IgG2 constant region (Fc), forming a construct dubbed GHSR1a/Fc. This construct was injected and electroporated into mouse gastrocnemius muscle. We found that *in vivo* expression of GHSR/Fc caused a reduction in AG but, interestingly, not UAG levels. This treatment prevented high fat diet induced weight gain, which was associated with an altered adipose gene expression profile and improved glucose clearance and insulin sensitivity. Our observations suggest that the GHSR1a/Fc fusion construct may find clinical use in treating obesity.

6.3 Materials and Methods

Construct design

All ghrelin receptor constructs were designed based on the mouse growth hormone secretagogue receptor sequence (Gene ID: 208188). GHSR regions and mouse IgG Fc fragment were produced by PCR amplification. Primers used for amplification of the N-terminal region were (6mNtF) GCG GGG TAC CAT GTG GAA CGC GAC GCC A and (6mNtR) GCG AGT ACT CGC GGG GAA CAG TGG CAG CAG TTC, the first extracellular loop (6mEC1F) GCG AAG CTT TTC CAG TTT GTC AGC GAG AGC TGC ACC TAC GCC CCC AGC GAG ACC GTC ACC TGC and (6mEC1R) CGA AGC TTG CAG AGC AGG TCG CCG AAG TTC CAG GGC CGA TAC TGC CAG AGG CGC GCG GGG AAC AGT GGC AGC AGT TC, the second extracellular loop (6mEC2F) GCG ACG GAT CCC CGG GAC ACC AAC GAG TGC CGC GCC ACC GAG TTC GCT GTG CGC TCT CCC AGC GAG ACC GTC ACC TGC AAC and (6mEC2R) GCGGGGATCCGTG CCG TTC TCG TGC TCC ACG CCC ACC AGC ACG GCG TAG GTG CAG CTC TCG CTG AC, and mouse IgG Fc fragment (mIgGF) GCG AGT ACT TGG CCC AGC GAG ACC GTC ACC TGC AAC and (mIgGR) GCG CTC GAG CAG GGA AGA AGT CTG TTA TCA TGC A. Each extracellular domain GHSR PCR product was cleaved with KpnI and ScaI while the mouse IgG Fc fragment was cleaved with ScaI and XhoI. This was ligated into the pSecTag2B vector (Invitrogen, ON Canada) at XhoI and ScaI using T4 DNA ligase.

***In vitro* and *in vivo* expression of the fusion constructs**

To establish the expression and secretion of the GHSR1a/Fc constructs, the rat skeletal muscle cell line was transfected using Lipofectamine 2000 (Invitrogen) with the designed plasmids. Cell extract and media were collected using RIPA cell extraction buufer. At the end of experiments, the gastrocnemius muscle was collected and extracted from treated mice to examine the *in vivo* expression. 2 ul of blood plasma was collected at the end of the study. All samples were separated by 8% acrylamide SDS gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membrane and probed anti-Myc (Millipore, ON Canada) at 1:3000 at 4 C for 16 hours followed by secondary rabbit ant-mouse HRP at 1:5000 at room temperature for one hour.

Immunofluorescence microscopy

To determine the localization and expression of transfected GHSR1a/Fc, cell lines were immunostained with anti IgG Fc antibody which only detects the Fc chain of the transfected protein. L6 cells were grown on coverslips and fixed for one hour at room temperature in 4% paraformaldehyde. They were then washed three times in phosphate-buffered saline followed by 15 minutes of blocking in 5% normal horse serum. Cells were incubated with primary biotinylated anti mouse Fc in blocking solution for two hours at room temperature (RT). Cells were then washed and treated with avidin conjugated Cy3 in blocking solution for 45 minutes in dark at RT. Coverslips were mounted on slides and visualized on a Zeiss Axioplan II microscope.

***In vivo* electroporation**

All animal work was approved by the St. Michael's hospital animal care facility, University of Toronto. Intramuscular electroporation was carried out as previously described (Soltani, Kumar et al. 2007). Plasmids were extracted using an endotoxin free plasmid kit (Qiagen Mississauga ON). Briefly, 50 µg of plasmid DNA was injected (25 µg per leg) intramuscularly into gastrocnemius of eight week old male C57/Bl6 mice. After the injection, an electrical current was applied using caliper electrodes (BTX, MA) on the muscle as follows; 8 pulses (pulse length 20 milliseconds) with 1 second intervals at 200V/cm. A conductive gel (aquasonic 100) was used to facilitate current delivery. Five animals per plasmid were group housed and plasmid injection/electroporation was administered weekly for the first three weeks of study.

Food intake and body weight measurement:

High fat diet (Research diets, North Brunswick, NJ, USA, containing 60% of kCal as fat) began on the day of the first DNA injection and continued until the end of the experiment 54 days later. Food consumption was measured by weighing of food basket in each cage every three days. Animals from each group were weighed individually every three days.

Glucose and insulin tolerance tests:

Intraperitoneal (IP) glucose and insulin tolerance tests were completed after 54 days of high fat diet. Animals were fasted overnight for 12 hours prior to tests. For the IPGTT, a single bolus injection of glucose at 1.5 mg/kg of mouse weight was administered ip. For the ITT, a single bolus injection of insulin at 0.75U/kg was injected ip. Tails tips were treated with topical anesthetic (EMLA, ON Canada) and blood samples were drawn from tail vein at 0, 10, 20, 30, and 60 minutes post injection. Blood samples (4-5 μ l) were analyzed by the glucose oxidase method using the Bayer Acensia Elite XL glucometer (Bayer, ON Canada).

Fat tissue collection

Post mortem analysis of fat tissues weight was completed by bi-lateral harvesting of fat pads and immediate weighing. The peritoneal fat tissue was snap frozen in liquid nitrogen for subsequent RNA extraction.

Real time qPCR

Visceral white adipose tissue was collected at the end of the experiment. Total RNA was extracted using the Trizol (Invitrogen) extraction phenol chloroform precipitation method as per manufacturer's protocol. Samples were treated with DNAase (Invitrogen) and cDNA was produced using random hexamers with reverse transcriptase.

Realtime PCR was conducted on the Bio-Rad CFX instrument (Bio-Rad, CA USA) using primers for leptin (forward: CCAAAACCCTCATCAAGACC, reverse: TGTCTCCACCACCGAAACTC), hormone sensitive lipase (forward: TGTCTCCACCACCGAAACTC, reverse TCTCCAGTTGAACCAAGCAGG TCA), PPAR γ (forward: GGAAAGACAACGGACAAATCAC, reverse: ATCCTTGGCCCTCTGAGATG), IL-1 (forward: TGTCTGAAGCAGCTATGGCAA, reverse: TGCTGCGAGATTTGAAGCTG) and TNF α (forward: TGATCGGTCCCCAAAGGGAT, reverse: TTGCTACGACGTGGGCTAC). Data was analyzed using Bio-Rad CFX manager software and relative expression was determined using the standard curve method with 18S as the normalization gene.

Hormone assays

Blood was collected at the end of the experiment in capillary tubes containing EDTA (Sarstedt, PQ Canada) from ad-libitum fed mice. Plasma levels of hormones involved in the regulation of energy metabolism were analyzed using the Milliplex hormone assay panel (Millipore, MA) including; active GLP-1, insulin, PYY, pancreatic polypeptide, and GIP (Millipore). AG and IGF-1 plasma levels were analyzed by enzyme-linked immunoassays (Cayman chemical, ON Canada and Millipore, respectively) and total ghrelin levels were measured using a radioimmunoassay (Phoenix Pharmaceuticals, CA USA).

Statistical Analysis

The relative changes in weight gain over time were analyzed using the two-way ANOVA with Bonferroni post hoc test to compare each group to the control group. Multiplex hormone assays analyzing each group were compared with the one-way ANOVA. Time points during IPGTT and ITT were examined by two-way ANOVA. All other comparisons between the control and GHS-R1a/Fc groups were analyzed with the Student's t-test.

6.4 Results

Validation of GHSR constructs

To assess the expression and secretion of the GHS-R1a/Fc fusion protein (Figure 26A), the plasmid vectors were transiently transfected into the L6 rat skeletal muscle cell line. The expression of the fusion constructs was examined by immunofluorescence using anti-IgG-Fc antibodies. Produced protein was detected throughout L6 cells (Figure 26B). At 48 hours after construct transfection, the medium and the cells were harvested separately and total protein was extracted. Western blot analysis showed that GHS-R1a/Fc was detected in both the cell lysate and culture media whereas the wild type (WT) GHSR, which contains the transmembrane domains, was only found in the cell lysate (55kD) (Figure 26C).

Effects of GHSR gene therapy on weight gain

To examine the impact of GHSR1a/Fc gene therapy on energy intake and weight gain, body weight and food consumption was measured in mice injected with the GHSR based constructs and control mice (injected with the Fc empty vector) fed a high fat diet. Animals treated with the GHS-R1a/Fc construct gained significantly less body weight compared to the control animals (Figure 27A). The weight differences began at 30 days post-injection ($21.0 \pm 0.195\text{g}$ vs. $23.8 \pm 0.433\text{g}$ in control, $p < 0.05$), and continued until the termination of the experiment at 54 days post first injection

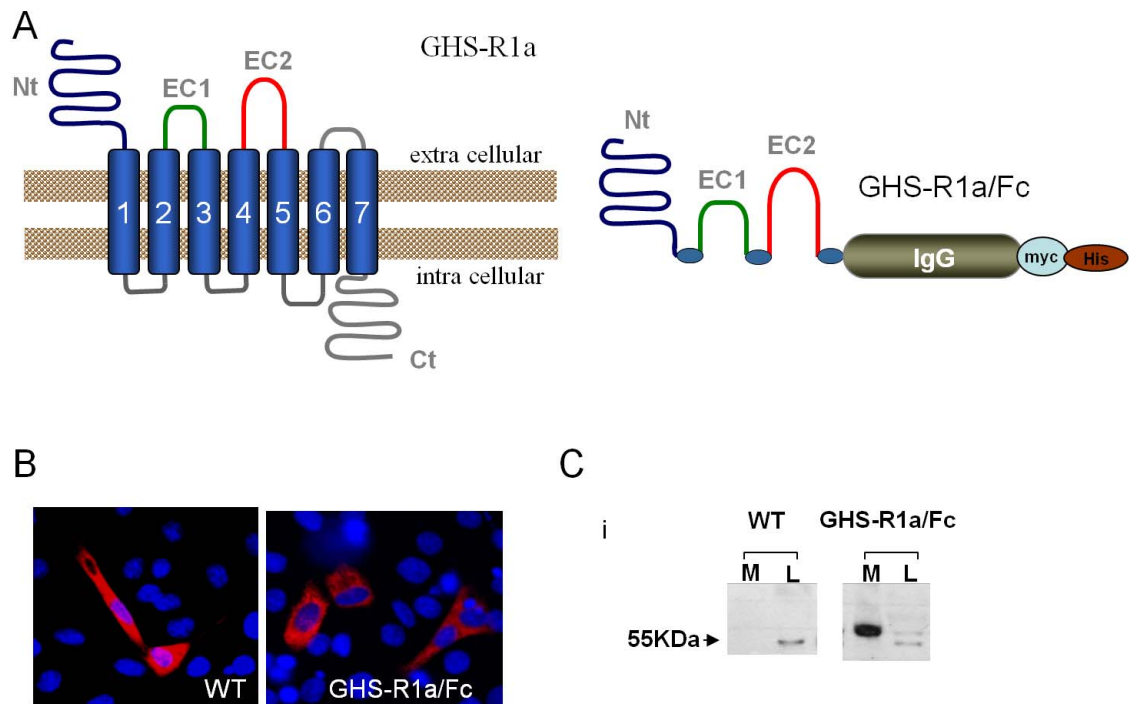
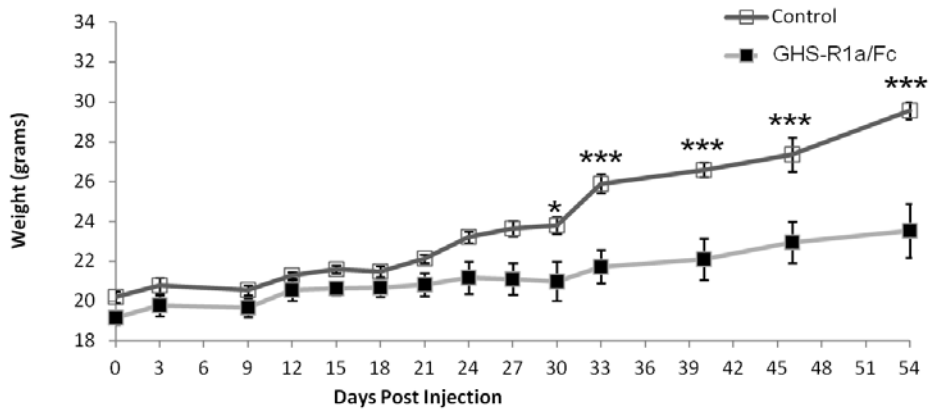
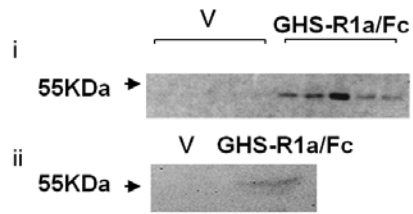


Figure 26 Validation of GHSR related constructs. Illustration of the full length (WT) and extracellular domain (GHS-R1a/Fc) constructs(A). Domains are identified as N-terminal (Nt), Extracellular domain 1 and 2 (EC1, EC2) and immune modulating FC immunoglobulin region. Fluorescent immunocytochemistry (red) using anti mouse Fc IgG was completed in L6 muscle cells transfected with WT and GHS-R1a/Fc plasmids (B).Western blots were conducted using anti Myc tag on cell lysates and culture media from L6 cells transfected with GHSR constructs.

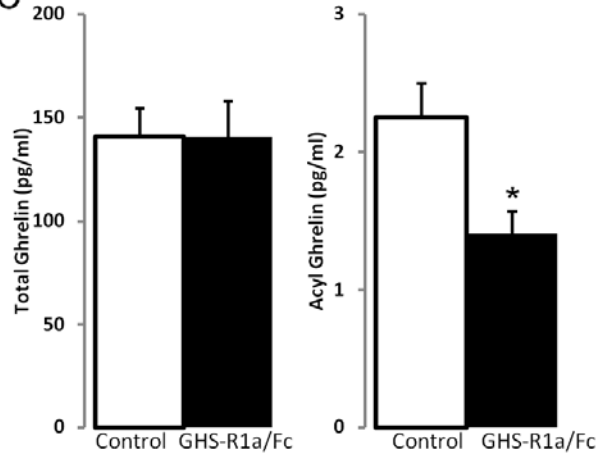
A



B



C



D

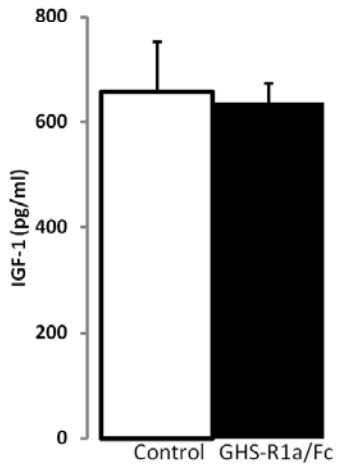


Figure 27 Effect of GHSR constructs on weight gain. Animals were placed on a high fat diet concurrent with the first gene transfer. GHS-R1a/Fc and control groups average weight (n=5) is indicated on the Y- axis for days post DNA treatment on the X axis (54 days) (A). Western blots were completed with anti Myc tag on of gastrocnemius muscle tissue lysate (Bi) and blood plasma (Bii) at the end of the study. Circulating levels of total (AG and UAG) and acyl specific (AG) as well as IGF-1 were assayed at the end of the study (C and D). * indicate $P < 0.05$, *** indicate $p < .0001$ with n=5.

($23.5 \pm 1.36\text{g}$ vs. $29.6 \pm .434\text{g}$ in control, $p < 0.001$), (Figure 27A). Although exogenous ghrelin administration was previously shown to have potent orexigenic effect, there were no differences the daily food and water consumption (data not shown).

In vivo expression of GHS-R1a/Fc was verified by western blotting of whole gastrocnemius muscle from treated mice, and expression of a 55kD protein corresponding to the produced protein was detected in these animals (Figure 27B i). To confirm the presence of the constructs in circulation, western blotting was performed on plasma from treated mice (Figure 27B ii). Finally, to confirm the ghrelin neutralizing effect of this treatment, the levels of AG and total ghrelin (primarily UAG) were measured in circulation. As shown in Figure 27C, expression of GHS-R1a/Fc did not alter the levels of total ghrelin (140.9 ± 30.2 pg/ml vs. 140.8 ± 37.94 pg/ml in control) but significantly reduced the levels of AG (1.41 ± 0.160 pg/ml vs. 2.25 ± 0.240 pg/ml in control, $p < 0.05$). To address whether reduced AG levels had an effect on the GH pathway, levels of the GH dependent insulin like growth factor-1 (IGF-1) were examined. IGF-1 is used as a marker of GH hormone levels as its levels are more stable in circulation. No difference was found for IGF-1 levels (Figure 27D).

Effects of GHS-R1a/Fc on adipose tissue

To determine the source of the reduced weight gain in the treated animals, fat pad mass and lean tissue mass (gastrocnemius muscle) was investigated. Visually, the amount of white adipose tissue (WAT) found in the peritoneum of treated mice was less than control (Figure 28A). This difference was quantified by the weighing of various fat pads.

Several fat stores were significantly smaller in the treated mice including retroperitoneal (0.476 ± 0.12 g vs. 0.948 ± 0.119 g in control, $p < 0.001$), peri-renal (0.44 ± 0.19 g vs. 0.734 ± 0.011 g in control, $p < 0.05$) and inguinal fat pads (0.396 ± 0.067 g vs. 0.722 ± 0.56 g in control, $p < 0.05$) (Figure 28B). Lean mass was not affected by treatment as determined by the equal gastrocnemius muscle weight (0.131 ± 0.016 g vs. 0.109 ± 0.011 g in control) (Figure 28B).

Since reduced caloric consumption was not responsible for the reduction in WAT, alterations in WAT mRNA expression of several metabolic genes were examined. Visceral WAT was examined for the adipokine leptin (a hormone that signals in response to fat cell anabolism), hormone sensitive lipase (HSL; an enzyme catalyzing the breakdown of triglycerides to fatty acids) and peroxisome proliferator-activated receptor- γ (PPAR γ ; a nuclear receptor that promotes adipogenesis) mRNA using quantitative RT-PCR. Treated animals had lower levels of leptin gene expression in WAT (0.10 ± 0.14 fold of control, $p < 0.05$) while HSL mRNA (1.76 ± 0.07 fold of control, $p < 0.05$) and PPAR γ mRNA (3.91 ± 0.378 fold of control, $p < 0.05$) expression were elevated (Figure 28C). In addition, mRNA levels of adipose derived proinflammatory cytokines, known to be elevated in obesity, were examined in WAT. Both interleukin-1 (IL-1) and tumor necrosis factor α (TNF α) were reduced in GHS-R1a/Fc compared to control (0.029 ± 0.016 for IL-1 and 0.15 ± 0.07 for TNF α fold of control, $p > 0.05$) (Figure 28C).

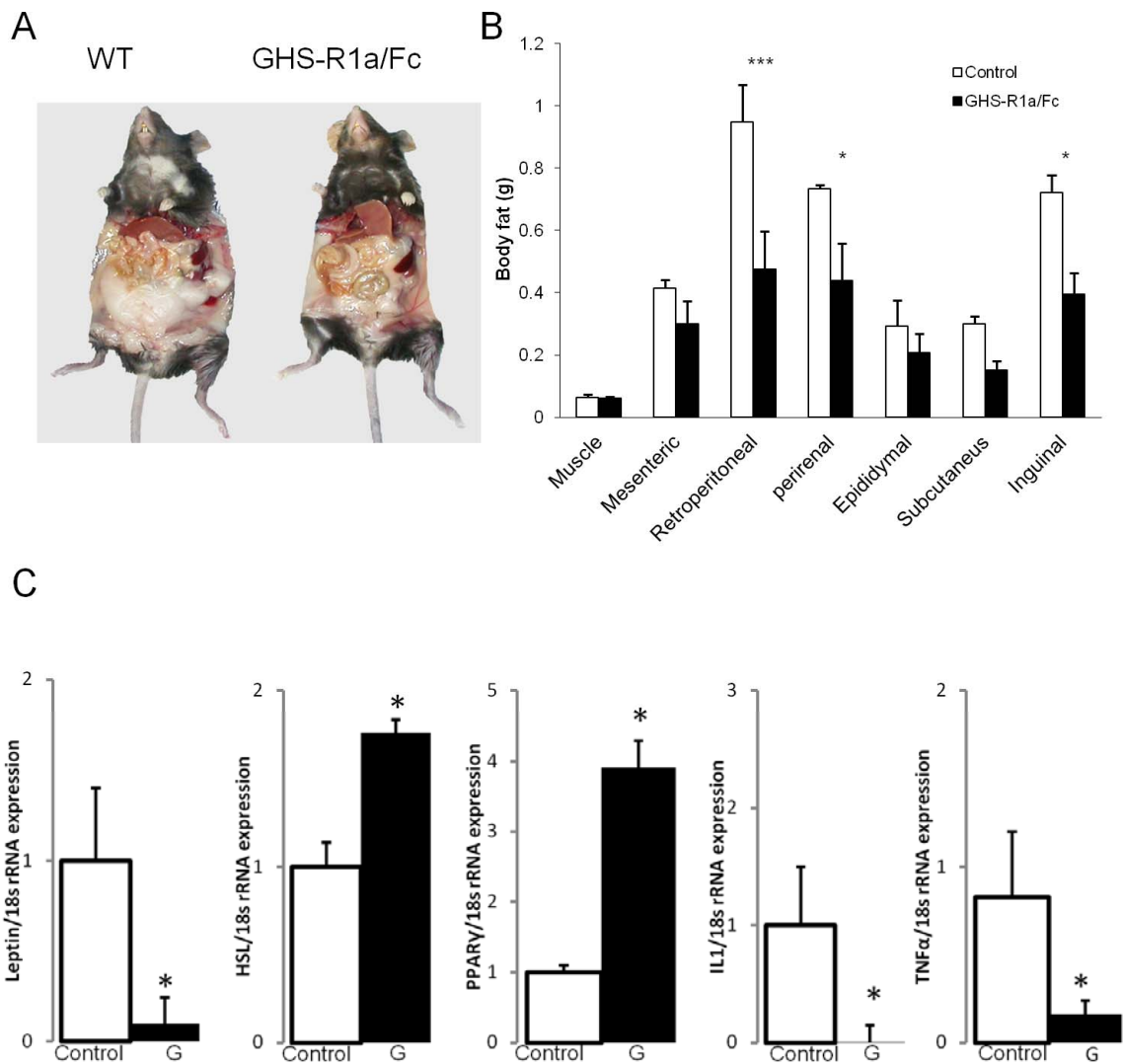


Figure 28 Effect of GHS-R1a/Fc on adipose tissue. Post experimentally, peritoneal fat pads were observed from sacrificed control and GHS-R1a/Fc treated animals (A). Fat pad mass and gastrocnemius muscle was quantified for indicated stores in control and GHS-R1a/Fc (B). White adipose tissue was collected and RT-qPCR in GHS-R1a/Fc relative to control was conducted for leptin, hormone sensitive lipase (HSL), PPAR γ , interleukin-1 (IL-1) and tumor necrosis factor α (TNF α) relative to 18s ribosomal RNA using the standard curve method (C). * indicates $P < 0.05$ and *** indicates $p < 0.0001$ with $n = 5$.

Effect of neutralizing ghrelin on glucose metabolism

Ghrelin has been shown to affect glucose homeostasis (Broglio, Arvat et al. 2001). To determine the effect of GHS-R1a/Fc treatment on glucose metabolism, intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT) were performed on control and treated mice at the end of the study. Treated mice had improved glucose tolerance with significantly lower blood glucose at 10 minutes (14.5 ± 1.52 mmol/L vs. 18.9 ± 1.48 mmol/L in control, $p < 0.01$) and 20 minutes (10.1 ± 0.968 mmol/L vs. 14.2 ± 0.989 mmol/L in control, $p < 0.05$) post-glucose injection (Figure 29A). The area under the curve in the IPGTT was also significantly reduced in the treated mice (516 ± 32.2 vs. 668 ± 22.1 in control, $p < 0.05$) (Figure 29B). In ITT, the area under the glucose curve was lower in treated animals (180 ± 19.1 vs. 230 ± 20.3 in control, $p < 0.05$) indicating increased insulin sensitivity (Figure 29C and 29D). These results suggest that reduced circulating AG improves insulin sensitivity and glucose tolerance in mice on a high fat diet.

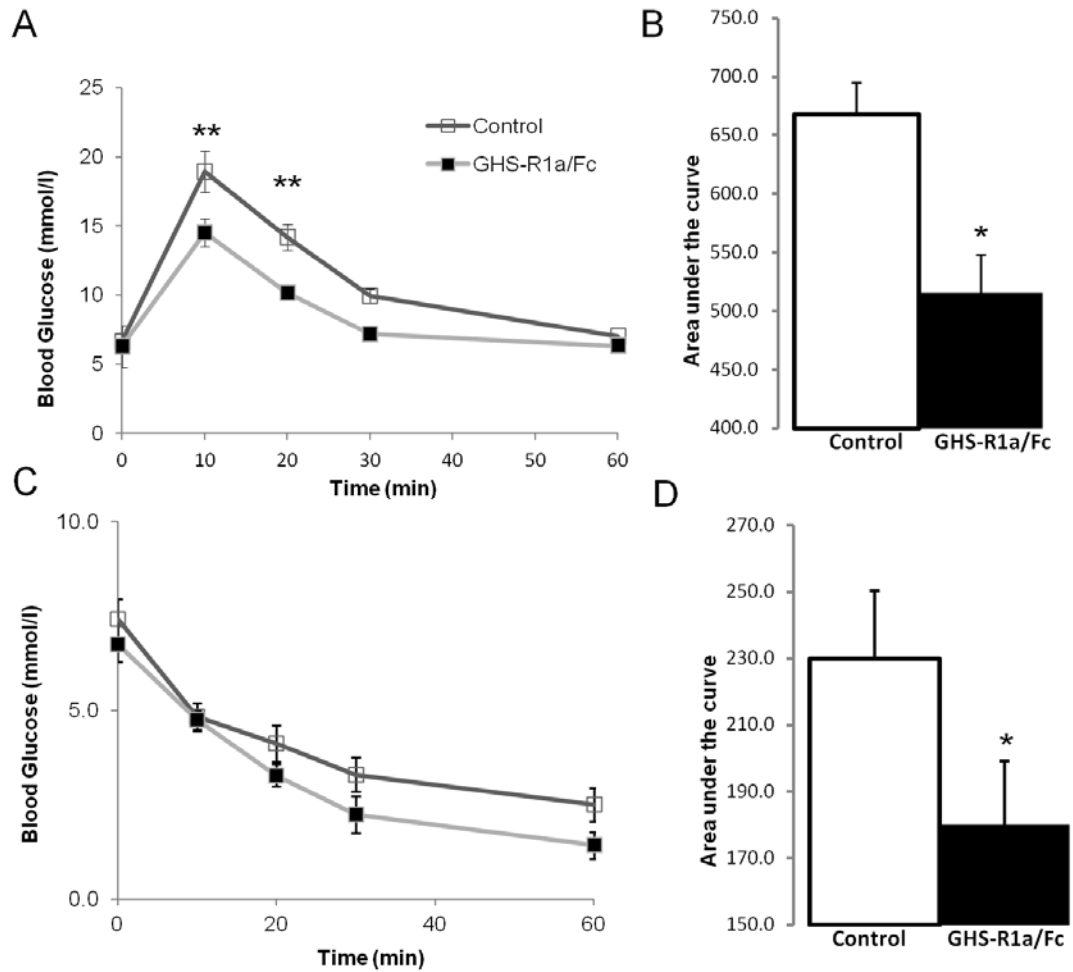


Figure 29 Effect of GHSR/Fc on glucose metabolism. Intra-peritoneal glucose and insulin tolerance tests were completed at the end of the study. Glucose measurements were taken at indicated time points after an IP injection of glucose in GHS-R1a/Fc and control animals (A). The total area under the curve for the glucose tolerance test was compared between control and GHS-R1a/Fc (B). Glucose measurements were taken after an IP injection of insulin in control and GHS-R1a/Fc animals (C). * indicates $P < 0.05$, ** indicates $p < .001$ with $n = 5$.

6.5 Discussion

In this study we used a non-viral gene transfer method to express a GHSR1a based construct and reduce AG in circulation. The custom plasmid was designed to incorporate the extracellular segments of the GHSR1a that interact with the acylated portion of ghrelin. In addition, the GHSR1a sequences were fused with IgG sequences to improve the stability and half-life of the complex in circulation (Soltani, Kumar et al. 2007). Initially, we confirmed expression and secretion of the construct *in vitro* by transfecting into the L6 muscle cell line. As expected, the full length GHS-R1a (WT) was not detected in culture media as it possesses the hydrophobic transmembrane domains that would retain it in the plasma membrane. Later, we confirmed the expression in both the muscle and plasma from GHS-R1a/Fc plasmid injected animals. To verify that the treatment led to altered ghrelin levels we measured both the total (AG + UAG) and acylated (AG) levels in the circulation. While no significant difference was observed in the levels of total ghrelin, AG was significantly reduced in treated animals. It is important to note, any differences in AG (5% of total ghrelin in circulation) occurring in the total ghrelin assay would likely be undetectable (Kojima, Hosoda et al. 1999; Ariyasu, Takaya et al. 2001). The lack of difference in the total ghrelin assay confirms the specificity of the GHS-R1a for binding only AG.

To examine the impact of neutralizing ghrelin on energy regulation, animals were placed on a HFD. Thirty days after the first plasmid treatment, animals treated with GHSR1a/Fc gained significantly less weight compared to control mice on HFD. The reduced weight gain in the GHSR1a/Fc group was maintained until the end of the study at

54 days post gene transfer, with a final weight gain reduction of over 20%. Our observed reduced weight gain and reduced circulating AG is in agreement with previous work examining the effects of ghrelin immunoneutralization (Zorrilla, Iwasaki et al. 2006).

We also designed constructs coding for the N-terminal region of the GHSR1a (Nt/Fc) and encoding the N-terminal and the first extracellular loop (Nt-EC1/Fc), but did not observe a change in weight gain with these constructs (data not shown). The GHS-R1a/Fc was the only plasmid that incorporated all three of the extracellular portions of the GHS-R1a (Nt, EC1 and EC2). Of particular importance was the incorporation of both the extracellular loop domains (EC1 and EC2) as these extracellular loops are thought to be the binding sites for AG to the GHS-R1a (Pedretti, Villa et al. 2006).

Despite the difference in weight gain, we did not observe any difference throughout the study in food consumed between the GHS-R1a/Fc treatment and control groups. Ghrelin's action on appetite occurs through binding the GHS-R1a in neurons within the arcuate nucleus of the hypothalamus (Cowley, Smith et al. 2003). While peripheral ghrelin administration has been shown to cross the blood brain barrier (BBB) and stimulate appetite (Banks, Tschop et al. 2002), a population of ghrelin producing neurons also exists within the arcuate nucleus (Kageyama, Kitamura et al. 2008). In the present study, due to the size of the GHS-R1a/Fc protein, it is unlikely that it was able to cross the BBB and neutralize hypothalamic ghrelin. Thus, our strategy may only be targeting peripheral ghrelin and its actions. In agreement with our study, ghrelin immunoneutralization studies also have no effect on food intake (Zorrilla, Iwasaki et al. 2006). Furthermore, studies that examined both ghrelin and GHSR1a embryonic knockout mice also saw no effect on feeding behaviour; however, when challenged with

a HFD, they were more likely to utilize fat as their energy substrate (Wortley, Anderson et al. 2004; Zigman, Nakano et al. 2005). Like these studies, we found a reduction in fat pads, primarily in the peritoneum.

As reduced food consumption was not the cause for the reduced fat mass, it is possible that the reduction in AG affected fat tissue metabolism. Indeed, a recent study indicated that ghrelin acts directly on adipocytes to prevent lipolysis (Davies, Kotokorpi et al. 2009). To determine the effect of reduced circulating ghrelin levels on adipose tissue, we measured the mRNA expression of fat metabolism genes in WAT. Not surprisingly, leptin, which is produced during fat accumulation and adipocyte differentiation (Houseknecht, Baile et al. 1998), was lower in the GHS-R1a/Fc treated animals. Of particular interest, HSL, a key enzyme in the catabolism of triglycerides to fatty acids (Yeaman 2004), was found to be elevated in the treated animals. This increased lipase expression is indicative of increased mobilization of fatty acids for energy substrate, which supports our finding that the treated animals were protected from adiposity while eating a HFD. This finding also agrees with indirect calorimetry experiments on ghrelin KO mice that were found to preferentially use fat as their energy substrate (Wortley, Anderson et al. 2004). PPAR γ mRNA levels were also elevated in the treated animals. While activation of this nuclear receptor typically leads to the differentiation and growth of adipose tissue, some evidence suggests that increased PPAR γ may partition fat away from visceral stores (Laplante, Sell et al. 2003). In accordance, our study showed that the most significant reduction on fat pad weight was in the visceral depots.

Our findings are in agreement with another group that showed low dose ghrelin administration in mice caused increased fat pad weight and altered adipocyte gene expression without an effect on feeding (Tsubone, Masaki et al. 2005). Taken together, our results indicate that reducing circulating AG with GHSR1a/Fc treatment leads to reduced fat stores and altered adipocyte gene expression.

Reducing AG with GHSR1a/Fc treatment significantly improved glucose tolerance and insulin sensitivity in mice submitted to a high fat diet regimen. These findings are in agreement with several studies suggesting that ghrelin promotes glucose homeostasis through inhibiting insulin release from the pancreas (Dezaki, Sone et al. 2006; Dezaki, Sone et al. 2008). While we did not observe any differences in circulating insulin levels, another possibility for the improved glucose clearance could stem from reduced hepatic glucose production in the GHSR1a/Fc treated animals since previous work has indicated that ghrelin promotes glucose production in hepatocytes (Gauna, Delhanty et al. 2005). It must also be considered that the improvements in glucose tolerance may be a secondary effect to the reduced visceral adiposity in the treated animals. It is known that increased adiposity can lead to insulin resistance, which is brought on by proinflammatory factors released from inflamed fat stores (Oliver, McGillicuddy et al. 2010). To determine the possible involvement of pro-inflammatory cytokines, we examined the mRNA expression of both IL-1 and TNF α in visceral adipose tissue. Both these genes had significantly lower expression in GHSR1a/Fc treated animals. While these findings do not indicate if ghrelin directly regulates pro-inflammatory cytokine expression, they support the overall result that depleting ghrelin

using the GHSR1a/Fc treatment led to an improvement in glucose tolerance, which could be beneficial in therapies for T2DM.

In our study we examined if the GHSR1a/Fc treatment had any impact on other metabolic hormones and found that none of the hormones examined (GLP-1, insulin, PYY, pancreatic polypeptide, and glucose insulinotropic peptide) were significantly altered (data not shown). As ghrelin is a known GH secretagogue (Kojima, Hosoda et al. 1999) and GH has effects on glucose metabolism and insulin sensitivity (Moller and Jorgensen 2009), the effects of ghrelin depletion on GH were examined. Since GH varies throughout the day in a pulsatile fashion, a more stable measurement of relative GH levels can be obtained by measuring circulating IGF-1. Interestingly, circulating IGF-1 levels were not affected by ghrelin neutralization and likely were not responsible for the reduced weight and improved glucose parameters. A similar lack of change in GH levels was observed in studies examining HFD in GHSR1a knockout mice (Zigman, Nakano et al. 2005).

In summary, we utilized a novel *in vivo* gene transfer of a secretable GHSR1a based construct to reduce the levels of active circulating ghrelin. This strategy prevented HFD induced body weight gain and insulin resistance. Of particular benefit, the reduced ghrelin levels were still present nearly 40 days after the last injection suggesting that long-term regulation of ghrelin levels is possible with minimal gene transfers. This reduction in ghrelin caused reduced body weight and adiposity without affecting appetite. Altered gene expression in adipose tissue suggests a shift to fat usage rather than storage and a reduction in the production of pro-inflammatory cytokines. In addition, we observed improved glucose clearance due to improved insulin sensitivity. The use of *in*

in vivo gene transfer to neutralize ghrelin may provide a new strategy for the treatment and management of obesity and T2DM.

Acknowledgement

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CHAPTER 7: OVERALL DISCUSSION

7.1 Summary of Findings

In this thesis, I have presented novel findings on both the mechanism of ghrelin secretion and the function of AG in energy metabolism as well as the feasibility of reducing AG in circulation as a strategy to reduce body weight and insulin resistance. In order to elucidate the detailed mechanisms of ghrelin secretion, I developed a novel primary culture of stomach cells that secrete ghrelin in a regulated manner. These cells proved to be an ideal system as they attached to untreated cell culture plates, secreted ghrelin both under basal and stimulated conditions, and responded to treatments in a consistent fashion. Using this model not only allowed the investigation of how individual factors (neurotransmitters, hormones, glucose, and metformin) affected ghrelin secretion, but also the intracellular pathways by which their activity occurred. From this work, and the work of other groups, it is clear that ghrelin cells are able to directly sense energy status in the body. This has great importance since ghrelin has been clearly demonstrated to regulate appetite and energy storage. To further understand ghrelin's role in energy regulation and to determine the feasibility of depleting ghrelin as a potential therapy, we designed and tested a ghrelin reducing decoy protein (based on the ghrelin receptor). Expressing this protein in mice led to a reduction in circulating AG and prevented diet induced weight gain independent of feeding, potentially through altering fat metabolism. While a discussion follows each of the published manuscripts in this thesis, a further detailed discussion will be presented in this section.

7.2 Neurotransmitters and Ghrelin Release

Our initial investigation into a potential role for neurotransmitters in ghrelin secretion stemmed from the fact that the stomach is heavily innervated by the enteric nervous system. In addition, several studies had demonstrated a potential role for vagal efferents (Williams, Cummings et al. 2003) and sympathetic innervations (Mundinger, Cummings et al. 2006) in ghrelin secretion *in vivo*. We demonstrated that NE stimulated ghrelin secretion while acetylcholine had no effect. While I was investigating these systems in the primary stomach culture, Zhao *et al* published a study investigating the role of neurotransmitters on ghrelin secretion from their tumor derived ghrelin cell line (Zhao, Sakata et al. 2010). Using their pancreatic derived (PG-1) ghrelin cell line, they found that both epinephrine and NE stimulated, while carbachol (a cholinergic agonist) had no effect on ghrelin secretion.

The failure of cholinergic agonists to produce an effect in both studies suggests that the parasympathetic nervous system may not have a direct role in the regulation of ghrelin secretion. This is in contradiction to a study where severing the vagus (a branch of the parasympathetic nervous system) prevented the fasting induced increase in ghrelin (Williams, Cummings et al. 2003). However, it must be noted that while preganglionic neurons are cholinergic, it is possible that the post-ganglionic vagal efferent fibres innervating the stomach ghrelin cells are not using acetylcholine as their neurotransmitter. In addition, since vagotomy is known to effect the release of several other hormones (reviewed in (Chang, Mashimo et al. 2003)), there are likely indirect effects of the vagotomy conducted in by Williams *et al*. that could be contributing to fasting ghrelin release.

A remaining question is whether an increase in sympathetic activity is expected in the viscera during the fasting state. Earlier studies in rat cardiac tissue demonstrated that fasting led to decreased sympathetic tone (Young and Landsberg 1977). Since fasting causes increased ghrelin secretion, this is at odds with our finding, and that of others, that NE acts to stimulate ghrelin secretion. However, several lines of evidence support an increase in sympathetic tone during the fasting state and this increase appears to be important for mobilizing energy stores. Increased sympathetic tone was shown to mobilize TG's from white adipose tissue during a prolonged fast (Migliorini, Garofalo et al. 1997). Furthermore, activation of sympathetic nerves in calves caused the breakdown of liver glycogen leading to increased circulating glucose (Edwards and Silver 1970). Taken together, this supports an important role for the sympathetic nervous system in restoring energy homeostasis in a depleted state and is in agreement with our finding that NE stimulates ghrelin secretion.

7.3 Insulin Suppresses Ghrelin Secretion

Using the primary stomach culture cell preparation, insulin was shown to inhibit ghrelin secretion. Given that insulin increases postprandially in response to glucose while ghrelin decreases under a similar situation, a role for insulin in the reduction of ghrelin secretion was predicted. Indeed, studies that examined ghrelin levels in humans found that insulin levels were negatively correlated with ghrelin levels (Ikezaki, Hosoda et al. 2002; Haqq, Farooqi et al. 2003). However, in these studies, hyperinsulinemia and ghrelin levels were examined in obese individuals. As there are many other metabolic

consequences in obesity, it is difficult to attribute a direct relationship between elevated insulin and lower ghrelin levels. Some clarification came from a study by McLaughlin *et al.* where ghrelin levels were compared in obese insulin resistant (hyperinsulinemic) and obese insulin sensitive (normal insulin) individuals (McLaughlin, Abbasi *et al.* 2004). In this study they observed lower ghrelin levels in obese insulin resistant compared to obese insulin sensitive individuals (McLaughlin, Abbasi *et al.* 2004), suggesting that hyperinsulinemia in obesity may be the causative factor in the reduction of ghrelin levels.

More direct evidence for insulin's suppressive effects on ghrelin secretion came from a study where ghrelin was examined while insulin was infused under glucose-clamped (euglycemia) conditions. This study clearly demonstrated the ability of insulin (independent of glucose effects) to reduce circulating ghrelin (Saad, Bernaba *et al.* 2002). A role of insulin in ghrelin secretion at the cellular levels was demonstrated by Iwakura *et al.* using their novel ghrelinoma derived cell line (MGN3-1) (Iwakura, Li *et al.* 2010). Specifically, they also found that insulin was able to reduce ghrelin secretion (Iwakura, Li *et al.* 2010), however they did not investigate the mechanism of this effect. These studies and our report on the effect of insulin in ghrelin secretion are in contradiction with a report by Zhao *et al.* using pancreatic derived PG-1 ghrelin cells (Zhao, Sakata *et al.* 2010). In that report, insulin failed to suppress ghrelin secretion and as discussed in Chapter 2, may have been due to a difference in the regulation of pancreatic ghrelin cells (PG-1) vs. stomach ghrelin cells (MGN3-1 and primary culture) or potentially from the use of the 100nM insulin dose.

One of the interesting findings that emerged from the examination of insulin on ghrelin secretion was a lack of effect in the higher (100 nM) insulin dose range. Our

subsequent experiments determined that prolonged exposure (24 hours) to 100 nM insulin dose led to decreased insulin receptor protein expression and impaired insulin signalling through the AKT pathway. We described this scenario as one of cellular insulin resistance since our *in vitro* setting does not comprise the myriad of altered factors of *in vivo* insulin resistance (increases in proinflammatory cytokines, leptin, glucagon, and circulating triglycerides).

A major question that arises from these results, is can ghrelin cells become insulin resistant in a situation of prolonged hyperinsulinemia *in vivo*? In support of this, oral glucose treatment in obese children, despite causing a much greater release in insulin, led to a similar percent drop in ghrelin compared to normal weight children (Bacha and Arslanian 2005). This suggests that some insulin resistance may be occurring at the level of the ghrelin cell. To control for the differences between obese and normal weight individuals, McLaughlin *et al.* compared obese insulin resistant (as measured by increased steady state plasma glucose) to obese insulin sensitive. They found that under a steady insulin infusion, both groups had a similar suppression in ghrelin (McLaughlin, Abbasi *et al.* 2004). This suggests that despite the prolonged hyperinsulinemia in this insulin resistant group, ghrelin cells maintain their ability to respond to insulin. That is, ghrelin cells continue to have insulin-induced suppression of ghrelin release even in insulin resistance. The lack of response we observed after the 100 nM insulin pre-incubation may be due to receptor desensitization or down-regulation. Indeed, chronic insulin incubation (10 nM for 16 hours) in rat hepatocytes also leads to decreased insulin receptor expression (Gavin, Roth *et al.* 1974). Whether or not it is the hyperinsulinemia or other factors that are responsible for the further suppression of ghrelin in humans

remains to be confirmed. One way to clarify the role of insulin would be to examine the ghrelin response to euglycemic, hyperinsulinemic pancreatic clamp in a similar obese-diabetic vs. obese-normal groups. This way, the acute response to insulin (while controlling glucose) could be determined. In addition, cohorts of non-obese individuals with T2DM could be examined in a similar manner. This would clarify the impact of insulin resistance on ghrelin secretion, independent of obesity.

7.4 High Glucose Inhibits Ghrelin Secretion

Another important aspect of insulin's regulation of ghrelin secretion is the role glucose plays. In our preliminary examination of the effect of glucose alone, we observed an inverse relationship between glucose concentration and ghrelin secretion. We found that higher concentrations of glucose (15 mM) had reduced ghrelin secretion relative to very low glucose (1 mM) (Figure 20), which was also demonstrated by Sakata *et al.* in primary mouse stomach cells (Sakata, Park et al. 2012). The ability of ghrelin cells to suppress secretion under elevated glucose levels may have important implications in the postprandial suppression of ghrelin secretion. At the other extreme, the ability of ghrelin cells to stimulate secretion in low glucose may be involved in the fasting induced increases in ghrelin levels. However it must be noted that 1mM glucose is well below physiological fasting glucose. Never the less, these findings support that ghrelin cells are able to sense the surrounding glucose environment. The exact intracellular mechanisms of how this occurs remain to be elucidated.

Elevated glucose stimulates insulin secretion, and it is possible that both glucose and insulin act together to inhibit ghrelin. Whether high glucose can potentiate the suppressive effect of insulin, or conversely, whether low-glucose can block the effect of insulin on ghrelin secretion is not clearly understood. To clarify, we incubated primary stomach culture cells with insulin in varying concentrations of glucose. Relative to basal (5mM) glucose, insulin treatment suppressed ghrelin secretion by ~50% regardless of the 3 tested glucose environments (1, 5 and 15mM). Statistically (using a two way ANOVA), glucose had no interaction with the effect of insulin on ghrelin secretion. This suggests that while glucose may have effects on ghrelin secretion, it is unable to alter the suppressive ability of insulin. This finding is in contrast to the study by Sakata *et al.* who found insulin was unable to suppress ghrelin secretion in a 5 or 10mM glucose environment (Sakata, Park et al. 2012). It should be noted that their study used a 100 nM dose of insulin, which we also found was unable to suppress ghrelin secretion due to dysregulated insulin receptor signalling (Chapter 2).

7.5 Glucagon Stimulates ghrelin secretion

In the literature, the role of glucagon in ghrelin secretion was sparsely investigated compared to that of insulin. Furthermore, there is conflicting data regarding the effect of glucagon on ghrelin secretion with studies showing reduction, stimulation or no effect at all. As indicated in the above sections, glucagon injections in humans caused a reduction in circulating ghrelin levels (Arafat, Otto et al. 2005; Soule, Pemberton et al. 2005). In contrast, isolated rat stomachs and rats directly perfused with glucagon in the femoral vein caused an increase in ghrelin secretion (Kamegai, Tamura et al. 2004;

Katayama, Shimamoto et al. 2007). Finally, an investigation by Zhao *et al.* in their pancreatic ghrelin PG-1 cell line indicated that glucagon (100 nM) had no effect on ghrelin secretion (Zhao, Sakata et al. 2010). This prompted our investigation of how glucagon would affect ghrelin secretion in the isolated rat primary stomach culture.

Our findings indicated that glucagon stimulated ghrelin secretion through the ERK and EPAC pathways. With regards to blocking the EPAC pathway using BFA, we acknowledge that BFA also inhibits vesicle transport from the ER to golgi and future work using more specific EPAC blockers is required. Stimulation of ghrelin secretion by glucagon is in agreement with glucagon's established role for mobilizing energy stores in situations of low energy availability. As indicated in the Chapter 3 discussion, an explanation for the reduced ghrelin secretion observed in the human studies may be from the secondary effects of glucagon injection. Indeed, glucagon injection also caused an increase in both circulating insulin and glucose (both factors that reduce ghrelin secretion). Strategies to clarify the role of glucagon in ghrelin secretion *in vivo* would need to clamp pancreatic insulin release while maintaining constant insulin and euglycemia.

One aspect that was examined in the glucagon study was the synergy between the stimulation of glucagon and NE. While examining how individual factors contribute to ghrelin is one of the strengths of the primary stomach cell preparation, it is understood that multiple factors could be in contact with ghrelin cells at one time. Such is the case with the sympathetic neurotransmitter NE and glucagon. The levels of sympathetic activation on the stomach and circulating glucagon both increase under fasting conditions and both independently lead to increased ghrelin secretion. To determine if these two

pathways stimulated ghrelin secretion in a synergistic fashion, ghrelin secretion was examined by co-incubation with both glucagon and NE. The combination of both glucagon and NE stimulated ghrelin secretion greater than the sum of their parts. While the precise mechanism of how this synergy occurs was not investigated in greater detail, it is possible that greater secretion was obtained through increased cAMP production. Since both glucagon and NE increased cAMP levels, any additional cAMP production from the NE treatment may have led to increased overall levels and ghrelin secretion.

Regardless of the exact mechanism, it is clear that both pathways share similarities in their cellular mechanisms leading to increased ghrelin secretion. However, it is also apparent that there were some differences. Despite the increase in cAMP observed after glucagon treatment, activation of the cAMP dependent protein kinase (PKA) was not a requirement for its action. While cAMP is known to activate other pathways including EPAC and ion channels (reviewed in (Skalhegg and Tasken 2000)), it is somewhat paradoxical that PKA was not important in glucagon stimulated secretion since it was essential for NE stimulated ghrelin secretion in primary stomach culture. One possible explanation for this may be the distinct cellular localization of the increase cAMP levels. Using a cAMP sensitive fluorescent resonance energy transfer (FRET) assay, DiPilato *et al.* demonstrated that cAMP increases occur in both a spatial and temporal fashion (DiPilato, Cheng et al. 2004). Since the cAMP assays conducted in the above manuscripts do not resolve the localization of increased cAMP activity, further work is required to determine the role of subcellular cAMP localization in ghrelin cells.

7.6 Metformin Inhibits Ghrelin Secretion

Anti-diabetic medications including biguanides (metformin), sulphonoureas (gliburide) and PPAR γ agonists (rosiglitazone), target a variety of pathways to reduce circulating glucose. Metformin is unique as it has been shown to reduce body weight (mean of 8kg weight loss) in obese patients with T2DM (Lee and Morley 1998). Metformin's main mechanism of action is through the AMP activated serine/threonine kinase, AMPK. As ghrelin cells appear to act as energy sensors through both hormones (insulin and glucagon) and nutrients (glucose), the direct effect of metformin on ghrelin secretion was examined. In the primary stomach culture, metformin reduced ghrelin secretion by activating the AMPK pathway. The downstream effects of AMPK activation that lead to reduced ghrelin secretion were not investigated; however, AMPK has several targets involved in cellular energy homeostasis that may play a role in regulating ghrelin secretion and proghrelin expression. One such target is the glucose transporter (GLUT4). While GLUT4 is typically expressed and regulated in muscle and adipose tissue, it is also enriched in purified ghrelin cells relative to non ghrelin stomach cells (Sakata, Park et al. 2012). Using primary human muscle myotubes, McGee *et al.* determined that AMPK activation led to increased GLUT4 production through deacetylation and transcription (McGee, van Denderen et al. 2008). Increased GLUT4 expression could enhance transport of glucose into the cell. Our work (and that of others) has shown that excess glucose leads to reduced ghrelin secretion, thereby providing a possible mechanism for metformin's effect on ghrelin secretion.

Even more striking than the effects of metformin on secretion, was the effect on proghrelin mRNA production (metformin caused a ~10-fold reduction in ghrelin

message). A possible pathway leading to the large reduction in proghrelin mRNA is the downstream target of metformin, mammalian target of rapamycin (mTOR) which is a potent regulator of transcription and translation (Mayer, Zhao et al. 2004). mTOR is a serine/threonine kinase that forms an activated complex (MTORC1) that is inhibited by AMPK (Kimura, Tokunaga et al. 2003). A previous study determined that injecting the mTOR inhibitor, rapamycin, stimulated proghrelin expression in gastric tissue and total ghrelin in secretion in mice (Xu, Li et al. 2009). This is surprising since suppressing mTOR should lead to decreased translation and transcription and perhaps lower proghrelin mRNA production. Nevertheless this *in vivo* work supports the idea that the mTORC pathway is part of the cellular machinery leading to regulation ghrelin secretion. To examine the role of mTOR in ghrelin secretion at the cellular level, the primary culture could be treated with rapamycin. In addition, to determine if AMPK is responsible for inactivating mTOR, phosphorylated levels of the S6 ribosomal protein (a downstream target of mTOR) could be examined after metformin or AICAR treatment.

Another possible target of activated AMPK that may be responsible for suppressing ghrelin is P13K. AMPK has been demonstrated to activate PI3K in the 3T3L1 adipocyte cell line (Tao, Gong et al. 2010). Also, we have demonstrated that PI3K is activated by insulin in the primary ghrelin culture, and that the insulin-dependent activation of PI3K is required for the suppression in ghrelin secretion. Never the less, the precise mechanism of metformin action on proghrelin mRNA production and ghrelin secretion require further investigation.

7.7 Reduction of Circulating Acylated Ghrelin

The discussion thus far has focused on the mechanisms regulating ghrelin secretion in the primary rat stomach culture. From this work it is clear that ghrelin cells possess the ability to sense and respond to energy cues. This is an important ability since downstream ghrelin functions will promote both the intake and storage of energy. While understanding the mechanism(s) of ghrelin secretion may aid in the discovery of therapies designed to inhibit or stimulate ghrelin release, strategies that reduce circulating ghrelin after release are also of interest. In the final manuscript presented, the effects of reducing circulating AG using *in vivo* expression of a novel ghrelin receptor-based decoy protein in mice were examined. This decoy was a secretable fusion protein comprised of the extracellular ligand binding portions of the GHSR1a and a mouse IgG Fc region (GHSR1a/Fc). Injection of this plasmid into the gastrocnemius muscle and electroporation led to plasmid uptake, synthesis, and secretion of the decoy protein, which in turn caused a reduction in the circulating AG. The primary outcome of this study was a reduction in HFD-induced weight gain compared to an empty vector control, which occurred 30 days after the treatments.

We predicted that reduced feeding would be responsible for this loss in weight gain (an expected outcome of reduced circulating ghrelin). Surprisingly, treated animals consumed the same amount of food as control animals (this was further confirmed with the use of metabolic cages). In the manuscript discussion we highlighted how this reduced HFD weight gain without reduced feeding was a common event in other ghrelin

depletion studies. It is interesting that the majority of studies demonstrating a potent orexigenic effect of ghrelin have been done through exogenous administration (both peripheral and central) and not through depleting endogenous ghrelin. In light of this, it may be that endogenous peripheral ghrelin is not critical for stimulating appetite. It is also important to note, that the animals in our study were not fasted or put in a situation where ghrelin levels would be increased. Having access to HFD *ad libitum* is known to increase the feeding frequency in mice compared to normal chow (Kohsaka, Laposky et al. 2007). This increased frequency could blunt the diurnal rhythm of ghrelin release, thereby further lowering the ability for ghrelin to regulate feeding behaviour. Regardless of the lack of appetite effects, improvements in body weight gain were obtained using this receptor decoy approach.

In addition to effects on body weight, the treated animals also had improved glucose tolerance and insulin sensitivity, which did not appear to be the result of increased insulin secretion as the levels of fasting insulin were similar between treated and control animals. This suggested that ghrelin's known effects on suppressing insulin secretion were not a factor in the improved glucose regulation we observed (reviewed in (Dezaki, Sone et al. 2008)). However, fasting insulin levels may not be the best measure, as studies in humans have indicated that ghrelin affects glucose stimulated insulin secretion rather than fasting insulin levels (Tong, Prigeon et al. 2010). Alternatively, the improved glucose regulation may be related to the reduced pro-inflammatory cytokines in the treated animals. Cytokines like IL-1, 6 and TNF α are produced in enlarged adipose tissue stores and are linked to impaired glucose metabolism (reviewed in (Maury and Brichard 2010)). The reduced mRNA expression of pro-inflammatory cytokines in the

white adipose tissue of GHSR1a/Fc treated animals may have contributed to the improved glucose metabolism. Whether the reduced cytokines were a direct result of the lower circulating ghrelin or an indirect effect of the lower adiposity remains to be determined. Little information is currently available on the role of ghrelin in the release of adipokines (cytokines, hormones etc), but studies using primary adipose tissue treated with ghrelin could potentially resolve this. Overall, the ability of the GHSR1a/Fc fusion construct to reduce ghrelin, weight, pro-inflammatory cytokine expression and improve glucose regulation under HFD provides promise for this strategy in the treatment of obesity.

While the results of this study demonstrate a proof of principle that the receptor decoy approach can reduce circulating ghrelin and have beneficial metabolic consequences, further work is now needed. The temporal nature of reduced ghrelin should be examined throughout the study to confirm that the reduced fasting ghrelin is not simply a product of increased adiposity. High fat diet was used to allow the animals to gain weight more rapidly however this experiment could also be done with normal chow to determine if appetite regulation is affected. Another key experiment is to examine whether this treatment can reduce body weight once obesity has developed.

An important question in this study is whether the use of a ghrelin receptor-based decoy protein is a feasible therapeutic approach. While progress has been made in the use of non-viral plasmid based gene therapy (reviewed in (Li and Huang 2006), further studies are required and underway to ensure their safety and efficacy in humans. The use of the GHSR1a/Fc fusion protein is however not restricted to plasmid transfection into the host. Therefore, this plasmid could conceivably be used to express the recombinant

decoy protein outside the animal (in a cell system), harvested, purified, then administered as a therapy. A similar approach is currently used in the anti-inflammatory drug etanercept (Embrel). This medication is also based on a fusion construct, where the extracellular domain of the TNF α receptor, connected to an IgG Fc fragment was constructed in a plasmid vector and expressed in Chinese hamster ovary cells (Peppel, Crawford et al. 1991). The produced protein was found to bind TNF α with high affinity and is currently being prescribed in the treatment of inflammatory diseases including rheumatoid arthritis and psoriasis (reviewed in (Thalayasingam and Isaacs 2011)). As such, we have submitted a patent application in the design and use of the ghrelin receptor based fusion protein.

7.8 Conclusion

Ghrelin stands out as an important component in the regulation of whole body metabolism. It achieves this function through its direct action on several systems both centrally and in the viscera. While much work has been done examining the many functions and mechanism of ghrelin action, less has been done regarding how ghrelin secretion is regulated at the cellular level. To this end, I developed a rat primary cell culture system that enabled an *in vitro* examination into the mechanism of ghrelin secretion. Many of the factors that were found to regulate ghrelin in this *in vitro* setting were also in agreement with the associations observed in human and animal models *in vivo*. My work extended these associations and indirect relationships into a direct and mechanistic understanding of how these factors regulate ghrelin secretion. Furthermore, the detailed report on cell preparation provided in Chapter 2 (Gagnon and Anini 2012) will enable our lab, and other groups, to test the how other hormones, nutrients and medications effect ghrelin secretion. In addition to these studies, we have also developed a novel strategy to reduce circulating ghrelin *in vivo*. This approach may not only be of use in future ghrelin based therapies, but also provides another tool for studying the effects of depleting ghrelin in adult animals. In conclusion, my work on elucidating the mechanisms of ghrelin secretion, and developing a novel method to regulate peripheral ghrelin levels, has demonstrated the importance of ghrelin in the regulation of whole body energy homeostasis.

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