The Role of Hydrogen Sulfide (H_2S) in Ghrelin Secretion and Appetite

by

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Abstract

Ghrelin is a stomach derived hormone that stimulates appetite. H_2S a gaseous signaling molecule also produced in the stomach has been implicated in the secretion of other metabolic hormones. We hypothesize that H_2S can directly suppress ghrelin secretion leading to a reduction in appetite. We tested this by treating ghrelin producing rat primary stomach culture with the H_2S donor molecule GYY4137 or H_2S synthesis inhibitor PPG. GYY4137 dose-dependently suppressed ghrelin secretion, while increasing cellular levels of phosphorylated AKT/Total AKT. Additionally, fluorescent immunocytochemistry demonstrated of that ghrelin cells co-localize with the H_2S producing enzyme, CSE and inhibition with PPG stimulated ghrelin secretion. In mice, a single IP injection with GYY4137 (30mg/kg) prolonged glucose induced suppression of ghrelin compared to saline control. This lower level of ghrelin was accompanied by a reduction in feeding ($P<0.05$). In conclusion, our data demonstrates that H_2S has a suppressive effect on ghrelin secretion and appetite.

Keywords

Ghrelin, hydrogen sulfide, gasotransmitter, hormone regulation, appetite

Abbreviations

AKT: Protein kinase B

ARC: Arcuate nucleus

BBB: Blood brain barrier

BMI: Body mass index

cAMP: Cyclic adenosine monophosphate

CAT: Cysteine aminotransferase

CBS: Cystathionine β-synthase

CRISPRi: Clustered regularly interspaced short palindromic repeat interference

CSE: Cystathionine γ-lyase

CVD: Cardiovascular disease

ERK: Extracellular signal-regulated kinase

FBS: Fetal bovine serum

GH: Growth Hormone

GHRH: Growth hormone releasing hormone

GHSR: Growth hormone secretagogue receptor

GHSR1a: Growth hormone secretagogue receptor 1a

GI: Gastrointestinal

GLP-1: Glucagon-like peptide-1

GOAT: Ghrelin O-acyl transferase

HDL: High density lipoprotein

IGF-1: Insulin-like growth factor 1

IP: Intraperitoneal

KO: Knockout

MST: 3-mercaptopyruvate sulfurtransferase

mTOR: Mammalian target of rapamycin

NDS: Normal donkey serum

NSAID: Nonsteroidal anti-inflammatory drug

NPY: Neuropeptide Y

P5P: Pyridoxal-5-phosphate

PFA: Paraformaldehyde

PI3K: Phosphoinositide 3-kinase

PKA: Protein kinase A

PPG: DL-propargylglycine

SP: Streptomycin/Penicillin

TBST: TRIS buffered saline

TFA: Trifluoroacetic acid

VEGF: Vascular endothelial growth facotr

Co-Authorship Statement

For the manuscript submitted in chapter 2, I prepared the first draft and edits with guidance and review of Dr. Gagnon.

For the research project shown in chapter 2, I completed all the animal work which included blood collections, IP injections and food measurements. I also completed a majority of the primary culture with the exception of the immunocytochemical stains and western blot data, which was provided by Laura Williams as part of her 4th year thesis project. Dr. Gagnon established the animal protocol and provided training on primary culture and animal handling techniques. Dr. Gagnon also provided the general goal of the project and guidance on writing the manuscripts.

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Introduction

1.1 Metabolic Disease Impacts in Canada

Two of the most prevalent metabolic diseases are obesity and type 2 diabetes mellitus. Obesity is defined as having a body mass index (BMI; height in cm/weight in kg^2) of 30 or higher while being overweight is defined by a BMI above 25. Obesity is associated with risk factors such as cardiovascular disease, high blood pressure, increased plasma triglycerides, hyperglycemia, lower high density lipoprotein (HDL)-cholesterol and an increased risk of developing type 2 diabetes mellitus (1). The latter is characterized by hyperglycemia and the inability for insulin to store glucose in peripheral tissues. Other symptoms associated type 2 diabetes mellitus are polydipsia, polyuria, blurred vision, fatigue, weight change and slow wound healing. Type 2 diabetes mellitus also shares many of the same comorbidities as obesity (2). Both chronic conditions have been increasing in prevalence and a 2015, Statistics Canada study reported that 3 comorbidities of obesity, heart disease, stroke and diabetes mellitus, are 3 of the top 10 leading causes of death for Canadians (3). The Canadian Obesity Network reported that in 2010 one in four adults and one in ten children in Canada were classified as obese. Not only that, but prevalence of this condition was increasing despite an estimated six billion dollars of the health care budget being used to help treat patients suffering from this disease (4). The current strategies used to treat obesity are proving ineffective on a national scale and thus research into the physiology of the digestive and endocrine systems is essential. Understanding how this systems function and interact will further our knowledge of metabolism allowing us to better understand the current health implications of metabolic diseases such as obesity. One such system that plays a major role in metabolic health is the gastrointestinal (GI) tract.

1.2 Gastrointestinal Hormones and Metabolism

The GI tract is responsible for the breakdown and absorption of nutrients for the body to use as energy. This process takes place through mechanical breakdown, enzymatic cleavage, and microbial fermentation of nutrients until they can be absorbed through the intestinal epithelium. The GI tract is comprised of the oral cavity, esophagus, stomach, small intestines, large intestines and rectum (5). Absorption is not the only function of the GI tract. The GI tract is also lined with many cells capable of nutrient sensing and hormone production called enteroendocrine cells (6). These cells sense the composition and quantity of nutrients ingested leading to regulation in the release of gastrointestinal hormones. Gastrointestinal hormones regulate various metabolic processes such as appetite, plasma glucose levels, digestion of nutrients, gut motility, and the storage or utilization of energy (6).

There is a large variety of enteroendocrine cell types found throughout the digestive system which have unique functions. Table 1 below is adapted from (6) and lists many of the enteroendocrine and pancreatic cells, where they are localized, what hormone they produce and secrete and the general effect of that hormone. Hormones send signals throughout the body which alter cells function, metabolism is one such process that is controlled by the regulation of multiple hormones. The hormone ghrelin can alter metabolism and stimulate appetite making it an important hormone to consider in metabolic diseases (6). It is the hormone of interest in this manuscript.

Table 1: Major metabolic hormones (Adapted from (6))

Cell Type	**Cell Localization**	**Major hormone released**	**Main function**
P/D1 cell/ε-cells	(X/A-like cells in rodents) (P/D1 cells in human Stomach) (Epsilon cells in pancreas)	**Ghrelin**	• Appetite control • Growth hormone secretagogue • Energy balance
β-cell	Pancreas	**Insulin**	• Glucose, carbohydrate, fats, and protein uptake in tissues (metabolism/storage)
α-cell	Pancreas	**Glucagon**	• Conversion of glycogen into glucose • Inhibits glycolysis
L-cell	Ileum, colon	**GLP-1**	• Incretin effect • Appetite control • Gut motility

1.3 Ghrelin the “Hunger Hormone”

Ghrelin was originally discovered as an endogenous ligand for the growth hormone secretagogue receptor (GHSR). This receptor starts the signaling pathway which results in the release of growth hormone (GH). Ghrelin was named for this pathway as “ghre” is the Proto-Indo-European term for grow (7). While its seminal discovery linked ghrelin with growth hormone secretion, continued work has implicated ghrelin in appetite and energy balance. This section will describe the production, action and dysfunction of ghrelin in metabolic diseases as well as how the secretion of this hormone is controlled.

1.3.1 Ghrelin Synthesis and Expression

Ghrelin was an unknown ligand when it was discovered to bind to the growth hormone secretagogue receptor 1a (GHSR1a). Purification and sequencing of this peptide showed it to be 28 amino acids in length (7). Ghrelin is expressed from the preproghrelin gene which can also create a 23-amino acid peptide known as obestatin (8). Ghrelin can be produced in either an acyl, or des-acyl form, the former having the octanoic moiety being added to the 3rd amino acid in the sequence, which is serine. The addition of the octanoic acid is through the enzyme ghrelin O-acyl transferase (GOAT) which has been found to be localized in the endoplasmic reticulum where the peptide undergoes posttranslational modification (9,10). Acylated ghrelin is capable of interacting with GHSR1a and is known as the active form of ghrelin as the function of des-acyl ghrelin is still unknown (11).

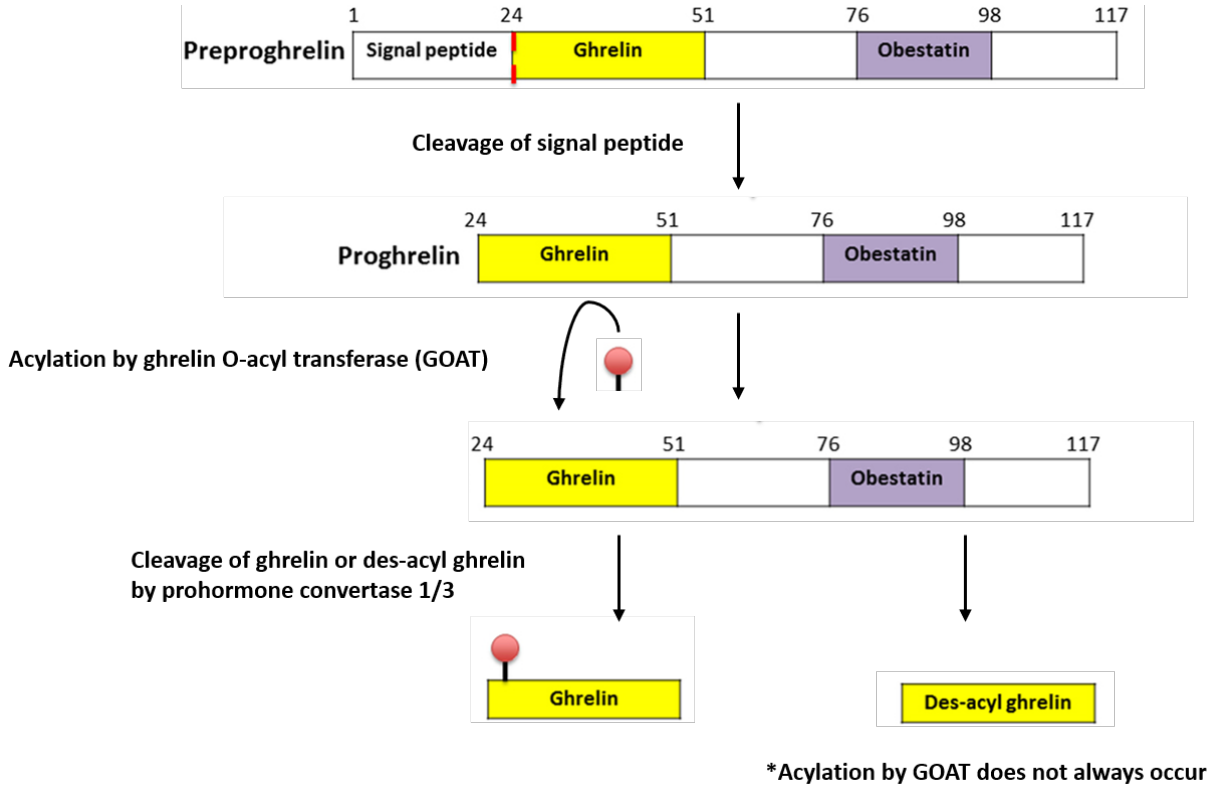

Figure 1: Preproghrelin and posttranslational processing (Adapted from (12))

When discovered, ghrelin was found to be produced mainly in the stomach. The enteroendocrine X/A-like cells of the upper fundus synthesize ghrelin and make up approximately 20% of the endocrine cells in the oxyntic glands of the fundus. X/A-like cells are also expressed throughout much of the proximal small intestines although in lesser concentrations than the stomach (13). Approximately 70% of plasma ghrelin concentrations are derived from the stomach, as when it is removed by gastrectomy ghrelin levels drop dramatically. The remaining stomach tissue can compensate, given time, by creating more ghrelin-producing cells or upregulating expression of ghrelin production, with the gastric fundus being the most capable tissue of providing this compensation (14). The ghrelin producing cells within the small intestine and colon, present in

the crypts and villi, are predominantly open type cells in contrast to the closed type cells present in the stomach tissue. Cells open to the luminal contents are likely nutrient sensing and have a separate pathway of regulation compared to the closed type cells (15). The endocrine pancreas also contains ghrelin producing ε-cells and the glucagon secreting α-cells can produce ghrelin as well (16). The ghrelin producing cells are in greater abundance in the pancreas during gestational development and decline postnatally, suggesting a role in development of the embryonic pancreas (17). Another well documented location of ghrelin expression is within the central nervous system. The hypothalamus, cerebral cortex and brainstem all contain ghrelin producing cells, which has been well documented using immunofluorescent techniques in rats and transgenic mice (18,19).

1.3.2 Functions of Ghrelin

As mentioned previously, ghrelin is a hormone that helps regulate appetite and energy metabolism. Most of this hormone is produced in the stomach and many of the effects it has relate to energy metabolism. However, it was originally discovered for activation of a receptor in the central nervous system, and expression of receptors in which ghrelin can bind are not exclusive to the digestive system. This section will discuss the various functions of ghrelin in the body with a focus on appetite and energy balance.

1.3.2.1 Growth Hormone Release

As mentioned previously, ghrelin was discovered as the ligand that binds to GHSR1a. This knowledge has been used to confirm the function of ghrelin in GH release in humans (7,20). Acyl-ghrelin is the only form of ghrelin that binds to this receptor on the anterior pituitary gland and stimulates a greater secretion of GH than growth hormone releasing hormone (GHRH) alone (11). Both ghrelin and GHRH target separate G-coupled receptors in separate structures in the brain. Ghrelin binds to GHSR1a in the arcuate nucleus of the hypothalamus, whereas GHRH binds to growth hormone releasing hormone receptor in the anterior pituitary gland (21). Treatment with either hormone will increase GH secretion but treatment with both hormones together provided a synergistic increase to GH secretion (22). GH is involved with many anabolic processes, primary by signaling the release of insulin-like growth factor 1 (IGF-1) from the liver. GH also stimulates gluconeogenesis in the liver and inhibits the uptake of glucose in peripheral adipose tissue while promoting glucose storage in skeletal muscles (23). Together these demonstrate the importance of ghrelin in the storage and utilization of energy through GH regulation.

1.3.2.2 Energy Mobilization and Storage

The indirect increase in secretion of GH and IGF-1 is not the only way in which ghrelin can regulate energy balance in the body. As ghrelin secretion is primarily controlled based upon energy state, it is not surprising that it has multiple effects on the availability of macronutrients. Ghrelin has an important role in regulating plasma glucose levels as it was discovered that

mRNA for GHSR was present in pancreatic cells (24). Insulin secretion from the pancreas helps manage plasma glucose. Ghrelin suppresses insulin secretion and reduces the glucose induced insulin response (25,26). This was later confirmed in a study showing that the suppression can be blocked using GHSR1a antagonists (27). This suppressive effect on insulin is activated through voltage dependent K^+ channels (28). Experiments isolating and examining the pancreatic artery and vein in mice show a greater level of ghrelin in the pancreatic vein and decreased suppression in isolates from ghrelin knockout (KO) mice (29). This suggests that pancreatic production of ghrelin is also actively regulating hormone secretion from the pancreas. Ghrelin may also have an indirect effect on insulin secretion by regulating the secretion of another pancreatic hormone which acts to raise blood glucose levels in a fasted state. Ghrelin directly stimulates glucagon secretion from α-cells of the pancreas (30). In addition to regulating insulin secretion, ghrelin has also been shown recently to have a role in reducing insulin sensitivity in peripheral tissues (31). Beyond its roles in insulin regulation, ghrelin also has roles in promoting gluconeogenesis in hepatic cells, as well as promoting storage of glucose in skeletal muscle and adipose tissue likely through upregulation of GLUT4 transporters in these tissues (32). This increased uptake of glucose into adipocytes may predispose fat accumulation. Indeed, treatments with ghrelin have also shown an increase in adiposity while appetite and food consumption between groups remained the same (33). Another study involving constant ghrelin infusion showed increased free fatty acids in subjects receiving the ghrelin treatment. However this may be the result of growth hormones effect on lipolysis in a fasted state (34,35) While both ghrelin and ghrelin receptor KO mice exhibit normal appetite and body weight on a chow diet, these KO animals are resistant to high fat diet induced obesity suggesting that ghrelin and ghrelin signaling are essential to the formation of adipose tissues (36,37).

1.3.2.3 Appetite Stimulation

Ghrelin has many functions in helping us store the nutrients we ingest, but ghrelin levels are highest in a fasted state and there is a relationship between ghrelin and appetite. Initial studies showed a dose dependent stimulation of appetite when ghrelin was injected intracerebroventricularly in rodents (33). The orexigenic effect ghrelin has was further examined by Wang *et. al* where they looked into how intraperitoneal (IP) injections of ghrelin dose-dependently increased appetite in mice (38). The function of neuropeptide Y (NPY)-releasing neurons in the arcuate nucleus (ARC) in stimulating appetite and increasing feeding responses is well understood (39). The group examined how increased ghrelin would affect activation of these neurons in the ARC by measuring *fos* activation via immunohistochemistry. They found that the IP injections of ghrelin showed activation of *fos* in cells and that 90% of those cells began expressing NPY mRNA in response to treatment. This confirmed that the appetite-stimulating effect of ghrelin is signalled through hypothalamic NPY-expressing neurons (38). As most ghrelin is produced peripherally, an interesting study showed that peripheral ghrelin can act in the central nervous system by crossing the blood brain barrier (BBB) using transporters (40). The rate at which peripheral ghrelin is transported across this barrier appears to be dependent upon nutrient status (41). Due to the limited ability for ghrelin to cross the BBB it is important that it can signal in the brain through other means. Ghrelin receptors can be found on the afferent portions of the vagus nerve. This afferent signalling is believed to stimulate appetite as vagotomy and pharmacological inhibition of the vagus nerve were shown to block the effects of ghrelin (42). Immunohistochemical stains of the ARC detecting *fos* and NPY expression confirmed that the orexigenic stimulation described previously can also be signaled through the vagus nerve (43). In contrast another group examined the effects of IP-injected ghrelin in rats having

undergone the most selective form of vagotomy and found the appetite stimulation was similar to the control group. (44). This maintained stimulation could be due to the ability for peripheral plasma ghrelin to cross the BBB causing the orexigenic effect of ghrelin through increased expression of NPY in the ARC. Ghrelin signalling through the vagus nerve has also been shown to inhibit the satiety-promoting effects of other gut hormones (45).

Beyond regulating appetite, ghrelin can stimulate food reward systems as well, which is reviewed in depth by Perelló *et al.* (46) . Of note is a study showing that reward-based feeding depended on the presence of the GHSR and GOAT enzyme using KO-mice. GOAT KO-mice showed decreased desire for high fat diet food, confirming that acyl ghrelin production is required to stimulate reward based feeding (47).

1.3.2.4 Other Systems Affected by Ghrelin

The expression of ghrelin receptors is also found in a variety of tissues and so here we will briefly discuss other roles ghrelin may play in the body. Ghrelin has other roles in the gastrointestinal tract, such as increasing gastric emptying which may be mediated through increased gut motility by binding to GHSR1a activating pathways in the enteric nervous system (46,47). Other metabolic hormones being regulated by ghrelin have been discussed and changes to their secretion could be involved in these modifications in gut motility (6). The effects are thought to be important in ghrelin's role of preparing the body for its next meal. In the cardiovascular system ghrelin has a role in vasodilation signaled through nitric oxide (50,51). It is also cardioprotective during ischemia and reperfusion by reducing apoptosis caused by reactive oxygen species and inflammation (52). Within the central nervous system, specifically

the hippocampus, ghrelin has been seen to have a neuroprotective effect on cells resulting in increased memory and memory retention. This is supported by the observation that ghrelin KO mice have memory deficits that can be reversed upon ghrelin administration (53). This promotion of neurogenesis could be through indirect effects such as the release of growth hormone or IGF-1 as both have been shown to produce similar effects (54,55). It is currently thought that exercise is an important lifestyle change that can prevent or slow the progression of Alzheimer's disease and other forms of dementia. It is suggested that an increase in ghrelin levels may mediate some of the beneficial effect of exercise on brain function (56). In the reproductive system ghrelin has shown an inhibitory effect on gonadotropin releasing hormone, which would decrease the secretion of follicle stimulating hormone and luteinizing hormone (57).

Table 2: Summary of the effects of ghrelin

Function	Effect
Growth Hormone Release	Increased
Insulin Secretion	Decreased
Gluconeogenesis	Increased
Adipose Tissue Formation	Increased
Appetite	Increased
Gastric Emptying	Increased
Vasodilation	Increased
Memory Formation	Increased

1.3.3 Ghrelin Levels in Metabolic Diseases

In the previous section, the function of ghrelin in the body was discussed. It is shown that ghrelin is primarily involved in regulating appetite, energy availability and metabolism. When a metabolic disease is present, one or more of these processes is altered along with the secretion of metabolic hormones. Therefore, understanding ghrelin regulation is important as it is sometimes

unclear if the change in ghrelin secretion is the cause or the result of the disease state and whether manipulating ghrelin levels would prove beneficial to patients.

1.3.3.1 Ghrelin in Obesity

A concern for many in society today is the continuous rise in obesity rates. As weight gain is normally the result of a higher intake in calories than those used, most treatments for obesity target diet and exercise with varied effect. As this is a growing health complication, an understanding of physiological mechanisms that control energy balance is important to develop new effective treatments for this disease.

Ghrelin is a regulator of appetite and energy metabolism and both basal and fasting plasma ghrelin levels are altered during obesity (58,59). One might expect that since ghrelin promotes appetite and energy partitioning to adipocytes, that ghrelin levels would be elevated in an obese state (33). However, the opposite is true, as pre-meal ghrelin levels are generally lower in obese individuals and negatively correlate with an individual's BMI (59). Lower ghrelin levels may be related to the increased energy availability in an obese state as there is increased adipose tissue providing a higher quantity of fatty acids for energy utilization. Ghrelin is involved with stimulating appetite and managing energy balance and the chronic surplus of available energy in obese individuals may be causing these decreased ghrelin levels. While ghrelin levels may be lower throughout the day, there appears to be a dysfunction in the post-meal percentage drop in ghrelin secretion in humans (58,60). This impaired drop in ghrelin may be partially responsible for the reports of increased appetite in obese individuals. Another suggestion is altered acylation of ghrelin in obese and diabetic individuals (61). Despite lower levels of total ghrelin there is an

increased ratio of acylated ghrelin to unacylated ghrelin which could be altering the hormones effect on appetite in obese individuals (61).

1.3.3.2 Ghrelin and Insulin Resistance

Type 2 diabetes mellitus is a comorbidity of obesity and is characterized by hyperglycemia, hyperinsulinemia and insulin resistance (62). Ghrelin has a suppressive effect on insulin and likewise insulin has been shown to have a suppressive effect on ghrelin secretion (63). Prior to this, studies have been conducted showing that ghrelin concentrations are not only decreased in obese individuals, but also suppressed further in individuals with insulin resistance (64). This suggests that ghrelin secretion could be further impaired in obese individuals who are also insulin resistant, as the pancreas increases insulin secretion to overcome the insulin insensitivity associated with type 2 diabetes mellitus. With these alterations in ghrelin in different diseased states, it is important to examine different regulators of the hormone to determine if restoring ghrelin secretion to normal levels can help reverse the diseased state.

1.3.4 Ghrelin Regulation

As ghrelin secretion can be altered in metabolic diseases, understanding how it is normally regulated is important. The location of ghrelin producing cells and how both ghrelin production and secretion are managed is essential to the study of this hormone. The next section will now expand upon those concepts by discussing the stimuli that signal these cells.

1.3.4.1 The Effect of Fasting and Feeding on Ghrelin Secretion

It is well established that plasma ghrelin levels rise in a fasted state and fall post meal. This has been shown in both rats and humans since ghrelin was first found to play a role in appetite regulation (33). The documentation of ghrelin levels during a circadian rhythm was done later by Cummings *et. al* which showed fluctuations in plasma ghrelin over the course of meal times with peaks right before each meal and troughs immediately after (65). An interesting finding has been that ghrelin regulation has been shown to maintain rising and falling patterns around expected mealtimes during a 24 hour fast (66). The fasted state is the primary reason why ghrelin increases prior to meals although this shows that it is not the sole regulator of ghrelin secretion. Ghrelin may promotes gluconeogenesis in a fasted state or the storage of macronutrients post-meal (32,67). All these functions support the necessity for a rise in ghrelin during a fasted state and the decline in ghrelin post meal.

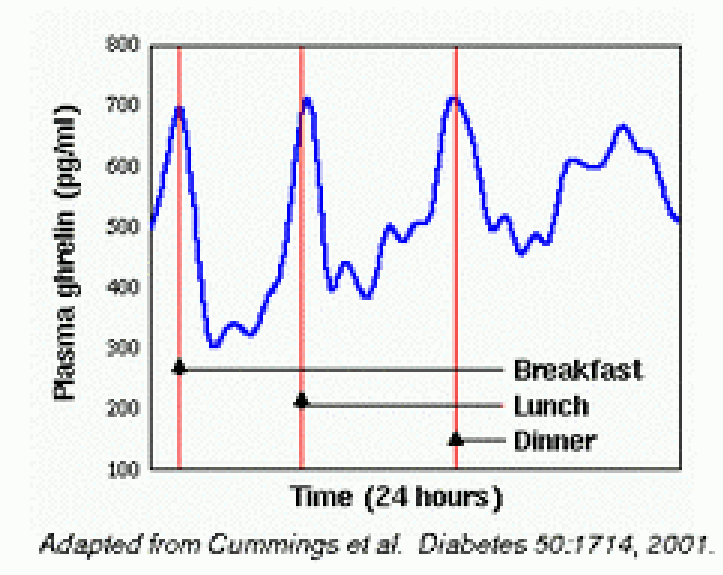

Figure 2: Plasma ghrelin fluctuations during a 24 hour cycle (65)

1.3.4.2 Hormones Regulation of Ghrelin

In response to meals, the secretion of several hormones is modulated, which could in turn regulate the secretion of ghrelin. Insulin is a hormone that is secreted post-meal from the pancreas and aids in storing and managing the large amount of glucose that has just entered the circulatory system. As such it has an inverse secretion pattern with ghrelin post-meal. Insulin has been shown to have a rapid inhibitory effect on ghrelin levels when injected into peripheral circulation (68). This suppressive effect was confirmed in cell culture, helping to discover the cellular mechanism that insulin triggers to suppress ghrelin secretion (63). Phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT) are two intracellular kinases known to be involved with many cell signaling pathways and have increased activity during the suppression of ghrelin secretion by insulin (63,69). Glucagon-like peptide-1 (GLP-1) an incretin hormone with functions such as satiety and a glucose-dependent increase in insulin secretion has also been shown to inhibit ghrelin secretion (70). Although this was initially thought to occur through an increase in insulin secretion, studies using a GLP-1 receptor agonist have shown a suppression of ghrelin in the absence of increased insulin levels potentially suggested that GLP-1 suppresses ghrelin via an independent pathway (71,72). Glucagon is another hormone involved with glucose homeostasis by promoting gluconeogenesis in the liver. This hormone was initially believed to suppress ghrelin in human studies (73). However, further investigation using ghrelin-secreting rat stomach primary culture confirmed glucagon’s direct stimulatory effect on ghrelin secretion (74).

1.3.4.3 Nervous Systems role in Ghrelin Secretion

The nervous system is responsible for receiving stimuli and sending signals throughout the body. The autonomic branch of the nervous system controls involuntary processes of the body such as vascular tension, inflammation and digestion. Norepinephrine is a neurotransmitter of the sympathetic nervous system that plays a role in ghrelin regulation. Norepinephrine is involved with our fight or flight responses and aids in mobilizing glucose to provide our body the energy it needs to allow us to survive stressful experiences. A way in which Norepinephrine raises plasma glucose is by increasing ghrelin secretion (63). Norepinephrine increases the expression of cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) and the secretion of ghrelin from rat stomach primary culture (63). This pathways importance was examined by the use atenolol and H89 which are inhibitors for β1-adrenergic receptors and PKA respectively. The use of either of the inhibitors removed the increase in ghrelin secretion observed with norepinephrine treatment (63). Norepinephrine, the neurotransmitter for the sympathetic nervous system, stimulates ghrelin secretion. The other half of the autonomic nervous system is the parasympathetic branch which helps manage the rest and digest functions of the body facilitating the processing and storage nutrients consumed. The main regulator of this system in the stomach is the vagus nerve and its role in ghrelin secretion has been examined. Vagotomised rats showed no change in post meal ghrelin suppression (42). However, the increase in ghrelin secretion during a fasted state was lost in these animals, indicating a role for stimulating ghrelin secretion in a fasted state (42). Another study has confirmed these findings and shown that stimulation of the vagus nerve to increase fasting ghrelin levels may be activated by thyrotropin releasing hormone in the central nervous system which is known to regulate the secretion of other metabolic hormones (75). Intracisternal injection of the thyrotropin releasing hormone analogue

increased peripheral plasma ghrelin and feeding in mice through activation of vagus nerve in the dorsal vagal complex (75). The increases in peripheral ghrelin secretion were lost when atropine, a cholinergic inhibitor, was delivered or a vagotomy was performed (75). This confirms the increase in ghrelin secretion is signaled through the parasympathetic nervous system, specifically the vagus nerve. Other studies have reinforced the involvement of branches of the autonomic nervous system. In one study, pharmacological agonists and antagonists and vagotomy confirmed that activation of adrenergic receptors increased ghrelin secretion, and vagotomy removed the stimulation of ghrelin secretion initially but, stimulation was increased 7 days after vagotomy (76). All of these findings together reinforce the complex involvement of the nervous system in regulation of appetite regulating hormones in the GI tract. The portion of the nervous system that stimulates the GI tract is known as the enteric nervous system. With the increased frequency of vagotomies over recent years, enteric control in the GI tract has been examined and has been seen to be capable of sensory and stimulatory effects independent of the central nervous system (77). The enteric nervous system has thus been called the second brain as it can send and receive stimuli independent of the central nervous system (78). The role of the enteric nervous system in controlling gut motility and secretions in the GI tract may also be expanded to endocrine hormone release and be primary responsible for regulating ghrelin secretion.

As seen in this section, many different stimuli can regulate the secretion of ghrelin, including other metabolic hormones. These stimuli in the digestive system end up signaling not just the regulation of one but multiple hormones and systems.

1.3.4.4 Macronutrient Suppression of Ghrelin Release

Just as fasting can stimulate the secretion of ghrelin, the ingestion of macronutrients has been shown to have an inhibitory effect on ghrelin secretion. The ingestion of actual nutrients is required to decrease ghrelin secretion, noncaloric water meals had no decrease in ghrelin (33). The previous study confirms that nutrient sensing is central to the inhibitory effect on ghrelin secretion. This was demonstrated again by the observation that rats receiving a glucose infusion with a pyloric clamp blocking entry to the duodenum showed no postprandial ghrelin drop compared to their unclamped counterparts (79). A study by Callahan *et. al* examined the effect of caloric content on postprandial ghrelin drops and found that higher calorie meals caused greater decline in blood ghrelin levels and longer feelings of fullness (80). The type of macronutrient also influenced ghrelin suppression. Carbohydrates and proteins had a stronger suppression of ghrelin secretion, while lipids had a smaller effect. It was also found that carbohydrates had a rebound effect where ghrelin levels rose back to higher plasma concentrations quicker. Protein meals not only triggered the largest decrease in ghrelin secretion, but they also led to a more prolonged suppression (81). This finding was further examined, and it was shown that when specific amino acids were ingested they had varying levels of ghrelin and appetite suppression (82). The amino acids containing sulfur groups were found to have the largest effect on both appetite and ghrelin (82). This led us to propose that these sulfur-containing amino acids are being metabolized into a molecule(s) signaling the suppression of appetite and ghrelin secretion. The molecule we believe is responsible for this is H_2S.

1.4 H_2S a Gasotransmitter

H_2S has long been thought of as a toxic gas (83). While colourless, it gives off a pungent smell, is highly flammable and can cause serious harm to humans in high concentrations. Despite this, it is produced by many tissues in our bodies. More recently it has been established as a gasotransmitter, a gaseous biological signaling molecule, responsible for regulatory functions in multiple systems. The previous section discussed a metabolic hormone, ghrelin, that is important for weight management and metabolic health. Our group as well as other studies have shown that H_2S can regulate other metabolic hormones such as insulin and GLP-1 (84,85). Our study was conducted to investigate if H_2S may affect ghrelin secretion.

1.4.1 Direct Effects of H_2S on Cellular Proteins

H_2S is a small gaseous molecule that can freely move through cellular membranes and react with proteins through a process called sulfhydration. Sulfhydration involves H_2S reacting with a free sulfhydryl (-SH) group and forming a persulfide (-SSH) group, therefore it can interact with any protein that contains a S atom. Sulfhydration will generally increase and maintain the active configuration of the target protein (86). Several target proteins will be discussed in the following sections.

1.4.1.1 K_{ATP} Channel Sulfhydration

Ion channels are a well documented target for sulfhydration by H_2S and K_{ATP} channels are one of the best examples of this. The SUR1 subunit is the target for sulfhydration as it contains a cysteine amino acid with a free sulfhydryl group near the opening of channel. H_2S can sulfhydrate this cysteine forming a persulfide group from the free sulfhydryl which then holds the channel in the open configuration (87). The opening of K_{ATP} channels by H_2S can cause vasorelaxation of vascular tissues, cardioprotective properties in the myocardium, inhibition of insulin release from β-cells, neuroprotection and decreased leukocyte efficiency. The understanding of H_2S's effect in these systems has been examined using a combination of K_{ATP} channel inhibitors (glibenclamide, gliclazide), H_2S donors (NaHS) and endogenous H_2S producing enzyme inhibitors (PPG) (88–93).

1.4.1.2 cAMP Activation

Increased production of cAMP by adenyl cyclase has also been investigated as a potential target for H_2S within cells. Increased cellular cAMP can then activate more PKA through phosphorylation and which can in turn regulate the activity of many other cellular processes, including increased activation of PI3K and AKT. The increased effects of long term potentiation in learning in memory from H_2S are attributed to the cAMP/PKA pathway (94,95). Other experiments concerning H_2S's effect in the nervous system involved increased neurotransmitter release. With the use of an adenyl cyclase inhibitor the increased neurotransmitter release was maintained despite decreased production of cAMP (96). This suggests that H_2S may directly

target PKA or another downstream molecule in this pathway such as PI3K or AKT to elicit the increased neurotransmission.

1.4.1.3 PI3K Activation

Direct sulfhydration of the PI3K enzyme has not yet been confirmed, yet it is currently under investigation. H_2S has been shown to increase both PI3K and AKT activity in both cardiac and neural cells of mice that have suffered a traumatic brain injury (97). This increased signaling was accompanied by decreased apoptosis and increased mitochondrial integrity (97). Improved cardiac function after an ischemic event has also been shown with the use of H_2S donors, which is reversed in the presence of PI3K inhibitors (98). PI3K and AKT are intracellular signaling molecules that have important roles in signaling cell survival and proliferation (69). As discussed previously, the PI3K pathway is involved in the suppression of ghrelin secretion by insulin and will be a pathway investigated as part of the manuscript in section 2 (63).

1.4.2 H_2S Signaling Effects

H_2S is readily available and actively produced in living systems. This portion of the manuscript will discuss the importance of H_2S as a signaling molecule and the effects elicited. With a better understanding of what H_2S can do, new technologies to modify its production and availability could be developed to alter those functions.

1.4.2.1 H_2S in the Endocrine System

As the hormone ghrelin is the focus of this manuscript, it is important to discuss the effects H_2S has in the endocrine system. H_2S-generating enzymes are found in abundance in cells within the pancreatic islets, which led to the investigation of the effects it may have on these endocrine cells (99,100). H_2S has been shown to inhibit glucose-stimulated insulin secretion from pancreatic β cells. This has been examined thoroughly with the use of both in vitro and in vivo experiments. The mechanism of insulin suppression is through the activation of K_{ATP} channels. This occurs by sulfhydration of the sulfhydryl group in K_{ATP} channels which maintains activation of the channel (87). Our group has recently expanded on the field of how H_2S can regulate hormones with a study showing that increasing H_2S production in the GI tract can increase GLP-1 secretion (85). H_2S's effects on the endocrine system are a new and interesting field. This manuscript expands the field to introduce how H_2S can modify ghrelin secretion.

1.4.2.2 H_2S in the Gastrointestinal System

A majority of H_2S is produced in the GI tract by the microbiome (101). Sulfate-reducing bacteria in the lumen of the GI tract produce H_2S and many cells of the stomach and small intestine contain enzymes responsible for H_2S production (102). The effects of microbially produced H_2S include cytoprotection, immune response regulation and regulation of the GI hormone GLP-1 mentioned previously (85,103). The endogenous effects of H_2S can occur from both microbial and enzymatic production of H_2S as both methods produce H_2S in the body. The primary role of H_2S on the GI tract has been gastric motility. In the gastric smooth muscle cells of guinea pigs

H_2S has shown a relaxing effect (104). This however may be tissue specific as muscle tone was increased when the same group examined the effect of H_2S on the gastric fundus (105). Smooth muscle relaxation has also been shown in jejunum and colon tissues from both humans and rodents, all of these effects appear to be mediated by the sulfhydration of K_{ATP} channels (106). Sulfhydration of K_{ATP} channels by H_2S also mediates an anti-inflammatory response in the GI tract, helping to reduce the development of gastric lesions (107). Although sulfhydration of K_{ATP} channels can explain the effects of H_2S on the gastric motility, the previously discussed stimulation in gastric motility from ghrelin could also be an indirect way in which H_2S controls gastric motility.

1.4.2.3 H_2S in Other Systems

The enzymes responsible for the endogenous production of H_2S are located in many tissues of the body, the most well documented being the cardiovascular system. An exciting finding is a negative inotropic effect in rat hearts during ischemia and reperfusion injury which could potentially translate to cardio protective effects in humans (108,109). Other effects include vasodilation of vasculature through the relaxation of smooth muscle cells in a variety of blood vessels and the occurrence of hypertension and vasoconstriction in the absence of endogenous H_2S production (110,111). H_2S also has regulatory effects on the proliferation of vascular tissues. Of note are the inhibition of growth and apoptotic effects on vascular smooth muscle cells and the stimulation of proliferation in the endothelial cells on the vascular tissues (112,113). In the nervous system H_2S can increase long term potentiation, a form of increased synaptic strength associated with increased memory consolidation (114). This process has been studied in rodents

and is known to be regulated by the stimulation of NMDA receptors, likely through an increase in cAMP production (94). Physiological levels of H_2S have been shown to enhance the activity of these pathways, although prolonged higher doses of H_2S have negative effects on long term potentiation (114,115). Anti-inflammatory effects of H_2S have been noticed and primarily explained by a decreased ability for leukocyte adherence and infiltration into the vascular endothelium (89). Other suggested mechanisms are increased expression of genes for heme oxygenase, through increased extracellular signal-regulated kinase (ERK) activation, which can reduce oxidative stress (116,117). H_2S has also been shown to have pro-inflammatory effects, and can increase pro-inflammatory cytokine production from monocytes (118). High levels of H_2S in the blood are seen in conditions such as sepsis and treatment using DL-propargylglycine (PPG), an inhibitor of endogenous H_2S production, improved survival of mice with sepsis, whereas the use of H_2S donors exacerbates the condition and increases mortality (119). The liver and kidney contain the highest expression of endogenous H_2S-producing enzymes leading to the study of H_2S in these organs. They primarily revolve around vascular tone, increasing the arterial buffer capacity in the liver and glomerular filtration rates in the kidney (120,121). Ischemia reperfusion injury to both of these tissues can also be partially reduced and reversed by treating with H_2S donors and from the natural production in these tissues (122–124). All of these effects and more are thoroughly reviewed in (103).

1.4.3 H_2S Production

The role H_2S plays in signaling functions in biological systems is important. This signaling process requires that H_2S first be produced. This section will discuss the methods of H_2S production that modify the concentration to appropriately signal different functions in the body.

1.4.3.1 Endogenous Enzymes

Endogenous H_2S-producing enzymes are primarily found within the cytosol of cells. H_2S produced via enzymes can act on the cell producing H_2S or diffuse out of the cell. Cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) are the enzymes that produce the majority of enzymatic H_2S. CSE is expressed more in peripheral tissues such as the vasculature, liver and stomach, whereas CBS is predominantly expressed in the nervous system. The major metabolites of these enzymes are L-cysteine and homocysteine. (125–129). As the stomach produces a majority of the ghrelin in the body, it is worth mentioning that CSE is the primary H_2S-producing enzyme in gastric cells and the primary mechanism for production in these cells (130). CSE functions by using L-cysteine as a substrate and water and pyridoxal-5-phosphate (P5P) as co-substrates to cleave the C-γ-S bond forming H_2S, NH_3 and pyruvate (103). Other reactions utilizing CSE to form H_2S use the catalytic cleavage of C-γ-S bonds but are less common in gastric cells. CBS will primarily facilitate the production of H_2S through the reaction of water and L-cysteine to form L-serine and H_2S (103). To a lesser degree 3-mercaptopyruvate sulfurtransferase (MST) and cysteine aminotransferase (CAT) produce H_2S, although they are tissue specific (131). These two enzymes work together, first CAT converts cysteine to 3-

mercaptopyruvate through a transaminase reaction with α-ketobutrate using P5P as a cofactor (132). MST will then transfer the sulfhydryl group from 3-mercaptopyruvate, forming pyruvate. This reaction accounts for the enzymatic formation of acid-labile sulfur in the mitochondria discussed in the next section and can be reduced to release free H_2S (103). This enzymatic production contributes to the approximately 50µM concentration of H_2S in blood (129). Other less prominent mechanisms for endogenous H_2S production as well as those discussed are further reviewed in (103).

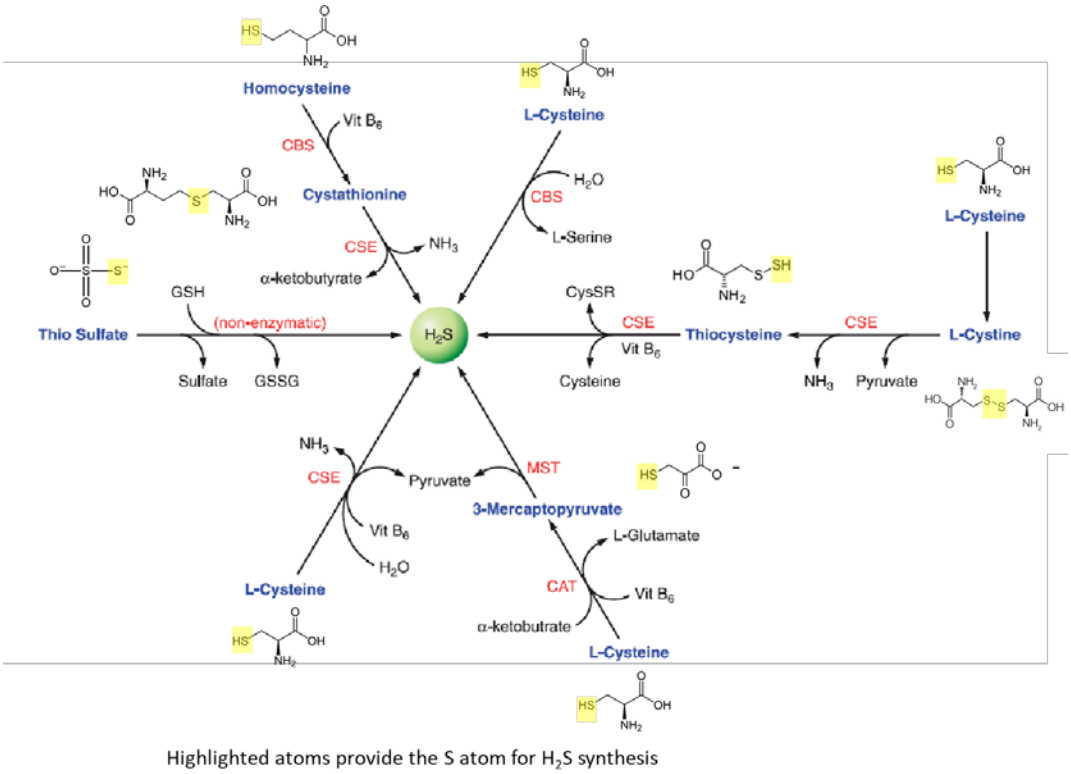

Figure 3: Pathways for endogenous H_2S production (Adapted from (103))

1.4.3.1.1 Availability of L-Cysteine

As endogenous H_2S in the models we are studying is produced enzymatically using the substrate L-cysteine, it is worth mentioning the availability of this substrate. L-cysteine can come from dietary sources, although it is considered semi-essential as it can also be made in the body through other sources. Endogenous formation of L-cysteine can be obtained through either protein degradation or the enzymatic conversion of methionine to L-cysteine (reviewed in (133)). This amino acid can then be used as a substrate to produce several products that have important functions in our bodies.

1.4.3.1.2 Other Effects of L-Cysteine

Aside from the capacity for L-cysteine to be enzymatically metabolized into H_2S, it can be metabolized into other products which may be producing some of the effects seen when using this amino acid as a treatment. L-cysteine is the rate limiting substrate in the formation of glutathione. Glutathione is a substrate for glutathione peroxidase which acts to reduce reactive oxygen species decreasing oxidative stress in our cells (134). Glutathione peroxidase contains a selenol group which acts as a reducing agent for hydrogen peroxide converting it into two molecules of water. The now oxidized form of glutathione peroxidase will act as an oxidizing agent for two molecules of glutathione forming glutathione disulfide. The second reaction uses glutathione as a substrate and allows glutathione peroxidase to reduce more reactive oxygen species present in the cell. Another product of L-cysteine metabolism is taurine, which requires numerous enzymatic steps. This amino acid has several functions but, also has the ability to

reduce oxidative stress (135). Taurine reduces oxidative stress by reducing the production of reactive oxygen species produced by the electron transport chain (ETC) in mitochondria. β-alanine depletes cellular taurine which reduces the function of the ETC and increases superoxide production from the mitochondria (136). Complex I and complex III activity is lower in taurine deficient cardiomyocytes and produce the majority of superoxides in the ETC (136). Treatment of cardiomyocytes with taurine and β-alanine together reduces the superoxide species generated by mitochondria and restores complex I and complex III activity (136). The suggested mechanism for how taurine improves ETC function is not known but it is suggested that it helps mediate the functions of complex I and complex III on the ETC.

All of these products from L-cysteine including H_2S can play a role in reducing cellular oxidative stress (137). Therefore, it was important to use an experimental design in our study that confirms that H_2S the cause of modulation of ghrelin secretion.

1.4.3.2 Microbial Production

H_2S can also be produced by microorganisms in the GI tract. This is in large part due to bacteria that reside in our gut microbiome. Hydrogen-consuming bacteria or hydrogenotrophs consume nutrients to generate products such as methane, acetyl-CoA and H_2S and maintain the environment of our microbiome. A specific group of hydrogenotroph known as sulfate-reducing bacteria use hydrogen or other organic compounds to reduce a sulfate group forming H_2S at millimolar concentrations in the lumen (102). The presence of H_2S in the lumen has been studied and shown to have beneficial effects for repairing ulcers and decreasing inflammation in the GI tract mucosa and, as such, is an important molecule for gut health (138). It is also interesting that

the majority of H_2S stored in our cells and freely available in plasma is lost in germ free mice which contain no microbiome (101). Due to this, microbial production of H_2S may have a substantial role in endogenous H_2S signaling. Noteworthy changes in physiology of germ free mice include decreased muscular tone in the GI tract, impaired immune function and impaired memory formation, as they have been previously discussed in the functions of H_2S signaling (139–142).

1.4.4 Metabolism and Storage of Endogenous H_2S

Endogenously-produced H_2S is involved in signaling. Thus, it is important to understand how this gaseous signaling molecule acts in the body. H_2S dissolved in cellular cytosol or blood plasma is constantly in equilibrium represented by $H_2S \leftrightarrow H^+ + HS^- \leftrightarrow 2H + S^{2-}$. The ratio of H_2S to HS^- is about equal in the cytosol of cells whereas in extracellular fluid or plasma the ratio of H_2S to HS^- is about 1:5. This molecule is lipid-permeable and can modify the activity of a variety of protein structures through sulfhydration. H_2S has a relatively short half-life spanning from seconds up to a minute yet is readily available due to production and the ability for it to be stored intracellularly (83).

1.4.4.1 Storage of Endogenous H_2S

When not free, H_2S can be stored within the cytoplasm of cells as bound sulfane sulfur. This classification includes thiosulfates, persulfides, thiosulfonates, polysulfides, polythionates, elemental sulfur as well as disulfides which contain a C-S bond adjacent to an unsaturated C.

Sulfane sulfur is characterized by covalent bond formation between two sulfur atoms and is therefore formed due to the oxidation of H_2S. Sulfane sulfur is commonly found bound to proteins such as albumin as H_2S will react with sulfur containing amino acids modulating the function of different intracellular proteins. The most common intracellular reducing agents for bound sulfane sulfur are glutathione and cysteine. Due to the relative stability of some of these molecules, H_2S can be stored in cells and tissues for longer periods than initially predicted. Some studies show that tissues containing the enzymatic machinery to produce H_2S generally store higher amounts of these bound sulfane sulfurs (143). Acid-labile sulfur is another intracellular store of H_2S within the mitochondria of cells. This store of H_2S is bound to iron containing enzymes through the S group of H_2S and is released under acidic conditions (144)

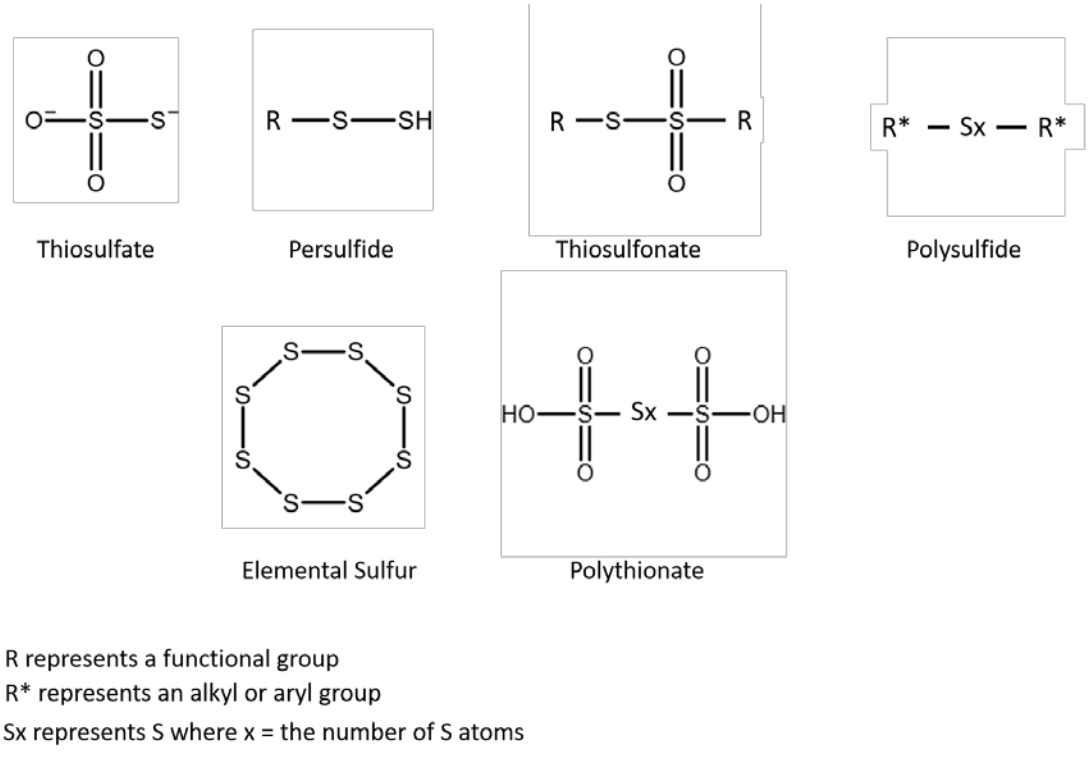

Figure 4: Sulfane sulfur molecules (103)

1.4.4.2 Elimination of Endogenous H_2S

Having stores of this bioactive molecule is important for signaling, although in higher concentrations it can become cytotoxic, and so removal of H_2S from the body is an important process. Scavenging of H_2S can occur with methemoglobin, hemoglobin by the ferric iron in the heme group or by reactions with sulfur atoms in disulfide containing molecules (145,146).

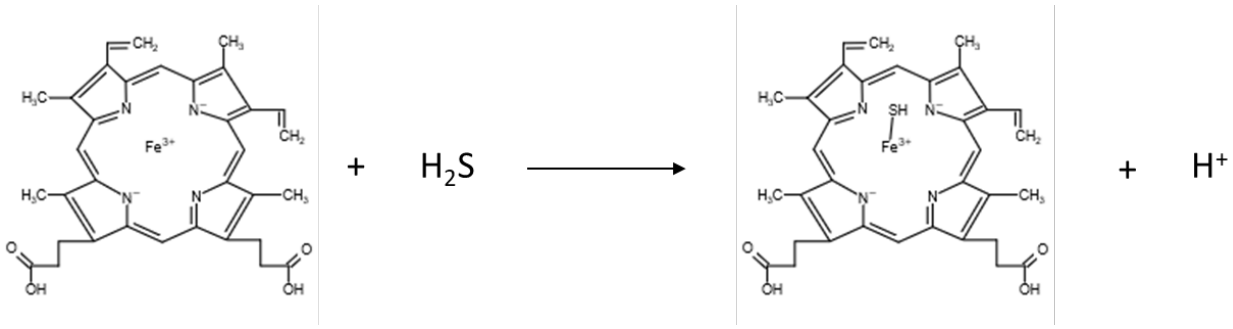

Figure 5: Scavenging of H_2S by ferric heme groups

The catabolism of H_2S into thiosulfate by the enzymes ethylmalonic encephalopathy protein 1 and sulfur quinone oxidoreductase can occur (147). This thiosulfate is further oxidized to sulfides/sulfates. Oxidation of sulfites to sulfates occurs readily by sulphate oxidase and thiosulfates in urine are a general biomarker for endogenous H_2S production (148). Methylation is a less common and very slow method of H_2S elimination (149). H_2S can also be exhaled through the lungs and has been measured in both breath and as free sulphates in flatulence (150).

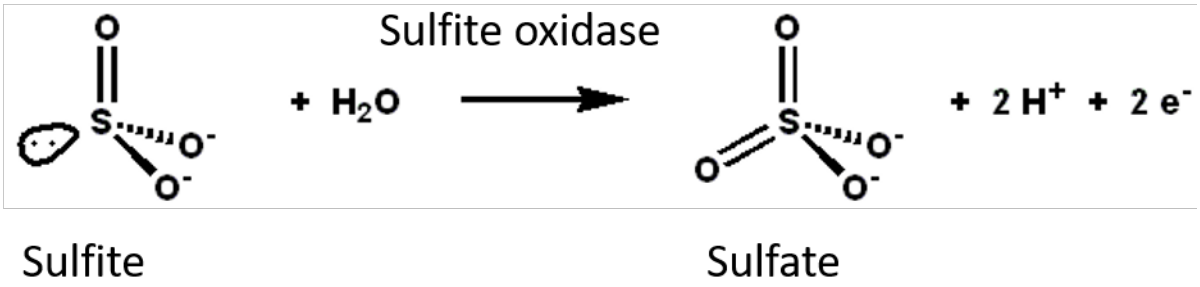

Figure 6: Oxidation of sulfites in the kidney

1.4.5 Hypothesis and Objectives

Ghrelin is a hormone with metabolic functions that is suppressed for a longer duration after meals high in protein. Sulfur containing amino acids that make up this protein have a greater suppression of ghrelin and appetite compared to other amino acids. Sulfur containing amino acids can be metabolized into H_2S, a gasotransmitter that can regulate the secretion of other hormones such as insulin and GLP-1. We hypothesize that H_2S can directly suppress ghrelin secretion, and that this will lead to a decrease in appetite.

This will first be examined via direct treatment of H_2S donor to primary gastric culture. Second, we confirmed the presence of endogenous H_2S producing enzyme in our cell model and tested the effect of inhibiting this enzymes production of H_2S. Third, we looked at the AKT signaling pathway to explore the cellular mechanism thought to be involved with ghrelin secretion. Finally, we conducted an in vivo experiment injecting H_2S donors to examine the post-prandial ghrelin response and determine if that modifies appetite. The following chapter is a submitted manuscript that contains experiments completed in this study.

2 Hydrogen sulfide suppresses ghrelin secretion *in vitro* and delays post-prandial ghrelin secretion while reducing appetite in mice

RESEARCH ARTICLE

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Short title: Hydrogen sulfide suppresses ghrelin secretion

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Abstract: Ghrelin is a stomach-derived hormone that regulates several metabolic functions including growth hormone release, appetite, adiposity, and gastric motility. Nutrients, the autonomic nervous system, and other metabolic hormones have all been implicated in the regulation of ghrelin secretion. Despite this, ongoing efforts to develop modulators of ghrelin secretion in human diseases are still underway. Hydrogen sulfide (H_2S) is a gaseous signaling molecule that is produced both endogenously in many tissues and by the gut microbiome. H_2S has established roles in cardiovascular and immune health, however more recently H_2S has been implicated in the regulation of metabolic hormone secretion. We hypothesized that H_2S is able to directly regulate ghrelin secretion and in turn, regulate appetite. We first demonstrated that GYY4137 (an H_2S donor molecule) directly suppresses ghrelin secretion in rat primary gastric culture, in part through the activation of the protein kinase B (AKT) pathway. We then demonstrated the colocalization of ghrelin positive gastric cells with the H_2S producing enzyme cystathionine-γ-lyase (CSE). While GYY4137 suppressed ghrelin secretion, inhibition of CSE caused a stimulation in ghrelin secretion in primary gastric culture. In mice, GYY4137 treatment prolonged the post-prandial drop of circulating ghrelin and caused reduced food consumption up to 4 hours after treatment. These results demonstrate for the first time a role for H_2S in the regulation of ghrelin and appetite. Modulating H_2S levels may be a novel approach to regulate ghrelin secretion in the treatment of metabolic diseases.

Keywords: ghrelin, gastrointestinal tract, cell biology, hormone secretion

2.1 Introduction

Ghrelin is a stomach-derived hormone that has metabolic functions throughout the body primarily in energy metabolism (7). Ghrelin has been shown to stimulate appetite, stimulate growth hormone secretion, promote adiposity and stimulate gut motility (151). As ghrelin plays many important roles in metabolism, understanding how this hormone is regulated is of key importance in metabolic health and disease. It is well documented that plasma ghrelin levels are highest prior to food consumption and are suppressed post-prandially. Accordingly, the circadian rhythm of circulating ghrelin fluctuates around meal times, suggesting that ghrelin plays an important role in meal cues and post-meal metabolism. There are several pathways involved in regulating ghrelin secretion. The type of macronutrient consumed will yield different levels and duration of ghrelin suppression with proteins and fats manifesting the longest suppression (81,82). The autonomic nervous system has also been implicated in regulating ghrelin secretion (63,76). In addition, our research and others has implicated metabolic hormones, including insulin and GLP-1 in the regulation of ghrelin (63,70).

The gasotransmitter hydrogen sulfide (H_2S) may have a role in the regulation of ghrelin secretion. H_2S is an endogenously produced (through several enzymatic pathways) gaseous signalling molecule. Research on H_2S has largely focused on its cardiovascular effects through vascular tone (103), with most studies indicating that H_2S is a vasodilator with cardioprotective, anti-apoptotic, and anti-inflammatory effects, reviewed in (152). Interestingly, several groups have demonstrated a role for H_2S in the regulation of insulin secretion, reviewed in (153). In endocrine beta cells, H_2S has been shown to modulate a variety of ion channels and cellular kinases (153). Recently, our group demonstrated a role for H_2S in the stimulation of the gastric hormone glucagon like peptide 1 (GLP-1). However, the role H_2S plays on other metabolic

hormones, including ghrelin, remains to be determined. When examining the production of H_2S from tissues in mice, the stomach was found to both express the H_2S-producing enzyme cystathionine-γ-lyase (CSE) and produce detectable levels of this gas (126). Since H_2S is produced in the stomach (the site of 70-80% of ghrelin production (14)), and is already known to regulate other gastric hormones (85,99), we hypothesized that H_2S would play a role in the regulation of gastric ghrelin. We investigated this hypothesis through a combination of cell and animal-based methods. In vitro, using primary rat stomach culture, we tested the effect of H_2S donors and CSE inhibitors on ghrelin secretion and cell signalling. In vivo, we examined the effect of these compounds on post-prandial ghrelin suppression and appetite in mice.

2.2 Material and Methods

2.2.1 Animals

Experiments were performed following the guidelines outlined by the Canadian Council on Animal Care guide to the Care and Use of Experimental Animals (CCAC, Ottawa, ON: Vol. 1, 2nd edition, 1993: Vol. 2, 1984) (154). Animal Protocols were approved by the Laurentian University Animal Care Committee. Pregnant female Sprague Dawley rats as well as male and female wild-type C57BL/6 mice aged 7-8 weeks were purchased from Charles River Laboratories (St. Constant, Quebec). The dams and pups were housed together until pups were sacrificed on postnatal day 8 for primary culture preparation. The mice were all singly housed in standard cages. All rodents were maintained on a 12-hour light/dark cycle in the Paul Field Animal Care Facility at Laurentian University.

2.2.2 Primary culture preparation

All cell culture media and reagents, unless otherwise stated, were obtained from Sigma Aldrich (Oakville, ON, Canada). Primary cell culture was prepared from male and female rat stomachs postnatal day 8 as indicated in (63). Briefly, stomachs from each litter were extracted, rinsed and enzymatically digested twice with collagenase. The final pellet was resuspended in 10ml of culture media (low glucose DMEM, 10% fetal bovine serum (FBS), 1% Streptomycin/Penicillin (SP)). Cells were plated in 6 or 12 well culture plates for ghrelin secretion, western blotting, or immunocytochemistry as indicated below.

2.2.3 Ghrelin secretion experiments

Secretion experiments were conducted using rat stomach primary culture seeded into 6-well culture plates (2 000 000 cells per well). 24 hours after initial plating the media was removed and replaced using a culture media containing 50 µM octanoic acid. After an additional 24 hours, cells were washed with wash buffer then incubated at 37℃ for 4 hours with treatments dissolved in 2 ml secretion media (low glucose DMEM 0.5% FBS 1% SP). The treatments (Cayman Chemicals, Ann Armor, MI): H_2S donor GYY4137, which releases H_2S via hydrolysis reactions in solution, CSE inhibitor DL-Propargyl Glycine (PPG) or phosphoinositide 3 kinase (PI3K) inhibitor LY294002 were dissolved in DMSO. Media with vehicle (DMSO) served as a control for baseline secretion and forskolin (which increases intracellular cAMP) was used as a positive control for acylated ghrelin secretion. Following the 4 hour incubation, the media was collected and spun down at 1000 x g for 5 minutes to remove floating cell debris. The supernatant was acidified using trifluoroacetic acid (TFA) to a final concentration of 0.1% and stored at -20 ℃ to prevent protease activity and loss of acylated ghrelin. The cell lysates were collected off the plate using a cell scraper in 500 µL of an acidic lysis buffer (1M HCl, 1% TFA

and 50 mM NaCl) and sonicated for 10 seconds on ice (155). The lysate was spun down at 13 000 x g at 4 ℃. The supernatant was separated from cell debris and stored at -20 ℃. The media and lysates were subjected to hydrophobic reverse phase resin chromatography (C-18 SepPak cartridges, Waters) according to manufacturer's instructions and eluted in 5 ml of 80% isopropanol in water containing 0.1% TFA. Samples were then dried in a vacuum concentrator. These dried samples were stored at -20 ℃ until analysis using a commercial acylated ghrelin enzyme immunoassay kit described below. Cell viability was determined under similar experimental conditions using the neutral red uptake assay described in (156).

2.2.4 Western blot

Primary stomach cells were seeded into 6-well culture plates (2 000 000 cells per well) and received treatments after 48 hours as indicated above. On the day of treatment, cells were washed with wash buffer and treated with secretion media alone or secretion media containing100 μM GYY4137 for 15 minutes at 37 ^{o}C, 5% CO_2. Each well received 100 μl of 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) and cells were collected, sonicated on ice (10 seconds, Power 4), and centrifuged (5 minutes, 13 000 x g, 4 ^{o}C). Protein concentration was measured using the Bradford protein methods and the expression levels of phosphorylated AKT and total AKT were analyzed by Western blotting. 20 μg of protein was loaded per lane on 4-12% acrylamide sodium dodecyl sulphate-polyacrylamide gels and run at 180 V. The protein was then transferred for 1.5 h at 200 V onto a polyvinylidene fluoride membrane, blocked in 5% w/v milk powder and 0.1% Tween 20 TRIS buffered saline (TBST) for 45-minutes, then incubated in primary antibodies (see table 1) in TBST containing 5% BSA overnight. After 3 x 5 minute washes in TBST, the

membrane was incubated with a horseradish peroxidase-conjugated secondary antibody at a concentration of 1:2000 for 1 hour at room temperature. Signal was developed by exposing the membrane to Luminata Forte Western HRP Substrate (Millipore Corporation, Millerica, Ma) for two minutes. Chemiluminescence signal was recorded using a BioRad Chemidoc XRS documentation apparatus and analyzed using QuantityOne program. Densitometry was used to analyze band intensity to compare phosphorylated AKT and total AKT protein expression between treatments.

2.2.5 Immunocytochemistry and Immunohistochemistry

Primary stomach culture cells (1ml of culture media) were plated at a concentration of 1000000 cells per mL on sterile glass cover slips in a 12 well cell culture dish. After 24 hours in culture, wells were washed 3 x 5 minutes with TBS containing 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Cells were washed again 3 x 5 minutes with TBS. Cells were then treated with 500 µl per well of a permeabilizing/blocking solution (5% normal donkey serum (NDS), 0.1% Triton X-100 in TBS) for 30 minutes.

Paraffinized fundus tissue sections of mouse C57 stomach were purchased from Zyagen. Tissue sections were deparaffinized as indicated in (157) excluding the microwave antigen retrieval.

Primary antibodies for ghrelin and CSE were added to the cover slips and tissue sections and incubated overnight in blocking solution at 4 ℃ as indicated in table 1. Cells and tissue were washed three times in TBS and then incubated in secondary antibodies (Table 1) in blocking buffer for 45 minutes in the dark. After 3 x 5 minute washes in TBS, the cover slip was placed cell-side down into Fluoroshield Mounting Medium with DAPI (Abcam, Toronto, Canada) and

fixed using clear nail polish. The cells were observed in a dark room using an inverted Zeiss Axioplan fluorescent microscope and images were captured using the Zeiss AxioVision software (Zeiss, Oberkochen, Germany).

2.2.6 In vivo H2S experiments

Separate experiments were conducted for the H_2S inhibitor (PPG) and donor (GYY4137) studies. Following a week-long acclimation period in the animal care facility, male and female mice were divided into control and treatment groups. All animals were fasted overnight (16 hours) to ensure high basal ghrelin levels. For CSE inhibitor experiments, PPG (30 mg/kg body weight) or saline was injected intraperitoneally (IP) 16 hours prior to glucose delivery, while the H_2S donor GYY4137 (30 mg/kg body weight) or saline was IP-injected 30 minutes prior to glucose delivery. Animals then received an oral glucose gavage (2 g/kg body weight in water) to elicit a nutrient-induced drop in circulating ghrelin (80). A small prick was made in the saphenous vein of the animal and blood was collected into EDTA coated capillary tubes for ghrelin measurements at 0, 30 and 60 minute timepoints after the oral glucose gavage. After 60 minute blood collection, pre-weighed food was returned and consumption was measured at 1, 2, 3, 4, and 24 hour timepoints. Blood samples were stored on ice and centrifuged at 6000 x g, 4 ℃ for 6 minutes. 20 µl of plasma was used in an enzyme immunoassay kit for acylated ghrelin.

2.2.7 Ghrelin Assays

Ghrelin levels in cell culture and plasma samples were determined using an acylated ghrelin enzyme immunoassay kit (Cayman Chemical, Ann Arbor MI) as per manufacturer's guidelines. Dried media and lysate samples were resuspended in immunoassay buffer immediately before assay. Ghrelin levels were determined for entire media and cell lysate from each sample. Results for various treatments were presented as a percent secretion

(media/media+cell) relative to untreated control. Acylated ghrelin was analyzed using 20µl of plasma diluted in assay buffer.

2.2.8 Data Analysis

All data are expressed as mean ± SEM. Studies comparing 2 groups were analyzed by student's *t*-test. Studies with multiple doses of the same treatment were analyzed by one-way ANOVA, followed by a Bonferroni post hoc test. Studies with 2 independent variables were analyzed by two-way ANOVA, followed by a Bonferroni post hoc test at individual time points where applicable. $P < 0.05$ was considered significant.

2.3 Results

The H_2S donor GYY4137 suppresses ghrelin secretion *in vitro*

To determine the direct effect of H_2S on ghrelin secretion, a slow releasing H_2S donor, GYY4137, or control media was applied to a rat stomach primary culture for 4 hours. The mean total of secreted ghrelin from primary culture controls was 146.9 ± 15.58 pg/ml. Overall, GYY4137 decreased ghrelin secretion ($P < 0.05$ overall one-way ANOVA), with the highest dose (500 µM) causing the largest decrease (0.47 ± 0.07 fold of control Fig. 7A). Cell viability examined by the neutral red assay was not affected by GYY4137 treatments (Fig. 7B).

As previous studies have demonstrated a role for the AKT pathway in ghrelin suppression (63), we next investigated AKT phosphorylation in rat stomach primary cultures treated with GYY4137. GYY4137 caused a significant increase in the levels of phosphorylated/total AKT compared to control ($P < 0.01$, Fig. 7C). To determine if the AKT

pathway is required for GYY4137 mediated ghrelin suppression, we co-incubated cells with GYY4137 and the PI3K inhibitor LY294002. In these experiments, the effect of GYY4137 was not completely blocked by LY294002 (no statistical interaction); however, the significant suppression of ghrelin secretion normally seen with GYY4137 alone is lost in the presence of 0.1 µM LY294002 (*P* <0.01 in post hoc analysis, Fig. 7D).

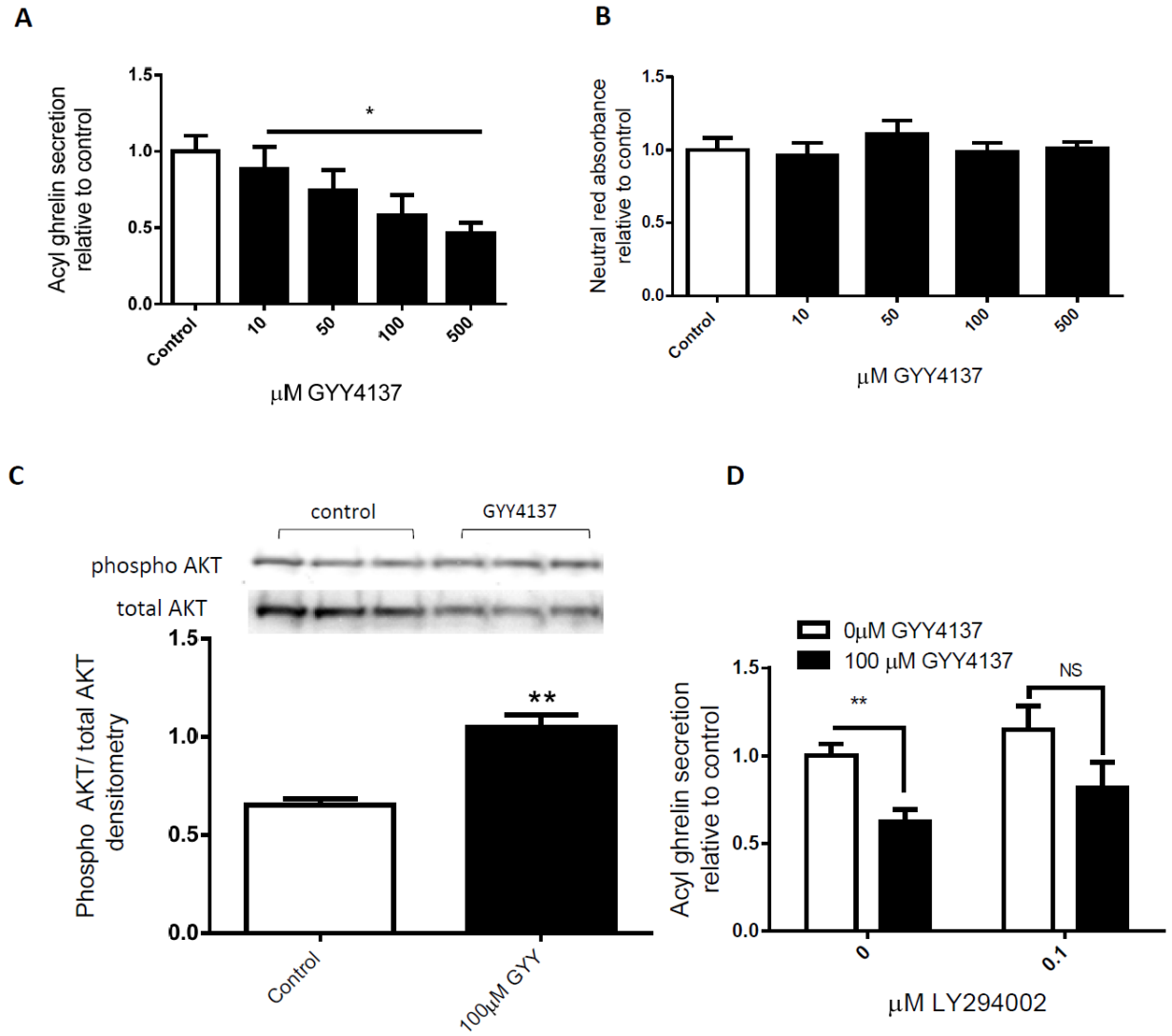

Figure 7: H2S suppresses ghrelin secretion in gastric primary culture.

Percent acyl ghrelin secretion (A) and neutral red absorbance (B) relative to control was examined after 4-hour treatments with GYY4137. Phosphorylated AKT and total AKT were analyzed using a Western blot in cells treated for 15 minutes with GYY4137(C). Percent acyl ghrelin secretion relative to control was examined after 4-hour treatments with GYY4137 and/or the PI3K inhibitor LY294002 (D). n = 3-6; * = $P < 0.05$ vs. control cells, ** = $P < 0.01$ vs. control cells.

Endogenous H_2S regulates Ghrelin *in vitro*

Since exogenous H_2S caused a suppression in ghrelin secretion, we next examined the role of endogenous H_2S in ghrelin regulation. To ensure the rat stomach primary culture was a suitable model to study endogenous H_2S production, we examined the protein expression of the H_2S-synthesizing enzyme CSE using fluorescent immunocytochemistry. CSE was expressed throughout the primary culture preparation (Fig. 8A). As such, all ghrelin positive cells in the primary culture preparation also co-expressed CSE (Fig. 8A). We then examined mouse stomach fundus using immunohistochemistry to examine abundance and distribution of ghrelin and CSE expressing cells (Fig. 8B). Cellular expression of cells producing ghrelin, CSE or co-localized expression were determined from a count of 100 cells (Fig. 8D). To ensure the fluorescent signal for CSE was specific to this enzyme, we completed a western blot of total cell lysate using the anti CSE antibody and detected a signal band at 44 kDa (corresponding to the correct molecular weight of CSE, Fig. 8C).

As we observed a high level of CSE signal in the primary culture preparation, we next determined the effect of inhibiting this enzyme on ghrelin secretion. Cells incubated with the

CSE inhibitor PPG had an overall increase in ghrelin secretion (P <0.001 one-way ANOVA), with the highest dose (10 mM) being 1.28 ± 0.06 fold of control (Fig. 8E) without any effect on cell viability as shown in the neutral red assay (Fig. 8F).

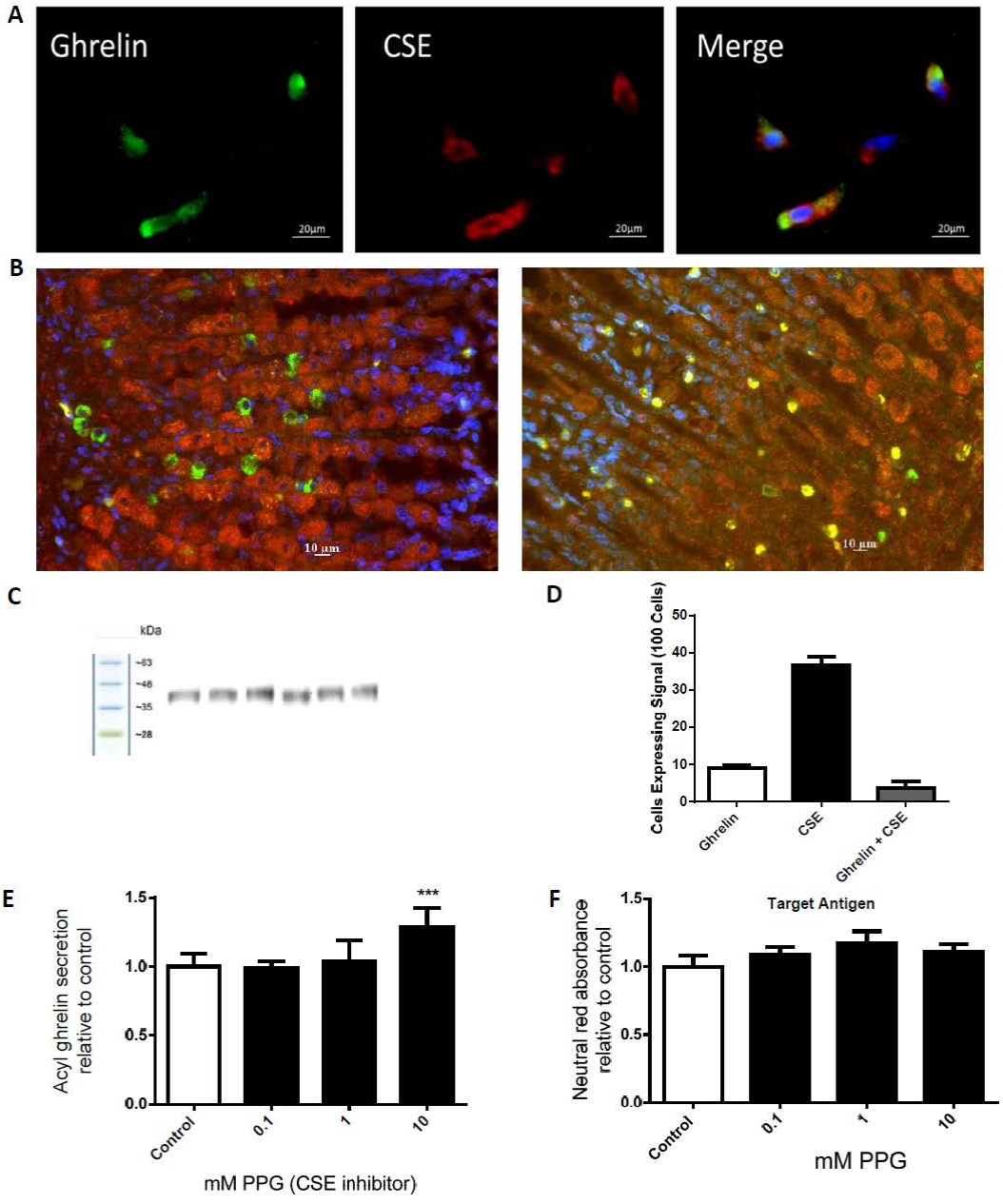

Figure 8: Inhibition of CSE stimulates ghrelin secretion.

Primary stomach culture and stomach fundus tissue were examined by fluorescent immunocytochemistry/immunohistochemistry for ghrelin (green), CSE (red), and nuclei (blue, in merge)(A, B). CSE MW was confirmed by western blot in primary stomach culture (C). Cells expressing ghrelin, CSE or both were counted in a 100 cell preparation to determine abundance and location within the fundus (D). Acylated ghrelin secretion from primary stomach culture was examined after 4-hour treatments with the CSE inhibitor PPG (E). Cell viability was examined under similar conditions (F). n = 6-9; *** = $P < 0.001$ vs. control cells.

In vivo effects of H2S on post-prandial ghrelin levels

All animal experiments were completed using both male and female mice. Since no statistically significant difference was observed between sex in all parameters investigated, we combined the male and female data sets. The mean basal plasma ghrelin levels of mice was 77.24 ± 4.35 pg/ml in the control group and 87.88 ± 8.23 pg/ml in the treatment group. To determine if the reduction of ghrelin secretion seen in the primary culture with the H_2S donor GYY4137 would translate to the vivo model, mice were given an IP injection containing either saline or 30 mg/kg GYY4137 prior to the nutrient gavage. While no difference in circulating ghrelin levels were observed at time 0 and 30 minutes between treatment and controls, GYY4137 significantly prolonged the post-prandial ghrelin suppression at 60 minutes with 0.83 ± 0.07 fold suppression for control (Male 0.90 ± 0.12, Female 0.75 ± 0.06) and 0.59 ± 0.07 fold suppression for treatment (Male 0.55 ± 0.07, Female 0.62 ± 0.13) (Fig. 9A, $P < 0.05$).

To determine if the increase in ghrelin secretion seen in our primary culture with the CSE inhibitor would translate to an in vivo model, mice were given an IP injection containing either saline or 30 mg/kg PPG prior to the nutrient gavage. While a trend of increased ghrelin levels

was seen 60 minutes after glucose in treated animals (Fig. 9B), this was not statistically significant ($P > 0.05$ two-way ANOVA treatment effect).

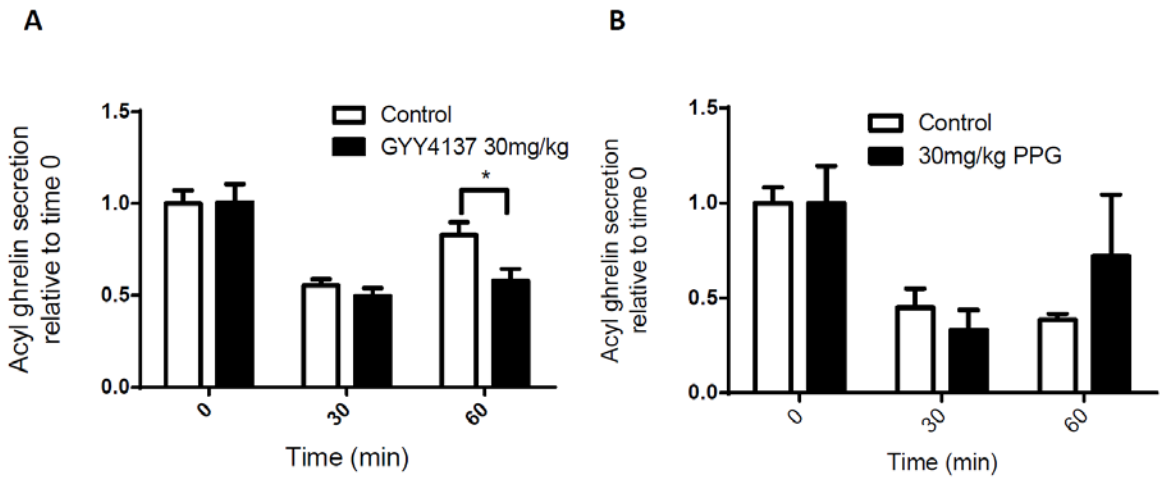

Figure 9: In vivo effects of H2S on post-prandial ghrelin suppression

Plasma acyl ghrelin was measured in mice receiving single injection of the H_2S donor GYY4137 (A) or the CSE inhibitor PPG (B) at 0, 30 and 60 minutes after and given an oral glucose gavage. n = 14 – 15 per group; * = $P < 0.05$ vs. control animals.

In vivo effects of H2S on food consumption

Since GYY4137 prolonged post-prandial ghrelin suppression in mice, we next evaluated its impact on food consumption. GYY4137 caused a significant reduction in cumulative food consumption (P<0.05 for treatment effect) with the 4 hour time point having the greatest difference at 1.04 ± 0.09 g in control (Male 1.02 ± 0.08, Female 1.04 ± 0.18) vs 0.77 ± 0.06 g in treatment (Male 0.85 ± 0.09, Female 0.69 ± 0.07) (Fig. 10A, P<0.01 post hoc test). When total food consumed was examined at the 24 hour mark, no difference in food consumption was observed (Fig. 10B). Similar to the lack of effect observed with PPG for ghrelin secretion, no effect was observed with PPG for food consumption (data not shown).

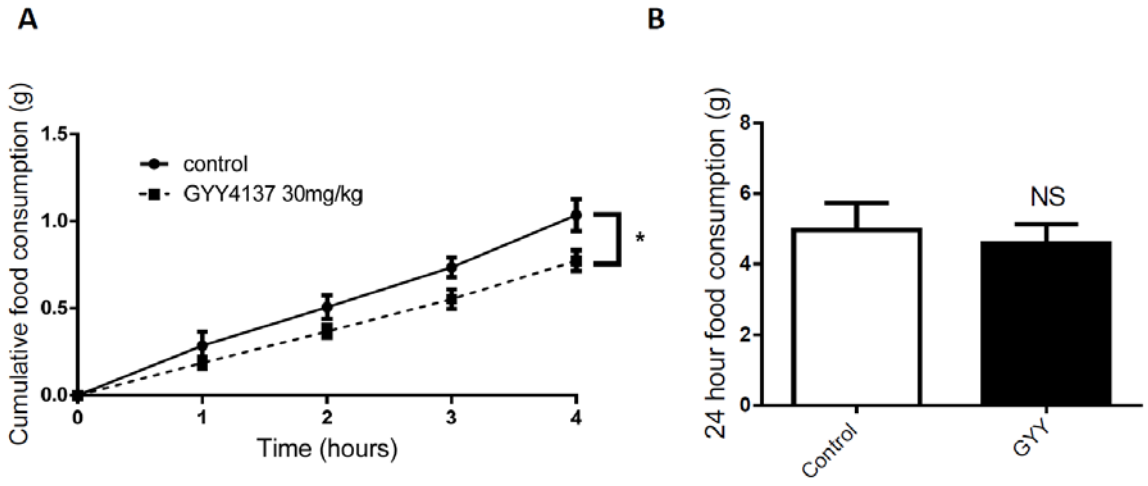

Figure 10: H2S reduces short term food consumption in mice.

Cumulative food consumption was examined in fasted mice after a single i.p. injection of the H_2S donor GYY4137 up to 4 (A) and 24 hours (B). n = 14 – 15 per group; * = $P < 0.05$ vs. control animals.

2.4 Discussion

In this study, we examined the role of H_2S in the regulation of ghrelin secretion and appetite. This was investigated through a combination of cell and animal-based studies using a H_2S donor, as well as an inhibitor of endogenous H_2S production.

Using primary stomach cultures, we determined that the H_2S donor GYY4137 causes a suppression in ghrelin secretion and an increase in the phosphorylation of AKT. We determined the use of the slow releasing H_2S donor GYY4137 would be the most effective H_2S donor to use as it can maintain steady H_2S concentrations for the length of both our primary culture and in

vivo experiments (158). H_2S readily diffuses from solution at room temperature and quick releasing H_2S donors would not be able to maintain media H_2S concentrations for our primary culture experiment duration (103). Our finding of increased AKT activation with reduced ghrelin secretion is in agreement with previous rat primary culture studies delineating the mechanism of insulin-induced ghrelin suppression (63). We recognize the lower total AKT levels in our GYY4137 treatments appear to be contribute to the increased ratio of phosphorylated AKT, although other studies have shown that AKT degradation occurs during vascular endothelial growth factor (VEGF) inhibition (159). GYY4137 may increase the degradation of AKT as it increases nitric oxide production which decreases VEGF release (160). The precise mechanism of how AKT phosphorylation is triggered by H_2S is not clear. Nevertheless, other groups have demonstrated the ability of H_2S to increase phosphorylated AKT levels in cancer cells (161) and endothelial cells (112). The latter being a required pathway for the proangiogenic effects of H_2S. When we co-incubated the primary culture with the H_2S donor and the kinase inhibitor LY294002, a partial loss of ghrelin suppression was observed. However, our analysis did not demonstrate significant interaction for LY294002, which suggests that pathways other than PI3K AKT may be involved in H_2S mediated ghrelin suppression.

Endogenous H_2S production from rat stomach primary culture was measured using a methylene blue experiment (data not shown). Measurable H_2S was unable to be quantified in our primary stomach culture as the quantity of cells produced from this method are not sufficient to measure H_2S production. This is due to the comparatively low levels of expression of H_2S producing enzymes in the stomach as opposed to the liver (130).

When examined in vivo, GYY4137 also caused lower levels of circulating ghrelin 60-minutes post-prandial. This prolonged drop is important since in humans ghrelin levels normally

rise 1-hour after oral glucose challenge (162). The ability of the H_2S donor to keep post-prandial ghrelin levels lower may lead to reduced feeding. Indeed, this delayed rise in ghrelin coincided with less food consumption over the next 4 hours. It should also be mentioned that while we predict that reduced ghrelin levels are responsible for the reduced food consumption in this treatment, additional experiments using ghrelin deficient mice would clarify this. This gap in food consumption between treatment and control eventually closed, as there was no difference in total food consumed at the 24 hour time point. Future work examining daily delivery H_2S donors will clarify whether prolonged food consumption effects are possible.

CSE and cystathionine-β-synthase (CBS) are the predominant H_2S producing enzymes in the body, although CBS is predominantly expressed in the central nervous system (125,163). As previous work demonstrated that inhibition of CSE reduced nearly all gastric H_2S production (126), we focused our examination of endogenous H_2S on CSE. We found CSE immunofluorescence throughout the stomach primary culture preparation. Expression was not restricted to ghrelin producing cells, however all ghrelin producing cells were positive for CSE. This suggests that the stomach possesses the ability to generate H_2S, and that ghrelin producing cells may be regulated by this gas in both an autocrine and paracrine manner. Furthermore, our detection of CSE enzyme in the stomach is in agreement with previous studies. Early work on CSE demonstrated that while the liver and kidney have the highest levels of CSE expression and activity, the stomach and intestine were the only other organs with detectable levels (130). As the H_2S donor treatments suppressed ghrelin, we predicted the inhibition of H_2S production would increase ghrelin. Indeed, when CSE was inhibited with PPG, we observed the expected increase in ghrelin secretion in vitro. Surprisingly, while we did see a trend towards increased 60 minute

rebound ghrelin levels in the PPG injected mice, this did not reach significance. It is possible that sampling the animals at 60 minutes to catch the post-prandial ghrelin rebound was insufficient, and that longer time course studies would give a clearer picture. Future work in larger animals (rats) will enable additional blood sampling to resolve this. Alternatively, some recent work has implicated the other predominant H_2S producing enzyme, CBS, in the stomach (164). An examination of the effects of CBS inhibitors on ghrelin levels would clarify the potential role of this enzyme in ghrelin regulation.

In this proof of concept study we investigated the role of both exogenous and endogenous H_2S in ghrelin regulation and physiology. Together these findings support the possibility that H_2S plays a suppressive role in ghrelin regulation. Future work should investigate if repeated H_2S delivery can mediate a sustained reduction in appetite. This could be done via chronic delivery of H_2S donors or diets enriched in precursors for endogenous H_2S production including cysteine and methionine. Another approach to increasing circulating H_2S may be through enhancing microbial H_2S production. Indeed, microbes are responsible for 50-80% of circulating H_2S (101). Our group has demonstrated that sulfur-containing prebiotics are able to enrich *Desulfovibrio piger* and elevate H_2S levels in the colon of mice, leading to increased GLP-1 secretion (85). While in the latter study ghrelin levels were not measured, it is possible that this colonic source of H_2S could have a significant impact on ghrelin cells.

In conclusion, this study demonstrates for the first time, a role for H_2S in the regulation of ghrelin and appetite in rodents. Future work will continue to elucidate the mechanisms of H_2S

action on ghrelin producing cells as well as the ability of H_2S to reduce appetite and stimulate weight loss through the ghrelin system.

3 Extended Discussion

3.1 Potential Applications of H_2S for Appetite Control

As seen in this study, the use of a H_2S donor can both decrease ghrelin secretion *in vitro* and delay post-prandial ghrelin secretion while reducing appetite in a mouse model. These findings support the idea that increased cellular H_2S production, or the supplementation of H_2S, may lead to prolonged appetite suppression. A study from our group using chondroitin sulfate, a prebiotic that can promote the growth of sulfate-reducing bacteria, and colonic H_2S production also showed an increased secretion of the appetite suppressing hormone GLP-1. This was accompanied by a decreased food consumption in obese mice given the prebiotic during the last 9 days of the appetite study (85). While we did not measure ghrelin levels in that study, these findings help support the idea that H_2S, whether of endogenous (through microbial or tissue-based production), or exogenous (through H_2S donors) sources, helps signal feelings of fullness and could promote sustained weight loss in obese individuals. In the present thesis, only short-term refeeding was examined. Indeed, our single injection did not yield sustained 24-hour reduced food consumption. Of interest would be a long-term appetite study where H_2S donors are given daily to monitor if appetite reduction can be sustained past the 24-hour period.

While the use of H_2S donors to modify ghrelin secretion is preclinical, there are some new therapies being tested containing H_2S releasing molecules. Nonsteroidal anti-inflammatory drugs (NSAID) are commonly used for chronic pain and have recently been shown to help treat specific cancers, although a common side effect of the drug is GI damage, including gastric lesions (165). A drug called ATB-346 was developed which combines a NSAID (Naproxen) with a H_2S donor. This compound is currently in phase 2 clinical trials and when tested not only

had greater apoptotic effects in melanoma cells but eliminated the side effect of gastric lesions associated with naproxen alone (166,167). Another current strategy that is undergoing clinical trials involves safely raising the bioavailable levels of H_2S in cardiovascular disease (CVD). It is currently known that patients undergoing heart failure have lower levels of H_2S, and that there are cardiac benefits from this gasotransmitter (108,168). SG1002 is an oral H_2S prodrug, and can safely raise H_2S levels in humans (169). The development of a safe treatments that increase bioavailability of this gasotransmitter could be a very effective tool not only in CVD but in other pathologies where elevated H_2S is desired. It would be interesting to see if the effect of these novel H_2S drugs on metabolic hormones, and by extension, on body weight and glucose metabolism.

3.2 The Effect of H_2S on Ghrelin: Future Perspectives

In this study we demonstrated that the H_2S donor GYY4137 suppresses ghrelin secretion causing a temporary decrease in food consumption. Although, this is the first step, additional experiments will need to be completed to fully implicate H_2S in ghrelin regulation and appetite.

While our data suggests that the AKT pathway is activated via H_2S and that plays a role in suppressing ghrelin release, additional experiments are required. The inhibitor LY294002 is a nonspecific inhibitor of PI3K and will prevent more than just the phosphorylation of AKT (170). The use of another PI3K inhibitor such as wortmannin or inhibitors of AKT like triciribine could increase the confidence in these results yet other experimental techniques would further strengthen this claim. Using a KO animal for the AKT gene would not be viable as the complete

KO of the AKT gene is lethal (171). Gene knockdown experiments, specifically transient gene knockdown with siRNA would be an effective way to study the involvement of AKT in our primary culture model. Unfortunately, the primary stomach cell preparation may not be the best cellular model for siRNA experiments as knockdown efficiency is often reduced in primary culture (172). Clustered regularly interspaced short palindromic repeat interference (CRISPRi) is another gene knockdown technique that could be used if transient knockdown is ineffective. This technique has been shown to have a higher knockdown efficiency in primary culture cells than transient knockdown (173). Gene knockdown techniques would effectively silence AKT expression and determine the AKT pathways involvement in H_2S induced ghrelin suppression.

To fully understand the effect H_2S has on ghrelin secretion a cellular mechanism must be identified. As mentioned in the introduction, H_2S has been shown to increase cAMP and PKA activity in cells which could potentially make them the target for sulfhydration as opposed to PI3K or AKT (95). To discover what the direct target of H_2S is in the signaling of ghrelin suppression, a modified biotin switch technique should be applied. An alkylating agent would be used to block reactivity of sulfhydryl groups present on proteins. Then the persulfide group will react with the added biotin conjugate allowing for measurement of sulfhydration between treatment groups (174). These proteins are then isolated and can be identified using a western blot to show the direct mechanism for the suppression of ghrelin secretion by H_2S in our rat stomach primary culture.

Another point that needs to be addressed when studying the effect of H_2S is the multitude of targets and the effects it has in vivo that were discussed previously. When injecting a H_2S donor such as GYY4137 H_2S will diffuse into all cells not only the gastric cells that secrete ghrelin. Alterations in inflammation and vascular tone can modify the stress response in mice which

could potentially manipulate appetite (89,111). H_2S can also regulate the secretion of GLP-1 and insulin altering the secretion of ghrelin (85,99). These confounding variables make the primary culture experiments conducted alongside the in vivo experiments essential to documenting the role that H_2S has in the regulation of ghrelin secretion. Aside from H_2S donors, the inhibitor PPG is not exclusively transported into gastric cells. PPG irreversible binds CSE effectively reducing H_2S production throughout the body (103). Unwanted effects of using PPG are similar to those seen in CSE KO mice (175). The CSE KO mice exhibit increased blood pressure and decreased vasorelaxation (110). All these variables will should be considered in future experimental design as the proper combination of experimental techniques can ensure the validity of our findings.

Future in vivo experiments will involve a study in high fat diet induced obese mice. Here, daily treatment with H_2S or supplementation of precursors of H_2S will be given to determine if H_2S can be used to help reverse an obese state. If that study shows that H_2S can reverse an obese state in mice, it would be important to research whether providing a prebiotic diet could increase H_2S production by the microbiome to the extent that it alters ghrelin release. Other future studies could include the use of CSE KO mice. The use of these mice would examine whether or not enzymatic production of H_2S in the stomach is responsible for ghrelin regulation.

4 Conclusion

H_2S is a gasotransmitter that has been examined thoroughly in biological systems and the role in the endocrine system is now being investigated. Ghrelin is one of many endocrine hormones that is primarily secreted from the stomach and is responsible for appetite stimulation, gut motility

and the nutrient metabolism. In our study we demonstrated a direct suppression of ghrelin secretion when treating our stomach primary culture with H_2S donors. This suppression of ghrelin secretion as well as a temporary reduction in appetite was also seen during an in vivo experiment examining the effects of H_2S donors in a mouse model on ghrelin secretion and appetite. Further examining the role H_2S has in ghrelin secretion and appetite will be a useful tool for learning about how this gasotransmitter can signal the regulation of endocrine hormones, and whether or not it can help alter or reverse different diseased states such as obesity.

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