IMPAIRED GHRELIN SECRETION IN OBESITY: THE KEY ROLE OF INSULIN RESISTANCE

by

Bader N. Alamri

Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
August 2017

DEDICATION

"He has not thanked Allah who has not thanked people."
Prophet Muhammed (PBUH)

To my parents, Fatima & Nasser, who think I'm getting a Nobel prize: I have not got it yet!
To my angel, my beloved wife, Arwa, who believes I'm genius: Thank you Google!
To my wise mentor, Dr. K. Al-Rubeaan, who keeps telling me I'm unguided missile: I've been guided.
Last but not least, to my great supervisor, Dr. Y. Anini, who had a hard time teaching me: not all physicians have I.Q. below average!

Bader

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ABSTRACT

Background: The stomach derived hormone, ghrelin, has emerged as a key player in the neuro-endocrine regulation of appetite and energy storage. Ghrelin increases food intake and adiposity through its action on the ghrelin receptor in appetite-regulating neurons of the hypothalamus. Ghrelin levels increase during periods of fasting and decrease after a meal is consumed. Although obesity is commonly associated with increases in food intake, basal concentration of ghrelin was found to be decreased in obese individuals. These findings suggest that obesity is linked with a dysregulation in ghrelin secretion.

Objective: The goal of the present study was to investigate the putative mechanisms underlying dysregulation of ghrelin secretion in obesity using the diet-induced obesity (DIO) mouse model.

Methods: We used the mouse model of high fat-diet induced obesity to investigate the glucose-suppressing effect of ghrelin secretion in normal weight and obese mice. We further delineated the intracellular pathways involved using primary culture of ghrelin cells. Since increased adipose tissue-derived inflammatory cytokines was linked with insulin resistance, we investigated the role of TNF-α in ghrelin secretion.

Results: Oral glucose significantly suppressed ghrelin secretion in normal weight mice by 60% at 30 min and 70% at 60 min ($p<0.001$, $p<0.001$ respectively, $n=6$), but failed to do so in obese animals. In primary cultures of gastric cells from lean mice, 10 nM insulin reduced ghrelin secretion by 40% ($P< 0.05$, $n=6$). In contrast, the effect of insulin was lost in gastric mucosal cells derived from DIO mice ($n =6$). The effect of insulin was found to be linked with decreased activation of Akt phosphorylation. Pre-treatment of ghrelin cells with TNF-α (10ng/mL) abolished the inhibitory effect of insulin (10 nM) on ghrelin secretion.

Conclusion: We demonstrate that impaired ghrelin suppression found in obesity is due to insulin resistance occurring at the level of ghrelin cells and involves adipose tissue-derived inflammatory cytokines.

LIST OF ABBREVIATIONS USED

AG	acyl ghrelin
AgRP	agouti-related protein
AKT	serine-threonine kinase
AN	anorexia nervosa
ARC	arcuate nucleus
BMI	body mass index
CAD	coronary artery disease
CART	cocaine and amphetamine regulated transcript
CCK	cholecystokinin
COPD	chronic obstructive pulmonary disease
CSF	cerebrospinal fluid
DIO	diet-induced obesity
DMH	dorsomedial hypothalamus
FSH	follicle-stimulating hormone,
GHRH	growth hormone releasing hormone
GHS-R	growth-hormone secretagogue receptor
GI	gastro-intestinal
GIT	gastrointestinal tract
GLP	glucagon-like peptide
GluR	glucagon receptors
GnRH	gonadotropin-releasing hormone
GOAT	ghrelin O-acyl transferase,
GSIS	glucose-stimulated insulin secretion
GTT	glucose tolerance test
HFD	high fat diet
i.c.v.	intracerbralventricular,
IR	insulin resistance
ISHH	in situ hybridization histochemistry
Kv	voltage-dependant K+
LFD	low fat diet
LH	luteinizing hormone
LHA	lateral hypothalamic area
MI	myocardial infarction
MS	metabolic syndrome
NE	Norepinephrine
NPY	neuropeptide-Y
OGTT	oral glucose tolerance test
PC	prohormone convertase
PG-1	pancreatic ghrelinoma
PKA	protein kinase A
PP	pancreatic polypeptide
PTX	Pertussis toxin

PVN	paraventricular nucleus
PYY	peptide tyrosine tyrosine
qPCR	quantitative PCR
s.c.	subcutaneously
SG-1	stomach ghrelinoma
SST	somatostatin
SST5	somatostatin receptor subtype-5
T2DM	type 2 diabetes
TEA	tetraethylammonium
TNF-α	Tumor necrosis factor-α
TSH	thyroid stimulating hormone
UAG	unacylated ghrelin
WHO	World Health Organization
WT	wild type
ZT	zeitgeber time
α-MSH	α-melanocyte stimulating hormone

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my sponsor, King Fahad Specialist Hospital-Dammam, Saudi Arabia, for their generous financial aid, and to the members of my supervisory committee, Dr. R. McLeod and Dr. R. Bouzayen. I would also like to thank my lab colleagues, Kyung Shin, Michael Landsman, William Gendron, and Melanie Simourd for their support, and Fatima Imran who helped review this manuscript.

I would like to express my gratitude to my internal medicine program director, Dr. I. Epstein, and the Chief Residents at the Department of Medicine for 2015, 2016, and 2017 (Marko, Alison, Kate, Keigan, Harrison, and Rachelle) for their immense help in accommodating my call shifts during my busy time in the laboratory.

Last but not least, I am indebted to the Clinician-Investigator Program (CIP) director, Dr. J. Berman, and the program manager, J. Kinney, for keeping me on track and making CIP such a wonderful experience.

CHAPTER 1 INTRODUCTION

1.1 OBESITY

1.1.1 EPIDEMIOLOGY AND MECHANISMS

Obesity is an endemic syndrome in most developed countries affecting up to 25% of adults in Canada, 36% of adults in the United States and more than 500 million people world-wide [2-4]. The WHO defines obesity as "abnormal or excessive fat accumulation that may impair health" [4] which can be measured on a population level using BMI. Individuals with a body mass index (BMI; weight in kilograms divided by height in meters squared) greater than or equal to 25 are classified as overweight and those with a BMI of 30 or above are classified as obese [5]. In the pediatric population BMI specific to age and gender with the cut-off point for classifying a weight status as obese set equal or greater to the 95th percentile [6, 7]. Although BMI is a convenient and cost effective mean to assess adiposity, but it lacks accuracy as it depends solely on weight to estimate fat percentage. This limitation is evident in muscular individuals with high BMI due to muscle mass rather than fat [8].

Childhood obesity is also increasing in both the developed world and in low-middle income countries. Nearly 18 million children under the age of 5 in Asia and 11 million children in Africa are overweight [4]. This global increase in the prevalence of obesity is shadowed by a corresponding rise in diabetes [4]. The WHO estimates that diabetes and other diseases associated with obesity such as cardiovascular disease and cancer are responsible for at least 2.8 million deaths every year [5].

Unsurprisingly given its prevalence and complexity, the economic burden of obesity is substantial. Hammons *et al.* reported excess medical spending related to obesity

to be $640 million within the United States alone. In addition to these direct costs, indirect costs attributed to obesity-related absenteeism totalled to almost six billion dollars [9].

Several treatment options and management strategies have been described to address obesity. These range from relatively conservative options, including counseling on life style changes and pharmacotherapy, to more invasive techniques used in select situations such as bariatric surgery [10]. Given this variety, it is important for health care professionals and researchers to understand the processes contributing to increased body weight and its association to metabolic disease so that treatment strategies can be tailored to patients effectively.

There is a common misconception that increased food intake is the only mechanism involved in developing obesity, however, research has demonstrated that the condition is the result of a complex and interconnected process. Factors that have been identified in contributing to obesity include environmental factors, including socioeconomic status, genetic factors as well as dysregulation of appetite and energy expenditure [10].

Several population level factors have also been determined as contributory to a global rise in obesity. These include an upsurge in the consumption of sugar-sweetened food, larger portion sizes, increased utilization of high calorie foodstuff, reduced work-related and leisure-time physical activities as well as increased prescribing of medications that cause weight gain as a side effect [10-13].

Several twin studies have confirmed the importance of genetics in the development of obesity. Estimates of BMI heritability vary up to 70%, suggesting about half of interindividual variations in BMI is related to genes, while the other half is due to environmental effects. Interestingly a demonstrable link has also been shown between

genetics and weight loss [14]. Hatoum *et al.* genotyped patients undergoing Roux-en-Y gastric bypass (RYGB) surgery to determine if gene makeup plays a role in weight loss [15]. They found a similar response to surgery amongst first-degree relative pairs but not in cohabitating or unrelated individuals, suggesting the role of a genetic factor [15].

Environmental factors can affect the function of genes without altering their sequence through epigenetic changes. There is a growing evidence that certain in-utero or early infancy exposures are associated with higher risk of developing obesity and metabolic diseases without DNA sequence alterations [16]. For example, after the Dutch Hunger Winter in 1944-1945, it was observed that children of women who were exposed to malnutrition during pre-natal development were more likely to be obese as adults compared to their unexposed siblings. Upon analysis, it was also observed that DNA coding regions involved with growth and metabolism in exposed individuals were methylated, confirming the role of epigenetic [16, 17].

The human body tightly controls weight by balancing between food intake and energy expenditure. In the arcuate nucleus of the hypothalamus, two groups of neurons interact with several gut hormones and neurotransmitters to achieve this stability [10]. The current consensus is that whole body energy status is conveyed from peripheral metabolic organs (including the GI, pancreas, and adipose tissue) to energy regulating regions in the hypothalamus [18].

Until recently, the mechanisms regulating energy balance were largely unknown. However, with the discovery of anorexic hormone leptin [19] in the mid 1990's, a major interest on the molecular mechanisms regulating energy balance has developed (reviewed in [18]). The consensus is that whole body energy status is conveyed from peripheral

metabolic organs (including the GI, pancreas, and adipose tissue) to energy regulating regions in the hypothalamus [18]. The importance of energy sensing in the periphery and the effects on body weight are seen in the development of severe obesity in the absence of the anorexic hormone leptin [20] and in Prader-Willi Syndrome due to elevated levels of the orexigenic hormone ghrelin [21]. Furthermore, dysregulation in either the production or the targeted signalling of energy regulating factors during obesity disrupts the normal balance of appetite regulation and metabolism [22]. To fully understand the importance of peripheral and central energy regulation, a detailed description is required. (Reviewed in ENERGY REGULATION).

1.1.2 ADIPOSITY AND ADIPOKINES

The long-established model of adipose tissue as simply energy storage was adapted in 1987 by the discovery of its endocrine function [23]. In the same year, Flier *et al.* reported decreased adipsin mRNA, a serine protease homolog, within fat cells of obese mice given glucose infusions compared to normal-weight mice [24] suggesting a role within the development of metabolic disorders.. The discovery of the obesity (*ob)* gene in mice and its product, leptin, in 1994 confirmed the role of adipose tissue as an endocrine organ [19]. Current understanding of the function of adipose tissue has expanded to include involvement in regulating energy homeostasis, appetite, reproduction and much more.

1.1.2.1 Obesity-induced inflammation

Adipose tissue is an active metabolic organ composed of several types of cells such as connective tissue matrix, nerve tissue, and immune cells alongside adipocytes (fat cells). [25, 26]. Throughout life, preadipocytes in the stromal-vascular fraction differentiate to adipocytes depending upon nutritional status. Adipose tissue is described based on either

its location, subcutaneous or visceral, or its function, white or brown for energy storage and thermogenesis, respectively [27]. Metabolic properties vary between these categories with visceral adipocytes found to have the highest metabolic activity whereas lower body adipocytes were observed to have the lowest [26].

Adipose tissue has also been shown to contribute towards obesity-related complications and associated inflammation through both metabolic and autocrine processes [28, 29]. In one study that looked at more than one hundred non-diabetic subjects, C-reactive protein concentration (an inflammatory marker) was significantly associated with circulating pro-inflammatory cytokines, particularly interlukin-6 and TNF-α. Interestingly obese subjects had higher levels of C-reactive protein and pro-inflammatory cytokines and tended to have more obesity-related complications such as hypertension and hypertriglyceridemia [30]. In another clinical study focused on obese women, a weight reduction as low as 10%, which targets mostly fat, was associated with a significant decrease in pro-inflammatory cytokines, reinforcing the interactivity between adipose tissue, pro-cytokines and obesity-induced inflammation [31].

The focus in the next section is the role of adipokines, the bioactive peptides secreted by adipose tissue in diabetes and insulin resistance. Adipokines include cytokines (e.g. IL-6 and IL-8), adiponectin, leptin, TNF- α, resistin and many others [25, 32].

1.1.2.2 Tumor necrosis factor-α (TNF-α)

In 1975, TNF- α was first labeled as an endotoxin-induced serum factor causing tumors necrosis [33]. Today, TNF- α is known for its multifaceted role in inflammation, immunity, apoptosis and insulin resistance [34].

There are two types of TNF- α receptors, type I and type II, which mediate signaling by interacting with intracellular adaptor proteins [34]. Most of the biological actions of TNF-α are mediated by TNF- α R1 with downstream activation of JNK and nuclear factor κB (NK- κB) (reviewed in [35]). Adipocytes express both receptor types [34] and are the main source of TNF-α, with greater gene expression in subcutaneous fat relative to visceral fat tissue [25, 32].

In both obese animals and humans, TNF- α mRNA and protein are highly expressed within adipose tissue compared to lean subjects [36, 37]. In explanted human adipose tissue a BMI of 45 was associated with higher levels of TNF-α compared to a BMI of 32 [25, 32].

The association between TNF-α, hyperinsulinemia and insulin resistance has also been a focus for intensive research [36, 37]. Chronic incubation of adipocyte cell lines with TNF-α has been shown to decrease insulin-stimulated uptake of glucose in a dose-dependent manner [38]. These results have been replicated *in vivo* with infusion of TNF-α in rats [39]. Interestingly, neutralization of TNF-α or its removal from culture media reverses insulin resistance and restores glucose hemostasis, indicating a transient effect [38, 39]. TNF-α-deficient and TNF-α receptor-knockout obese mice have also exhibited improved insulin sensitivity and enhanced insulin receptor signaling in comparison to control obese subjects [40]. These findings have also been demonstrated in clinical settings where the administration of TNF-α antibodies over 4 weeks in obese patients with type 2 diabetes mellitus showed no improvement in either insulin sensitivity or glucose clearance [41] whereas there was a significant reduction in serum insulin level and an associated decrease in TNF-α mRNA expression in obese subjects who lost 17% percent of their body weight, which indicated resolving of hyperinsulinemia caused by insulin-resistance [37].

In addition to its role in insulin resistance TNF-α has several different metabolic effects. In rats, TNF-α infusion for one day increased free fatty acids (FFAs) by 70%, and infusion for 4 days showed a 150% increase, an effect that is theorized to contribute towards TNF-α-induced insulin resistance. In the same study, TNF-α infusion was shown to decrease mRNA expression and down regulate several proteins involving FFAs as well as glucose uptake in a time dependent manner. Interestingly, TNF-α infusion also induced select cytokines and chemokine genes in adipose tissue that are involved in immune and inflammatory responses [42]. These findings confirmed the role of TNF-α in inducing insulin resistance both *in vitro* and *in vivo* (Figure 1).

1.1.2.3 Leptin

One of the peptides secreted by adipose tissue that regulates appetite and energy homeostasis is leptin. Leptin release is positively correlated with overall adiposity and nutritional abundance, where leptin secretion from visceral fat is higher than from subcutaneous tissue [25, 32]. Although most circulating leptin is secreted from white adipose tissue, some studies have reported leptin mRNA expression in brown adipose tissue as well [43, 44]. Leptin is encoded on *ob* gene and either its deficiency or resistance results in obesity from both significant increases in food intake as well as reductions in energy expenditure [19, 45].

Several proposed functions of leptin have been reported in addition to its known effects on appetite and metabolism (reviewed in [46]). In this section, we will mention some relevant physiological functions.

Hyperinsulinemia was reported in both *ob/ob* and *db/db* mice with leptin deficiency and resistance, respectively, suggesting that leptin may play a role in insulin secretion

suppression [47]. Kieffer *et al.* reported leptin receptor expression in both mice primary β -cells culture and insulinoma cell lines [48]. In both humans and rodents collective evidence confirmed the suppressive effect of leptin on glucose-induced insulin secretion [49-51]. In contrast, leptin levels were elevated in healthy subjects during euglycemic-hyperinsulinemic clamp, suggesting insulin has a stimulatory action on leptin secretion [52]. However, despite the promising therapeutic effect of leptin in diabetes and obesity its clinical use still limited [53, 54].

In *ob/ob* leptin deficient mice, markers of inflammation and acute phase reactants, such as serum amyloid A (SAA), are elevated. In addition to weight and food intake reduction after 18 days of leptin replacement, SAA and other inflammatory markers also reduced significantly, suggesting a pro-inflammatory role [55].

Leptin has many interactions with other metabolites and hormones including other adipokines. In a human subject study, TNF- α and leptin were measured in BMI-matched diabetic and non-diabetic subjects. TNF- α levels were positively and independently associated with leptin levels. Similar results have been shown in rodents, suggesting that TNF- α may also play a role in increasing leptin [56, 57]

Adiponectin, as will be discussed in the subsequent section, is decreased in obesity. In *ob/ob* mice with an obese phenotype, adiponectin was reduced alongside elevations in insulin, glucose and triglyceride levels. Furthermore, after leptin administration, adiponectin levels normalized and expression in white adipose tissue increased [55], suggesting that leptin may play a role in adiponectin secretion.

Figure 1: Role of TNF- α signaling in insulin resistance

TNF- α phosphorylates serine residues of IRS-1 through JNK pathway activation. Another mechanism is through reduction of insulin-induced tyrosine phosphorylation in IRS-1. This will inhibit the downstream signaling of insulin.

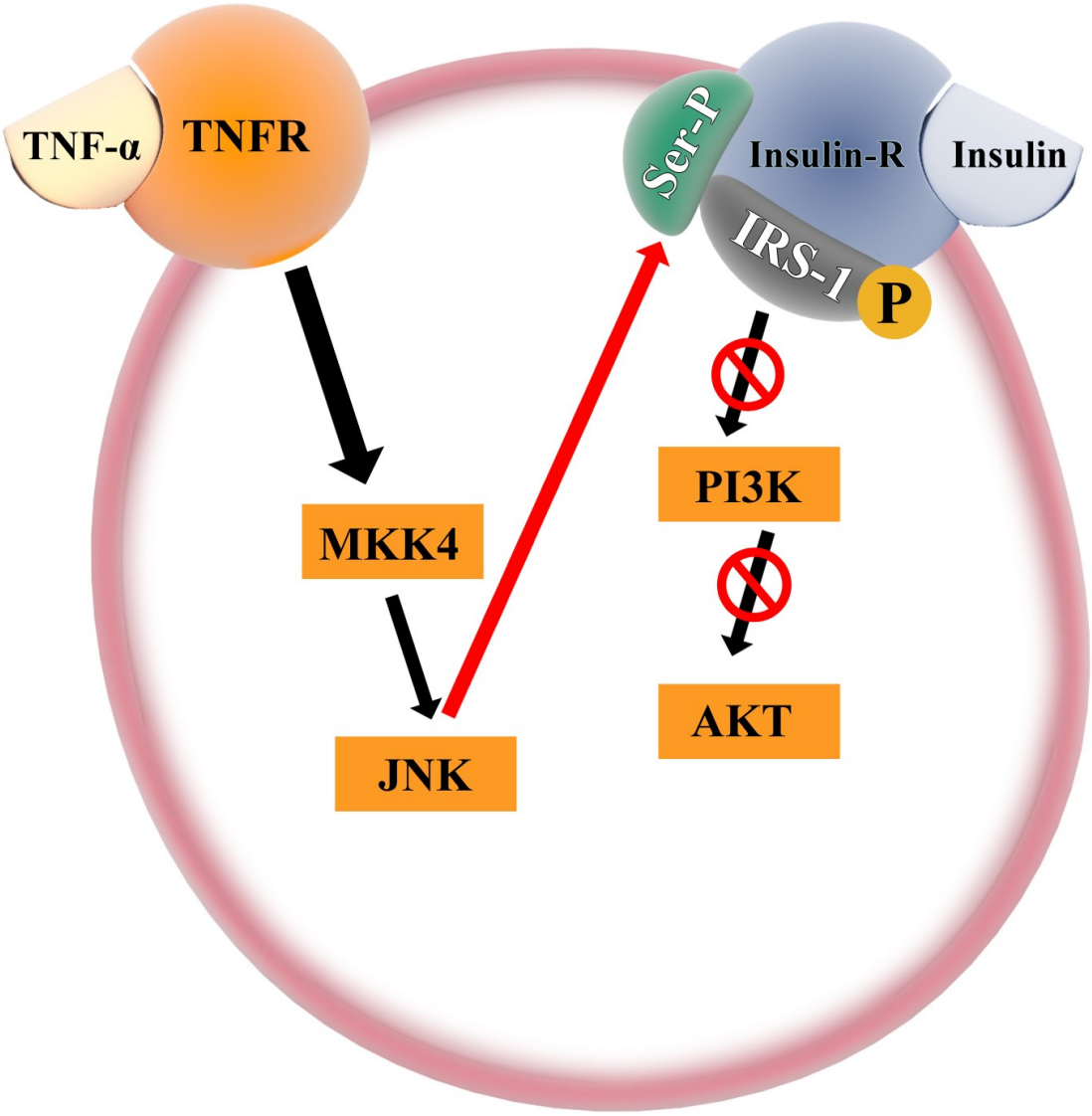

Figure 1 Role of TNF-alpha signaling in insulin resistance

1.1.2.4 Adiponectin

Adiponectin and TNF-α are similar in structure which suggests an evolutionary link [58]. Most adipokine levels increase with high body fat, however adiponectin uniquely is inversely related to body mass index (BMI) [59]. In BMI-matched diabetic and non-diabetic subjects, adiponectin was lower among diabetics compared to control. Additionally, levels were also lower in diabetics with coronary artery disease (CAD) in comparison to the diabetic subjects without complications, suggesting a role for adiponectin in the prevention of vascular disease in patients with diabetes [60].

Numerous studies have investigated the relation between adiponectin and insulin sensitivity. In wild-type mice and type 2 diabetes mice models, intraperitoneal injection of adiponectin resulted in a significant decrease in glycaemia that was not associated with a rise in serum insulin level but rather with a reduction in hepatic glucose output [61]. These results were replicated in type 1 diabetes mice models suggesting that adiponectin can affect glucose hemostasis in insulin-resistant and insulin-deficient models.

Adiponectin levels inversely correlate with insulin resistance progression and the development of type 2 diabetes in both humans and animals [58, 60, 62]. In obese euglycemic rhesus monkeys, circulating adiponectin levels in the presence of hyperinsulinemia were lower compared to lean monkey, whereas leptin levels were relatively elevated. After obese monkeys developed type 2 diabetes, leptin levels equated to levels seen in lean monkeys. However, adiponectin remained lower than control, suggesting that adiponectin may have a role in developing insulin resistance [62]. In contrast however, acute administration of insulin in lean and obese human subjects has also been shown to result in significantly decreased adiponectin levels, indicating insulin may

play a role in adiponectin suppression [63]. One proposed explanation for this seeming contradiction is that adiponectin expression may differ based on adipose tissue site. Expression is lower in omental adipose tissue relative to subcutaneous fat, which could explain the correlation between increased visceral fat and insulin resistance [58, 64].

1.2 ENERGY REGULATION

1.2.1 Central Energy Regulation

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

1.2.1.1 Hypothalamus

The hypothalamus is a relatively small structure comparable to roughly the size of an almond that secretes a large amount and variety of peptide hormones as well as neurotransmitters that are crucial to normal function. The axis between the hypothalamus and the anterior pituitary gland has been thoroughly described with tropic hormones such as thyroid stimulating hormone (TSH), growth hormone releasing hormone (GHRH) and gonadotropin releasing hormone (GnRH). Direct evidence for an energy regulatory component within the hypothalamus came over 80 years ago when Hetherington observed in rats that causing lesions in the hypothalamus led to developing obesity [65]. Later research showed that the hypothalamus contains several distinct regions (nuclei) that are involved in energy balance. Due to its proximity to the median eminence (a region devoid of the blood brain barrier) the hypothalamus is considered a circumventricular organ and is able

to receive input from circulating hormones and nutrients (reviewed [66]). The regions, the signaling molecules produced and their function in regulating energy homeostasis will be discussed.

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 [67] and reduce energy expenditure[68] . The other group produces α-melanocyte stimulating hormone (α-MSH) derived from POMC as well as cocaine and amphetamine regulated transcript (CART), which are known to inhibit appetite [69, 70] and stimulate energy expenditure [71]. These opposing roles serve to maintain energy levels in situations where there is both low and high energy availability. Once activated, projections from neurons within the ARC can activate other hypothalamic nuclei including the paraventricular nucleus (PVN), the lateral hypothalamic area (LHA) and the dorsomedial hypothalamus (DMH) (reviewed in [72]). Within these nuclei further projection of orexin-A neurons may then exert their appetite regulating effects throughout the brain [73].

1.2.1.2 Brainstem

The brainstem (in particular, the nucleus of the solitary tract) has extensive connections with both the hypothalamus and other brain regions [74]. In addition, the brainstem receives afferent input from the vagus nerve which has extensive connections to metabolic organs and the gastro-intestinal (GI) tract (reviewed [75]). Vagal afferents send input from chemoreceptors in taste buds and along the GI tract

[76], from mechanoreceptors along the GI tract [77] and from metabolic hormones through receptors contained within the vagal afferents [78, 79].

1.2.1.3 Reward Based Eating

While the reward associated with eating is known to increase with an energy deficit [80], it is clear from the obesity epidemic that reward based eating still occurs in the absence of energy need. Indeed, mice given free access to high fat/high sugar diet (which then had the expected changes in the homeostatic neuropeptides within the hypothalamus) were still hyperphagic [81]. The pathways associated with food rewards are now beginning to be understood. Many of the neurotransmitters that are associated with pleasure and addiction are implicated with food reward including increased dopamine levels [82] and opioids [83]. Antagonists of opioids have also been shown to reduce palatable food choices in humans, confirming its important role in the food reward pathway[84].

1.2.2 Appetite Regulation

The increased prevalence of obesity is a population-based problem; however, it must also be looked at as an individual energy balance subject. Energy hemostasis is defined as the balance between energy intake and expenditure. Even a positive balance as little as 0.5% can lead to increase obesity overtime [85]. The control of appetite and body weight is therefore tightly regulated by the hypothalamus and its peripheral inputs from different GI hormones (Figure 2) [86, 87].

GI hormones provide signals about body energy status to the hypothalamus to inhibit or stimulate food intake to regulate body weight. The hedonic appetite pathway is an exception as it is related to the reward system of the ingested food regardless of energy

homeostasis [88]. Many appetite-regulation hormones have receptors in hypothalamic nuclei with demonstrated cross interaction [87].

The pancreatic polypeptide-fold peptides, including peptide tyrosine tyrosine (PYY) and pancreatic polypeptide (PP), are involved in gut-hypothalamus appetite regulation. PYY is secreted by the L-cells in the GI tract, where it is widely distributed. The PPY immunoreactivity levels are low in the proximal small intestine and gradually increase to reach high levels in the rectum [89]. In response to food intake, the basal levels of serum PPY increase which suggests a role in appetite suppression [90]. This is confirmed by the observation that injecting PPY peripherally in mice reduces food intake and inhibits weight gain. It is thought that PPY acts centrally through the activation of its receptors (Y2R) in the arcuate nucleus of the hypothalamus, as demonstrated by Y2R-knockout mice administered PPY failing to inhibit food intake [91] In addition to appetite suppression PPY is linked to several other effects such as an anorexic effect, enhanced glucose-induced insulin release and lipolysis [87, 92].

Figure 2: Appetite regulation by hypothalamus and different hormonal inputs.

The only known peripheral hormone to increase appetite is ghrelin.

PP: pancreatic polypeptide, GLP-1: glucagon-like peptide 1, CCK cholecystokinin, PPY: peptide tyrosine tyrosine.

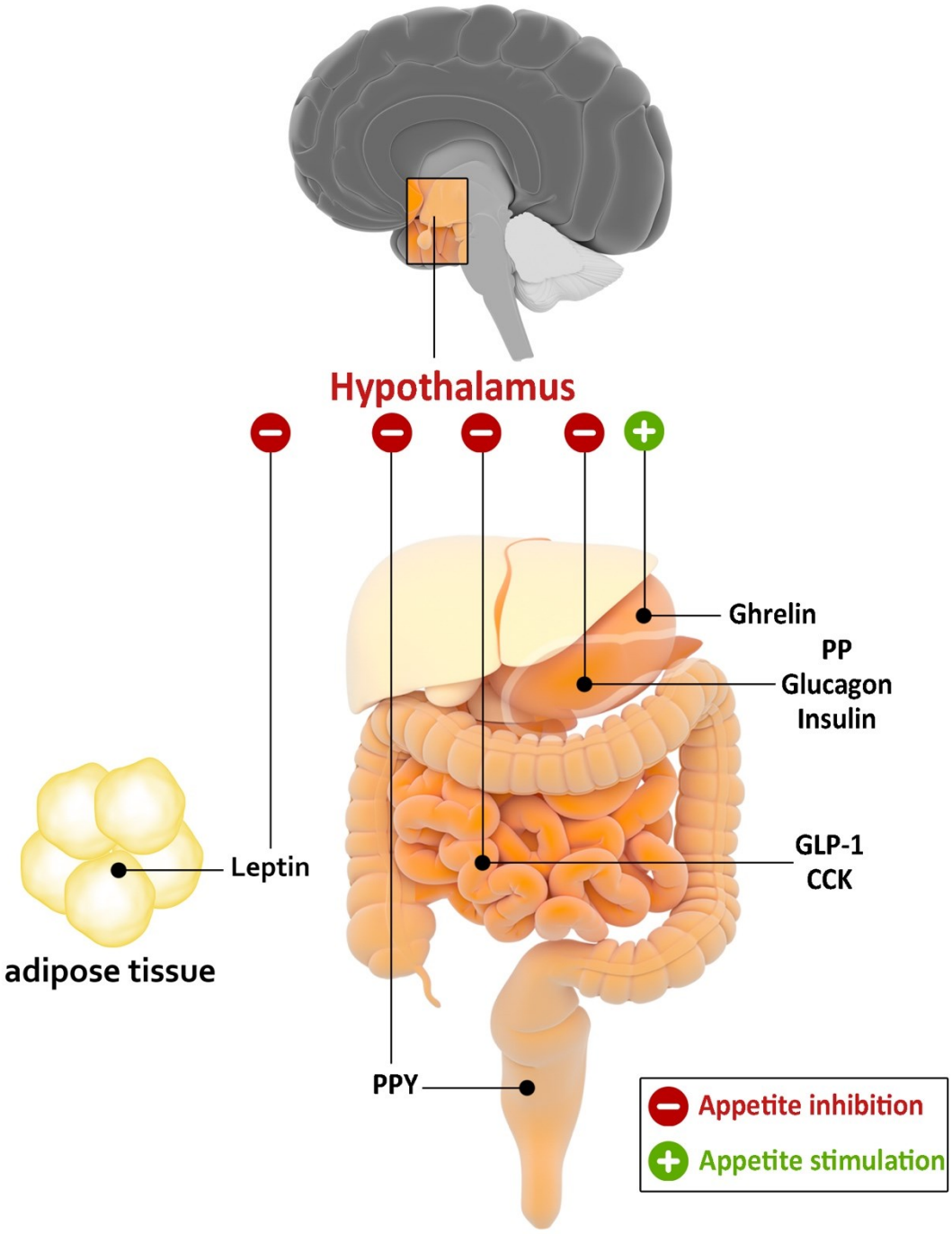

Figure 2 Appetite regulation by hypothalamus and different hormonal inputs

Another hormone from the same family is PP which in addition to its anorexic effects also decreases gastric emptying rate in a dose-dependent manner, decreases ghrelin mRNA expression in stomach and increases both gallbladder contraction as well as pancreatic exocrine secretion. The anorexic effects are mediated by the activation of Y2 receptors in hypothalamus [87, 93, 94].

Proglucagon-derived peptides are another peptide family that is involved in appetite regulation. They include glucagon, glucagon-like peptide 1 and 2 (GLP-1 and GLP-2) and oxyntomodulin [95]. In response to hypoglycemia glucagon is secreted from pancreatic α cells and opposes the action of insulin [95]. It has been shown in both human and animal studies that glucagon infusions decrease food intake [96, 97]. By using c-fos immunohistochemistry, Parker *et al.* showed increased neuronal activity in mice hypothalami and brainstems after peripheral administration of glucagon [97]. Co-administration of glucagon and GLP-1 had anti-obesity and anti-diabetic effects in diet-induced obese mice [98], implying a therapeutic use when the hormones were combined.

In response to food intake, GLP-1 is secreted from L cells in intestines alongside PYY. GLP-1 receptors (GLP-1R) are widely distributed and are found in the pancreas, hypothalamus, and the heart [87, 99, 100]. GLP-1 plasma levels have also been shown to increase and exhibit anorexic effects after food intake by inhibiting glucagon secretion and improving glucose-induced insulin release. In clinical settings, GLP-1 agonists (*i.e.*: exenatide) are associated with decreased body weight in diabetic subjects and offer an advantage over many anti-diabetic agents which commonly cause weight gain as a side effect.[101].

Another gut hormone that is involved in appetite regulation is cholecystokinin (CCK) which was named after its function as a regulator for gallbladder contraction. CCK was the first gut hormone found to be involved in appetite regulation after Gibbs *et al.* investigated its role as an appetite suppressant in mice in 1973 [102]. CCK is secreted after food intake from the small intestine I cells [103] and the CCK 1 and CCK 2 receptors, formerly known as CCK A and B receptors, are distributed throughout the GIT and the nervous system, respectively [87, 104].

Since the discovery of the metabolic and hormonal functions of adipose tissue, there has been a particular research focus on its role with energy regulation, and particularly with regards to the hormone leptin [25]. With the discovery that leptin has demonstrable receptors within the hypothalamus, it has emerged within research as an important peripheral hormone in energy homoeostasis [105]. In diabetic and obese *db/db* mice, a mutation in leptin receptors within the hypothalamus was identified [45] and in leptin-deficient obese mice, administration of leptin twice daily resulted in decreased food intake, fat mass as well as decreased plasma glucose levels [106]. In a human subject study, Farooqi *et al.* investigated the effect of a daily subcutaneous injection of leptin on 3 obese leptin-deficient children. All subjects subsequently lost significant fat mass and reduced lipids and insulin levels [107]. Both mice and human subject data clearly demonstrated the importance of leptin in glucose and body weight control. However, obesity in humans is usually associated with high levels of leptin, which may result in leptin resistance by impaired signalling transduction and thus limit its therapeutic use [87, 108].

One of the main hormones in glucose and energy control is insulin. Insulin is secreted from pancreatic β cells in response to meal intake, mainly carbohydrates [109].

Although the hypoglycemic effect is the main function for insulin, its receptors are widely distributed in the hypothalamus which indicates an additional central role [110]. In rats, an intracerebroventricular (i.c.v.) administration of insulin significantly decreased food intake compared to saline infusion, confirming its anorexic effect [111].

Glucose itself is an important appetite-modulator independently from its hormone-stimulating effects. In the hypothalamus and brainstem, glucose-excited and glucose-inhibited neurons fire at varying rates based on glucose levels. These neurons interact with each other and other neuronal pathways to help regulate appetite [112] .

1.3 GHRELIN

1.3.1 Structure and Localization

Ghrelin is a 28 amino-acid polypeptide discovered in 1999 by Kojima [113] and is a growth-hormone secretagogue receptor (GHS-R) ligand [114] with an important role in energy regulation. Acting centrally on the pituitary and hypothalamus ghrelin increases growth hormone secretion and hunger, respectively [113].

Rat and human ghrelin are very similar in molecular structure with a difference of only two amino acids [113, 115]. Ghrelin is mainly secreted by endocrine X/A like cells (ghrelin cells) in the stomach fundus, however many other organs also express it as well. These include the pancreas, duodenum, colon, kidney, ovary and liver [113, 115-117], and ghrelin receptors (GHS-R) are widely distributed as well [116].

Sakata *et al.* studied ghrelin-producing cells in adult rat gastrointestinal tracts (GIT) using immunohistochemistry and in situ hybridization. In keeping with previous findings ghrelin cells were highly dense in stomach compared to the rest of the GIT. These cells were localized in mucosal layer but not in the myenteric plexus [117]. In stomach, mucosal

layer ghrelin cells were highest in the glandular base and decreased towards the glandular neck. In the rest of the GIT, however, they were spread within crypts and the villi epithelia [117]. Surprisingly, there was no variance in immunoreactivity amongst ghrelin cells across the GIT even though they were phenotypically different. In the stomach, ghrelin cells were smaller and rounded (closed-type), but in the duodenum, ilium, cecum, and colon ghrelin cells exhibited mixed morphology including closed-type, elongated, and cells in contact with lamina (open-type) [117]. No immunoreactivity differences were observed between the different types of ghrelin cells were found. The presence of both open and closed type ghrelin cells in the GIT may suggest different physiological stimulations either directly with nutrients in the lumen or through hormones and nutrients in blood circulation. Two subsequent studies confirmed the increased density of ghrelin cells in the mucosal layer of animal stomachs [118, 119]. In a human study by Maksud *et al.*, morbidly obese patients without type 2 diabetes (T2DM) or metabolic syndrome (MS) had higher ghrelin-immunoreactivity cell density in oxyntic mucosa compared with patients who were overweight, lean patients, and morbidly obese with T2DM or MS, suggesting a compensation mechanism to overcome a resistance [120].

Preproghrelin, a 117 amino-acid polypeptide that is encoded on the ghrelin gene, is cleaved by prohormone convertase (PC) 1/3 to produce ghrelin and obestatin (Figure 3) [121]. PC1/3 is colocalized with ghrelin in ghrelin-producing cells in mice stomach and it is the only enzyme from the PC family able to cleave proghrelin *in vivo* [121]. PC1/3 is a member of the endoproteolytic enzymes family (proprotein convertase subtilisin/kexin-PCSK), alongside with PC2 and PC5, which are found in endocrine tissues and related to energy and nutrient homeostasis [122]. In order for PC1/3 to become biologically active, it

must undergo a maturation process where it is converted from proPC1/3 to active PC1/3 in endoplasmic reticulum. Interestingly, decreased PC1/3 activity is associated with early-onset obesity in human (reviewed in details in [122]).

By adding octanoic group on the 3rd serine residue ghrelin becomes acylated (acyl ghrelin (AG)), it is the biological active form [113]. This modification is produced by the ghrelin O-acyl transferase (GOAT), a membrane-bound O-acyl transferase [123, 124]. Similar octanoylation is conserved across many vertebrate species [125] and the acylation is hypothesized to occur prior to the cleavage of proghrelin to ghrelin [121]. The highest level of GOAT in human is found in the stomach and pancreas [124]. Alongside the acylated form which activates the GHS-R, an unacylated ghrelin (UAG) is also secreted and it is the most abundant form in the blood circulation. It is unclear why UAG is the most abundant form, but it may have other undiscovered actions through GHS-R independent pathway [113, 127-129]. The de-acylation of ghrelin can be due to many factors, including: unstable acyl moiety in plasma, the exposure to platelet activating factor (PAF) acetylehydrolase, carboxypeptidase, cholinesterase, and others (reviewed in [130]).

Initially, taught to be inactive, UAG has now shown to have many other roles independent of GHSR activation [131]. In addition to those 2 forms of ghrelin, obestatin (a product of proghrelin cleavage) was shown to have opposing functions to ghrelin where it acts as anorexic hormone by decreasing food intake and body weight [132]. However, these proposed effects are controversial, as many studies were unable to reproduce these results [133, 134].

Figure 3. Proghrelin structure and derived peptides

A 117-polypeptide precursor is produced as preproghrelin. An octanoic acid moiety is added - either during or post-translationally- to the 3rd serine of the ghrelin peptide. The precursor is processed by proprotein convertase subtilisin/kexin type 1 (PCSK1) to produce the 28-amino acid peptide, ghrelin. The active form of ghrelin can become unacylated. Obestatin is also produced from the proghrelin precursor.

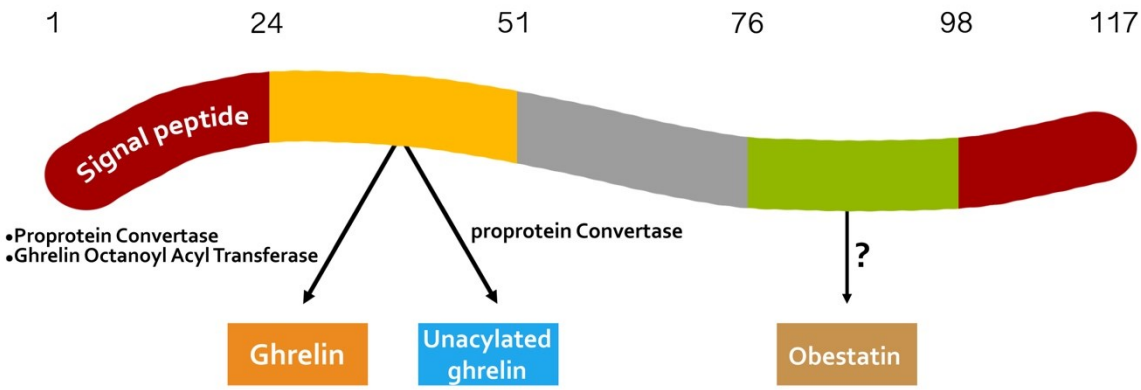

Figure 3 Proghrelin structure and derived peptides

1.3.2 Ghrelin Receptor and Signaling

Ghrelin receptor, GHS-R, is expressed in almost all body tissues, including the central nervous system, liver, spleen, placenta, muscles, pancreas, and the GIT, particularly the stomach fundus which is the main site of ghrelin secretion [116]. There are several physiological functions of GHS-R which include the release of GH and other hormones,, stimulation of food intake, glucose metabolism and cardiovascular protection as well as many others [135].

Both ghrelin mRNA and GHS-R are expressed in pancreatic β cells and inhibit glucose insulin-release. In hypothalamus and pituitary, ghrelin increases appetite and GH secretion, respectively (reviewed briefly in Role of ghrelin in glucose hemostasis , Role of Ghrelin in GH secretion, and Role of ghrelin in appetite regulation) [126, 136].

Two types of GHS-R have been identified so far, GHS-R1a and GHS-R1b [114]. Most of the physiological actions of ghrelin are mediated through GHS-R1a (especially in the pancreas and central nervous system), while type 1b is considered a non-functional receptor [116].

In the arcuate nucleus of the hypothalamus ghrelin mediates its orexigenic effect through GHS-R1 by stimulating NPY/AgRP neurons [137]. The signalling of GHS-R1a is complex and is reviewed in details by Briggs *et al.* [138]. Briefly, by increasing intracellular calcium concentration $[Ca^{2+}]_i$ in NPY neurons, ghrelin activates AMP-activated kinase (AMPK) which in turn increases ATP production. Downstream action of ghrelin-induced AMPK activation includes AMPK-CPT1-UCP2 pathway activation, increased acyl-CoA concentration and increased fatty acid oxidation. The end result for these pathways and its byproducts is sustained NPY/AgRP neuronal activation during

starvation and stimulation of food intake [137-139]. Interestingly, adult mice with ablated NPY/AgRP neurons die of starvation, which might be explained by the interruption of ghrelin signalling in the arcuate nucleus [140].

In pancreatic β cells, ghrelin mediates its insulinostatic effect through GHS-R-cAMP/TRMP2 signaling [141]. Pertussis toxin (PTX)-treated mice were used to study GHS-R G-protein coupling in β cells. It is known that PTX reduces the ability of the α-subunit of G_i ($G\alpha_i$) family to bond to G-protein-coupled receptors. The insulinostatic effect of exogenous and endogenous ghrelin was attenuated in PTX-treated mice, but ghrelin-induced growth hormone release was unchanged. Hence, the G-coupling for the ghrelin action on insulin release is different from that of GH release [142]. In another study, Dezaki *et al.* explored the cAMP signaling and insulinotatic effects of ghrelin in pancreatic β cells [143]. It is established that cAMP is produced by adynalte cyclase, and the latter is inhibited by PTX-sensitive G_i – proteins. Incubation of rat pancreatic islets with phosphodiesterase inhibitor (IBMX) and 8.3 mmol/L glucose resulted in a small increase in cAMP productions, but higher concentration (22 mmol/L) of glucose had a more significant increase in cAMP. Adding exogenous ghrelin to glucose-treated islets inhibited cAMP productions, and this inhibition was augmented by GHS-R antagonist. Neither exogenous ghrelin, nor GHS-R antagonist had any effect on glucose-induced cAMP productions in islets isolated from PTX-treated rats [143]. This collective evidence indicated that GHS-R activation in β cells resulted in attenuation of cAMP (**Error! Reference source not ound.**).

Figure. 4: GHSR signaling in the pancreatic β cell

Signaling pathway of ghrelin in pancreatic β cell. The level of cAMP is suppressed by ghrelin through high [ghrelin]/[SST] ratio and $G\alpha_i$ signaling. The conductance of Kv2.1 channel is enhanced by decreased cAMP level, which leads to decreased [Ca^+]. The end result is attenuation of insulin release.

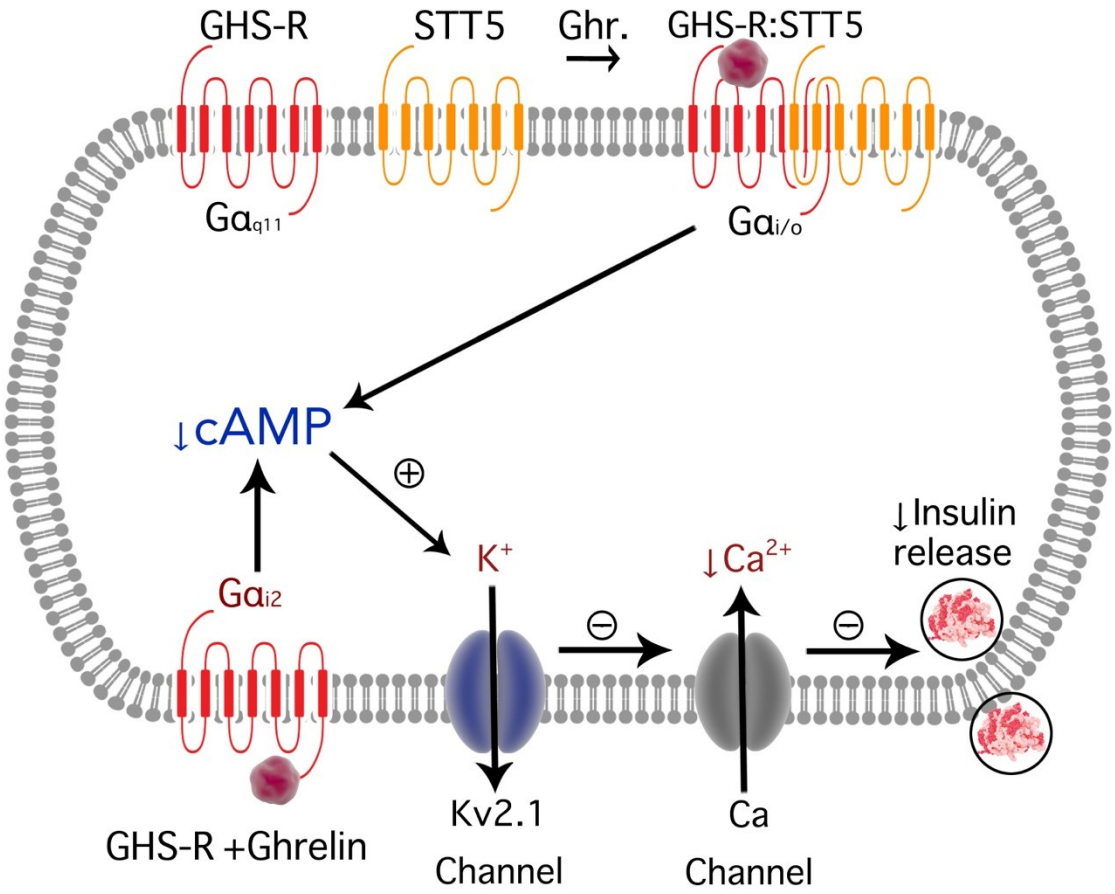

Figure 4 GHSR signaling in the pancreatic β cell

1.3.3 Roles of Ghrelin

1.3.3.1 Role of Ghrelin in GH secretion

GH is secreted in a pulsatile manner under the influence of somatostatin and growth hormone releasing hormone (GHRH) [144, 145]. Although GH pulses are related to GHRH peaks, about a third of GH pulses are disassociated from GHRH, suggesting that other factors contribute to GH release from pituitary [146, 147]. In 4 adult men with GHRH receptor mutation and GHRH resistance, GH oscillated in the same manner as the control despite overall GH deficiency, supporting the hypothesis of other factors role in GH release [148]. A different human study on familial short stature found a missense mutation in GHSR [149].

In animal studies, transgenic rats with attenuated GHS-R expression in the arcuate nucleus of the hypothalamus had decreased basal secretion of GHRH, suggesting an interaction between GHRH and ghrelin systems [150]. A significant correlation was observed in a clinical study between GH and acylated ghrelin secretion in eight healthy human subjects [151]. In a clinical randomized trial on older healthy adults, the oral administration of ghrelin memetic, MK-677, resulted in an approximately two-fold increase in GH from baseline [152]. In rats, ghrelin-induced GH secretion was attenuated by GHS-R1a antagonist, confirming the role of ghrelin receptors in GH secretion [153]. These findings from clinical and animal studies reinforce the role of ghrelin and its receptors in GH secretion and amplitude.

1.3.3.2 Role of ghrelin in appetite regulation

Studies on both human and rodents showed a peak of ghrelin secretion after fasting and a post-prandial suppression [154-156]. It is believed the pre-prandial ghrelin surge is

important in initiating food intake [156]. This mechanism has been proposed as a cause for hyperphagia seen in Prader-Willi syndrome. This syndrome is a genetic disorder characterized by hyperphagia-induced obesity, short stature, mental retardation, GH deficiency and hypogonadism [157]. When compared to obese control subjects, Prader-Willi patients had more than four-fold higher fasting ghrelin levels than control subjects [21].

In animal studies, centrally administration of ghrelin in rats increased food intake significantly more than administration of GHRP-6, a GHS-R agonist. In addition to increased food intake, chronic ghrelin administration resulted in weight gain without affecting general activity [158]. In GH-deficient rat model, i.c.v. infusion of ghrelin induced food intake, suggesting feeding stimulation by ghrelin is GH-independent [158]. Sun *et al.* investigated the role of GHS-R in ghrelin-induced food intake. In GHS-R-knockout mice, despite the normal phenotype compared to wild type (WT), acute administration of ghrelin did not increase food intake, which confirms the orexigenic effect of ghrelin is dependent on GHS-R expression [159]. Feeding behaviour was decreased significantly in a dose-dependent manner in mice after administering a ghrelin antagonist, either centrally or peripherally [160]. Interestingly, mice with simultaneous deficiency in ghrelin and GHS-R had a significantly lower body mass compared to WT, with a minimum decrease in food intake [161].

1.3.3.3 Role of ghrelin in obesity

As shown in previous studies, ghrelin plays a major role in controlling body weight and food intake. Its action, release, and suppression are modulated through many cellular pathways, receptors and other hormones. Thus, tight control of ghrelin is important to

maintain normal energy hemostasis. Nevertheless, ghrelin secretion and suppression can be dysregulated in metabolic diseases such as diabetes and obesity [162].

Daily injection of ghrelin decreased fat utilization and increased body weight in rats [154]. As discussed previously, higher levels of plasma ghrelin were found in Prader-Willi syndrome patients, a syndrome which is characterized mainly by hyperphagia-induced obesity [21].

Unexpectedly, plasma ghrelin levels were inversely correlated to BMI [163, 164]. Moreover, the post prandial ghrelin suppression was found to be blunted in obese adults compared to normal weight individuals [165] . The same finding was observed among Hispanic obese adolescents [166].

The coexistence of increased adiposity and glucose intolerance (secondary to insulin resistance) makes it difficult to study ghrelin effects on both conditions separately. However, not all obese individuals have insulin resistance or the same degree of insulin sensitivity. McLaughlin and her colleagues studied ghrelin concentrations amongst two groups of obese individuals who were otherwise healthy: insulin resistant and sensitive [167]. In body weight and BMI- matched analysis, mean plasma ghrelin concentration was lower among obese subjects with insulin-resistance compared to matched-BMI subjects who were insulin-sensitive [167]. In another study on normal and overweight children, ghrelin dynamic was studied with the oral glucose tolerance test (OGTT). In obese children, fasting ghrelin level was lower, while fasting insulin level was higher compared to normal weight subjects. Ghrelin levels were negatively correlated with insulin sensitivity and a higher insulin sensitivity index was associated with a more reduced ghrelin level [168]. Those results show that ghrelin level variability is obesity-independent and is

inversely affected by insulin resistance. Interestingly, the same results were seen in X/A cells from rodent primary stomach cultures that were treated with high insulin dose and developed (IR) [1].

1.3.3.4 Role of ghrelin in glucose hemostasis

In 11 healthy young men, the administration of IV acylated ghrelin increased serum glucose levels compared to placebo after 15 minutes of injection. Concurrently, a significant reduction in insulin levels was observed after 30 minutes [169] that continued for more than 100 minutes [169]. In many studies, ghrelin and GHS-R were identified in pancreas islets [114, 170-172] and an *in vivo* animal study done on mice revealed higher concentration of acylated and desacylghrelin in the pancreatic vein compared to the pancreatic artery [173]. Using perfused rat pancreas, Dezaki *et al.* studied the effect of endogenous ghrelin on insulin secretion. The glucose-induced insulin release was augmented by infusing GHS-R antagonist ([D-Lys3]-GHRP-6) or anti-ghrelin antiserum. The deacylated ghrelin form (desacylghrelin) did not alter glucose-induced insulin release. Both treatments had no effect on basal insulin secretion [173]. Gastrectomized and control rats were studied to investigate if the enhancement of glucose-induced insulin release is due to blockade of circulating rather than pancreatic ghrelin. As expected, gastrectomized rats had significantly lower acylated ghrelin level than control. By injecting GHS-R antagonist in both, gasrectomized rats had a significant increase in plasma insulin level comparable to the control group. This result confirmed that the enhancement in glucose-induced insulin release is due to a blockage of local ghrelin, as the administration of exogenous ghrelin to control rats decreased plasma insulin level [173]. In perfused pancreas of ghrelin knockout rats, glucose-induced insulin release was higher compared to

the wild type with no difference in basal insulin release between both, confirming previous results of ghrelin insulinostatic effect [173, 174].

Ghrelin was also found to increase hepatic glucose production in a mice study [175]. In porcine primary hepatocyte culture, glucose output was increased after 20 minutes of AG incubation by 132% compared to control [176]. The hepatic glucose release was lost after 40 minutes of incubation with AG. On contrary, incubation with UAG inhibited hepatic glucose output after 20 minutes and was in a dose-dependent manner. Intriguingly, equimolar UAG reversed AG-induced hepatic glucose release [176] to the control level independently from GHS-R activation [176]. In another study, rats injected with subcutaneous ghrelin had a significant weight gain despite comparable food intake with the control group [177]. In ghrelin-treated rats, liver glycogen synthase kinase was suppressed while the activator of gluconeogenesis protein (PGC1α) expression was significantly enhanced [177]. This result was supported by a different study on mice where insulin-induced suppression of hepatic gluconeogenesis was reduced by ghrelin administration [175].

While s.c. acyl ghrelin injections failed to stimulate food intake in GHS-R knockout (KO) mice with limited expression in hindbrain, it did increase fasting glucose to wild-type level, suggesting that ghrelin affects glucose metabolism through central nervous system signaling as well [178].

As expected, GOAT-KO mice had higher glucose tolerance and enhanced glucose-induced insulin release compared to wild type (WT). But in a calorie restriction state, GOAT-KO mice developed severe fasting hypoglycemia compared to WT despite equal weight lose suggesting the importance of ghrelin to survive malnutrition [179].

As mentioned before, many human and animal studies have demonstrated ghrelin insulnostatic effect on pancreatic β cells. By infusing acylated ghrelin to healthy non-diabetic volunteers, Tong *et al.* showed that glucose-induced insulin release was suppressed in a dose-dependent manner. A significant reduction in C-peptide release in response to IV glucose was observed as well [136]. Following adjustment for insulin sensitivity, acylated ghrelin decreased glucose disappearance rate after IV glucose infusion, a finding not seen with UAG infusion [136, 180, 181].

In mice, intraperitoneal administration of GHS-R antagonist significantly decreased fasting blood glucose level [126] and enhanced insulin response to glucose tolerance test (GTT) [126]. On the other hand, ghrelin administration increased fasting blood glucose by 10-20 mg/dl after 30 min. The increase in blood glucose was fully reversed by the administration of a GHS-R antagonist. Concurrent administration of ghrelin with glucose during GTT attenuated insulin response compared to control values [126].

Overall, these studies strongly indicate the essential role of endogenous ghrelin in glucose metabolism and hemostasis, either directly by its insulinostatic effect or through glucose disposal peripherally. Also, these results suggest the interference with ghrelin signaling can help improve glycemic control in obesity and type 2 diabetes [182].

1.3.3.5 Role of ghrelin in insulin secretion

Dezaki *et al.* studied the effect of ghrelin on insulin release in isolated pancreatic rat cells and intracellular calcium concentration $[Ca^{2+}]_i$. By adding GHS-R antagonist ([D-Lys3]-GHRP-6) or anti-ghrelin antiserum (SPA) to media, insulin release was increased significantly. This effect was reduced by removing external calcium, suggesting that endogenous ghrelin is suppressing Ca^{2+} -dependent insulin release [126]. Pancreatic islets

$[Ca^{2+}]_i$ was measured using fura-2 microfluorimetry, and the first phase $[Ca^{2+}]_i$ responses to different glucose levels were significantly augmented in the presence of GHS-R antagonist or anti-ghrelin antiserum [126].

In mouse β cell line (MIN6) cells transfected with a fluorescent-translocation biosensor, intracellular cAMP $[cAMP]_i$ surged in an oscillatory manner by increasing glucose concentration from 3 to 11 mmol/L [143]. These oscillations were inhibited by ghrelin administration, and a ghrelin washout reversed its inhibitory effect, implying complete reversibility [143]. In rat single β cells, protein kinase A (PKA), a cAMP-dependant enzyme, was increased with glucose concentration rise and inhibited by ghrelin treatment [143]. These collective findings suggested ghrelin-induced insulinostatic effect via cAMP-dependant pathway.

In electrophysiological studies, glucose (8.3 mmol/L) induced repetitive action potentials in single β cells. Ghrelin administration decreased both frequency and amplitude of action potentials [142]. By treating single β cells with tetraethylammonium (TEA), a voltage-dependant K^+ (Kv) channels blocker, glucose-induced insulin release was increased. However, ghrelin failed to inhibit glucose-induced insulin release in the presence of TEA, suggesting the ghrelin-induced attenuation of insulin release is Kv channels dependant [142].

In pancreatic β cells, both ghrelin and somatostatin (SST) inhibit glucose-stimulated insulin secretion (GSIS). Park *et al.* hypothesized that ghrelin-induced attenuation of GSIS is by noncanonical coupling of GHS-R1a with Gα(i/o) rather than Gα(q11) by a formation of heteromer with somatostatin receptor subtype-5 (SST5) [183]. By studying this mechanism in INS-1SJ, Park *et al.* demonstrated that ghrelin-GHS-R

complex failed to inhibit GSIS in the absence of STT5. However, in the presence of STT5, GHS-R formed heteromers with STT5 and ghrelin-induced GSIS attenuation was achieved through Gα(i/o)-dependent pathway [183] (**Error! Reference source not found.** and REF _Ref487571158 \h * MERGEFORMAT Figure 5).

1.3.3.6 Role of ghrelin in cardiovascular disease

Both GHS-R and ghrelin mRNA have been identified in myocardium [116], suggesting a role in cardiovascular physiology. In 50 healthy obese and lean men, a significant association was reported between high endogenous ghrelin levels and greater cardiovascular indexes in both groups [184]. The same finding was obtained by injecting ghrelin to healthy individuals [185]. In another clinical study on heart failure patients, exogenous administration of ghrelin decreased mean arterial pressure and systemic vascular resistance with improvement in stroke volume index [186]. In rats with heart failure, ghrelin administration improved left ventricular function [187]. In a different animal study where hearts were subjected to ischemia/reperfusion injury, acylated ghrelin significantly decreased infarct size and had a protective effect [188]. In a myocardial infarction (MI) mice model, ghrelin knockout group had a significant arrhythmia-induced mortality rate compared to WT. Interestingly, ghrelin administration before inducing MI in ghrelin knockout mice reduced arrhythmia and improved prognosis [189]. These results indicate a protective function for endogenous and exogenous ghrelin against malignant arrhythmia post MI. Lilleness *et al.* reviewed ghrelin effects on cardiovascular system in details [190].

1.3.3.7 Role of ghrelin in reproduction

Ghrelin and its receptor (GHS-R) are expressed in ovary and placental tissue [116], and ghrelin peptide was shown to cross placenta which raises the question of the effect of ghrelin on female reproduction and fertility [191, 192]. Furuta *et al.* studied the effect of ghrelin on luteinizing hormone (LH) in rats. An intracerebroventricular (i.c.v.) administration of ghrelin in ovariectomized female rats had a significant suppressive effect on pulsatile LH secretion and frequency. As expected, increased GH level was also observed. These changes were not seen with an intraventricular cerebrospinal fluid (CSF) infusion [193]. In addition to confirming the result of the previous study, Fernández-Fernández *et al.* did *in vitro* experiments to look at ghrelin effects on the hypothalamus and pituitary. In hypothalamic explants from ovariectomized female rats, incubation with ghrelin markedly decreased gonadotropin-releasing hormone (GnRH) release [194]. In contradiction to *in vivo* findings, ghrelin increased both LH and follicle-stimulating hormone (FSH) in pituitaries obtained from ovariectomized female rats. When pituitaries were incubated with GnRH and ghrelin, there was no change in basal and GnRH-stimulated gonadotropin secretion [194]. These findings suggest that ghrelin-induced LH suppression *in vivo* was due to predominant inhibition of GnRH release from hypothalamus. In another study on male rats, even subcutaneous administration of unacylated ghrelin (UAG) was able to inhibit LH serum level to the same amount as with ghrelin administration, suggesting GHS-R independent pathway [195].

In humans, IV administration of 50 μg acylated ghrelin to healthy young male individuals induced significant LH suppression compared to placebo group. Testosterone level was lower in ghrelin-treated group as well [196]. In a different single-blind, placebo-

controlled, randomized, crossover study, Kluge *et al.* showed that IV acylated ghrelin administration in healthy women significantly decreased both LH and FSH [197].

To investigate the role of ghrelin on embryo development, Kawamura *et al.* studied mouse preimplantation embryos in the presence of fluorescent-labeled ghrelin in culture media. Fluorescent signals could be detected in blastocysts incubated with fluorescent ghrelin indicating that ghrelin receptors (GHS-R) are expressed in early developmental stages. This result was confirmed by the detection of ghrelin and GHS-R mRNA in those embryos. When comparing embryos cultured in human tubal fluid (control), ghrelin-treated embryos were developmentally retarded and blastocysts with fewer cells [198]. This effect was blocked by incubating the embryos with GHS-R antagonist, confirming the effect of ghrelin through its receptor. This study suggested that ghrelin can inhibit embryo development in case of malnutrition to reserve energy [198].

In a human study comparing ghrelin levels in appropriate for gestational age (AGA) and intrauterine growth restricted (IUGR) neonates, Cortelazzi *et al.* reported a higher level of ghrelin in IUGR [199].

Fasting ghrelin levels were studied during pregnancy, and found to be highest by week 18, followed by a gradual decline to reach the lowest value in the late third trimester [200]. After delivery, fasting ghrelin levels were significantly higher compared to week 16. These changes in serum ghrelin were not significantly correlated to maternal weight, but were negatively linked to placental growth hormone (PGH) [200]. This can be explained by the switching from pituitary GH, which is stimulated by ghrelin, to the predominant PGH in late pregnancy [201].

Martin *et al.* examined the effect of ghrelin deficiency on mice fertility. In mice that were exposed to ghrelin deficiency *in utero* had reduced fertility and smaller offspring compared to wild type [202]. Despite the similar number of ovarian follicles and embryo produced, wild-type embryos transferred to mice uteri that were exposed to ghrelin deficiency *in utero* had 60% in implantation rate [202]. This result suggested that ghrelin is playing a major role in offspring fertility [202].

1.3.3.8 Role of ghrelin in different clinical diseases

A surprising relationship between *H. pylori* infection and ghrelin-producing cells has been described in the literature. After treatment and eradication of *H. Pylori* infection, patient's total body weight and BMI increases, while no significant change in these parameters with eradication failure [203, 204]. This finding motivated many researchers to look for ghrelin cells in relation to *H. pylori* infection as a modulator for the weight gain after eradication. In 10 human subjects who were *H. pylori* positive, a fasting ghrelin level before and after patients had been cured was measured. Interestingly, fasting ghrelin level increased by 75% after *H. pylori* eradication [205]. The same finding was seen in a larger study by Osawa *et al.*, who measured plasma ghrelin and examined gastric mucosa in 110 *H.pylori*-positive and 50 *H.pylori*-negative individuals [206]. Using RT-PCR, the *H.pylori*-positive group had a lower ghrelin mRNA expression compared to *H.pylori*-negative group in corpus mucosa. Even the numbers of ghrelin-producing cells between the 2 groups were significantly different, with lower numbers in *H.pyolri*-positive subjects. These findings suggest that decreased serum ghrelin level among *H.pylori*-infected subjects is related to the reduced ghrelin-producing cells in the stomach [206].

The role of ghrelin in anorexia nervosa (AN), a psychiatric disorder characterized by extreme concern about body shape and weight, has been a focus of many clinical studies over the years (reviewed in [207]). In AN women, plasma ghrelin levels and ghrelin/obestatin ratios were significantly higher than healthy women [208]. Compared to constitutionally thin women who had higher ghrelin levels than healthy subjects, ghrelin levels were significantly higher among AN patients, suggesting that ghrelin level is not only affected by body fat mass but by feeding behaviour as well [209]. In addition to higher fasting plasma ghrelin levels, AN is associated with central resistance as demonstrated by Holsen *et al.* using functional magnetic resonance imaging [210]. In a small pilot study to investigate the therapeutic role of ghrelin in AN, an intravenous infusion of ghrelin for 14 days increased hunger sensation and energy intake by 20%. These effects could be related to the central action of ghrelin in increasing appetite and/or peripheral effects, as the GI symptoms in those patients improved as well [211]. Although this study was small in number (only 5 patients), it suggested a potential therapeutic use for ghrelin in AN patients.

In chronic diseases that are associated with weight loss, ghrelin administration was shown to improve appetite to increase weight gain. In chronic obstructive pulmonary disease (COPD) patients with cachexia, 3-week infusions of ghrelin increased food intake and lean body mass, improved muscle strength and functional capacity [212]. In cancer patients, where cachexia is linked to anorexia, high dose of ghrelin agonist administration resulted in improvement of appetite and reverse of muscle catabolism [213].

Most hypothalamus and pituitary lesions and adenomas are associated with increased body weight [214]. In a recent publication by Andrusiewicz *et al.*, they reported different expression levels of ghrelin mRNA and GHS-R1, with the highest expression in non-

functioning tumors and somatotropinomas, respectively [215]. In children with craniopharyngioma with adequate hormone replacement, ghrelin suppression following oral glucose intake was delayed compared to weight-matched control [216]. These results suggest a possible role in pathogenesis and the associated obesity.

1.4 GHRELIN SECRETION

1.4.1 Nutrients Regulate Ghrelin Synthesis and Secretion

The effect of ghrelin in increasing body weight is independent from GH secretion. By injecting ghrelin daily in wild mice, their fat mass increased without change in lean or bone mass. Compared to GH-treated mice, ghrelin-treated mice had also less fat utilization [154]. The suppression of ghrelin postprandially was demonstrated not to be related to stomach stretch, but rather to re-feeding or glucose administration, as a water meal did not decrease ghrelin level [154, 217].

Ghrelin suppression after a meal is affected by the caloric contents that have been ingested. With the same level of plasma glucose among different caloric groups, the ghrelin suppression was proportionate to caloric intake [218]. Regarding the effect of macronutrients on ghrelin suppression, a human study showed higher suppression of ghrelin after high-carbohydrate compared to high-fat meals [219]. In this study, high-carbohydrate intake has more satiating effect compared to fat, which can be related to higher suppression of ghrelin [219]. Those findings have been shown in animal models as well [220]. On the other hand, high protein and high fat meals induced higher prolonged ghrelin suppression compared to carbohydrate and glucose intake [221, 222]. This result can be explained by delayed gastric emptying by protein and fat ingestion [223].

The role of stomach and duodenum in postprandial ghrelin suppression is an area of interest especially with the increased popularity of weight-loss surgeries (e.g. gastric bypass). In a human study, Cummings et. al. found lower total ghrelin level (AUC) in 24 hours in a group who had a proximal Roux-en-Y gastric bypass compared to normal-weight control by 77% [224]. Interestingly, after diet-induced weight loss, total ghrelin in 24 hours increased. Those findings show the importance of ghrelin in energy homeostasis and body weight control [224, 225]. To study the role of duodenum in postprandial ghrelin suppression, Overduin et. al. used animal models with proximal duodenum and jejunal catheters to infuse different macronutrients. There were no difference in ghrelin suppression between the sites, but higher suppression was observed after glucose infusions compared to fat, amino acids, and saline [226]. Despite a higher suppression of ghrelin after carbohydrate ingestion, this suppression exhibits a rebound phenomenon after 3 hours. Ghrelin increased by 37% from baseline after marked decrease following a carbohydrate meal in the second 3-hour period. Such rebound was not observed after protein or lipid in ingestions [227]. This overshooting of ghrelin can be an explanation for the high glycemic index carbohydrate ingestion and associated rebound hunger [227, 228]. In rat isolated stomach, adding amino acids to basal media resulted in a 30% reduction in ghrelin secretion, but no difference with intralipid perfusion [229].

Studying macronutrient intake on ghrelin secretion as discussed above in isolation of other hormones would not provide clear mechanisms for this effect. From those clinical and animal studies, there was an association between ghrelin and different neurotransmitters and gut hormones, either proceeding or following ghrelin suppression [126, 155, 220, 227, 230, 231]. Thus, *in vitro* models to study ghrelin release, action and

suppression are essential. Such models include ghrelin-producing tumour cell lines such as pancreatic ghrelinoma (PG-1), stomach ghrelinoma (SG-1) [232] and MGN3-1 cell lines[233], in addition to a primary culture of dispersed rodents stomach mucosa [1, 234]. Those models are very important in studying cellular and molecular aspects of ghrelin physiology, signalling and its interactions with different hormones. The following section will summarize some relevant examples of such interactions.

1.4.2 Neurotransmitters Regulating Ghrelin Secretion

1.4.2.1 Norepinephrine

Earlier studies were done to investigate the role of sympathetic nervous system in ghrelin secretion. In streptozotocin (STZ)-diabetic rats, Mundinger *et al.* reported an increase in plasma ghrelin concentration after electrical sympathetic nerve stimulation of postganglionic axons. This increase in ghrelin was associated with a rise in norepinephrine concentration in portal venous plasma. The same result was observed with a pharmacological stimulation of sympathetic nerves by tyramine [235].

Using rat primary stomach culture, Gagnon *et. al.* studied the role of nervous system on ghrelin release. There was no effect on ghrelin secretion when incubated with 10 μM of acetylcholine. However, a dose-dependent increase in ghrelin secretion was observed with Norepinephrine (NE) [1] which also showed an increase in cAMP and protein-kinase A (PKA) activity. This effect was blocked by pre-treatment with Atenolol (β1-adrenergic receptor antagonist. This experiment confirmed that ghrelin release is mediated through β1-adrenergic receptors [1]. In a stomach ghrelinoma cell line, SG-1, Zhao *et al.* reported an increase in acylated and unacylated ghrelin secretion by adrenergic agonists, epinephrine and NE. Both ghrelin forms were decreased in cells, suggesting that adrenergic

agonists stimulate secretion but not synthesis [232]. However, in an animal model study, ghrelin had no effect on NE release from the hypothalamus [236] (Figure 5).

1.4.2.2 Acetylcholine

Since the vagus nerve provides parasympathetic inputs to the GIT and play a major role in appetite regulation [237], it is very important to understand the effect of its neurotransmitter (acetylcholine) on ghrelin secretion.

In isolated rat stomach, Sherestha *et al.* demonstrated that perfusion of insulin decreased basal ghrelin secretion as confirmed by previous studies. Interestingly, acetylcholine perfusion increased ghrelin basal level by 37% [229]. In a different approach, the administration of an acetylcholine antagonist, atropine, in healthy human subjects resulted in a significant reduction in ghrelin basal level [238]. In sham-operated rats, ghrelin levels were increased after 24 or 48 hours of food deprivation. Whereas there was no increase in plasma ghrelin levels in vagotomised rats that underwent the same period of food deprivation [239]. Administration of atropine to sham-operated rats after food deprivation resulted in a significant reduction in ghrelin levels [239].

All these results suggested an important role of the vagal neurotransmitter (acetylcholine) through its muscarinic receptors to increase ghrelin basal levels in response to energy deficit.

1.4.3 Taste Receptors and Ghrelin Secretion

The identification of bitter taste receptors in GIT and their effects on GI endocrine cells secretion opened the question for their role in detecting nutrition in GIT [240]. A family of bitter taste receptors (T2R), including α-gustducin and α-transducin, are G-protein coupled receptors that have been identified in mouse ghrelin-producing cells in

stomach [241]. To study the role of these receptors on ghrelin secretion, Janssen *et al.* administered a mixture of bitter agonist by oral gavage in wild type (WT) and α-gustducin knockout mice. Ghrelin plasma levels were similar in both genotypes after water gavage. Interestingly, both acylated and total ghrelin plasma levels increased and peaked after 40 minutes of oral gavage in WT mice. In contrast, plasma level of acyl ghrelin was significantly lower in α-gustducin knockout mice compared to WT, but no difference in total ghrelin levels in both genotypes was observed[241]. Increased ghrelin level in WT mice was associated with increased food intake, but not after T2R agonists gavage in α-gustducin or GHS-R knockout mice [241]. In the same experiment, gastric emptying was significantly delayed in WT mice treated with T2R agonists compared with water gavage. Pretreatment of WT mice with CCK and GLP-1 antagonists could not reverse gastric emptying delay by T2R agonists, suggesting a direct effect from bitter taste receptors in ghrelin-producing cells independent from CCK and GLP-1 [241].

1.4.4 Circadian Rhythm and Ghrelin Secretion

The link between fasting and food intake to ghrelin secretion has been known for years. Cummings *et al.* studied ghrelin secretion rhythm in healthy human subjects. It was found that plasma ghrelin levels surge before meal intake and reach a nadir by 1 hour postprandially [156]. As expected, insulin secretion was reciprocal to plasma ghrelin levels, increasing 3 to 7 folds after food consumption. Despite post-prandial suppression of ghrelin, ghrelin levels increased progressively throughout the day, with the highest level in 0100. Overnight, ghrelin levels continuously decreased to reach a trough before rising again at 0600 [156].

Ghrelin activating enzyme, GOAT, is also expressed in a circadian rhythm. In WT mice, circadian oscillations of both gastric GOAT and ghrelin mRNA expression levels were observed reaching the maximum at *zeitgeber time* (ZT) 4 [242]. These oscillations were followed by a rise in total and acylated ghrelin in a circadian manner [242]. Circadian oscillations of gastric ghrelin were abolished in mice with intrinsic circadian clock factor, Bmall- knockout. On the other hand, ghrelin rhythmicity in ghrelinoma cell lines was triggered in the presence of epinephrine or peptone, mimicking fasting and post prandial states, respectively. [242].

In animal studies, Horvath *et al.* investigated ghrelin mRNA expression in mice hypothalami throughout the 24-hour day. Ghrelin mRNA in the hypothalamus increased 2.5-fold during the dark period, and this increase was associated with a parallel rise in an intrinsic circadian clock factor, Bmall [243]. This finding indicates that ghrelin is involved in the hypothalamic circadian system.

1.4.5 Hormonal Regulation of Ghrelin Secretion

1.4.5.1 Insulin

Ghrelin release pre- and post prandially is inversely related to insulin level as ghrelin is suppressed by high insulin in plasma [156]. This reciprocal relation is expected, giving the role of insulin in controlling glucose homeostasis and satiety. Unexpectedly, fasting ghrelin concentrations were 27% lower in obese human subjects compared to lean [163]. We know from a previous study by Gagnon *et al.* on primary rodent stomach culture that insulin can inhibit ghrelin secretion directly through ghrelin cells insulin receptors [1]. Moreover, NE-induced ghrelin release was suppressed by insulin treatment. This inhibition was mediated by AKT phosphorylation and increased cAMP level. Proghrelin mRNA

expression was not changed by insulin treatment [1]. When the cells were treated with high-insulin concentration (100 nm) for 24-hour period, insulin resistance (IR) was developed and ghrelin secretion was not suppressed by insulin, but was still increased by NE. In IR-treated cells, insulin receptors were down regulated and insulin failed to activate AKT phosphorylation. These results showed that ghrelin-secreting cells can develop IR *in vitro* and the importance of insulin in regulating ghrelin release [1], which explain why obese individuals have a blunted suppression of ghrelin after food intake [244]. Similarly, to how insulin can affect ghrelin's release, ghrelin also has insulinostatic effect on islet β-cells [142]. The effect of ghrelin on insulin and glucose hemostasis has been discussed in detail in different sections (Role of ghrelin in glucose hemostasis and Effects of Obesity and Diabetes).

1.4.5.2 Glucagon

In human studies, administration of glucagon (either intramuscularly or subcutaneously) decreased serum ghrelin concentrations [245-247]. These studies could not provide clear evidence on the role of glucagon in ghrelin suppression, as glucagon administration was associated with an increase in insulin and glucose as well. Previous studies showed that insulin and glucose inhibit ghrelin release [1, 248].

In order to study glucagon effect on ghrelin release independently from other factors, Gagnon *et al.* used the primary culture of rat gastric cells model. To confirm the expression of glucagon receptors (GluR) on ghrelin- producing cells, double-immunofluorescence staining was used and showed co-localization of ghrelin with GluR [249]. Then, the cells were treated with glucagon in different concentrations (1,10, and 100 nM). Over 4-hour period, a significant increase in ghrelin secretion was observed which

was blocked by the presence of a GluR antagonist. Proghrelin mRNA expression was significantly increased after 24-hour incubation with 10 nM of glucagon. The effects of glucagon on ghrelin-producing cells were mainly mediated by increasing cAMP level through EPAK dependent pathway [249]. Unexpectedly, using protein kinase A (PKA) inhibitors did not affect glucagon-stimulated ghrelin release, indicating a PKA-independent pathway [249]. Calcium was shown to play a role in glucagon-stimulated ghrelin release as the preincubation of cells with thapsigargin significantly reduced ghrelin secretion by glucagon. Another finding from this study is that NE and glucagon work synergistically to stimulate ghrelin secretion [249].

In a different direction, Chuang et al. studied the effect of ghrelin on glucagon release [250]. Ghrelin receptors (GHS-R) were highly expressed in mouse pancreatic α-cells. By using quantitative PCR (qPCR) and in situ hybridization histochemistry (ISHH), a higher level of GHS-R mRNA was isolated form pancreatic α-cells than in hypothalami or peripheral organs. Injecting acyl-ghrelin in male C57BL7/J mice subcutaneously (s.c.) was associated with increased plasma glucagon in a dose-dependent manner. Although an increase in plasma glucose was associated with acyl-ghrelin injection, no significant increase in plasma insulin levels were observed [250]. When using GHR-S null littermates, acyl-ghrelin infusion did not affect plasma glucose, insulin, or glucagon levels [250]. These results are showing a bidirectional relation between ghrelin and glucagon-producing cells and their importance in glucose hemostasis (Figure 5).

1.4.6 Effects of Obesity and Diabetes on Ghrelin Secretion

In obesity, ghrelin levels are generally reduced compared to normal weight subjects, and negatively correlated to body fat mass [163]. Interestingly, the numbers of

ghrelin-positive cells are higher among obese subjects compared to non-obese controls. However, GOAT-positive cells are reduced as is active plasma levels of ghrelin among obese individuals. These findings suggested a dysregulation of ghrelin on the tissue level in obesity [251].

ghrelin resistance was observed in DIO mice in the hypothalamus as demonstrated by Briggs *et al.* experiment on mice [252, 253]. Ghrelin resistance was elicited by observing food intake after ghrelin administration in chow-fed control and DIO mice. As expected, ghrelin infusion stimulated food intake in control but not in DIO mice, suggesting a ghrelin resistance in the obese group. By counting Fos-immunoreactive neurons, it was found that ghrelin failed to stimulate the neurons in arcuate nucleus, establishing ghrelin resistance in the hypothalamic level [253].

In one clinical study on obese polycystic ovary syndrome patients, ghrelin administration decreased insulin levels, however, ghrelin-induced GH secretion was blunted compared to lean control [254]. In another human study, acute administration of ghrelin in obese patients did not affect insulin or glucose levels, but in contrast to the previous study, stimulated GH secretion [255]. The data of ghrelin administration effect in human obesity are conflicting, but overall indicting abnormal responses. This conflict can be explained by different body weights, insulin resistance or associated diseases among research participants.

As reviewed before, ghrelin and insulin are reciprocally related, where ghrelin inhibits insulin release from pancreatic β cells and increases plasma glucose levels (Role of ghrelin in glucose hemostasis). In a study on children with obesity or metabolic syndrome, homeostasis model assessment of insulin resistance (HOMA-IR) was negatively

correlated to total and UAG [256]. One of the explanations for IR in type 2 DM is increased ghrelin-induced free fatty acid release [257, 258]. In type 1 DM, which is mainly characterized by insulin deficiency, ghrelin concentrations were correlated inversely with the chronicity of the disease, suggesting a role in developing complications [259].

In type 2 DM, ingestion of a typical mixed meal resulted in comparable ghrelin dynamic to control subjects, but the ghrelin circadian rhythm was not observed, pointing to dysregulation in the circadian ghrelin secretion among those patients [260].

In STZ-diabetic rats, a significant decrease in stomach ghrelin-immunopositive cells and ghrelin receptor mRNA was observed after one month of diabetes onset. However, a subsequent increase in ghrelin cells and GHS-R mRNA was seen in 2 and 3 month-period after diabetes onset [261]. This increase can be explained by diabetes-induced cachexia in those rats with high blood glucose over a longer period, which in turn stimulated ghrelin secretion. In a different study on the same diabetic-rat model, the preproghrelin mRNA increased significantly in the stomach, but not in the duodenum and colon compared to control [262]. Although the second highest concentration of preproghrelin mRNA is in duodenum, the significant increase in stomach tissue suggests that stomach ghrelin-producing cells are the main source of ghrelin in diabetic rats [262].

Figure 5 effects of different hormonal stimulation on ghrelin secretion.

Insulin inhibits ghrelin secretion through AKT pathway and decreasing cAMP production. AMPK is another pathway involved in supressing ghrelin secretion via activation of mTOR1, and treatment with metformin block this effect. NE and glucagon are acting to increase ghrelin secretion through different pathways, including cAMP/PKA, EPAK (exchange protein directly activated by cAMP) and MAPK.

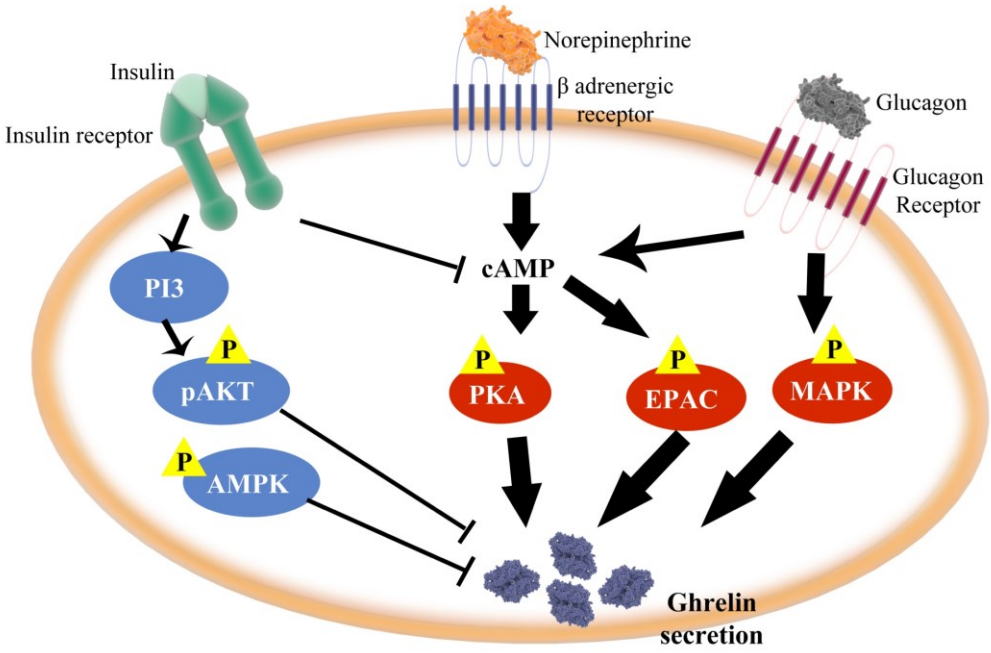

Figure 5 effects of different hormonal stimulation on ghrelin secretion

CHAPTER 2 METHODOLOGY

2.1 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. Mice were maintained in a conventional rodents holding facility at the IWK in vivo laboratory with a 12-h light/dark cycle (6 AM/6 PM). Mice were fed either a standard rodent chow diet (12 kcal% fat, 2016, Teklad, Madison, WI), or a high-fat diet, (HFD; 60 kcal% fat, D12492, Research Diets, Inc., New Brunswick, NJ) for 12 weeks. Lean mice were fed standard rodent chow diet throughout the study period, while the DIO mice were switched to HFD at 4 weeks of age. Body weight was monitored weekly. At 16 weeks of age, mice were euthanized by Isoflurane overdose, for isolation of gastric mucosa cells to establish primary cultures. All mice in these studies were sacrificed after a 4-h food deprivation starting at the beginning of the light cycle (9 AM–10 AM).

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 scarified at post-natal day (PND) 8 as described in [1].

2.1.1.1 Blood parameters

Blood samples for glucose measurement were obtained via the tail vein. Blood glucose levels were measured using a hand-held glucometer (OneTouch, LifeScan Inc., Milipitas, CA). Blood samples for ghrelin levels were obtained via cardiac puncture prior to euthanasia. Blood samples were placed on ice and treated with the cysteine protease

inhibitor PHMB (p-hydroxymercuribenzoate). Plasma was separated by centrifugation at 1500 × *g,* acid stabilized, as described in [1] and stored at −80 °C. Acylated ghrelin was determined using active ghrelin enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) as per the manufacturer's guidelines.

2.1.1.2 Primary cell culture preparation

All cell culture media and reagents, unless otherwise stated, were obtained from Sigma-Aldrich (Oakville, Ontario, Canada).

2.1.1.2.1 New born rat primary culture

Primary cell culture was prepared from mixed pups (male and female), because there was no difference between genders for basal ghrelin percentage secretion (11.9 ± 1.37% male and 12.38 ± 1.24% female). Mixed pups were anesthetized with isoflurane. From each litter, three separate cellular preparations comprised of three animals each were prepared. The stomach ghrelin cell extraction protocol was as described in [1, 249, 263]. 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 were then aspirated, and stomachs were minced into small pieces using surgical scissors. Stomachs were resuspended in 5 ml of wash buffer and incubated at room temperature for 5 min. The stomachs were then subjected to enzymatic digestion in 5 ml of wash buffer supplemented with type 1A collagenase (6 mg/ml) with orbital shaking at 37 C for 20 min. Five milliliters of wash buffer were added, and digestion mixture was incubated at room temperature for 5 min. The supernatant was collected, centrifuged at 200 × *g* for 10 min and set aside as digestion fraction no. 1. The remaining

undigested material was treated to a second 20-min digestion in collagenase (3 mg/ml) followed by an additional 5-min incubation with 100 µl of 5 mM EDTA. Cells were rinsed with 5 ml of DMEM 10% fetal bovine serum (FBS) and passed through a 200 µM nylon cell strainer (VWR, Ontario, Canada) into a 50-ml tube along with digestion fraction no. 1. The filtrate was spun down at 200 × *g* for 10 min. 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 min. The final cell pellet was then resuspended in 10 ml of culture media. Cell count was determined using a hemocytometer. Cells were plated in six-well cell culture plates for ghrelin secretion.

2.1.1.2.2 Primary Culture of Ghrelin Cells from Adult Mice

In vitro models have been used to study ghrelin secretion. Of which are ghrelinoma cell lines from either stomach (SG-1 and MGN3-1) or pancreas (PG-1) [232, 233]. Although these cell lines have been validated for ghrelin secretion, they are derived from tumor cells and do not represent the normal physiology and dynamic of ghrelin secretion. Hence, primary stomach culture model considered the gold standard to study ghrelin secretion *in vitro.*

We validated the rat primary culture model in our lab and we demonstrated that these cells secrete ghrelin in a regulated manner [1, 234, 249]. Although this cell model is excellent for investigating the cellular pathways involved in ghrelin synthesis and section, it has some limitations. The main limitation is that it is difficult to compare the results obtained from new born rat cells with the mouse models of obesity and diabetes. Thus, to complement the rat cell studies, we developed and validated a primary culture of adult mouse ghrelin cells. Male C57BL/6J mice aged 8–12 week were used. The primary cell

culture was prepared as described by Sakata *et al.* [264]. Briefly, the stomachs were quickly excised rinsed and turned inside-out and incubated in the digestion solution [2.5 mg of Dispase II (Roche Diagnostics, Indianapolis, IN) per 1 ml of PBS] per stomach at 37°C for 90 min. The mucosal layer of each stomach was isolated and the cell suspension centrifuged at 1,000g for 3 min, after which the cells were collected by filtering through a 100-µm nylon mesh. The cells were counted using a hemocytometer and diluted to a final concentration of 1×10^5 cells/ml.

2.2 GHRELIN SECRETION EXPERIMENTS

Secretion experiments were completed in six-well cell culture plates (BD Falcon, Mississauga, Ontario, Canada). Cells were plated at 1×10^6 cells per well in 2 ml of culture media. Treatments were started 48 h after cells were put in culture. On the day of secretion experiment, cells were washed with PBS and incubated with different treatments dissolved in 2 ml of low glucose (1000 mg/liter), phenol red-free DMEM, and 0.5% FBS for 4 h. After incubation, culture media were collected and spun at 1000 × *g* for 5 min to remove any floating cell debris. The supernatant was acidified with 200 µl of 1% trifluoroacetic acid (TFA) and kept on ice to prevent protease activity and loss of the acylated ghrelin. Cells were scraped in 500 µl of an acidic lysis buffer (1 M HCl, 1% TFA, and 50 mM NaCl) and were sonicated for 5 seconds on ice. The lysate was spun down for 5 min at 11,000 × *g* at 4C. The supernatants from the culture media and cell extract were then subjected to hydrophobic reverse phase resin chromatography (C-18 Sep-Pak cartridges; Waters, Milford, MA) 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 was more than 85%. Pellets

were kept in −80 C 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 [1, 249].

2.3 GHRELIN ASSAYS

Total ghrelin (acyl and deacyl) level was determined using total ghrelin Elisa (Phoenix Pharmaceuticals, Burlingame, CA) as per the manufacturer's guidelines. This assay recognizes both acylated and nonacylated ghrelin with a minimum detection limit of 10 pg/ml. Acylated ghrelin was determined using active ghrelin enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) as per the manufacturer's guidelines. This assay recognizes only the acylated form of ghrelin as the antibody used recognizes the N-terminal of the acylated ghrelin (cross-reactivity with nonacylated 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.

2.4 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 min at room temperature. Cells were rinsed 3 × 5 min in TBS before being permeabilized in 0.1% Triton X-100 for 15 min. Cells were blocked for 20 min in a TBS blocking buffer

containing 0.1% Triton X-100 and 5% normal donkey serum (Vector Labs, Burlington, Ontario, Canada). Antibodies for TNF-α receptors (Abcam, Cambridge, MA) and proghrelin (Santa Cruz Biotechnology, Inc., 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 min at room temperature. Coverslips were then mounted on slides in Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Labs) and visualized using a Zeiss Axioplan 2 fluorescent microscope (Zeiss, Oberkochen, Germany).

2.5 WESTERN BLOTTING

Cells were plated into 10-cm plates at 4×10^6 cells per plate and allowed to grow for 24 h at 37C, 5% CO2. Insulin treatments (10 nM) were prepared in Krebs-Ringer buffer with 10 mM HEPES (pH 7.4) and given to cells for 15min at 37 C. 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, Guelph, Ontario, Canada). Cells were sonicated, and protein was quantified using the Bradford method. Forty micrograms of protein extract were denatured and separated in 8% acrylamide sodium dodecyl sulfate Tris tricine gels at 100 V. Gels were transferred for 1 h at 100 V to polyvinylidene fluoride membranes. Western blottings were blocked in 5% milk and 0.1% Tween 20. Primary antibodies for AKT, phosphorylated AKT, and insulin receptor (β-subunit) were obtained from Cell Signaling (Danvers, MA) and actin from Abcam. Secondary horseradish peroxidase-conjugated antibodies were obtained from GE Healthcare (Piscataway, NJ). Chemiluminescence was measured using

the Bio-Rad Chemidoc XRS and analyzed using the one-dimensional software from Bio-Rad.

2.6 DATA ANALYSIS

All data are expressed as mean ± SEM. Statistical differences were tested using Prism 6 (GraphPad Software, Inc., San Diego, CA). Student's “t”-test was used to compare differences between two sets of means. One-way ANOVA followed by Tukey's post-hoc multiple comparisons test was used to compare multiple groups. $P < 0.05$ was considered statistically significant.

CHAPTER 3 RESULTS

3.1 HIGH FAT DIET MOUSE MODEL OF OBESITY

To study the mechanisms underlying obesity-associated impairment in ghrelin secretion, we used a diet induced obesity (DIO) mouse model. C57BL/6 mice fed HFD for 12 weeks (DIO mice) had higher body weights compared to those fed standard rodent chow (lean/control mice) (Figure 6). Fasting blood glucose was higher in DIO mice compared to lean mice (Figure 7).

Mice were submitted to oral glucose tolerance test (OGTT) (2 gm/kg). DIO mice had a significant increase in blood glucose level compared to lean mice after 10 min of OGTT (14 ± 0.3 mM vs 11 ± 0.8 mM, $p<0.05$). After 20 min of OGTT, DIO mice exhibited gradual decrease in blood glucose levels, however, they were still significantly higher than control group (10 ± 0.6 mM vs 8 ± 0.5 mM, $p<0.05$). Even after one hour of administering oral glucose, a significant difference was observed between both groups (DIO 7 ± 0.8 mM vs LFD 5 ± 0.6 mM, $p<0.05$)

These findings confirm that the DIO mice were overweight and developed glucose intolerance which was evident during the first hour of OGTT.

Figure 6: Body weight comparison between DIO and lean mice.

Body weights of low-fat diet (LFD; left) and high-fat diet (HFD; right) mice were measured in grams. HFD had a significantly higher body weight compared to LFD, and exhibited obesity phenotype.

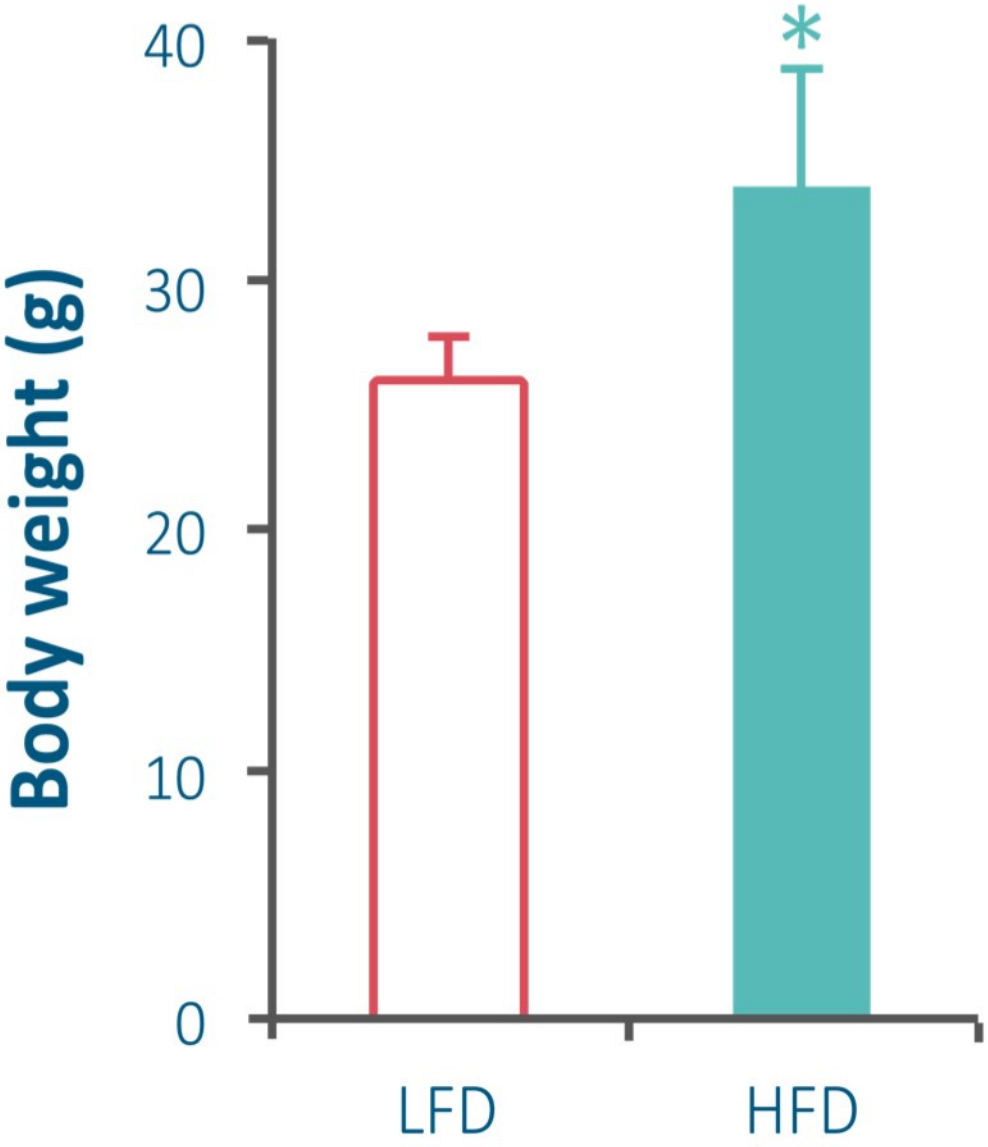

Figure 6 body weight comparison between DIO and lean mice

Figure 7: impaired glucose intolerance in DIO mice indicating development of diabetes and insulin resistance.

Blood glucose levels in LFD and DIO mice were measured from tails, before and after oral glucose (2 gm/kg) administration. Glucose levels were measure at 0, 10, 20, 30 and 60 min, and presented in mM. HFD mice (green line; upper) had high fasting blood glucose levels compared to LFD mice, indicating impaired fasting glucose. After oral glucose administration, HFD mice reached the peak level after 10 min, reaching >13 mM. In all measured blood glucose levels, HFD exhibited higher levels than LFD mice, even after 60 min. This indicates development of diabetes among DIO mice. (* $p<0.05$)

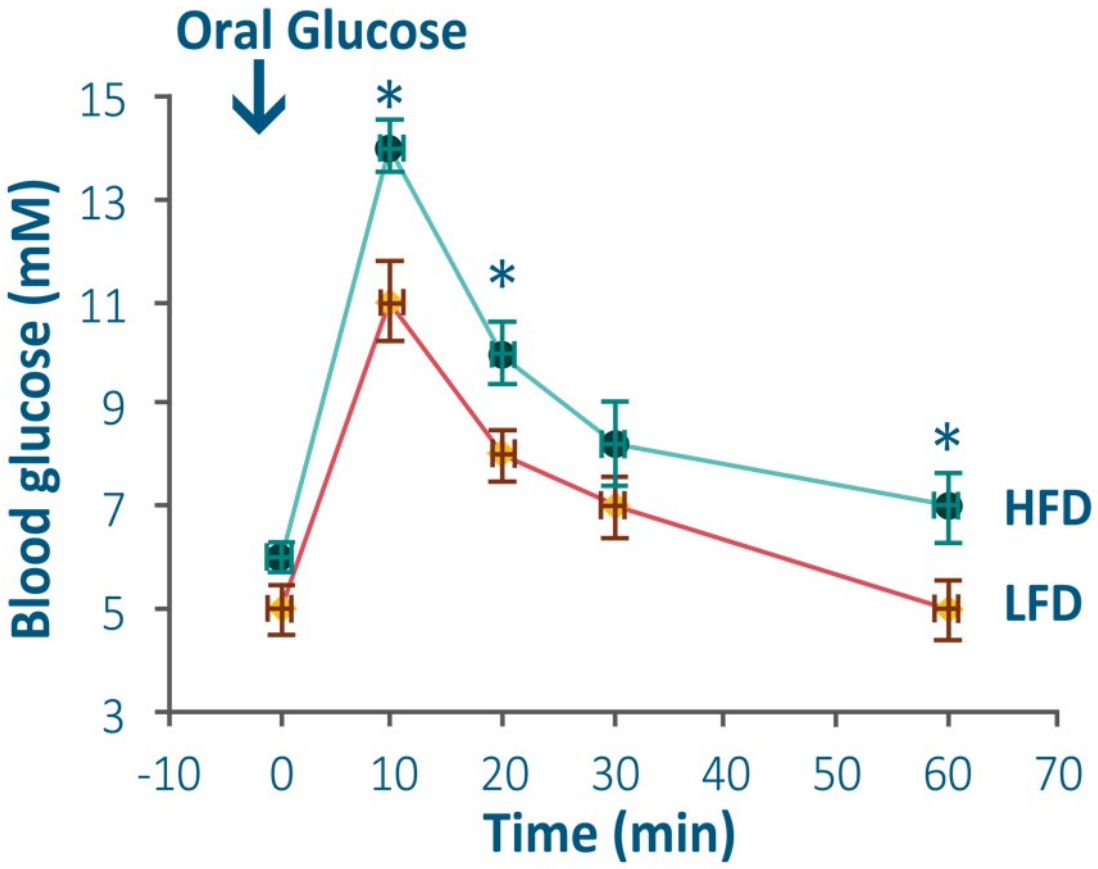

Figure 7 impaired glucose intolerance in DIO mice indicating development of diabetes and insulin resistance. ($p<0.05$)*

3.2 GHRELIN SECRETION IN DIO MICE

We then used DIO model to measure fasting and postprandial acyl-ghrelin secretion in obesity.

After overnight fasting, acyl-ghrelin levels were significantly higher in lean compared to DIO mice (1.62±0.21; 1.26±0.13, respectively. n=6). In keeping with previous reports, glucose administration resulted in acyl-ghrelin reduction in LFD mice measured after 60 and 120 mins. However, acyl-ghrelin reduction after OGTT was blunted in DIO mice at the same time points (0.69±0.1; 0.81±0.11 vs. 1.18±0.09; 1.21±0.9; respectively. $p<0.05$, n= 6) (Figure 8).

This result confirmed previous studies on lower fasting ghrelin levels and impaired post-prandial suppression in obesity.

Figure 8: impairment of acyl ghrelin secretion among DIO mice.

Fasting and postprandial Acyl ghrelin (AG) in HFD (green) and LFD (red) mice, measured at 0, 60 and 120 min. After an overnight fasting, lean mice had a significant higher level of circulating acyl-ghrelin compared to obese group (1.62±0.21; 1.26±0.13, n=6). Unlike LFD mice, glucose administration failed to suppress acyl-ghrelin levels in DIO mice after 60 and 120-min (0.69±0.1; 0.81±0.11 vs. 1.18±0.09; 1.21±0.9; respectively. $p<0.05$, n= 6).

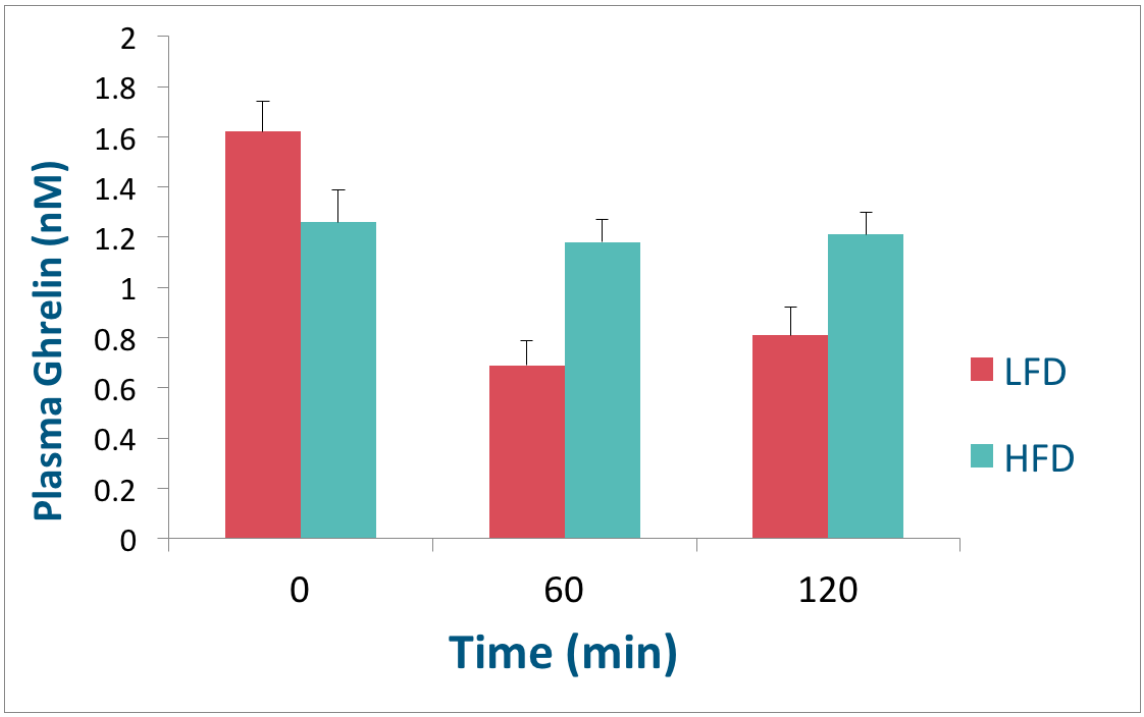

Figure 8 impairment of acyl ghrelin secretion among DIO mice

3.3 ALTERED RESPONSIVENESS OF GHRELIN CELLS TO INSULIN IN DIO MICE

Initially, we measured acylated and unacylated ghrelin *in vitro*. However, since both forms secretions were correlated, we continued measuring unacylated form. Unacylated Ghrelin (UAG) secretion in response to insulin mediating insulin suppression of ghrelin secretion was tested in primary cultures of dispersed gastric mucosal cells generated from either lean or DIO mice. In preparations from lean mice, 10 nM insulin reduced ghrelin secretion by 40% ($P < 0.05$, $n=6$). (Figure 9 A). In contrast, the effects of insulin on UAG release were lost in gastric mucosal cells derived from DIO mice ($n =6$) (Figure 9 B).

These results indicate that insulin suppression of ghrelin secretion is impaired in DIO mice indicating that the impairment of postprandial ghrelin secretions is partially due to insulin resistance.

Figure 9: comparison of insulin-induced ghrelin suppression between (DIO) and lean mice stomach primary cultures.

Primary culture of dispersed gastric mucosal cell from LFD (A) and DIO (B) mice. 10 nM of insulin incubation induced significant suppression of unacylated ghrelin secretion by 60% ($P < 0.05$, $n=6$). However, insulin failed to induced ghrelin suppression from DIO mice, indicating involvement of insulin resistance in postprandial blunted ghrelin suppression among DIO group.

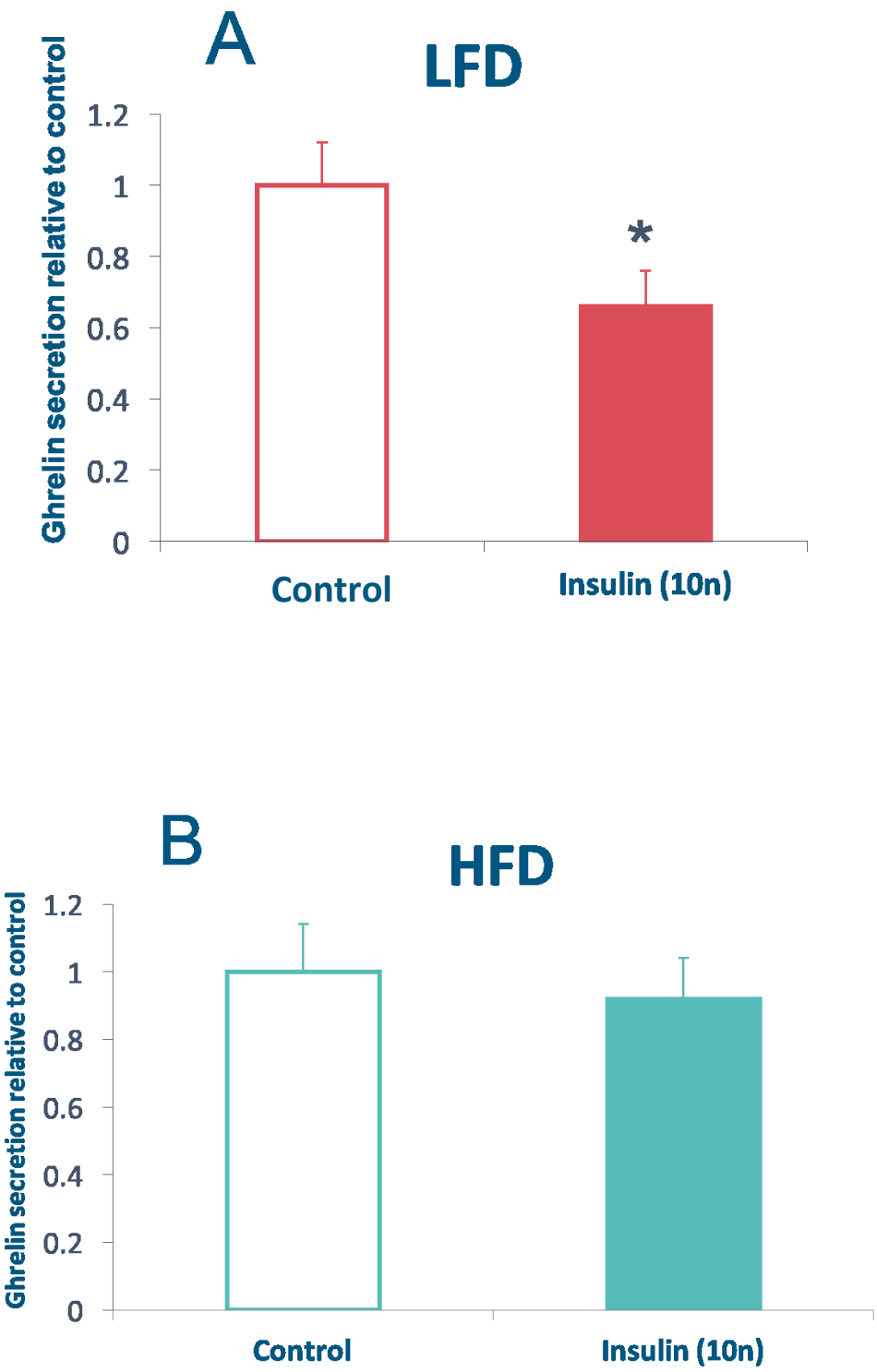

Figure 9 comparison of insulin-induced ghrelin suppression between lean (A) and DIO (B) mice stomach primary cultures.

3.4 Insulin Signaling in DIO

In our previous studies, we demonstrated that insulin inhibits ghrelin secretion via its action on the insulin receptor, leading to the activation of Pi3K and phosphorylation of AKT. In the same study, MAPK inhibitor did not affect insulin-induced ghrelin suppression [1]. To demonstrate that insulin can activate the AKT pathway in the primary stomach culture of lean and obese mice, cells were incubated with insulin (10 nM) for 15 min, and pAKT was measured via Western blotting. Insulin treatments significantly increased pAKT levels relative to total AKT (10.2 ± 3.2-fold of control, $P < 0.01$), in lean mice, whereas insulin failed to activate Akt primary stomach culture of obese mice (Figure 10).

These results indicate that ghrelin-producing cells from DIO mice developed insulin resistance, as the insulin-induced phosphorylation of AKT was suppressed. This interruption of downstream insulin signaling was observed in cells form DIO mice only, explaining the impairment of insulin-induced ghrelin secretion in this model.

Figure 10: the insulin-induced ghrelin suppression is mediated through phosphorylation of AKT.

Insulin-induced AKT phosphorylation was observed in primary cell culture from LFD mice (10.2 ± 3.2-fold of control, $P < 0.01$), whereas HFD-derived primary cell culture failed to respond to insulin activation of AKT as shown by measuring Western blotting densitometry of pAKT/AKT before and after 10 nM insulin treatment for 15 min. The figure data is a mean of 3 experiments and a representative Western blotting is shown.

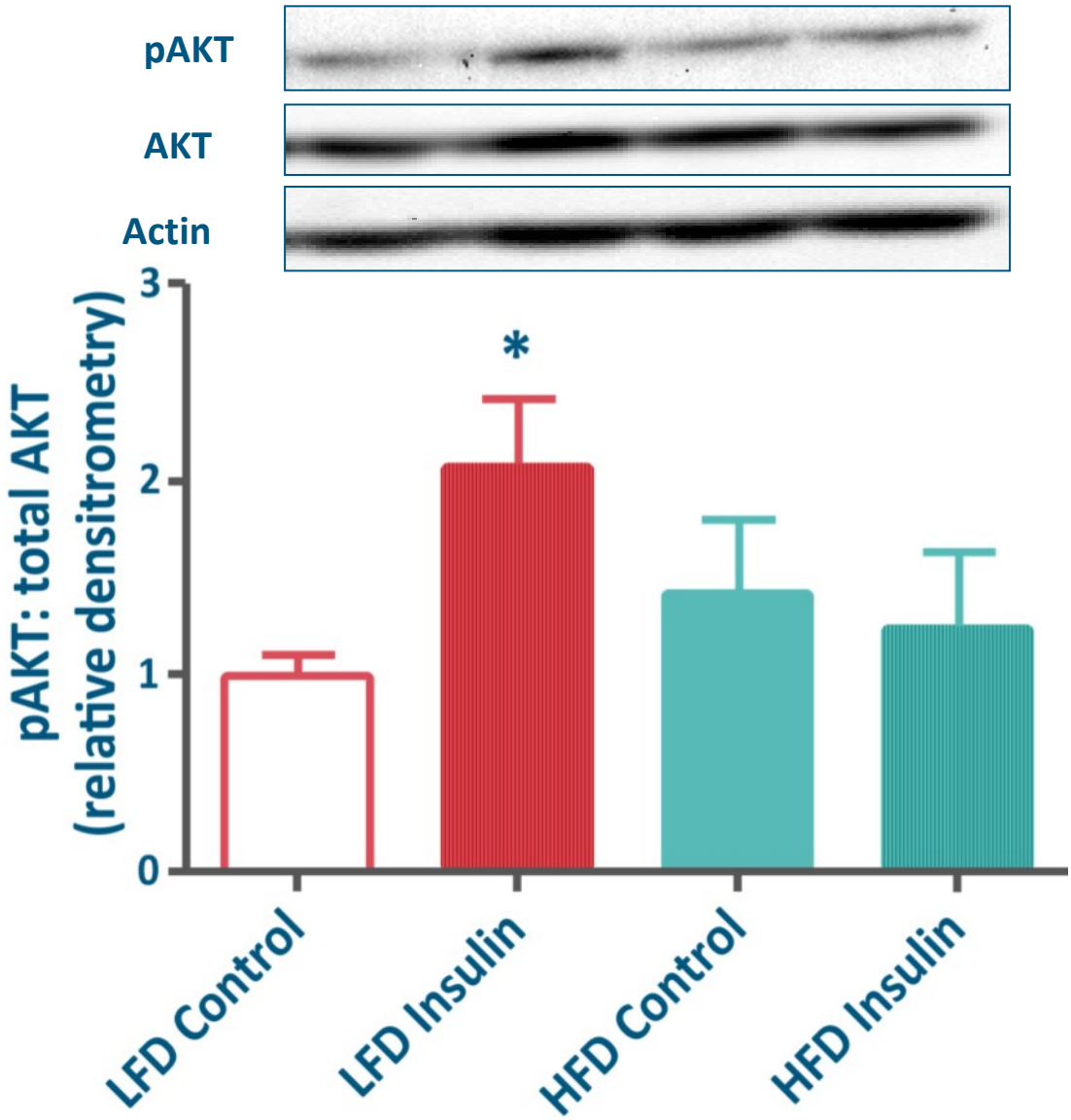

Figure 10 the insulin-induced ghrelin suppression is mediated through phosphorylation of AKT.

3.4.1 Role of Inflammatory Cytokines in Ghrelin Secretion

Inflammation is emerging as an important factor in obesity and the development of diabetes. We have now demonstrated that postprandial suppression of ghrelin secretion is reduced or completely blunted in rodents with obesity and insulin resistance (Figure 8) consistent with findings in humans with obesity and T2D. As enhanced levels of cytokines and, most notably, tumor necrosis alpha (TNF-α), are common in obesity, we hypothesized they may exerts direct regulatory effects on X/A cells to reduce ghrelin secretion and may contribute to the dysregulated ghrelin secretion observed in obesity.

We first confirmed the expression of both TNF-α receptors in Ghrelin cells using double immunofluorescence. As shown in Figure 11, both TNF receptor types, 1 and 2, were detected in ghrelin-producing cells (red), and ghrelin peptide was identified in the same cell as well (green). Both colures (red and green) were seen on the same cells. Quantitative RT-PCR confirmed the presence of both TNF receptor type (Figure 11). These results confirmed co-localization of TNF-α receptors and ghrelin in X/A cells.

Secondly, we examined the effect of TNF-α on ghrelin secretion. The effect of TNF-α alone or in combination with insulin was investigated in rat ghrelin primary culture. As shown in Figure 12, relative secretion of ghrelin in the presence of insulin (10 nM) was significantly reduced. While TNF-α alone had no effect on ghrelin secretion, pretreatment with TNF-α (10 ng/mL) blocked insulin-induced suppression of ghrelin that was observed before TNF-α treatment. This result strongly support our hypothesis of TNF-α role in impaired insulin-induced suppression of ghrelin in obesity.

Figure 11: ghrelin-producing cells from rat stomach primary culture express TNF receptors, 1 and 2.

Double immunofluorescence of ghrelin-producing cells (X/A) detecting co-localization of ghrelin (green) and TNFR1, and TNFR2 (red). Quantitative RT-PCR confirmed the presence of TNF receptors in X/A cells.

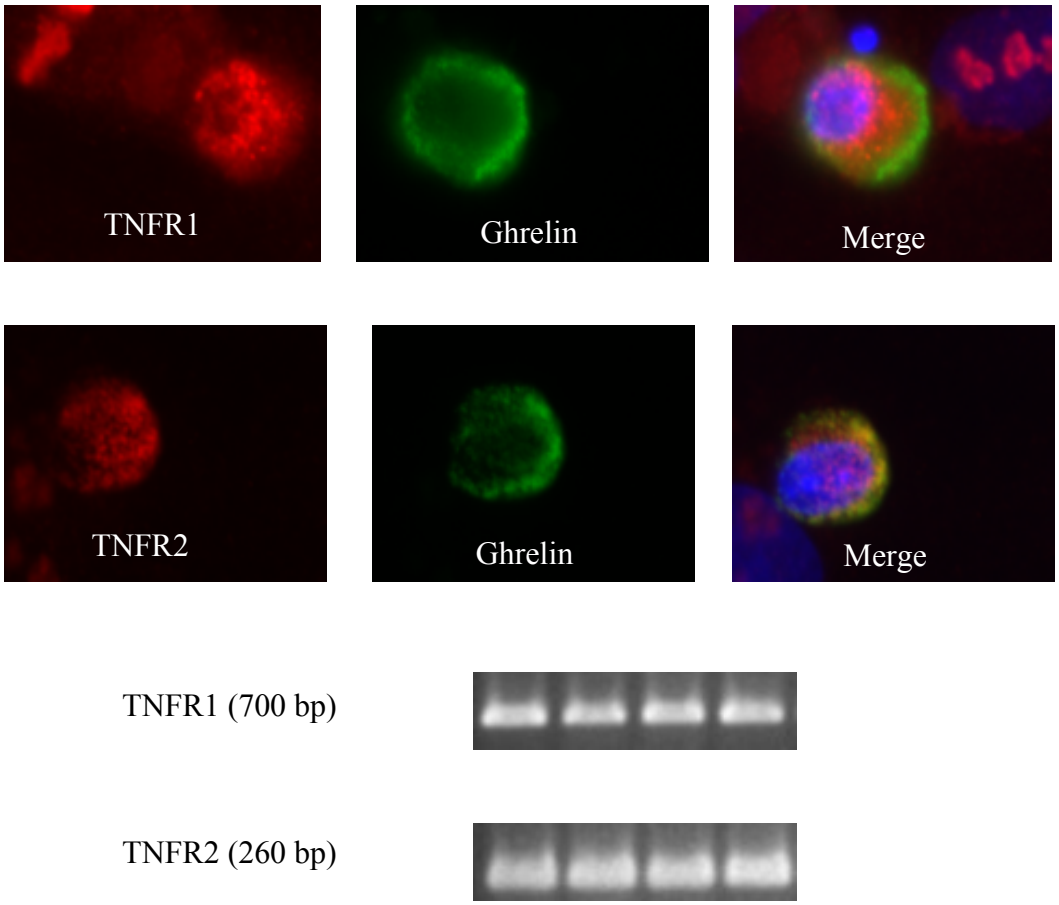

Figure 11 ghrelin-producing cells from rat stomach primary culture express TNF receptors, 1 and 2.

Figure 12: TNF-α blocking insulin-induced suppression of ghrelin in primary cell culture of ghrelin cells.

Relative ghrelin secretion from rat primary cell culture was significantly reduced after insulin (10 nM) treatemnt. However, this effect was blocked by pre-treatemnt with TNF-α (10 ng/mL). TNF-α alone failed to attenuate ghrelin secretion.

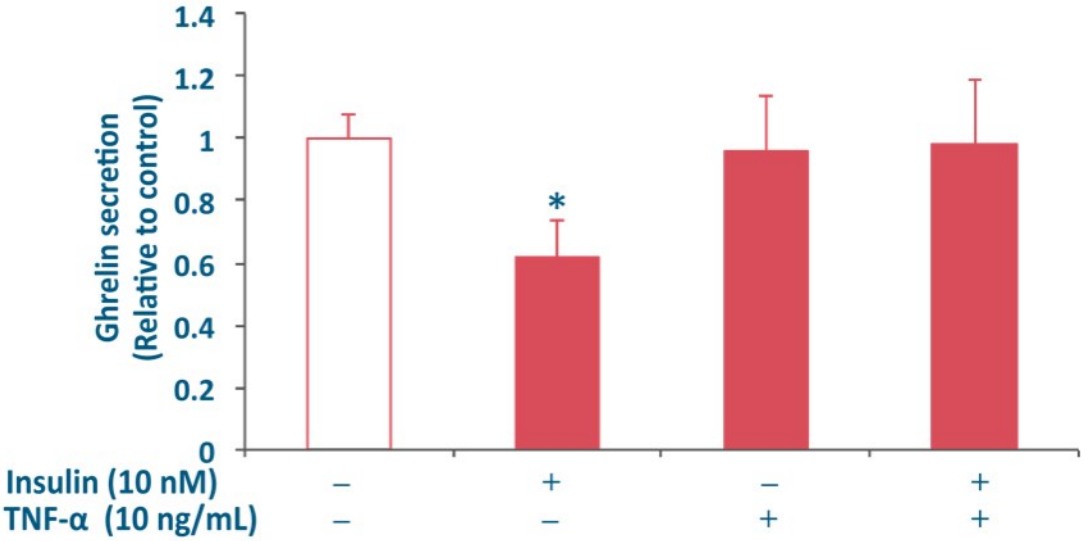

Figure 12 TNF-α blocking insulin-induced suppression of ghrelin in primary cell culture of ghrelin cells.

CHAPTER 4 DISCUSSION

4.1 FASTING AND POST PRANDIAL GHRELIN LEVELS

Ghrelin is known for its orexigenic effect, along with being a GH secretagogue [113, 265]. Since its discovery in 1999, ghrelin has been reported in many physiological and pathological conditions, particularly in metabolic syndrome and diabetes mellitus (reviewed in [257]). Unexpectedly, ghrelin levels were frequently reported among obese individuals to be reduced after fasting compared to lean control, with impaired suppression after food intake [244, 266-268]. Many researchers have hypothesized that such low levels associated with obesity could be due to overall body resistance to ghrelin. This resistance could be related to stomach ghrelin-producing cells with impairment of norepinephrine-induced secretion, resulting in lower ghrelin level among obese subjects [269]. Apart from the peripheral resistance hypothesis, central ghrelin resistance has been reported in the hypothalamus as well [253]. One evolutionary explanation for such resistance in obesity (food abundance) is to protect the body from high set point of ghrelin secretion in case of a following famine. In support for this hypothesis is what Briggs *et al.* reported in obese ghrelin-KO mice, where weight gain after a loss was attenuated compared to WT mice [270, 271].

However, the hypothesis of peripheral or central ghrelin resistance does not fully explain the lower ghrelin levels in obesity and the reversibility after weight reduction. In fact, some studies showed an increase in ghrelin positive cells in morbidly obese subjects, suggesting an impairment of ghrelin secretion rather than reduced cell numbers [120]. Other studies have shown the reason behind impermanent of ghrelin is not increased body weight per se, but rather the associated insulin resistance [167, 168, 272].

Many hormones are involved in the regulation of ghrelin secretion, including insulin and norepinephrine. By studying ghrelin-producing cells from stomach primary culture, our group has demonstrated that those cells are expressing both insulin receptor subunits, α and β [1]. Incubation of stomach primary culture with insulin resulted in a significant reduction in ghrelin secretion, as we will discuss later. In the same study, Gagnon *et al.* reported that norepinephrine stimulated ghrelin secretion through β1-adrenergic receptor, while acetylcholine had no effect, contradicting previous studies [1, 229].

In the present study, we used a mouse model of high fat-diet induced obesity (DIO) to investigate the mechanisms leading to impaired ghrelin secretion in obesity. This mice model has been used for obesity and diabetes-related research for the last few decades, due to its ability to develop hyperinsulinemia, hyperglycemia and obesity, like human, with high fat diet [273]. However, metabolic syndrome is not observed in this model with normal activity and low-fat diet. Even the fat deposition mainly mesenteric, which is known in human to be one of the diabetes risk factors [273, 274].

Compared to control mice (fed low fat diet), DIO mice have higher body weight, high blood glucose levels after fasting period, and impaired oral glucose tolerance response, indicating development of insulin resistance in this model. After the validation of the model in our hands with development of metabolic syndrome, we explored ghrelin secretion (fasting and postprandial) in lean vs obese mice. After fasting period, ghrelin levels in DIO mice were significantly lower compared to lean group. Not only the basal secretion was reduced, but also post prandial suppression was impaired in DIO mice. Our results confirmed the previously reported findings in humans and rodents [267, 269].

In humans, Bach *et al.* reported an inverse correlation between fasting ghrelin and fasting insulin levels among normal weight and overweight subjects, with the latter group having lower insulin sensitivity and higher insulin levels as a result [168]. In this study, ghrelin levels measured from DIO mice after fasting are reduced compared to controls, and this is in keeping with previous reports.

Dardzinska *et al.* reported an interesting result in obese individuals with metabolic syndrome [244]. No statistical significant difference in fasting or post-prandial UAG between obese and lean subjects. Yet, the main difference was observed in the active form, AG, after meal intake. Most studies investigated the total ghrelin suppression, rather than the active form, after meals. However, the studies are contradicting regarding the influence of obesity on AG and UAG, but consistently reporting significant decreased levels after fasting and blunted suppression after food intake [267, 275]. In our study, we measured AG *in vivo*. However, since both were consistently correlated in our previous studies on primary cell culture, we continued measuring UAG.

Our DIO model had an evidence of insulin resistance based on the impaired glucose levels before and after OGTT. Unlike the lean mice, DIO model exhibited impaired ghrelin suppression after oral glucose administrations, which indicates a dysregulation in ghrelin secretion dynamics, which could be explained by insulin resistance.

One of the limitations in our study is that we depended on the weight solely to conclude increased fat mass in DIO mice. Visually, the adipose tissue was more in DIO mice viscera, but quantitative methods like DEXA would exclude increased lean mass in this model. However, this model of DIO has been validated and accepted by scientific community to study obesity and MS [273].

To explore the cellular mechanism leading to impaired postprandial ghrelin secretion, we prepared primary cultures of ghrelin cells from stomach of lean and obese mice and studied the effect of insulin.

4.2 INSULIN-INDUCED GHRELIN SUPPRESSION IS IMPAIRED IN DIO MICE

As discussed before, higher circulating insulin levels were associated with lower fasting ghrelin concentrations. By using primary stomach culture from DIO and control mice, we have demonstrated the direct effect of insulin treatment on ghrelin producing-cells, which confirms the premise of insulin resistance action on the level of ghrelin cells. As shown in Figure 9, a significant suppression was observed after 4-hour period of 10 nM insulin incubation. The dose of insulin was determined based on a previous study published by our group, where the highest suppression of ghrelin was observed with 10 nM of insulin [1]. From previous studies, we know that high incubation with insulin (100 nM) for 24 hours could induce IR in primary cell cultures with decreased insulin receptors expression [1, 276]. In this study, we did not measure insulin receptors expression in ghrelin-producing cells, but we believe the observed result of impaired ghrelin suppression by insulin is related to decreased insulin receptors expression secondary to high circulating insulin and insulin resistance among DIO mice. This believe is supported by the fact of impaired OGTT in DIO mice.

Insulin receptor activation induces complex downstream signaling, involving PI3K, AKT and mTOR pathways. Briefly, insulin receptor activation induces phosphorylation in insulin receptor substrate-1 (IRS-1), which in turn activates PI3K pathway to

phosphorylate PIP2 to produce the second messenger PIP3. The latter activates AKT pathway, and mTORC2 is required for full activation of AKT (reviewed in details in [277]). Most of the insulin actions is mediated through AKT pathway activation, especially AKT2 [278] (Figure 5).

We measured AKT phosphorylation in both primary cell culture groups, before and after insulin treatment. In keeping with literatures, the activation on AKT was only observed in control primary stomach culture incubated with insulin, but not with DIO cell culture, suggesting an inhibition of insulin downstream signaling in these cells. Other pathway that can be involved in this impairment is mTORC1. While mTORC2 enhances AKT phosphorylation that is required for insulin signaling, mTORC1 is involved in insulin resistance through serine/threonine phosphorylation of IRS-1 [279]. On the other hand, AMPK was reported to inhibit mTORC1 [280]. The notion of mTORC1 involvement in impaired ghrelin suppresion is supported by previous study done by Gagnon *et al.* where metformin-induced AMPK enhancement was used to inhibit ghrelin secretion [234]. This pathway in ghrelin-producing cells requires further investigations.

4.3 TNF- α PREVENTS INSULIN-INDUCED GHRELIN SUPPRESSION

Obesity-related inflammation is mainly driven by adipokines actions [29]. Some of those adipokines are pro-inflammatory cytokines, including: interleukins and TNF-α. In human and animal studies, results have shown a strong association between TNF-α and insulin resistance, *in vivo* and *in vitro* [34, 40-42].

The role of TNF-α in developing insulin resistance has been reported in many studies [38, 39, 281]. As known, the phosphorylation of tyrosine (tyr) residues of IRS-1 is

essential for downstream signaling of insulin receptor. One of the mechanisms of TNF-α-induced IR is through reduction of insulin-induced tyr phosphorylation [282]. The other pathway is involving JNK activation, where the latter phosphorylates serine residues of IRS-1 and inhibit insulin signaling [283]. Interestingly, Ozes *et al.* reported that TNF-α-induced IR is involving mTOR/AKT pathway that we discussed before [284]. In that study, rapamycin, an mTOR inhibitor, blocked TNF-α-induced serine phosphorylation.

In the current study, TNF-α alone failed to inhibit ghrelin secretion from primary stomach culture, eliminating the possibility of TNF-α-dependent effect in ghrelin suppression. As shown before, insulin treatment induced a significant suppression of ghrelin. However, insulin failed to suppress ghrelin in pre-treated cell culture with TNF-α, confirming that insulin signaling in ghrelin-producing cells was blocked by TNF-α action. In humans and rodents, high fat diet induced inflammation in hypothalamus, as well as ghrelin resistance in appetite control center, suggesting a central inflammation-induced ghrelin resistance [253, 285]. Pro-inflammatory cytokines, including TNF-α, were reported to be elevated in hypothalamus with high fat diet [286]. Naznin *et al.* studied the effect of obesity-induced inflammation on impaired ghrelin secretion and reported downregulation of GHS-R expression in the inferior ganglion of vagus nerve (nodose ganglion), suggesting a resistance in afferent vagal signaling. interestingly, TNF-α and other cytokines were found to be high in the nodose ganglion [287].

From the previous discussion, most of the studies on TNF-α role in IR and ghrelin secretion were investigating central or peripheral ghrelin resistance, excluding the main source of ghrelin production, stomach ghrelin-producing cells. As far as we know, no

previous studies have looked at the effect of TNF-α on insulin-induced ghrelin suppression in a primary cell cultures, to investigate the effect on ghrelin-producing cells.

In this present study, we did not measure the weight of DIO mice livers or investigate the fat depositions in hepatocytes. It is known that fat-induced hepatocyte inflammation is linked to high TNF-α secretion [288], which may contribute to the impaired insulin signaling as well.

4.4 Role of glucose

Despite the evidence of TNF-α involvement in impaired insulin-induced suppression of ghrelin, we cannot exclude other factors *in vivo*, including glucose toxicity. From previous studies, we know that high glucose can induce insulin resistance through different mechanisms including β-linked N-acetylglucosamine (GlcNAcylation) pathway in adipocytes and muscle cells (reviewed in [289]) with impairment of the insulin-regulated glucose transporter 4 GLUT-4.

In primary cultured rat myoblasts, acylated ghrelin did not alter GLUT-4 expression [290].However, ghrelin gene deletion or GHS-R antagonist reversed rapamycin-induced suppression of GLUT-4 membrane translocation in muscle cells, indicating the role of ghrelin in rapamycin-induced hyperglycemia [291].

In clinical studies, high circulating glucose was associated with impaired glucose sensing in pancreatic β cells and induced insulin resistance as well (reviewed in [292]).

As far as we know, the effects of high glucose on insulin resistance and insulin-regulated glucose transporters has never been studied on ghrelin-producing cells.

4.5 CONCLUSION

In addition to many other physiological functions, ghrelin is a key hormone in appetite regulation, and its secretion impairment is associated with metabolic syndrome; obesity, and diabetes. Despite being the only known peripheral orexigenic hormone, ghrelin levels are unexpectedly reduced among obese individuals with insulin resistance, indicating it is affected, rather than effector, in obesity and insulin resistance.

Many studies have demonstrated the effects of different macronutrients, hormones and metabolites on ghrelin secretion and dynamics, including insulin and norepinephrine. In human and animal studies, insulin found to inhibit ghrelin secretion, and believed to be the main reason for post-prandial ghrelin suppression. While energy excess (food intake) associated with insulin surge that suppresses ghrelin, norepinephrine release, derived by hunger, stimulate ghrelin secretion. In obesity, both insulin and norepinephrine effects on ghrelin secretion are impaired.

Our present study has replicated some of the previous researchers' results on the effect of obesity on reduced fasting and impaired post-prandial suppression of ghrelin. However, we have demonstrated that insulin resistance on the level of ghrelin-producing cells is playing a major role in such impairment.

The other interesting finding is the role of TNF-α in insulin resistance and impairment of ghrelin secretion from ghrelin-producing cells. This role on ghrelin secretion from stomach primary cell culture is novel and required further investigation to elucidate the involved pathways and possibly to achieve novel obesity and IR treatments.

We can conclude from our present study that insulin-induced ghrelin suppression impairment not only occurs on central and axis levels, but ghrelin-producing cells are

directly involved and exhibit insulin resistance. The role of TNF-α on ghrelin secretion from stomach primary cell culture is novel and required further investigation to elucidate the involved pathways and possibly to achieve novel obesity and IR treatments.

The regulation of ghrelin secretion is complex, and is affected by many hormones and nutrients through different pathways. For the future research directions in clinical setting, it will be interesting to look at the effect of anti-TNF-α medications and ghrelin secretion in both, insulin sensitive and resistance individuals, may provide a new treatment target.

Another clinically relevant area is to study the role of ghrelin in PCOS patients and the role of pro-inflammatory cytokines in such patients.

Many patients with hypothalamus or pituitary tumors and injuries are experiencing increased weight gain after treatment, which may involve ghrelin-related mechanisms. In addition to investigating the cellular TNF-α role, *in vitro* studies are needed to explore the pathways involved, and if TNF-α is impairing insulin signaling by interfering with AKT phosphorylation.

Current ghrelin cell lines are difficult to study and not representing normal physiology. Gastric organoids are emerging now as a physiological model to study GI peptides including ghrelin [293]. Such model will provide further details on normal ghrelin secretion and interactions with other hormones.

High circulating glucose levels and its associated hyperglycemia-induced oxidative stress could be another process to explore in ghrelin-producing cells, independent of the insulin resistance or obesity-induced inflammation.

REFERENCES

1. Gagnon, J. and Y. Anini, *Insulin and norepinephrine regulate ghrelin secretion from a rat primary stomach cell culture.* Endocrinology, 2012. **153**(8): p. 3646-56.

2. Ogden, C.L., et al., *Prevalence of Obesity Among Adults and Youth: United States, 2011-2014.* NCHS Data Brief, 2015(219): p. 1-8.

3. Shields, M., et al., *Fitness of Canadian adults: results from the 2007-2009 Canadian Health Measures Survey.* Health Rep, 2010. **21**(1): p. 21-35.

4. World Health, O., *Global status report on noncommunicable diseases 2014*. 2014: World Health Organization.

5. WHO. *Obesity and Overweight*. Fact sheet 311 2012; Available from: http://www.who.int/mediacentre/factsheets/fs311/en/index.html.

6. Nguyen, D.M. and H.B. El-Serag, *The epidemiology of obesity.* Gastroenterol Clin North Am, 2010. **39**(1): p. 1-7.

7. Kuczmarski, R.J., et al., *2000 CDC Growth Charts for the United States: methods and development.* Vital Health Stat 11, 2002(246): p. 1-190.

8. Nevill, A.M., et al., *Relationship between adiposity and body size reveals limitations of BMI.* Am J Phys Anthropol, 2006. **129**(1): p. 151-6.

9. Hammond, R.A. and R. Levine, *The economic impact of obesity in the United States.* Diabetes Metab Syndr Obes, 2010. **3**: p. 285-95.

10. Heymsfield, S.B. and T.A. Wadden, *Mechanisms, Pathophysiology, and Management of Obesity.* N Engl J Med, 2017. **376**(3): p. 254-266.

11. Popkin, B.M. and C. Hawkes, *Sweetening of the global diet, particularly beverages: patterns, trends, and policy responses.* Lancet Diabetes Endocrinol, 2016. **4**(2): p. 174-86.

12. Church, T.S., et al., *Trends over 5 decades in U.S. occupation-related physical activity and their associations with obesity.* PLoS One, 2011. **6**(5): p. e19657.

13. Apovian, C.M., et al., *Pharmacological management of obesity: an endocrine Society clinical practice guideline.* J Clin Endocrinol Metab, 2015. **100**(2): p. 342-62.

14. Bray, M.S., et al., *NIH working group report-using genomic information to guide weight management: From universal to precision treatment.* Obesity (Silver Spring), 2016. **24**(1): p. 14-22.

15. Hatoum, I.J., et al., *Heritability of the weight loss response to gastric bypass surgery.* J Clin Endocrinol Metab, 2011. **96**(10): p. E1630-3.

16. van Dijk, S.J., et al., *Recent developments on the role of epigenetics in obesity and metabolic disease.* Clin Epigenetics, 2015. **7**: p. 66.

17. Tobi, E.W., et al., *DNA methylation signatures link prenatal famine exposure to growth and metabolism.* Nat Commun, 2014. **5**: p. 5592.

18. Flier, J.S., *Obesity wars: molecular progress confronts an expanding epidemic.* Cell, 2004. **116**(2): p. 337-50.

19. Zhang, Y., et al., *Positional cloning of the mouse obese gene and its human homologue.* Nature, 1994. **372**(6505): p. 425-32.

20. Farooqi, I.S., et al., *Effects of recombinant leptin therapy in a child with congenital leptin deficiency.* N Engl J Med, 1999. **341**(12): p. 879-84.

21. Cummings, D.E., et al., *Elevated plasma ghrelin levels in Prader Willi syndrome.* Nat Med, 2002. **8**(7): p. 643-4.

22. Levin, B.E., *Central regulation of energy homeostasis intelligent design: how to build the perfect survivor.* Obesity (Silver Spring), 2006. **14 Suppl 5**: p. 192S-196S.

23. Siiteri, P.K., *Adipose tissue as a source of hormones.* Am J Clin Nutr, 1987. **45**(1 Suppl): p. 277-82.

24. Flier, J.S., et al., *Severely impaired adipsin expression in genetic and acquired obesity.* Science, 1987. **237**(4813): p. 405-8.

25. Kershaw, E.E. and J.S. Flier, *Adipose tissue as an endocrine organ.* J Clin Endocrinol Metab, 2004. **89**(6): p. 2548-56.

26. Frayn, K.N., et al., *Integrative physiology of human adipose tissue.* Int J Obes Relat Metab Disord, 2003. **27**(8): p. 875-88.

27. Montanari, T., N. Pošćić, and M. Colitti, *Factors involved in white-to-brown adipose tissue conversion and in thermogenesis: a review.* Obes Rev, 2017. **18**(5): p. 495-513.

28. Trayhurn, P. and J.H. Beattie, *Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ.* Proc Nutr Soc, 2001. **60**(3): p. 329-39.

29. Esposito, K., et al., *Role of adipokines in the obesity-inflammation relationship: the effect of fat removal.* Plast Reconstr Surg, 2006. **118**(4): p. 1048-57; discussion 1058-9.

30. Yudkin, J.S., et al., *C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue?* Arterioscler Thromb Vasc Biol, 1999. **19**(4): p. 972-8.

31. Ziccardi, P., et al., *Reduction of inflammatory cytokine concentrations and improvement of endothelial functions in obese women after weight loss over one year.* Circulation, 2002. **105**(7): p. 804-9.

32. Fain, J.N., et al., *Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans.* Endocrinology, 2004. **145**(5): p. 2273-82.

33. Carswell, E.A., et al., *An endotoxin-induced serum factor that causes necrosis of tumors.* Proc Natl Acad Sci U S A, 1975. **72**(9): p. 3666-70.

34. Ruan, H. and H.F. Lodish, *Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-alpha.* Cytokine Growth Factor Rev, 2003. **14**(5): p. 447-55.

35. Chen, G. and D.V. Goeddel, *TNF-R1 signaling: a beautiful pathway.* Science, 2002. **296**(5573): p. 1634-5.

36. Hotamisligil, G.S., N.S. Shargill, and B.M. Spiegelman, *Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance.* Science, 1993. **259**(5091): p. 87-91.

37. Hotamisligil, G.S., et al., *Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance.* J Clin Invest, 1995. **95**(5): p. 2409-15.

38. Hotamisligil, G.S., et al., *Tumor necrosis factor alpha inhibits signaling from the insulin receptor.* Proc Natl Acad Sci U S A, 1994. **91**(11): p. 4854-8.

39. Lang, C.H., C. Dobrescu, and G.J. Bagby, *Tumor necrosis factor impairs insulin action on peripheral glucose disposal and hepatic glucose output.* Endocrinology, 1992. **130**(1): p. 43-52.

40. Uysal, K.T., et al., *Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function.* Nature, 1997. **389**(6651): p. 610-4.

41. Ofei, F., et al., *Effects of an engineered human anti-TNF-alpha antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM.* Diabetes, 1996. **45**(7): p. 881-5.

42. Ruan, H., et al., *Profiling gene transcription in vivo reveals adipose tissue as an immediate target of tumor necrosis factor-alpha: implications for insulin resistance.* Diabetes, 2002. **51**(11): p. 3176-88.

43. Moinat, M., et al., *Modulation of obese gene expression in rat brown and white adipose tissues.* FEBS Lett, 1995. **373**(2): p. 131-4.

44. Cinti, S., et al., *Immunohistochemical localization of leptin and uncoupling protein in white and brown adipose tissue.* Endocrinology, 1997. **138**(2): p. 797-804.

45. Chen, H., et al., *Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice.* Cell, 1996. **84**(3): p. 491-5.

46. Margetic, S., et al., *Leptin: a review of its peripheral actions and interactions.* Int J Obes Relat Metab Disord, 2002. **26**(11): p. 1407-33.

47. Dubuc, P.U., *The development of obesity, hyperinsulinemia, and hyperglycemia in ob/ob mice.* Metabolism, 1976. **25**(12): p. 1567-74.

48. Kieffer, T.J., R.S. Heller, and J.F. Habener, *Leptin receptors expressed on pancreatic beta-cells.* Biochem Biophys Res Commun, 1996. **224**(2): p. 522-7.

49. Emilsson, V., et al., *Expression of the functional leptin receptor mRNA in pancreatic islets and direct inhibitory action of leptin on insulin secretion.* Diabetes, 1997. **46**(2): p. 313-6.

50. Poitout, V., et al., *Inhibition of insulin secretion by leptin in normal rodent islets of Langerhans.* Endocrinology, 1998. **139**(3): p. 822-6.

51. Kulkarni, R.N., et al., *Leptin rapidly suppresses insulin release from insulinoma cells, rat and human islets and, in vivo, in mice.* J Clin Invest, 1997. **100**(11): p. 2729-36.

52. Utriainen, T., et al., *Supraphysiological hyperinsulinemia increases plasma leptin concentrations after 4 h in normal subjects.* Diabetes, 1996. **45**(10): p. 1364-6.

53. Paz-Filho, G., C.A. Mastronardi, and J. Licinio, *Leptin treatment: facts and expectations.* Metabolism, 2015. **64**(1): p. 146-56.

54. Xu, Y. and Q. Tong, *Central leptin action on euglycemia restoration in type 1 diabetes: Restraining responses normally induced by fasting?* Int J Biochem Cell Biol, 2017. **88**: p. 198-203.

55. Frühbeck, G., et al., *Normalization of adiponectin concentrations by leptin replacement in ob/ob mice is accompanied by reductions in systemic oxidative stress and inflammation.* Sci Rep, 2017. **7**(1): p. 2752.

56. Mantzoros, C.S., et al., *Leptin concentrations in relation to body mass index and the tumor necrosis factor-alpha system in humans.* J Clin Endocrinol Metab, 1997. **82**(10): p. 3408-13.

57. Grunfeld, C., et al., *Endotoxin and cytokines induce expression of leptin, the ob gene product, in hamsters.* J Clin Invest, 1996. **97**(9): p. 2152-7.

58. Chandran, M., et al., *Adiponectin: more than just another fat cell hormone?* Diabetes Care, 2003. **26**(8): p. 2442-50.

59. Arita, Y., et al., *Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. 1999.* Biochem Biophys Res Commun, 2012. **425**(3): p. 560-4.

60. Hotta, K., et al., *Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients.* Arterioscler Thromb Vasc Biol, 2000. **20**(6): p. 1595-9.

61. Berg, A.H., et al., *The adipocyte-secreted protein Acrp30 enhances hepatic insulin action.* Nat Med, 2001. **7**(8): p. 947-53.

62. Hotta, K., et al., *Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in rhesus monkeys.* Diabetes, 2001. **50**(5): p. 1126-33.

63. Yu, J.G., et al., *The effect of thiazolidinediones on plasma adiponectin levels in normal, obese, and type 2 diabetic subjects.* Diabetes, 2002. **51**(10): p. 2968-74.

64. Fisher, F.M., et al., *Differences in adiponectin protein expression: effect of fat depots and type 2 diabetic status.* Horm Metab Res, 2002. **34**(11-12): p. 650-4.

65. Hetherington, A.W. and S.W. Ranson, *Experimental Hypothalamico-Hypophyseal Obesity in the Rat.* Experimental Biology and Medicine, 1939. **41**(2): p. 465-466.

66. Cone, R.D., et al., *The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis.* Int J Obes Relat Metab Disord, 2001. **25 Suppl 5**: p. S63-7.

67. Stanley, B.G., et al., *Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity.* Peptides, 1986. **7**(6): p. 1189-92.

68. Billington, C.J., et al., *Effects of intracerebroventricular injection of neuropeptide Y on energy metabolism.* Am J Physiol, 1991. **260**(2 Pt 2): p. R321-7.

69. Lambert, P.D., et al., *CART peptides in the central control of feeding and interactions with neuropeptide Y.* Synapse, 1998. **29**(4): p. 293-8.

70. Fan, W., et al., *Role of melanocortinergic neurons in feeding and the agouti obesity syndrome.* Nature, 1997. **385**(6612): p. 165-8.

71. Pierroz, D.D., et al., *Effects of acute and chronic administration of the melanocortin agonist MTII in mice with diet-induced obesity.* Diabetes, 2002. **51**(5): p. 1337-45.

72. Kalra, S.P., et al., *Interacting appetite-regulating pathways in the hypothalamic regulation of body weight.* Endocr Rev, 1999. **20**(1): p. 68-100.

73. Peyron, C., et al., *Neurons containing hypocretin (orexin) project to multiple neuronal systems.* J Neurosci, 1998. **18**(23): p. 9996-10015.

74. van der Kooy, D., et al., *The organization of projections from the cortex, amygdala, and hypothalamus to the nucleus of the solitary tract in rat.* J Comp Neurol, 1984. **224**(1): p. 1-24.

75. Berthoud, H.R., *The vagus nerve, food intake and obesity.* Regul Pept, 2008. **149**(1-3): p. 15-25.

76. Eram, M. and W.C. Michel, *Heterogeneous distribution of taste cells in facial and vagal nerve-innervated taste buds.* Neuroscience, 2006. **138**(1): p. 339-50.

77. Berthoud, H.R., P.A. Lynn, and L.A. Blackshaw, *Vagal and spinal mechanosensors in the rat stomach and colon have multiple receptive fields.* Am J Physiol Regul Integr Comp Physiol, 2001. **280**(5): p. R1371-81.

78. Buyse, M., et al., *Expression and regulation of leptin receptor proteins in afferent and efferent neurons of the vagus nerve.* Eur J Neurosci, 2001. **14**(1): p. 64-72.

79. Date, Y., et al., *The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats.* Gastroenterology, 2002. **123**(4): p. 1120-8.

80. Lowe, M.R., et al., *Comparison of verbal and pictorial measures of hunger during fasting in normal weight and obese subjects.* Obes Res, 2000. **8**(8): p. 566-74.

81. la Fleur, S.E., et al., *A free-choice high-fat high-sugar diet induces changes in arcuate neuropeptide expression that support hyperphagia.* Int J Obes (Lond), 2010. **34**(3): p. 537-46.

82. Radhakishun, F.S., J.M. van Ree, and B.H. Westerink, *Scheduled eating increases dopamine release in the nucleus accumbens of food-deprived rats as assessed with on-line brain dialysis.* Neurosci Lett, 1988. **85**(3): p. 351-6.

83. Gosnell, B.A. and M.J. Majchrzak, *Centrally administered opioid peptides stimulate saccharin intake in nondeprived rats.* Pharmacol Biochem Behav, 1989. **33**(4): p. 805-10.

84. Yeomans, M.R., et al., *Effects of nalmefene on feeding in humans. Dissociation of hunger and palatability.* Psychopharmacology (Berl), 1990. **100**(3): p. 426-32.

85. Hagan, S. and K.D. Niswender, *Neuroendocrine regulation of food intake.* Pediatr Blood Cancer, 2012. **58**(1): p. 149-53.

86. Neary, N.M., A.P. Goldstone, and S.R. Bloom, *Appetite regulation: from the gut to the hypothalamus.* Clin Endocrinol (Oxf), 2004. **60**(2): p. 153-60.

87. Suzuki, K., C.N. Jayasena, and S.R. Bloom, *Obesity and appetite control.* Exp Diabetes Res, 2012. **2012**: p. 824305.

88. Yu, Y.H., et al., *Metabolic vs. hedonic obesity: a conceptual distinction and its clinical implications.* Obes Rev, 2015. **16**(3): p. 234-47.

89. Ekblad, E. and F. Sundler, *Distribution of pancreatic polypeptide and peptide YY.* Peptides, 2002. **23**(2): p. 251-61.

90. Adrian, T.E., et al., *Human distribution and release of a putative new gut hormone, peptide YY.* Gastroenterology, 1985. **89**(5): p. 1070-7.

91. Batterham, R.L., et al., *Gut hormone PYY(3-36) physiologically inhibits food intake.* Nature, 2002. **418**(6898): p. 650-4.

92. Sloth, B., et al., *Effects of PYY1-36 and PYY3-36 on appetite, energy intake, energy expenditure, glucose and fat metabolism in obese and lean subjects.* Am J Physiol Endocrinol Metab, 2007. **292**(4): p. E1062-8.

93. Asakawa, A., et al., *Characterization of the effects of pancreatic polypeptide in the regulation of energy balance.* Gastroenterology, 2003. **124**(5): p. 1325-36.

94. Kojima, S., et al., *A role for pancreatic polypeptide in feeding and body weight regulation.* Peptides, 2007. **28**(2): p. 459-63.

95. Sinclair, E.M. and D.J. Drucker, *Proglucagon-derived peptides: mechanisms of action and therapeutic potential.* Physiology (Bethesda), 2005. **20**: p. 357-65.

96. Bagger, J.I., et al., *Effect of Oxyntomodulin, Glucagon, GLP-1, and Combined Glucagon +GLP-1 Infusion on Food Intake, Appetite, and Resting Energy Expenditure.* J Clin Endocrinol Metab, 2015. **100**(12): p. 4541-52.

97. Parker, J.A., et al., *Glucagon and GLP-1 inhibit food intake and increase c-fos expression in similar appetite regulating centres in the brainstem and amygdala.* Int J Obes (Lond), 2013. **37**(10): p. 1391-8.

98. Pocai, A., et al., *Glucagon-like peptide 1/glucagon receptor dual agonism reverses obesity in mice.* Diabetes, 2009. **58**(10): p. 2258-66.

99. Wei, Y. and S. Mojsov, *Tissue-specific expression of the human receptor for glucagon-like peptide-I: brain, heart and pancreatic forms have the same deduced amino acid sequences.* FEBS Lett, 1995. **358**(3): p. 219-24.

100. Shughrue, P.J., M.V. Lane, and I. Merchenthaler, *Glucagon-like peptide-1 receptor (GLP1-R) mRNA in the rat hypothalamus.* Endocrinology, 1996. **137**(11): p. 5159-62.

101. Cummings, D.E. and J. Overduin, *Gastrointestinal regulation of food intake.* J Clin Invest, 2007. **117**(1): p. 13-23.

102. Gibbs, J., R.C. Young, and G.P. Smith, *Cholecystokinin decreases food intake in rats.* J Comp Physiol Psychol, 1973. **84**(3): p. 488-95.

103. Liddle, R.A., et al., *Cholecystokinin bioactivity in human plasma. Molecular forms, responses to feeding, and relationship to gallbladder contraction.* J Clin Invest, 1985. **75**(4): p. 1144-52.

104. Wank, S.A., *Cholecystokinin receptors.* Am J Physiol, 1995. **269**(5 Pt 1): p. G628-46.

105. Bjørbaek, C. and B.B. Kahn, *Leptin signaling in the central nervous system and the periphery.* Recent Prog Horm Res, 2004. **59**: p. 305-31.

106. Halaas, J.L., et al., *Weight-reducing effects of the plasma protein encoded by the obese gene.* Science, 1995. **269**(5223): p. 543-6.

107. Farooqi, I.S., et al., *Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency.* J Clin Invest, 2002. **110**(8): p. 1093-103.

108. Munzberg, H., *Leptin-signaling pathways and leptin resistance.* Forum Nutr, 2010. **63**: p. 123-32.

109. Polonsky, K.S., B.D. Given, and E. Van Cauter, *Twenty-four-hour profiles and pulsatile patterns of insulin secretion in normal and obese subjects.* J Clin Invest, 1988. **81**(2): p. 442-8.

110. Corp, E.S., et al., *Localization of 125I-insulin binding sites in the rat hypothalamus by quantitative autoradiography.* Neurosci Lett, 1986. **70**(1): p. 17-22.

111. Air, E.L., et al., *Acute third ventricular administration of insulin decreases food intake in two paradigms.* Pharmacol Biochem Behav, 2002. **72**(1-2): p. 423-9.

112. Gonzàlez, J.A., F. Reimann, and D. Burdakov, *Dissociation between sensing and metabolism of glucose in sugar sensing neurones.* J Physiol, 2009. **587**(1): p. 41-8.

113. Kojima, M., et al., *Ghrelin is a growth-hormone-releasing acylated peptide from stomach.* Nature, 1999. **402**(6762): p. 656-60.

114. Guan, X.M., et al., *Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues.* Brain Res Mol Brain Res, 1997. **48**(1): p. 23-9.

115. Date, Y., et al., *Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans.* Endocrinology, 2000. **141**(11): p. 4255-61.

116. Gnanapavan, S., et al., *The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans.* J Clin Endocrinol Metab, 2002. **87**(6): p. 2988.

117. Sakata, I., et al., *Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract.* Peptides, 2002. **23**(3): p. 531-6.

118. Zhao, C.M., et al., *Characterization of obestatin- and ghrelin-producing cells in the gastrointestinal tract and pancreas of rats: an immunohistochemical and electron-microscopic study.* Cell Tissue Res, 2008. **331**(3): p. 575-87.

119. Sakata, I., et al., *Localization of ghrelin-producing cells in the stomach of the rainbow trout (Oncorhynchus mykiss).* Zoolog Sci, 2004. **21**(7): p. 757-62.

120. Maksud, F.A., et al., *Density of ghrelin-producing cells is higher in the gastric mucosa of morbidly obese patients.* Eur J Endocrinol, 2011. **165**(1): p. 57-62.

121. Zhu, X., et al., *On the Processing of Proghrelin to Ghrelin.* Journal of Biological Chemistry, 2006. **281**(50): p. 38867-38870.

122. Dhanvantari, S., *The genetics of obesity meets basic cell biology through prohormone convertase 1/3.* Endocrinology, 2014. **155**(7): p. 2343-5.

123. Yang, J., et al., *Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone.* Cell, 2008. **132**(3): p. 387-96.

124. Gutierrez, J.A., et al., *Ghrelin octanoylation mediated by an orphan lipid transferase.* Proc Natl Acad Sci U S A, 2008. **105**(17): p. 6320-5.

125. Kojima, M. and K. Kangawa, *Ghrelin: structure and function.* Physiol Rev, 2005. **85**(2): p. 495-522.

126. Dezaki, K., et al., *Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca2+ signaling in beta-cells: implication in the glycemic control in rodents.* Diabetes, 2004. **53**(12): p. 3142-51.

127. Hosoda, H., et al., *Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue.* Biochem Biophys Res Commun, 2001. **279**(3): p. 909-13.

128. Sun, Y., J.M. Garcia, and R.G. Smith, *Ghrelin and growth hormone secretagogue receptor expression in mice during aging.* Endocrinology, 2007. **148**(3): p. 1323-9.

129. Chakrabarti, S., *Unacylated ghrelin: a gut-limb connection.* Diabetes, 2015. **64**(4): p. 1097-8.

130. Satou, M. and H. Sugimoto, *The study of ghrelin deacylation enzymes.* Methods Enzymol, 2012. **514**: p. 165-79.

131. Baragli, A., et al., *Neuroendocrine and metabolic activities of ghrelin gene products.* Peptides, 2011. **32**(11): p. 2323-32.

132. Zhang, J.V., et al., *Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake.* Science, 2005. **310**(5750): p. 996-9.

133. Nogueiras, R., et al., *Effects of obestatin on energy balance and growth hormone secretion in rodents.* Endocrinology, 2006. **148**(1): p. 21-6.

134. Seoane, L.M., et al., *Central obestatin administration does not modify either spontaneous or ghrelin-induced food intake in rats.* J Endocrinol Invest, 2006. **29**(8): p. RC13-5.

135. Yin, Y., Y. Li, and W. Zhang, *The growth hormone secretagogue receptor: its intracellular signaling and regulation.* Int J Mol Sci, 2014. **15**(3): p. 4837-55.

136. Tong, J., et al., *Ghrelin suppresses glucose-stimulated insulin secretion and deteriorates glucose tolerance in healthy humans.* Diabetes, 2010. **59**(9): p. 2145-51.

137. Andrews, Z.B., *Central mechanisms involved in the orexigenic actions of ghrelin.* Peptides, 2011. **32**(11): p. 2248-55.

138. Briggs, D.I. and Z.B. Andrews, *Metabolic status regulates ghrelin function on energy homeostasis.* Neuroendocrinology, 2011. **93**(1): p. 48-57.

139. Kohno, D., et al., *Ghrelin raises [Ca2+]i via AMPK in hypothalamic arcuate nucleus NPY neurons.* Biochem Biophys Res Commun, 2008. **366**(2): p. 388-92.

140. Luquet, S., et al., *NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates.* Science, 2005. **310**(5748): p. 683-5.

141. Kurashina, T., et al., *The β-cell GHSR and downstream cAMP/TRPM2 signaling account for insulinostatic and glycemic effects of ghrelin.* Sci Rep, 2015. **5**: p. 14041.

142. Dezaki, K., M. Kakei, and T. Yada, *Ghrelin uses Galphai2 and activates voltage-dependent K+ channels to attenuate glucose-induced Ca2+ signaling and insulin release in islet beta-cells: novel signal transduction of ghrelin.* Diabetes, 2007. **56**(9): p. 2319-27.

143. Dezaki, K., et al., *Ghrelin attenuates cAMP-PKA signaling to evoke insulinostatic cascade in islet beta-cells.* Diabetes, 2011. **60**(9): p. 2315-24.

144. Hartman, M.L., et al., *Temporal structure of in vivo growth hormone secretory events in humans.* Am J Physiol, 1991. **260**(1 Pt 1): p. E101-10.

145. Thorner, M.O., et al., *Growth hormone-releasing hormone and growth hormone-releasing peptide as therapeutic agents to enhance growth hormone secretion in disease and aging.* Recent Prog Horm Res, 1997. **52**: p. 215-44; discussion 244-6.

146. Frohman, L.A., et al., *Measurement of growth hormone-releasing hormone and somatostatin in hypothalamic-portal plasma of unanesthetized sheep. Spontaneous secretion and response to insulin-induced hypoglycemia.* J Clin Invest, 1990. **86**(1): p. 17-24.

147. Nass, R., B.D. Gaylinn, and M.O. Thorner, *The role of ghrelin in GH secretion and GH disorders.* Mol Cell Endocrinol, 2011. **340**(1): p. 10-4.

148. Maheshwari, H.G., et al., *Pulsatile growth hormone secretion persists in genetic growth hormone-releasing hormone resistance.* Am J Physiol Endocrinol Metab, 2002. **282**(4): p. E943-51.

149. Pantel, J., et al., *Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature.* J Clin Invest, 2006. **116**(3): p. 760-8.

150. Mano-Otagiri, A., et al., *Growth hormone-releasing hormone (GHRH) neurons in the arcuate nucleus (Arc) of the hypothalamus are decreased in transgenic rats whose expression of ghrelin receptor is attenuated: Evidence that ghrelin receptor is involved in the up-regulation of GHRH expression in the arc.* Endocrinology, 2006. **147**(9): p. 4093-103.

151. Nass, R., et al., *Evidence for acyl-ghrelin modulation of growth hormone release in the fed state.* J Clin Endocrinol Metab, 2008. **93**(5): p. 1988-94.

152. Nass, R., et al., *Effects of an oral ghrelin mimetic on body composition and clinical outcomes in healthy older adults: a randomized trial.* Ann Intern Med, 2008. **149**(9): p. 601-11.

153. Zizzari, P., et al., *Endogenous ghrelin regulates episodic growth hormone (GH) secretion by amplifying GH Pulse amplitude: evidence from antagonism of the GH secretagogue-R1a receptor.* Endocrinology, 2005. **146**(9): p. 3836-42.

154. Tschop, M., D.L. Smiley, and M.L. Heiman, *Ghrelin induces adiposity in rodents.* Nature, 2000. **407**(6806): p. 908-13.

155. Tschop, M., et al., *Post-prandial decrease of circulating human ghrelin levels.* J Endocrinol Invest, 2001. **24**(6): p. RC19-21.

156. Cummings, D.E., et al., *A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans.* Diabetes, 2001. **50**(8): p. 1714-9.

157. Burman, P., E.M. Ritzén, and A.C. Lindgren, *Endocrine dysfunction in Prader-Willi syndrome: a review with special reference to GH.* Endocr Rev, 2001. **22**(6): p. 787-99.

158. Nakazato, M., et al., *A role for ghrelin in the central regulation of feeding.* Nature, 2001. **409**(6817): p. 194-8.

159. Sun, Y., et al., *Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor.* Proc Natl Acad Sci U S A, 2004. **101**(13): p. 4679-84.

160. Asakawa, A., et al., *Antagonism of ghrelin receptor reduces food intake and body weight gain in mice.* Gut, 2003. **52**(7): p. 947-52.

161. Pfluger, P.T., et al., *Simultaneous deletion of ghrelin and its receptor increases motor activity and energy expenditure.* Am J Physiol Gastrointest Liver Physiol, 2008. **294**(3): p. G610-8.

162. Zigman, J.M., S.G. Bouret, and Z.B. Andrews, *Obesity Impairs the Action of the Neuroendocrine Ghrelin System.* Trends Endocrinol Metab, 2016. **27**(1): p. 54-63.

163. Tschop, M., et al., *Circulating ghrelin levels are decreased in human obesity.* Diabetes, 2001. **50**(4): p. 707-9.

164. Shiiya, T., et al., *Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion.* J Clin Endocrinol Metab, 2002. **87**(1): p. 240-4.

165. le Roux, C.W., et al., *Postprandial plasma ghrelin is suppressed proportional to meal calorie content in normal-weight but not obese subjects.* J Clin Endocrinol Metab, 2005. **90**(2): p. 1068-71.

166. Mittelman, S.D., et al., *Obese adolescents show impaired meal responses of the appetite-regulating hormones ghrelin and PYY.* Obesity (Silver Spring), 2010. **18**(5): p. 918-25.

167. McLaughlin, T., et al., *Plasma ghrelin concentrations are decreased in insulin-resistant obese adults relative to equally obese insulin-sensitive controls.* J Clin Endocrinol Metab, 2004. **89**(4): p. 1630-5.

168. Bacha, F. and S.A. Arslanian, *Ghrelin suppression in overweight children: a manifestation of insulin resistance?* J Clin Endocrinol Metab, 2005. **90**(5): p. 2725-30.

169. Broglio, F., et al., *Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans.* J Clin Endocrinol Metab, 2001. **86**(10): p. 5083-6.

170. Date, Y., et al., *Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion.* Diabetes, 2002. **51**(1): p. 124-9.

171. Volante, M., et al., *Expression of ghrelin and of the GH secretagogue receptor by pancreatic islet cells and related endocrine tumors.* J Clin Endocrinol Metab, 2002. **87**(3): p. 1300-8.

172. Wierup, N., et al., *The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas.* Regul Pept, 2002. **107**(1-3): p. 63-9.

173. Dezaki, K., et al., *Blockade of pancreatic islet-derived ghrelin enhances insulin secretion to prevent high-fat diet-induced glucose intolerance.* Diabetes, 2006. **55**(12): p. 3486-93.

174. Dezaki, K., H. Sone, and T. Yada, *Ghrelin is a physiological regulator of insulin release in pancreatic islets and glucose homeostasis.* Pharmacol Ther, 2008. **118**(2): p. 239-49.

175. Heijboer, A.C., et al., *Ghrelin differentially affects hepatic and peripheral insulin sensitivity in mice.* Diabetologia, 2006. **49**(4): p. 732-8.

176. Gauna, C., et al., *Ghrelin stimulates, whereas des-octanoyl ghrelin inhibits, glucose output by primary hepatocytes.* J Clin Endocrinol Metab, 2005. **90**(2): p. 1055-60.

177. Barazzoni, R., et al., *Ghrelin enhances in vivo skeletal muscle but not liver AKT signaling in rats.* Obesity (Silver Spring), 2007. **15**(11): p. 2614-23.

178. Scott, M.M., et al., *Hindbrain ghrelin receptor signaling is sufficient to maintain fasting glucose.* PLoS One, 2012. **7**(8): p. e44089.

179. Zhao, T.J., et al., *Ghrelin O-acyltransferase (GOAT) is essential for growth hormone-mediated survival of calorie-restricted mice.* Proc Natl Acad Sci U S A, 2010. **107**(16): p. 7467-72.

180. Tong, J., et al., *Physiologic concentrations of exogenously infused ghrelin reduces insulin secretion without affecting insulin sensitivity in healthy humans.* J Clin Endocrinol Metab, 2013. **98**(6): p. 2536-43.

181. Tong, J., et al., *Acute administration of unacylated ghrelin has no effect on Basal or stimulated insulin secretion in healthy humans.* Diabetes, 2014. **63**(7): p. 2309-19.

182. Gagnon, J., et al., *Neutralizing circulating ghrelin by expressing a growth hormone secretagogue receptor-based protein protects against high-fat diet-induced obesity in mice.* Gene Ther, 2015. **22**(9): p. 750-7.

183. Park, S., et al., *Modification of ghrelin receptor signaling by somatostatin receptor-5 regulates insulin release.* Proc Natl Acad Sci U S A, 2012. **109**(46): p. 19003-8.

184. Tritos, N.A., et al., *Association between ghrelin and cardiovascular indexes in healthy obese and lean men.* Clin Endocrinol (Oxf), 2004. **60**(1): p. 60-6.

185. Nagaya, N., et al., *Hemodynamic and hormonal effects of human ghrelin in healthy volunteers.* Am J Physiol Regul Integr Comp Physiol, 2001. **280**(5): p. R1483-7.

186. Nagaya, N., et al., *Hemodynamic, renal, and hormonal effects of ghrelin infusion in patients with chronic heart failure.* J Clin Endocrinol Metab, 2001. **86**(12): p. 5854-9.

187. Nagaya, N., et al., *Chronic administration of ghrelin improves left ventricular dysfunction and attenuates development of cardiac cachexia in rats with heart failure.* Circulation, 2001. **104**(12): p. 1430-5.

188. Frascarelli, S., et al., *Effect of ghrelin and synthetic growth hormone secretagogues in normal and ischemic rat heart.* Basic Res Cardiol, 2003. **98**(6): p. 401-5.

189. Mao, Y., et al., *Ghrelin prevents incidence of malignant arrhythmia after acute myocardial infarction through vagal afferent nerves.* Endocrinology, 2012. **153**(7): p. 3426-34.

190. Lilleness, B.M. and W.H. Frishman, *Ghrelin and the Cardiovascular System.* Cardiol Rev, 2016. **24**(6): p. 288-297.

191. Angelidis, G., et al., *Ghrelin: new insights into female reproductive system-associated disorders and pregnancy.* Reprod Sci, 2012. **19**(9): p. 903-10.

192. Nakahara, K., et al., *Maternal ghrelin plays an important role in rat fetal development during pregnancy.* Endocrinology, 2006. **147**(3): p. 1333-42.

193. Furuta, M., T. Funabashi, and F. Kimura, *Intracerebroventricular administration of ghrelin rapidly suppresses pulsatile luteinizing hormone secretion in ovariectomized rats.* Biochem Biophys Res Commun, 2001. **288**(4): p. 780-5.

194. Fernández-Fernández, R., et al., *Effects of ghrelin upon gonadotropin-releasing hormone and gonadotropin secretion in adult female rats: in vivo and in vitro studies.* Neuroendocrinology, 2005. **82**(5-6): p. 245-55.

195. Martini, A.C., et al., *Comparative analysis of the effects of ghrelin and unacylated ghrelin on luteinizing hormone secretion in male rats.* Endocrinology, 2006. **147**(5): p. 2374-82.

196. Kluge, M., et al., *Ghrelin suppresses secretion of luteinizing hormone in humans.* J Clin Endocrinol Metab, 2007. **92**(8): p. 3202-5.

197. Kluge, M., et al., *Ghrelin suppresses secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in women.* J Clin Endocrinol Metab, 2012. **97**(3): p. E448-51.

198. Kawamura, K., et al., *Ghrelin inhibits the development of mouse preimplantation embryos in vitro.* Endocrinology, 2003. **144**(6): p. 2623-33.

199. Cortelazzi, D., et al., *Circulating levels of ghrelin in human fetuses.* Eur J Endocrinol, 2003. **149**(2): p. 111-6.

200. Fuglsang, J., et al., *Ghrelin and its relationship to growth hormones during normal pregnancy.* Clin Endocrinol (Oxf), 2005. **62**(5): p. 554-9.

201. Caufriez, A., et al., *Placental growth hormone as a potential regulator of maternal IGF-I during human pregnancy.* Am J Physiol, 1990. **258**(6 Pt 1): p. E1014-9.

202. Martin, J.R., et al., *Maternal ghrelin deficiency compromises reproduction in female progeny through altered uterine developmental programming.* Endocrinology, 2011. **152**(5): p. 2060-6.

203. Fujiwara, Y., et al., *Long-term effect of Helicobacter pylori eradication on quality of life, body mass index, and newly developed diseases in Japanese patients with peptic ulcer disease.* Hepatogastroenterology, 2002. **49**(47): p. 1298-302.

204. Azuma, T., et al., *Eradication of Helicobacter pylori infection induces an increase in body mass index.* Aliment Pharmacol Ther, 2002. **16 Suppl 2**: p. 240-4.

205. Nwokolo, C.U., et al., *Plasma ghrelin following cure of Helicobacter pylori.* Gut, 2003. **52**(5): p. 637-40.

206. Osawa, H., et al., *Impaired production of gastric ghrelin in chronic gastritis associated with Helicobacter pylori.* J Clin Endocrinol Metab, 2005. **90**(1): p. 10-6.

207. Atalayer, D., et al., *Ghrelin and eating disorders.* Prog Neuropsychopharmacol Biol Psychiatry, 2013. **40**: p. 70-82.

208. Monteleone, P., et al., *Plasma obestatin, ghrelin, and ghrelin/obestatin ratio are increased in underweight patients with anorexia nervosa but not in symptomatic patients with bulimia nervosa.* J Clin Endocrinol Metab, 2008. **93**(11): p. 4418-21.

209. Tolle, V., et al., *Balance in ghrelin and leptin plasma levels in anorexia nervosa patients and constitutionally thin women.* J Clin Endocrinol Metab, 2003. **88**(1): p. 109-16.

210. Holsen, L.M., et al., *Abnormal relationships between the neural response to high- and low-calorie foods and endogenous acylated ghrelin in women with active and weight-recovered anorexia nervosa.* Psychiatry Res, 2014. **223**(2): p. 94-103.

211. Hotta, M., et al., *Ghrelin increases hunger and food intake in patients with restricting-type anorexia nervosa: a pilot study.* Endocr J, 2009. **56**(9): p. 1119-28.

212. Nagaya, N., et al., *Treatment of cachexia with ghrelin in patients with COPD.* Chest, 2005. **128**(3): p. 1187-93.

213. Molfino, A., et al., *Ghrelin: from discovery to cancer cachexia therapy.* Curr Opin Clin Nutr Metab Care, 2014. **17**(5): p. 471-6.

214. Daousi, C., et al., *Endocrine and neuroanatomic features associated with weight gain and obesity in adult patients with hypothalamic damage.* Am J Med, 2005. **118**(1): p. 45-50.

215. Andrusiewicz, M., et al., *Expression of ghrelin and ghrelin functional receptor GHSR1a in human pituitary adenomas.* Pol Arch Intern Med, 2017. **127**(3): p. 163-169.

216. O'Gorman, C.S., et al., *Delayed ghrelin suppression following oral glucose tolerance test in children and adolescents with hypothalamic injury secondary to craniopharyngioma compared with obese controls.* Int J Pediatr Obes, 2011. **6**(3-4): p. 285-8.

217. Williams, D.L., et al., *Meal-related ghrelin suppression requires postgastric feedback.* Endocrinology, 2003. **144**(7): p. 2765-7.

218. Callahan, H.S., et al., *Postprandial suppression of plasma ghrelin level is proportional to ingested caloric load but does not predict intermeal interval in humans.* J Clin Endocrinol Metab, 2004. **89**(3): p. 1319-24.

219. Monteleone, P., et al., *Differential responses of circulating ghrelin to high-fat or high-carbohydrate meal in healthy women.* J Clin Endocrinol Metab, 2003. **88**(11): p. 5510-4.

220. Sanchez, J., et al., *The inhibition of gastric ghrelin production by food intake in rats is dependent on the type of macronutrient.* Endocrinology, 2004. **145**(11): p. 5049-55.

221. Bowen, J., M. Noakes, and P.M. Clifton, *Appetite regulatory hormone responses to various dietary proteins differ by body mass index status despite similar reductions in ad libitum energy intake.* J Clin Endocrinol Metab, 2006. **91**(8): p. 2913-9.

222. Brennan, I.M., et al., *Effects of fat, protein, and carbohydrate and protein load on appetite, plasma cholecystokinin, peptide YY, and ghrelin, and energy intake in lean and obese men.* Am J Physiol Gastrointest Liver Physiol, 2012. **303**(1): p. G129-40.

223. Ma, J., et al., *Effects of a protein preload on gastric emptying, glycemia, and gut hormones after a carbohydrate meal in diet-controlled type 2 diabetes.* Diabetes Care, 2009. **32**(9): p. 1600-2.

224. Cummings, D.E., et al., *Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery.* N Engl J Med, 2002. **346**(21): p. 1623-30.

225. Cummings, D.E. and M.H. Shannon, *Ghrelin and gastric bypass: is there a hormonal contribution to surgical weight loss?* J Clin Endocrinol Metab, 2003. **88**(7): p. 2999-3002.

226. Overduin, J., et al., *Role of the duodenum and macronutrient type in ghrelin regulation.* Endocrinology, 2004. **146**(2): p. 845-50.

227. Foster-Schubert, K.E., et al., *Acyl and Total Ghrelin Are Suppressed Strongly by Ingested Proteins, Weakly by Lipids, and Biphasically by Carbohydrates.* The Journal of Clinical Endocrinology & Metabolism, 2008. **93**(5): p. 1971-1979.

228. Roberts, S.B., *High-glycemic index foods, hunger, and obesity: is there a connection?* Nutr Rev, 2000. **58**(6): p. 163-9.

229. Shrestha, Y.B., K. Wickwire, and S.Q. Giraudo, *Direct effects of nutrients, acetylcholine, CCK, and insulin on ghrelin release from the isolated stomachs of rats.* Peptides, 2009. **30**(6): p. 1187-91.

230. Muller, T.D., et al., *Ghrelin.* Mol Metab, 2015. **4**(6): p. 437-60.

231. Stengel, A. and Y. Taché, *Interaction between gastric and upper small intestinal hormones in the regulation of hunger and satiety: ghrelin and cholecystokinin take the central stage.* Curr Protein Pept Sci, 2011. **12**(4): p. 293-304.

232. Zhao, T.J., et al., *Ghrelin secretion stimulated by {beta}1-adrenergic receptors in cultured ghrelinoma cells and in fasted mice.* Proc Natl Acad Sci U S A, 2010. **107**(36): p. 15868-73.

233. Iwakura, H., et al., *Establishment of a novel ghrelin-producing cell line.* Endocrinology, 2010. **151**(6): p. 2940-5.

234. Gagnon, J., E. Sheppard, and Y. Anini, *Metformin directly inhibits ghrelin secretion through AMP-activated protein kinase in rat primary gastric cells.* Diabetes Obes Metab, 2012. **15**(3): p. 276-9.

235. Mundinger, T.O., D.E. Cummings, and G.J. Taborsky, *Direct stimulation of ghrelin secretion by sympathetic nerves.* Endocrinology, 2006. **147**(6): p. 2893-901.

236. Brunetti, L., et al., *Effects of ghrelin and amylin on dopamine, norepinephrine and serotonin release in the hypothalamus.* Eur J Pharmacol, 2002. **454**(2-3): p. 189-92.

237. Browning, K.N., S. Verheijden, and G.E. Boeckxstaens, *The Vagus Nerve in Appetite Regulation, Mood, and Intestinal Inflammation.* Gastroenterology, 2017. **152**(4): p. 730-744.

238. Maier, C., et al., *The cholinergic system controls ghrelin release and ghrelin-induced growth hormone release in humans.* J Clin Endocrinol Metab, 2004. **89**(9): p. 4729-33.

239. Williams, D.L., et al., *Vagotomy dissociates short- and long-term controls of circulating ghrelin.* Endocrinology, 2003. **144**(12): p. 5184-7.

240. Chen, M.C., et al., *Bitter stimuli induce Ca2+ signaling and CCK release in enteroendocrine STC-1 cells: role of L-type voltage-sensitive Ca2+ channels.* Am J Physiol Cell Physiol, 2006. **291**(4): p. C726-39.

241. Janssen, S., et al., *Bitter taste receptors and α-gustducin regulate the secretion of ghrelin with functional effects on food intake and gastric emptying.* Proc Natl Acad Sci U S A, 2011. **108**(5): p. 2094-9.

242. Laermans, J., et al., *Role of the clock gene Bmal1 and the gastric ghrelin-secreting cell in the circadian regulation of the ghrelin-GOAT system.* Sci Rep, 2015. **5**: p. 16748.

243. Horvath, T.L., et al., *Ghrelin-immunopositive hypothalamic neurons tie the circadian clock and visual system to the lateral hypothalamic arousal center.* Mol Metab, 2012. **1**(1-2): p. 79-85.

244. Dardzińska, J.A., et al., *Fasting and postprandial acyl and desacyl ghrelin levels in obese and non-obese subjects.* Endokrynol Pol, 2014. **65**(5): p. 377-81.

245. Arafat, M.A., et al., *Glucagon inhibits ghrelin secretion in humans.* Eur J Endocrinol, 2005. **153**(3): p. 397-402.

246. Soule, S., et al., *Prandial regulation of ghrelin secretion in humans: does glucagon contribute to the preprandial increase in circulating ghrelin?* Clin Endocrinol (Oxf), 2005. **63**(4): p. 412-7.

247. Lin, S.D., et al., *[Effects of glucagon on plasma ghrelin level in type 2 diabetes mellitus].* Zhonghua Yi Xue Za Zhi, 2009. **89**(14): p. 967-9.

248. Sakata, I., et al., *Glucose-mediated control of ghrelin release from primary cultures of gastric mucosal cells.* Am J Physiol Endocrinol Metab, 2012. **302**(10): p. E1300-10.

249. Gagnon, J. and Y. Anini, *Glucagon Stimulates Ghrelin Secretion Through the Activation of MAPK and EPAC and Potentiates the Effect of Norepinephrine.* Endocrinology, 2013. **154**(2): p. 666-74.

250. Chuang, J.C., et al., *Ghrelin directly stimulates glucagon secretion from pancreatic alpha-cells.* Mol Endocrinol, 2011. **25**(9): p. 1600-11.

251. Ritze, Y., et al., *Gastric ghrelin, GOAT, leptin, and leptinR expression as well as peripheral serotonin are dysregulated in humans with obesity.* Neurogastroenterol Motil, 2016. **28**(6): p. 806-15.

252. Sato, T., et al., *Physiological roles of ghrelin on obesity.* Obes Res Clin Pract, 2014. **8**(5): p. e405-13.

253. Briggs, D.I., et al., *Diet-induced obesity causes ghrelin resistance in arcuate NPY/AgRP neurons.* Endocrinology, 2010. **151**(10): p. 4745-55.

254. Fusco, A., et al., *Effects of ghrelin administration on endocrine and metabolic parameters in obese women with polycystic ovary syndrome.* J Endocrinol Invest, 2007. **30**(11): p. 948-56.

255. Alvarez-Castro, P., et al., *Effect of acute ghrelin administration on glycaemia and insulin levels in obese patients.* Diabetes Obes Metab, 2006. **8**(5): p. 555-60.

256. Pacifico, L., et al., *Acylated and nonacylated ghrelin levels and their associations with insulin resistance in obese and normal weight children with metabolic syndrome.* Eur J Endocrinol, 2009. **161**(6): p. 861-70.

257. Churm, R., et al., *Ghrelin function in human obesity and type 2 diabetes: a concise review.* Obes Rev, 2017. **18**(2): p. 140-148.

258. Vestergaard, E.T., et al., *Acute peripheral metabolic effects of intraarterial ghrelin infusion in healthy young men.* J Clin Endocrinol Metab, 2011. **96**(2): p. 468-77.

259. Polkowska, A., I. Szczepaniak, and A. Bossowski, *Assessment of Serum Concentrations of Ghrelin, Obestatin, Omentin-1, and Apelin in Children with Type 1 Diabetes.* Biomed Res Int, 2016. **2016**: p. 8379294.

260. Nuttall, F.Q., R.M. Almokayyad, and M.C. Gannon, *The ghrelin and leptin responses to short-term starvation vs a carbohydrate-free diet in men with type 2 diabetes; a controlled, cross-over design study.* Nutr Metab (Lond), 2016. **13**: p. 47.

261. Sönmez, M.F., et al., *The Effects of Long-Term Diabetes on Ghrelin Expression in Rat Stomachs.* Adv Clin Exp Med, 2015. **24**(3): p. 401-7.

262. Masaoka, T., et al., *Enhanced plasma ghrelin levels in rats with streptozotocin-induced diabetes.* FEBS Lett, 2003. **541**(1-3): p. 64-8.

263. Gagnon, J., E. Sheppard, and Y. Anini, *Metformin directly inhibits ghrelin secretion through AMP-activated protein kinase in rat primary gastric cells.* Diabetes Obes Metab, 2013. **15**(3): p. 276-9.

264. Sakata, I., et al., *Characterization of a novel ghrelin cell reporter mouse.* Regul Pept, 2009. **155**(1-3): p. 91-8.

265. Chaudhri, O., C. Small, and S. Bloom, *Gastrointestinal hormones regulating appetite.* Philos Trans R Soc Lond B Biol Sci, 2006. **361**(1471): p. 1187-209.

266. le Roux, C.W., et al., *Postprandial plasma ghrelin is suppressed proportional to meal calorie content in normal-weight but not obese subjects.* J Clin Endocrinol Metab, 2004. **90**(2): p. 1068-71.

267. Mittelman, S.D., et al., *Obese Adolescents Show Impaired Meal Responses of the Appetite-Regulating Hormones Ghrelin and PYY.* Obesity, 2010. **18**(5): p. 918-925.

268. Shiiya, T., et al., *Plasma Ghrelin Levels in Lean and Obese Humans and the Effect of Glucose on Ghrelin Secretion.* The Journal of Clinical Endocrinology & Metabolism, 2002. **87**(1): p. 240-244.

269. Uchida, A., et al., *Altered ghrelin secretion in mice in response to diet-induced obesity and Roux-en-Y gastric bypass.* Mol Metab, 2014. **3**(7): p. 717-30.

270. Briggs, D.I., et al., *Calorie-restricted weight loss reverses high-fat diet-induced ghrelin resistance, which contributes to rebound weight gain in a ghrelin-dependent manner.* Endocrinology, 2013. **154**(2): p. 709-17.

271. Cui, H., M. López, and K. Rahmouni, *The cellular and molecular bases of leptin and ghrelin resistance in obesity.* Nat Rev Endocrinol, 2017. **13**(6): p. 338-351.

272. Huang, L., et al., *Increased acyl ghrelin but decreased total ghrelin and unacyl ghrelin in Chinese Han people with impaired fasting glucose combined with impaired glucose tolerance.* Peptides, 2014. **60**: p. 86-94.

273. Collins, S., et al., *Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics.* Physiol Behav, 2004. **81**(2): p. 243-8.

274. Rebuffé-Scrive, M., et al., *Regional fat distribution and metabolism in a new mouse model (C57BL/6J) of non-insulin-dependent diabetes mellitus.* Metabolism, 1993. **42**(11): p. 1405-9.

275. Morash, M.G., et al., *Tissue distribution and effects of fasting and obesity on the ghrelin axis in mice.* Regul Pept, 2010. **163**(1-3): p. 62-73.

276. Lim, G.E., et al., *Insulin regulates glucagon-like peptide-1 secretion from the enteroendocrine L cell.* Endocrinology, 2009. **150**(2): p. 580-91.

277. Boucher, J., A. Kleinridders, and C.R. Kahn, *Insulin receptor signaling in normal and insulin-resistant states.* Cold Spring Harb Perspect Biol, 2014. **6**(1).

278. Cho, H., et al., *Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta).* Science, 2001. **292**(5522): p. 1728-31.

279. Destefano, M.A. and E. Jacinto, *Regulation of insulin receptor substrate-1 by mTORC2 (mammalian target of rapamycin complex 2).* Biochem Soc Trans, 2013. **41**(4): p. 896-901.

280. Agarwal, S., et al., *AMP-activated Protein Kinase (AMPK) Control of mTORC1 Is p53- and TSC2-independent in Pemetrexed-treated Carcinoma Cells.* J Biol Chem, 2015. **290**(46): p. 27473-86.

281. Nieto-Vazquez, I., et al., *Insulin resistance associated to obesity: the link TNF-alpha.* Arch Physiol Biochem, 2008. **114**(3): p. 183-94.

282. Hotamisligil, G.S., *Inflammatory pathways and insulin action.* Int J Obes Relat Metab Disord, 2003. **27 Suppl 3**: p. S53-5.

283. White, M.F., *Insulin signaling in health and disease.* Science, 2003. **302**(5651): p. 1710-1.

284. Ozes, O.N., et al., *A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1.* Proc Natl Acad Sci U S A, 2001. **98**(8): p. 4640-5.

285. Thaler, J.P., et al., *Obesity is associated with hypothalamic injury in rodents and humans.* J Clin Invest, 2012. **122**(1): p. 153-62.

286. De Souza, C.T., et al., *Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus.* Endocrinology, 2005. **146**(10): p. 4192-9.

287. Naznin, F., et al., *Diet-induced obesity causes peripheral and central ghrelin resistance by promoting inflammation.* J Endocrinol, 2015. **226**(1): p. 81-92.

288. Braunersreuther, V., et al., *Role of cytokines and chemokines in non-alcoholic fatty liver disease.* World J Gastroenterol, 2012. **18**(8): p. 727-35.

289. Copeland, R.J., J.W. Bullen, and G.W. Hart, *Cross-talk between GlcNAcylation and phosphorylation: roles in insulin resistance and glucose toxicity.* Am J Physiol Endocrinol Metab, 2008. **295**(1): p. E17-28.

290. Han, L., et al., *Effects of Ghrelin on Triglyceride Accumulation and Glucose Uptake in Primary Cultured Rat Myoblasts under Palmitic Acid-Induced High Fat Conditions.* Int J Endocrinol, 2015. **2015**: p. 635863.

291. Xu, G., et al., *Ghrelin contributes to derangements of glucose metabolism induced by rapamycin in mice.* Diabetologia, 2012. **55**(6): p. 1813-23.

292. Porter, J.R. and T.G. Barrett, *Monogenic syndromes of abnormal glucose homeostasis: clinical review and relevance to the understanding of the pathology of insulin resistance and β cell failure.* Journal of Medical Genetics, 2005. **42**(12): p. 893.

293. McCracken, K.W., et al., *Modelling human development and disease in pluripotent stem-cell-derived gastric organoids.* Nature, 2014. **516**(7531): p. 400-4.