The role of intestinal hydrogen sulfide on GLP-1 secretion and downstream metabolism

By

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Abstract

Metabolic hormones released from the gastrointestinal tract are core regulators for digestion, satiety, gut motility, and glucose homeostasis. Advancements in the management of type 2 diabetes and obesity have recently included the use of incretin hormone-based therapies, such as glucagon-like peptide-1 (GLP-1) analogs. GLP-1 can enhance glucose-dependent insulin secretion and stimulate satiety after a meal. The release of this incretin is stimulated by the entry of nutrients in the lumen or circulation. In addition, microbial metabolites have recently emerged as a new group of potent stimulators of GLP-1 secretion. Hydrogen sulfide (H_2S), produced from sulfate-reducing bacteria (SRB) of the distal ileum and colon, are localized in the same niche as GLP-1 secreting L-cells. The physiological role of endogenous H_2S is well established in many biological systems, however it is not well understood how or if H_2S can affect the enteroendocrine system. Therefore, we hypothesized that microbial H_2S from the distal GI tract can potentially alter GLP-1 secretion and downstream metabolism, and further investigated the potential mechanisms involved in the process. In murine colonic L-cells (GLUTag), we demonstrated that H_2S donors (NaHS and GYY4137) significantly enhanced GLP-1 secretion and that the process was regulated through the p38 MAPK signaling pathway. In male C57BL/6 mice, a 4-week chondroitin sulfate prebiotic diet successfully elevated SRB and colonic H_2S levels, enhanced the GLP-1 response, and reduced food intake. Together, this study demonstrated a direct role for H_2S in the stimulation of GLP-1 and a potential role for sulfur prebiotics to increase the H_2S producing SRB as a means to enhance GLP-1 and improve metabolism.

Keywords: Diabetes, gut microbiome, glucagon-like peptide-1, metabolism, hormones

Abbreviations

Akt: Protein kinase B

CAMP: Cyclic adenosine monophosphate

CCK: Cholecystokinin

CH_4: Methane

DPPIV: Dipeptidyl peptidase IV

EEC: Enteroendocrine cell

EPAC-2: cAMP-regulated guanine nucleotide exchange factor II

ERK 1/2: Extracellular signal-regulated kinase

FXR: Farnesoid X receptor

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GF: Germ free

GI: Gastrointestinal

GIP: Glucose dependant insulinotropic polypeptide

GLP: Glucagon-like Peptide

GPR: G-coupled protein receptor

GRPP: Glicentin-related pancreatic polypeptide

H_2S: Hydrogen Sulfide

IBD: Inflammatory bowel disease

IBS: Irritable bowel syndrome

K_{ATP}: ATP-sensitive potassium channel

LCFA: Long chain fatty acid

MAPK: Mitogen-activated protein kinase

MCFA: Medium chain fatty acid

NSAID: Non-steroidal anti-inflammatory drug

NTS: Nucleus Tractus Solitarius

OA: Osteoarthritis

PYY: Peptide Tyrosine Tyrosine

SCFA: Short chain fatty acid

SGLT: Sodium glucose linked transporter

SRB: Sulfate Reducing Bacteria

STZ: Streptozotocin

T2DM: Type 2 Diabetes Mellitus

TGR5: G protein-coupled bile acid receptor 5

Co-Authorship Statement

For the review paper published and shown in the section 1.7, I completed the manuscript with guidance and review of Dr. Gagnon.

For the research project shown in chapter 2, I completed all the cell culture and animal work which included blood collections, glucose tolerance tests, weight and food measurements, and maintaining food levels every day during the study. Nancy Fynn-Sackey provided some initial cell culture data about the effects of H_2S donors NaHS and GYY4137 on GLUTag cells as part of her 4^{th} year thesis project. Dr. Gagnon established the animal protocol and provided training on the blood collection and glucose tolerance test techniques. Dr. Gagnon also provided the general goal of the project and guidance on writing the manuscripts.

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1. Introduction

1.1 Gastrointestinal hormones & Metabolism

The gastrointestinal (GI) tract is the passage beginning from the mouth, down the esophagus, into the stomach and intestines, and ending at the anus. The breakdown of food consumed is accomplished through the release of enzymes from accessory digestive organs, the contraction of muscles that propels the food (peristalsis) through the GI tract, and the action of GI hormones. Along the GI tract are various types of cells that sense the presence of nutrients in the GI lumen or in circulation. Among the cells of the intestinal epithelium, are enteroendocrine cells (EEC). Most EECs release hormones in response to food-related stimuli, which govern metabolic processes that include digestion, glucose control, satiety, and gut motility (1,2). These particular EECs have direct contact with the luminal contents through their microvilli-covered apical surfaces (3,4). Once stimulated, hormone-containing granules are exocytosed through the basolateral membrane and into the connecting capillary network of the lamina propria, or they may act locally on surrounding cells (5). Hormones can act directly on tissues/organs, or they may bind to receptors on neurons that can send metabolic information to the central and peripheral nervous system (5). The rate and quantity of GI hormones released are determined by patterns of meal intake, the type of nutrient stimuli, and the localization of the endocrine cell (1). Generally, gut hormone levels increase after a meal, however ghrelin and somatostatin are some of the exceptions that peak before meals (6). Table 1 highlights many of the key gut hormones, their localization, and their main physiological roles in humans (although there could be many secondary sites of synthesis and effect).

Table 1. Key metabolic hormones (Adapted from (1))

Enteroendocrine Cell Type	Cell Localization	Major hormone released	Main function
L-cell	Ileum, colon	**GLP-1**	• Incretin effect • Appetite control • Gut motility
		GLP-2	• Enterocyte growth • Enhance intestinal function/blood flow
		PYY	• Water/electrolyte regulation • Appetite regulation
K-cell	Duodenum, jejunum	**GIP**	• Incretin effect • Inhibit gastric secretion/motility
G-cell	Stomach, duodenum	**Gastrin**	• Acid secretion • Gut motility
S-cell	Duodenum, Jejunum	**Secretin**	• Water regulation • Bicarbonate secretion
I-cell	Duodenum, jejunum	**CCK**	• Gall bladder secretion • Pancreatic enzyme secretion • Appetite control
P/D1 cell/Epsilon cells	(P/D1 cells in Stomach) (Epsilon cells in pancreas)	**Ghrelin**	• Appetite control • Growth hormone secretagogue • Energy balance
	Adipose tissue	**Leptin**	• Appetite control • Energy balance
Alpha-cell	Pancreas	**Glucagon**	• Conversion of glycogen into glucose • Inhibits glycolysis
Beta-cell	Pancreas	**Insulin**	• Glucose, carbohydrate, fats, and protein uptake in tissues (metabolism/storage)

1.2 Insulin stimulating GI hormones: Incretins

Insulin is well known for its essential metabolic role in the reduction of blood glucose. Insulin is released from the beta cells of the pancreas, and promotes the uptake of glucose and its conversion into glycogen in tissues, and halts the glucose production from the liver (7). Inversely to the effect of insulin, glucagon increases the hepatic glucose output and stimulates the breakdown of glycogen into glucose (8). Diabetes mellitus encompasses the metabolic disorders (microvascular and macrovascular diseases) caused by long-term exposure to high circulating glucose (9). This can occur from a dysfunction of insulin secretion (Type 1), tissues becoming unresponsive to insulin's function, termed insulin resistance (Type 2), or an insulin resistance developed during pregnancy due to hormonal changes (Gestational)(9). The development of insulin analogs (molecules that mimic insulin action) or insulin secretagogues (molecules that enhance insulin release) are the cornerstones of managing the many forms of diabetes.

There are many factors and regulators involved in the control of insulin secretion. Beta cells can sense changes in concentration of glucose and other nutrients in circulation, and will release insulin based on these rising levels (7). Insulin secretion can also be enhanced through incretin-dependent mechanisms (10). Incretins are GI hormones that are released post-prandially (after a meal) from EECs such as L-cells and K cells. These hormones travel in circulation to the pancreas where they can bind directly to surface receptors of beta cells and enhance glucose-stimulated insulin secretion (10). There are 2 incretin hormones: Glucagon-like Peptide-1 (GLP-1, from the L-cell) and Gastric Inhibitory Peptide (GIP, from the K cell) (10).

K cells are found in the crypts (invaginations) of the duodenum and jejunum (10,11). GIP is a 42 amino acid incretin, that also has a secondary role of inhibiting acid secretion from the stomach (12–14). The regulation of GIP is mainly through the entry of nutrients in the duodenum,

although neural stimulation by the vagus nerve has been suggested to be involved as well (15). Some of these nutrient stimuli include carbohydrates, glucose, lipids, amino acids, and long-chain fatty acids (4,10,16–19). Postprandial GIP levels peak between 10-20 min after food ingestion (16,18–20). While both GIP and GLP-1 are able to enhance insulin secretion, more attention has been given to GLP-1, as it has had greater therapeutic value in the treatment of type 2 diabetes mellitus (T2DM) (21–25).

GLP-1 is derived from the cleavage of proglucagon (a pro-hormone) by proteases in the L-cell, where there are more abundantly located in the ileum and colon (26,27)(Fig. 1). An N-terminal cleavage of proglucagon liberates glicentin (28), which can be further cleaved into oxyntomodulin and glicentin-related pancreatic polypeptide (GRPP) (29,30). A C-terminal cleavage liberates GLP-2, and the middle portion can be truncated to form GLP-1 (31,32). GLP-1 exists in two major forms in circulation: GLP-$1_{1\text{-}37}$, and a truncated GLP-$1_{7\text{-}37}$ that is more abundant and potent (32,33). Alpha cells from the pancreas can also produce small quantities of GLP-1 from the cleavage of proglucagon by proprotein convertases expressed in pancreatic islets. Alpha cells are interspread with beta cells, therefore, studies discussing the local alpha cell GLP-1 release describe a paracrine role for GLP-1 on beta cell regeneration (34). Rat and human islets produce small quantities of GLP-1 (~3.5 fmol/islet) (34), however picomolar concentrations of GLP-1 are required to activate the GLP-1R on islet beta cells to increase insulin secretion (35). The mechanisms of GLP-1 release from the alpha cell and L-cell are different, therefore, this study will focus on the L-cell derived GLP-1 release.

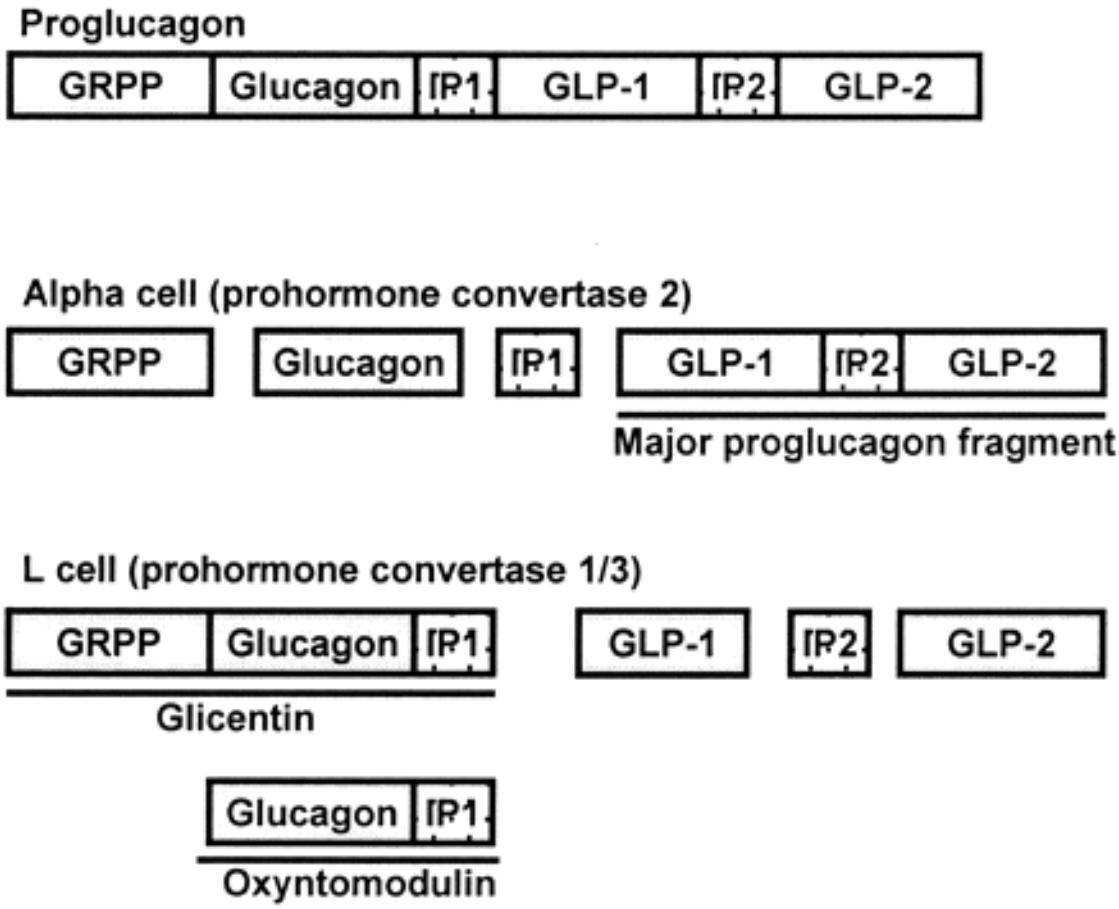

Figure 1. Proglucagon posttranslational processing (Adapted from (36)**).**

1.3 Mechanisms of GLP-1 secretion and action on the pancreas

There are two phases of postprandial GLP-1 release: the initial secretion within 15 min of food intake, and a second phase that occurs later in digestion after 90 – 120 min (37). L-cells are located in the distal small intestine of humans and rodents (38), therefore the initial GLP-1 release is proposed to be stemming from neural stimulation, and the later secretion from luminal or circulating nutrients binding directly to the cell's surface receptors (39). Nutrients including fatty acids, glucose, and bile acids can bind to their respective receptors on the L-cell and stimulate the release of GLP-1 vesicles by activating certain intracellular pathways (Fig 2).

1) Short chain fatty acids (SCFAs) from microbial fermentation and medium/long chain fatty acids (MCFAs and LSCFAs) from the breakdown of food can bind to fatty acid receptors on the surface of the L-cell. These nutrient receptors have been identified as GPR120 (40), GPR119 (41), GPR40 (42), and GPR41(43,44), and GPR43(43). Fatty acids binding to their receptor results in an elevation of intracellular Ca^{2+} ions, and the subsequent activation of a Ca^{2+}-sensitive transient receptor potential channel M5 (TRPM5) (40,42). TRMP5 stimulates the entry of Na^{+} ions, causing a membrane depolarization and the release of GLP-1 vesicles from the cell.

2) L-cells also express sweet taste receptors that can respond to various sweet molecules including natural sugars, sweeteners, and sweet proteins (45). These receptors can recruit sodium glucose cotransporters (SGLT-1), that can uptake the monosaccharide coupled to a Na^{+} ion into the L-cell (46). Once the monosaccharide is metabolized into ATP, the resulting energy triggers the ATP-sensitive potassium (K_{ATP}) channel. The K_{ATP} channel allows the entry of K^{+} ions and release of Na^{+} ions, causing the membrane to depolarize.

L-type calcium channels are activated from the depolarization, and allow an influx of Ca^{2+} ions and the subsequent exocytosis of GLP-1 vesicles (47).

3) Bile acid is another well-established stimulus of GLP-1 secretion (48). L-cells express a G protein-coupled bile acid receptor GPBAR1(TGR5) and a nuclear receptor farnesoid X receptor (FXR) (49). Once activated, these induce an elevation of intracellular cyclic adenosine monophosphate (cAMP). Similarly to the previous two nutrient-stimulating pathways, cAMP causes a rise in Ca^{2+} ions, and the subsequent secretion of GLP-1 vesicles (49).

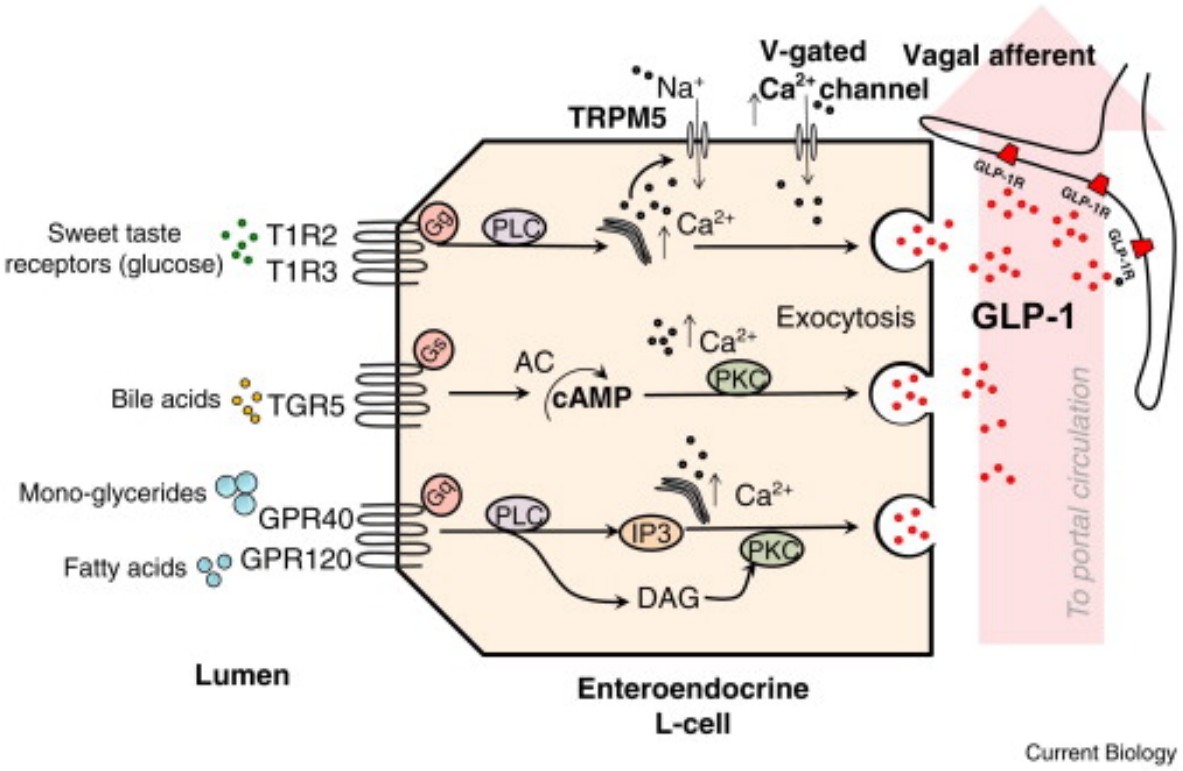

Figure 2. Intracellular mechanisms of GLP-1 secretion within the L-cell (Adapted from (50)**)**

Once GLP-1 is released from the L-cell it may enter the lamina propria and diffuse into the capillaries. Circulating GLP-1 level can be regulated by the enzymatic activity of dipeptidyl peptidase IV (DPPIV) (51,52). In the context of insulin secretion, GLP-1 binds directly to the GLP-1 receptor on the surface of the beta cell and causes the release of its insulin vesicles. G protein coupled receptor activation leads to the activation of adenylate cyclase, the elevation of cAMP, the activation of protein kinase A (PKA), and cAMP-regulated guanine nucleotide exchange factor II (Epac2) (10,53). Similar to glucose, GLP-1 acts by closing the K_{ATP} channel, causing the membrane to depolarize and an influx of Ca^{2+} ions (53). The rise of Ca^{2+} ions will stimulate the exocytosis of insulin vesicles into circulation (53).

This peptide also possesses many other metabolic functions that include slowing gastric emptying (how quickly food leaves the stomach) to induce satiety, and reducing glucagon release from the alpha cells (54–56). Therefore, GLP-1 has an essential role to play in the treatment of T2DM and more recently discovered obesity. GLP-1 agonists such as Exenatide (Byetta/Bydureon) and Liraglutide (Victoza/Saxenda) are currently on the market as non-insulinic options for T2DM. Many studies have administered Liraglutide (57–61) and Exenatide (62,63) at different doses, and have shown significant glycemic benefits in both rodents and humans. These products have many advantages for T2DM patients: they can be taken in combination with insulin medication, do not have any risks of causing hypoglycemia, and have been shown to help the patient lose weight. Recently, Liraglutide has been approved as a treatment for obesity in Canada. The role of GLP-1 in food intake and obesity will be reviewed in the subsequent section.

1.3 GLP-1's action in the brain

Mechanoreceptors and chemoreceptors dispersed along the GI tract can detect the entry of food and nutrients, and signal the information through the vagus nerve to the brain (Reviewed in (64)). However, GI hormones such as GLP-1 are also essential appetite regulators. Because GLP-1 has a short half-life in circulation, it has been suggested that this peptide binds to peripheral receptors on the vagal nerve (65) and transmits metabolic information to the nucleus tractus solitarius (NTS) in the brainstem (66–70), which then signals the hypothalamic nuclei(66,71). However, the complete mechanism involving GLP-1 and satiety is not completely understood.

There are other hormones that can also play a role in hunger and food intake, including peptide tyrosine tyrosine (PYY), leptin, ghrelin, and others. However, GLP-1 is the only GI hormone to have an approved analog used for weight loss. Liraglutide (marketed under Saxenda in Canada) has become an attractive medication for T2DM because of its secondary effect of weight loss, as previously mentioned.

In summary, digestion, glycaemia, and food intake are all regulated in part by gut hormones like GLP-1. The entry of nutrients in the GI tract, dispersed peripheral neurons, and other hormones may regulate the secretion of GLP-1. Because GLP-1 is a key player of insulin secretion and core to the progression of T2DM, there is an important need in diabetes research on other ways GLP-1 levels can be enhanced. The intestinal microbiota has recently emerged as an important contributor to our metabolism and many biological functions.

1.4 Gut Microbiota & Metabolism

Among the diverse community of EECs that are dispersed throughout our GI tract, lives another important cellular community: the gut microbiome. The human colonic microflora is composed of thousands of species, however, each individual's microbial community interpersonally differs from another (72). Only approximately 160 species are conserved throughout each individual as a core gut microbiome (72). As reviewed in (73), the gut microbiota contribute to many essential processes including: complementing mammalian enzymes in the breakdown of non-digested material, protecting from the entry of pathogens, stimulating the immune system, and eliminating toxins. Also reviewed in (74), a potential role of this gut community on human health has been demonstrated in a variety of studies comparing the fecal microbiota of healthy individuals to patients with obesity, diabetes, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), colon cancers, and others (75).

The gut microbiome emerged in obesity research due to differences of microbial composition between obese and lean individuals. One of the first major studies examining these microbial differences used a genetic model of obesity (ob/ob) in mice, and observed different cecal bacterial communities between obese and lean wild type (76). Further investigation revealed that bacteria specialized in energy extraction were more abundant in the ob/ob mice than in the lean group. These results suggested that the composition of the microbiota can promote or resist obesity in these mice (76). Another important study confirming the relationship between bacteria and obesity was one that inoculated germ-free mice (GF) with the luminal contents of lean or obese mice (77). GF mice that received the microbiota from obese donors gained more weight than the lean group, with equivalent food intake. GF mice are also resistant to diet-induced obesity and are significantly leaner than conventional mice. Differences in obese and lean

individual microbial communities were also examined in humans, and further studies determined diet interventions can shape and specialize the bacterial community (78). Recent research has also demonstrated that the obese microbiome has a depletion of the phylum of *Bacteroidetes*, a reduction of overall bacterial diversity, and species specialized in carbohydrate and lipid-utilization (79).

Therefore, the composition of the gut microbiota may predispose an individual to obesity, and contribute to it with enhanced absorption of lipids and carbohydrates. However, what mechanisms link the microbiome to obesity and other metabolic complications?

1.5 Bacterial metabolites

Bacteria in the GI tract metabolize undigested host derived dietary components for their own metabolic needs (Reviewed in (80)). The small intestine of humans is capable of digesting approximately 90% of proteins/carbohydrates (81–84), while the rest are left to bacterial degradation . Although the stomach and small intestine are efficient in their digestion, a significant portion of these compounds may reach the large intestine where the microbiota is most abundant (84,85). Microbial metabolites are the resulting by-products of the breakdown of these undigested materials, where the highest microbial activity and accumulation of metabolites can be seen in the large intestine. As previously described, EECs are dispersed throughout the GI tract and can come into contact with these products. A review by Neuman et al. (86) has described how some gut bacteria and their metabolites can alter the endocrine system, however this introduction will focus specifically on the crosstalk between L-cells and potential microbial metabolites.

Several recent studies have suggested that the presence or absence of certain microbial groups can alter GLP-1 release. GF and antibiotic treated mice have elevated GLP-1 levels (87).

Probiotics containing butyrate-producing bacteria given to mice also increased GLP-1 level (88). Beneficial increases of GLP-1 levels in rats (89) and humans (90) have also been correlated with changes in the composition of the intestinal microbiota. Many other bacterial metabolites are present in the gut from colonic microbial metabolism, including polyphenols, secondary bile acids, and microbial gases (91). However, relatively little is known on the mechanisms behind metabolite-induced GLP-1 secretion.

Microbial gases are largely produced in the colon, and these include hydrogen (H_2), hydrogen sulfide (H_2S), and methane (CH_4). Hydrogenotrophs include members like sulfate reducers, methanogens, and acetogens that compete for the hydrogen to use for their own metabolism (Reviewed in (92)). The existence of these bacteria are essential to the proliferation of other gut microbiota members because the accumulation of protons (produced by fermentative bacteria) can limit or be detrimental to the growth of the gut microflora (Reviewed in (92)). Hydrogen is cross-fed between species and reduced to hydrogen sulfide or methane. The resulting gas from these bacterial metabolic processes are released through the lungs, flatus, or absorbed in the colonic tissue (92). Healthy humans can produce liters of gas per day, therefore it is likely these gases can reach high concentrations in the lumen of the colon (93).

1.6 Sulfate-reducing bacteria and hydrogen sulfide

Sulfate-reducing bacteria (SRB) are the main bacterial producers of H_2S in the intestine (94). SRB are obligate anaerobic bacteria and belong to a few taxonomic groups under the δ-Proteobacteria phylum, namely: gram-negative mesophilic SRB, Gram-positive spore-forming SRB, bacterial thermophilic SRB, and archaeal thermophilic SRB (95) . SRB use dissimilatory sulfate reduction, a form of anaerobic respiration that uses sulfate as the electron acceptor and

hydrogen (or organic compounds) as electron donors to synthesize H_2S (96,97). This reaction is summarized as followed:

$$4H_2 + {SO_4}^{2-} + H^+ \rightarrow \boldsymbol{HS^-} + 4H_2O$$

Sulfate may be provided by the host through an inorganic source (i.e. sulfite, sulfates) or organic sources (i.e. sulfated mucins or dietary intake) (94). High levels of H_2S can be toxic, however the colonic tissues contain efficient detoxifying enzymes and storage mechanisms to eliminate H_2S before it is released from the body (98). The concentration of H_2S in the lumen varies depending on substrate (sulfate) availability, and the abundance/activity of SRB. H_2S is also produced ubiquitously in many other tissues through non-microbial processes (endogenous H_2S), and many studies have examined its physiological importance in other systems, as thoroughly reviewed by (99). The known and potential roles of H_2S in GI hormone regulation and downstream glucose homeostasis and diabetes are presented in the subsequent review article.

1.7 Implications of Hydrogen Sulfide in Glucose Regulation: How H_2S Can Alter Glucose Homeostasis through Metabolic Hormones

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Abstract

Diabetes and its co-morbidities continue to be a major health problem worldwide. Understanding the precise mechanisms that control glucose homeostasis, and their dysregulation during diabetes, are a major research focus. Hydrogen sulfide (H_2S) has emerged as an important regulator of glucose homeostasis. This is achieved through its production and action in several metabolic and hormone producing organs including the pancreas, liver, and adipose. Of importance, H_2S production and signaling in these tissues is altered during both type 1 and type 2 diabetes mellitus. This review first examines how H_2S is produced both endogenously and by gastrointestinal microbes, with a particular focus on the altered production that occurs during obesity and diabetes. Next, the action of H_2S on the metabolic organs with key roles in glucose homeostasis, with a particular focus on insulin, is described. Recent work has also suggested that the effects of H_2S on glucose homeostasis go beyond its role in insulin secretion. Several studies have demonstrated important roles for H_2S in hepatic glucose output and adipose glucose uptake. The mechanisms of H_2S's actions on these metabolic organs are described. In the final part of this review, future directions examining the roles of H_2S in other metabolic and glucoregulatory hormone secreting tissues are proposed.

Introduction

Hydrogen sulfide (H_2S) is a colorless gas that is produced both endogenously by a variety of mammalian cells and by the sulfate-reducing bacteria in the lower gastrointestinal tract. H_2S has emerged as an important gasotransmitter that regulates several systems including the cardiovascular, GI, immune, endocrine, and nervous systems (reviewed in detail in (99)). One area of recent interest is the potential role that H_2S may play in glucose regulation and metabolic health. Indeed, several groups have demonstrated that H_2S levels are altered obese and diabetic circulation (100,101) and tissues (102,103). The precise mechanisms of how H_2S can drive metabolic changes are beginning to be understood. A major factor in the regulation of glucose metabolism is the secretion and action of metabolic hormones. These hormones include insulin, glucagon, leptin and Glucagon like peptide-1 to name a few. Several groups have already described the action of H_2S on insulin secretion (104–106). Furthermore, recent work has demonstrated the effects of H_2S on downstream hormone signaling action (107). These studies, and others, suggest that H_2S may be a potential target in the treatment of metabolic diseases through modulating metabolic hormone secretion and signaling. The goal of this review is to describe the roles of H_2S in the regulation of metabolic hormone secretion, with a particular focus on insulin, and the downstream signaling of these hormones in the regulation of energy homeostasis.

H_2S Production

Although the presence of H_2S in the body has been known for some time, the precise locations of its production remain an active area of research. H_2S is produced by a large variety of cell types

in the body (here named endogenous) and by host microbes including the sulfate-reducing bacteria in the GI tract. The main enzymatic machineries in the endogenous production of H_2S are the cystathionine metabolizing cystathionine-β-synthase (CBS) (108) and cysthanionine γ-lyase (CSE)(109). Other enzymes such as 3-mercaptopyruvate sulfurtransferase (MST) and cysteine aminotransferase (CAT) are also important in specific tissue types (110). CSE activity is much higher than CBS in peripheral tissues, while CBS mainly predominates in the brain (111,112). The precise mechanisms involving the production of endogenous H_2S are thoroughly reviewed by Wang in (99). Once H_2S is produced in the cell, it can act on different cellular pathways or be stored for later release. H_2S can store its sulfur group with iron (acid labile sulfur) (113) or in sulfane sulfur (a persulfide)(114) in mammalian tissues. When required, and under the appropriate conditions, this bound sulfur can be released as S^{2-}, HS^-, or H_2S (115).

In addition to endogenous generation, H_2S can be produced from microorganisms in the GI tract. The gut microbiota aids in the decomposition and harvest of nutrients from food, a crucial step in energy production. Primary fermenters break down protein and complex carbohydrates into short-chain fatty acids (e.g., acetate, proprionate, and butyrate) that are an important energy source, and gases (e.g., hydrogen, carbon dioxide) that are released or absorbed by the system. Hydrogenotrophs, or H_2-consuming bacteria, are essential in keeping luminal hydrogen levels low, and stabilizing the environment for these primary fermenters. Among the groups of hydrogenotrophs are methanogens (produce methane), acetogens (produce acetate), and sulfate-reducing bacteria (produce H_2S). Sulfate reducing bacteria use hydrogen or organic compounds as electron donors and use sulfate as their terminal electron acceptor leading to a large production of H_2S. This process is known as dissimilatory sulfate and can lead to mM concentrations of H_2S in the lumen (116). Sulfur sources from diet can originate from amino

acids, preservatives, food additives (carrageenan), or as dietary supplements (chondroitin sulfate)(116). Microbial produced H_2S is a significant contributor to the bodies H_2S pool, as germ free mice have between 50-80% less H_2S in their tissues and circulation (117). Microbial H_2S has been associated with both maintaining gastric health, and being implicated in disease. Several groups have shown that H_2S regulates various physiological functions including maintenance of GI barrier function and injury repair (118). Some earlier studies have suggested that H_2S may be involved in the etiology of ulcerative colitis (119). However, more recent work points towards a protective role(120). Regardless of its source, H_2S has emerged as a regulator of glucose metabolism. The mechanisms of this action are described below.

Importance of H_2S in diabetes and insulin regulation

Insulin is one of the most researched, and clinically important, of the metabolic hormones. Strategies that seek to enhance insulin secretion and sensitivity are the cornerstone of diabetes treatment. Insulin biosynthesis is regulated by many physiological events, however the main driver of its secretion is circulating glucose, such that after a meal is consumed, the levels of insulin spike in circulation. Insulin then acts on a variety of tissues in the body, including, but not limited to, adipose, liver, and muscle. The cells are activated through the insulin receptor which then leads to increased translocation of glucose transporters to the membrane and glucose uptake. During the development of type 2 diabetes mellitus (T2DM), insulin signaling in the target tissues is impaired, and in order to overcome this resistance, the β-cells of the pancreas begin to proliferate and produce more insulin. In cases where the pancreas is unable to produce sufficient insulin to regulate the rising glucose levels, T2DM develops. In this scenario, a variety of treatments that act to increase insulin levels or enhance insulin signaling are employed.

Nevertheless, additional strategies to enhance insulin levels and signaling are of great interest in the treatment of diabetes and metabolic disease.

The investigation of hydrogen sulfide's potential involvement in glucose metabolism began in 1990 when Hayden and colleagues showed that a low dose (75ppm) H_2S exposure increased circulating glucose in postpartum rats (121). Later on, several groups began to investigate how H_2S levels fluctuate in metabolic disease. Human studies that have examined circulating H_2S in T2DM have found them to be reduced. Jain and colleagues found that T2DM individuals had significantly lower H_2S compared to age matched non diabetics (100). Whiteman and colleagues confirmed these findings and further demonstrated that adiposity was negatively correlated with H_2S (101). This is of particular interest since obesity is one of the principal causes of T2DM. Unfortunately, the mechanisms driving these changes in circulating H_2S or their effects on glucose metabolism were not investigated. As such, it is unclear whether the altered circulating H_2S observed in obese individuals is a driving force in their metabolic disease. A more mechanistic understanding of how H_2S can alter glucose metabolism has come to light through the examination of glucoregulatory hormones such as insulin and its target tissues. These pathways and their role in glucose homeostasis are described below.

H_2S production and function in the pancreas

The first evidence that H_2S was produced in the pancreas, and that it played a role in the regulation of insulin secretion, came from Yang and colleagues. Using the INS-1 cell line, they demonstrated that β cells express the enzymatic machinery required to produce H_2S, including CSE, and can produce high levels of H_2S which blocks glucose-stimulated insulin secretion

(106). This was later confirmed in another β cell model, Min6 (122). Yang and colleagues also demonstrated that treating INS-1 cells with H_2S, or overexpressing CSE, stimulated apoptosis (105). In addition, other groups have demonstrated the mRNA expression of both CSE and CBS in the rat pancreas, and that streptozocin-induced diabetes (a model of type 1 diabetes) causes increased mRNA expression of CBS and increased H_2S production (102). Using a rodent model of obese diabetic (the Zucker diabetic fatty rat), Wu and colleagues demonstrated that the animals impaired glucose metabolism was due to an overproduction of pancreatic H_2S and impaired insulin secretion (104). Together, these studies suggest that increases in H_2S may be responsible for a reduction in insulin secretion and ultimately the impaired glucose clearance that occurs in diabetes. However, other groups have suggested that the elevated H_2S production from the β cell is occurring as a result of elevated circulating glucose, and that H_2S is acting as a pancreatic brake, which may protect these insulin producing cells from being over stimulated by chronic hyperglycemia (123). Indeed, it was later demonstrated that mice on a high fat diet lacking CSE have significantly worse islet glucotoxicity compared WT animals (124). This protective role for H_2S in β cell apoptosis occurs through H_2S mediated activation of thioredoxin, a system responsible for controlling redox homeostasis that protects β-cells from glucotoxicity. The difference in reports of the protective vs toxic effect of H_2S in the pancreas may be due to the cell/animal model being used (whole animal vs cell studies, and type 1 vs type 2 diabetes models). Nevertheless, H_2S is produced in the pancreas and this appears to have important implications in insulin secretion and glucose homeostasis. How this gasotransmitter can elicit its effects on the cell is discussed below.

Mechanism of H_2S action in the pancreas

The earliest reports on the intracellular target of H_2S in insulin regulation was found to be an opening of the K_{ATP} channel (106). When glucose enters the β-cell, it generates ATP, causing the closure of ATP sensitive K_{ATP} channels and opening of calcium channels leading to depolarization, and thus insulin secretion (125). When K_{ATP} channels are kept open by H_2S, the β cell is hyperpolarized and insulin secretion is suppressed. Based on this, several groups have demonstrated that compounds that suppress the production of H_2S can increase the secretion of insulin from β cells (106,122). The precise mechanisms involved the opening of this channel remains an active area of research. It has been suggested that direct binding of H_2S to cysteine residues in proteins (sulfhydration) may be a potential mechanism (126). Using the patch clamp method coupled with channel subunit mutagenesis, Jiang and colleagues demonstrated the importance of the rvKir6.1/rvSUR1 subunits in mediating K_{ATP} channel opening (127). It should be noted however that the above studies on the precise mechanisms of H_2S on the K_{ATP} have not been done in the β cell.

Voltage-dependent calcium channels (VDCCs) in the β-cells control the movement of calcium, a crucial step in glucose stimulated insulin release. One of the early studies examining the effect of H_2S in β-cells found that NaHS (an H_2S donor) caused a decrease in the calcium oscillations caused by glucose, which ultimately led to reduced insulin secretion (122). Using whole mouse islets, Tang and colleagues demonstrated (via patch clamp) that L-type VDCC current density is inhibited by the H_2S donor NaHS, and that islets from mice lacking CSE had reduced L type VDCC activity (127). Of interest, these reports of decreased VDCC activity in β-cells and islets are in contrast to the increased calcium concentrations that result from H_2S in cerebellar granule

neurons (128). This difference suggests that H_2S may regulate similar intracellular pathways in distinct manners depending on the cell type.

In addition to ion channel activities, H_2S may also regulate insulin secretion through the modulation of intracellular kinases. Several of these kinases are known to be modulated during the secretion of insulin and other hormones including PI3K, ERK, AKT and MAPK. H_2S mediated suppression of insulin secretion has been shown to cause phosphorylation of p38 MAPK (105). Indeed, activation of the MAPK/JNK pathway is a known mechanism of impaired insulin release from the β cell (129). More studies are required to determine if additional cell signaling pathways are altered through the activity of H_2S.

H_2S effects on metabolic tissues

The description thus far focused on the production and effects of H_2S in the insulin secreting β cell. A vital part of glucose homeostasis is the function of the insulin sensitive metabolic organs, including adipose tissue, liver and muscle, and their interaction with the brain.

One of the principle targets of insulin is the adipocytes. Insulin promotes the storage of excess glucose, and its conversion to fat, leading to increased adiposity; a major risk factor for the development of metabolic disease. Several groups have demonstrated that adipose tissue produces H_2S, and that gasotransmitter production and signaling in the adipocytes is altered during obesity. Feng and colleagues were the first group to describe the expression of CBS and CSE, and production of H_2S from rat adipocytes (130). In this report they demonstrated that H_2S impairs insulin mediated glucose uptake, and that high fructose-induced diabetes led to increased production of H_2S in epididymal adipose tissue; an effect that could be blocked by inhibiting

CSE. This result points toward a negative effect of H_2S on glucose metabolism in the adipocytes. Interestingly, circulating levels of H_2S are lower in obese humans (101), suggesting a disconnect between the increased production observed in the rodent adipose tissue. Some groups have demonstrated a positive role for H_2S in glucose metabolism in the adipocytes. One study in 3T3L1 adipocytes found that H_2S is required for vitamin D induced GLUT4 translocation and glucose uptake (131). Another positive role for H_2S in adipose tissue metabolism appears to be its role in reducing inflammatory cytokine production from resident adipose macrophages. These cytokines are a known causal factors in the development of insulin resistance in adipose and other metabolic tissues (132). In one study, macrophages isolated from mice with diet-induced obesity produced less H_2S and more cytokines than macrophages from lean mice (103). Based on these reports, it may be important that future work in adipose tissue (from obese subjects) separate the adipocytes from the stromal vascular fraction. Several studies have also shown a role for the H_2S /CSE system in perivascular adipose tissue, although most of this work has described its importance in vascular tone (reviewed in (133)) rather than glucose homeostasis.

Another key organ in the regulation of glucose metabolism is the liver. During an elevated circulating glucose scenario, insulin acts on the liver to stimulate glucose uptake and its conversion to glycogen and fatty acids for storage. In a low glucose scenario, pancreatic glucagon acts on the liver to promote the production or liberation of glucose through gluconeogenesis or glycogenolysis, respectively. Dysregulation of insulin signaling in the liver (hepatic insulin resistance) is a common phenomenon in T2DM (reviewed in (134)). The mRNA expression of both CSE and CBS was demonstrated in the liver of rats, and was found to increase after inducing type 1 diabetes with Streptozotocin (STZ) (102). Later on it was demonstrated that overexpressing CSE in hepatocytes leads to reduced glycogen content. In this study, it was also

shown that CSE KO animals (lower H_2S) have a reduction in endogenous glucose production (135). A recent study by Ju and colleagues demonstrated a mechanism by which H_2S may directly stimulate gluconeogenesis. They found that pyruvate carboxylase (a key enzyme in gluconeogenesis) is sulfhydrated by H_2S, which leads to increased activity and glucose production (107). These findings seem to indicate that H_2S production in the liver causes enhanced glucose release, an effect that would aggravate the hyperglycemia observed in diabetes.

Surprisingly, there is a paucity of studies that have examined the role of H_2S in skeletal muscle, let alone, skeletal muscle glucose uptake. This may be due in part to the low, or non-detectable levels of the H_2S producing enzymes in rodent models (in contrast to the higher levels found in human muscle, reviewed in (136). Nevertheless, future work should, at the very least, examine the effects of H_2S donors since H_2S may act on muscle tissue via its circulating stores.

Other hormones and future work

While H_2S plays important roles in the metabolism of hormones like insulin and glucagon, a variety of other metabolic hormones remain to be examined. One emerging area holding potential for this is the gastrointestinal endocrine system. Here, a variety of enteroendocrine cells secrete numerous peptide hormones that play important roles in glucose homeostasis and energy metabolism. Some important candidates are the insulin-stimulating incretin hormones; glucose-dependent insulinotropic polypeptide (GIP) and glucagon like peptide-1 (GLP-1). Recently, Bala and colleagues examined the role of endogenous H_2S in a GI endocrine cell line, STC-1 (137). This cell line secretes the GLP-1 and the anorexic hormone peptide YY (PYY). They found that

H_2S donors and L-cysteine impaired oleic acid stimulated GLP-1 and PYY secretion. While their primary focus was on the modulatory effect of H_2S on oleic acid stimulated hormone secretion, their results support that further investigation of the H_2S on GI hormone secretion and signaling is warranted. Do GI endocrine cells produce their own H_2S, and is the altered H_2S level observed in obesity responsible for the dysregulation in GI hormone secretion (138)? Of importance, GLP-1 therapies have become a major tool in the treatment of type 2 diabetes (139) and recently obesity (140). Therefore, the role H_2S has in GLP-1 and other endocrine cells may be an additional mechanism by which this gasotransmitter can regulate glucose homeostasis.

2. Hydrogen sulfide stimulates the secretion of GLP-1 and improves glycaemia in male mice

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Abstract

Recently, the gastrointestinal (GI) microbiome, and its metabolites, has emerged as a potential regulator of host metabolism. However, to date little is known on the precise mechanisms of how this regulation occurs. Hydrogen sulfide (H_2S) is abundantly produced in the colon by sulfate-reducing bacteria (SRB). H_2S is a bioactive gas that plays regulatory roles in many systems including metabolic hormone regulation. Importantly, this gas metabolite is produced in close proximity to the glucagon like peptide-1 (GLP-1) secreting cells in the gut epithelium. GLP-1 is a peptide hormone that plays pivotal roles in both glucose homeostasis and appetite regulation. We hypothesized that H_2S can directly regulate GLP-1 secretion. We first demonstrated that H_2S donors (NaHS and GYY4137) directly stimulate GLP-1 secretion in murine L-cells (GLUTag) and that this occurs through p38 MAPK without affecting cell viability. We then increased SRB in mice by administering a prebiotic chondroitin sulfate containing diet for 4 weeks. Chondroitin sulfate treated mice had elevated *Desulfovibrio piger* levels in the feces and increased colonic H_2S concentration. Chondroitin sulfate treated animals also had enhanced GLP-1 secretion, improved oral glucose tolerance, and reduced food consumption. These results indicate that H_2S plays a stimulatory role in GLP-1 secretion, and that sulfate prebiotics can enhance GLP-1 release and its downstream metabolic actions.

Introduction

Obesity, and its associated metabolic co-morbidities including cardiovascular disease, type 2 diabetes mellitus (T2DM), nonalcoholic fatty liver disease, and others (73), continue to affect a growing proportion of the world. A recently approved treatment in both T2DM and obesity are incretin based therapies. These gastrointestinal hormones, including glucagon-like peptide-1 (GLP-1), are released from enteroendocrine cells in the GI tract and regulate many of our metabolic functions such as appetite, glucose homeostasis, and gastric motility, reviewed in 141–144. Due to its appetite suppressing and insulinotropic actions, GLP-1 based therapies are now an attractive non-insulinic options for patients with T2DM (58,60,145) and morbid obesity (7, 9).

In the last decade, the gastrointestinal microbiome has emerged as a potential regulator of overall metabolism. Metabolic disorders such as obesity and T2DM have been closely linked with changes in gut microbiota (147–149). Indeed, the gut microbiome has the ability, when transplanted, to alter energy homeostasis and weight gain (150). Importantly, interactions between the gut microflora and the enteroendocrine system have been recently demonstrated. Short-chain fatty acids (SCFAs), end products of intestinal bacterial fermentation, significantly increase the release of postprandial plasma GLP-1 in humans and mice (151,152). Indole, an end product of bacterial tryptophan breakdown, stimulates GLP-1 secretion in acute treatments *in vitro* (153). Many other bacterial metabolites are present in the gut from colonic microbial metabolism, including polyphenols, secondary bile acids, and sulfur compounds (91); however, relatively little is known of the interaction of these bacterial metabolites and the local enteroendocrine cells of the GI tract.

Hydrogen sulfide (H_2S) is a bacterial metabolite that is produced by sulfate-reducing bacteria (SRB) in the colon, and endogenously in many mammalian cell types. The endogenous

production of H_2S (in nanomolar amounts) is known as an essential signal transmitting molecule, termed "gasotransmitter", that acts on a variety of systems including cardiovascular, nervous, muscular, and endocrine (99,154,155). Of key importance, millimolar concentrations of microbial H_2S can be found within the lumen of the colon (99,116,156). This environment also contains the highest proportion of GLP-1 secreting L-cells (38). Despite this proximity, and the known role of H_2S as an endocrine modulator, very little is understood regarding the potential crosstalk between the H_2S and GLP-1. The goal of this study was to investigate the role of H_2S in the regulation of GLP-1. In vitro, this was done directly by using H_2S donors on the murine GLP-1 secreting GLUTag cell line. In vivo, a previously validated SRB prebiotic diet containing chondroitin sulfate was used to increase SRB and H_2S production. GLP-1 secretion, food consumption, and glucose tolerance were then examined in mice with enhanced SRB and H_2S levels.

Materials and methods

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). Animal protocols were approved by the Laurentian University Animal Care Committee. Male wild-type C57BL/6 mice aged 5-6 weeks were purchased from Charles River Laboratories (St. Constant, Quebec) and singly housed in standard cages on a 12-h light/dark cycle in the Paul Field Animal Care Facility at Laurentian University.

Diet and study design

Following a week-long acclimatization period on chow diet, 26 male mice were randomly divided into two groups. Animals were fed a diet *ad libitum* low in fermentable carbohydrates (20% wt/wt fat and 47% wt/wt sucrose) with or without chondroitin sulfate (3% wt/wt) for 4 weeks (custom diet prepared by Envigo Teklad Diets, Madison WI) as previously done (157). Animals had access to water *ad libitum.* Body weight and food intake were recorded daily and fecal material was collected and snap frozen every 2 days. Blood was collected every two weeks after an oral glucose gavage (OGTT) for glucose and GLP-1 measurements.

Once the study was complete, the mice were anesthetized with 5% isoflurane (500-1000 ml/min) until the animal was unresponsive to toe pinch, as per Animal Care approved Protocol. Cardiac puncture was performed to retrieve up to 1 ml of blood. Cervical dislocation was done as a secondary method of euthanasia to ensure animal is deceased.

Oral glucose tolerance test (OGTT)

An oral glucose tolerance test (OGTT) was performed at 0, 2, and 4 weeks of the study. Overnight-fasted mice received an oral gavage of D-(+)-glucose (2g/kg body wt). A small incision was made at the distal end of the lateral tail vein and blood was collected into EDTA coated capillary tubes for GLP-1 measurements at 0, 10, and 60 min after glucose gavage (158). An aprotinin (protease inhibitor), diprotin A (DPP-4 inhibitor), and additional EDTA cocktail were supplemented to the capillary tubes (10% v/v) to prevent degradation of the target hormone. Blood glucose was measured during the experiment using a glucometer (OneTouch Verio) at 0, 10, 60, and 90 min after glucose administration.

GLP-1 analysis

Blood samples were collected and kept in ice from the OGTT experiment. Approximately 60 uL of whole blood was centrifuged at 6000*g for 10 minutes at 4°C. 25uL of plasma was examined for total GLP-1 using a commercial competitive ELISA kit (Sigma Aldrich, St-Louis, MO)**.**

Fecal microbe genomic analysis

Fecal material was collected from the mice by grasping the skin around the neck and positioning the mouse upright until a fecal pellet was excreted directly into the DNA extraction tube, or by placing the animal in a clean cage and collecting the fecal sample with sterile forceps. Approximately 2-5 stool pellets were collected per mouse per collection period (~40 – 100mg). After collection, samples were stored at -80 °C until analysis. Genomic DNA was extracted from equal fecal weight (30mg) samples using FavorPrep™ Stool DNA Isolation Mini Kit as directed by the manufacturer (Favorogen Biotech Corporation), and the DNA samples were eluted into 50 uL aliquots. The concentration and purity of the resulting DNA was determined by 260 and 280 nm spectrophotometry and stored at -20°C.

Targeted real-time PCR

Fecal DNA was examined for *Desulfovibrio piger (D. piger)* and total bacterial genomic DNA (16S). The previously validated *D. piger* primer sequence originated from Rey *et al.*: DpigGOR1_fwd 5’ -AAAGGAAGCACCGGCTAACT-3′, DpigGOR1_rev 5′-CGGATTCAAGTCGTGCAGTA- 3′ (45). A previously validated bacterial universal 16S rRNA gene primer was acquired from Ritz *et al.*: 1048F 5’ – GTGSTGCAYGGYTGTCGTCA – 3’, 1194R 5’- ACGTCRTCCMCACCTTCCTC – 3’ (159).

Quantitative real-time PCR reactions (10 uL) contained 2uL of 10x diluted template DNA (80 ng final concentration), 5uL 2X SensiFast SYBR No-ROX supermix (Bioline Inc., Tauton, MA),

0.5 uL of forward and reverse primers (500 nM final concentration of each), and 2.5 uL sterile H2O. As directed by the manufacturer, PCR was comprised of an initial denaturation step of 95°C for 1 min, followed by 40 cycles of 95°C for 15s, 55°C for 15s, and 72°C for 15s. PCR controls included a no-template control (NTC) reaction, replacing the template with PCR grade water. PCR standards were made from gel quantified PCR products for *D. Piger*. Standards, samples, and NTCs were run in triplicates, and CT values were averaged. Quantification was completed using the standard curve method with *D. piger* being normalized to total bacteria (ribosomal 16s).

Hydrogen sulfide measurement

Fecal and colonic content hydrogen sulfide levels were quantified using the methylene blue method (160). Colonic contents were collected post-sacrifice. The material (~0.1g) was homogenized with 1% Zinc acetate trapping solution, 20 mM N,N-dimethyl-p-phenylenediamine sulfate in 7.2 N HCl, 30 mM $FeCL_3$ in 1.2 N HCl, and incubated for 30 min. Samples were centrifuged, and the clear upper phase was analyzed at 670 nm in comparison to a calibration curve of standard H_2S solutions (161,162). H_2S levels were expressed in µM/gram wet weight of feces or colonic contents.

Cell culture and GLP-1 Secretion

Mouse GLP-1 secreting GLUTag cells (Passage 10-30; a kind contribution from Dr. Drucker, Lunenfeld-Tanenbaum Research Institute, Toronto, ON) were grown as previously described by Gil-Lozano and Brubaker (163). GLUTag cells were seeded in 6-well plates (1,000,000 cells per well) and received treatment upon reaching approximately 80% confluence after 48 hours. Cells were treated for 2 hours (164) with H_2S donors: sodium hydrosulfide (NaHS; Fisher Scientific,

Mississauga Ontario) or GYY4137 (Cayman Chemicals, Ann Armor, MI), dissolved in low serum DMEM (0.5% FBS; GE Healthcare Life Sciences). Media with vehicle (water for NaHS experiment, DMSO for GYY4137 experiment) served as a control for baseline secretion, and forskolin was used as a positive control for GLP-1 secretion (data not shown). Total cellular protein content was assayed using the Bradford method, and no differences were observed (data not shown). Following the incubation, the collected media was acidified using trifluoroacetic acid to a final concentration of 0.1% and purified through a Sep-Pak C18 as per manufacturers' instructions (Waters, Germany). The retained material was eluted with 80% Isopropanol, 0.1% TFA, and then dried down using the Integrated Speedvac System**.** The dried purified samples were stored at -20°C until analysis. A commercial total GLP-1 competitive ELISA kit (Sigma Aldrich, St-Louis, MO) was used to quantify GLP-1 levels from dried samples.

Under similar experimental conditions, cell viability was determined by neutral red uptake assay, as described by (165). This technique is based on the ability of viable cells to incorporate neutral red dye in the lysosomes, after the cells have been treated with NaHS, GYY4137, or control media.

Western blot

GLUTag cells were seeded into 6-well plates for 48 hours. Cells were treated for 10 min in serum free media containing vehicle or an H_2S donor. Cells were lysed in lysis buffer (Cell Signaling Technologies, Danvers, MA Technologies) containing a phosphatase inhibitor (PhosphoSTOP by Roche), and a protease inhibitor (EDTA Free CompleteMini by Roche). Protein content was quantified by Bradford method (50 µg protein per well loaded) separated on an 8% denaturing SDS-PAGE Tris-glycine gel, transferred to a polyvinylidene fluoride (PVDF) membrane, blocked with 5% skim milk in Tris-buffered saline, and incubated overnight at 4°C

with the primary polyclonal rabbit antibody (phosphor- or total- p38 MAP kinase; Cell Signaling Technologies, Danvers MA). After washing in TBS, the membrane was incubated for 2 hours at room temperature with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Cell Signaling Technologies, Danvers, MA). The proteins were visualized using a Luminata Forte chemiluminescence HRP substrate (Millipore Corp, Billerica, MA), and luminescence was detected using the BioRad chemi doc XRS system (for antibodies, see Table 2). Total cognate antibodies (AKT and p38 MAPK) were used as an internal loading control by dividing the ratio of phospho-protein levels by the total protein levels. The phospho/total ratios of treated sampled were compared to those of the untreated samples to create the figures annotated “Relative to Control”.

Table 2. List of antibodies

Peptide/Protein Target	Antigen Sequence	Name of Antibody	Manufacturer	Species Raised in; Monoclonal or Polyclonal	Dilution Used	RRID
Phosphor-p38 MAP kinase	T180/Y182	Phosphor-p38 MAPK	Cell Signaling Technologies	Rabbit; polyclonal	1:1000	AB_10080263
P38 MAP Kinase		P38 MAPK	Cell Signaling Technologies	Rabbit; polyclonal	1:1000	AB_2225340
Phosphor-AKT	S-473	Phosphor-AKT	Cell Signaling Technologies	Rabbit; polyclonal	1:1000	AB_330713
AKT pan		AKT	Cell Signaling Technologies	Rabbit; polyclonal	1:1000	AB_2315049

Statistical analysis

All data are expressed as mean ± SEM. All measures were tested for normality; sets that passed the normality test were assessed using parametric techniques. 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 or more independent variables (i.e., time and treatment doses) were analyzed by two-way ANOVA, followed by Bonferroni post hoc tests at individual time points where applicable. $P < 0.05$ was considered significant.

Results

H_2S donors stimulate GLP-1 secretion *in vitro*

To determine the direct effect of H_2S on GLP-1 secretion, GLUTag cells were treated with an H_2S donor (NaHS or GYY4137), or control media for 2 hr. Sodium hydrosulfide (NaHS) increased GLP-1 secretion ($P < 0.05$ one-way ANOVA), with the highest dose of NaHS (10 mM) causing a 1.98 ± 0.2 fold increase (Fig. 3*A*). Lower doses of GYY4137 also caused a significant increase of GLP-1 levels ($P < 0.05$ one-way ANOVA), with the highest dose of GYY4137 (100uM) producing a 2.2 ± 0.21 fold increase of GLP-1 (Fig. 3B*).* All H_2S treatments used did not reduce cell viability, as shown by the neutral red assay (Fig. 3C).

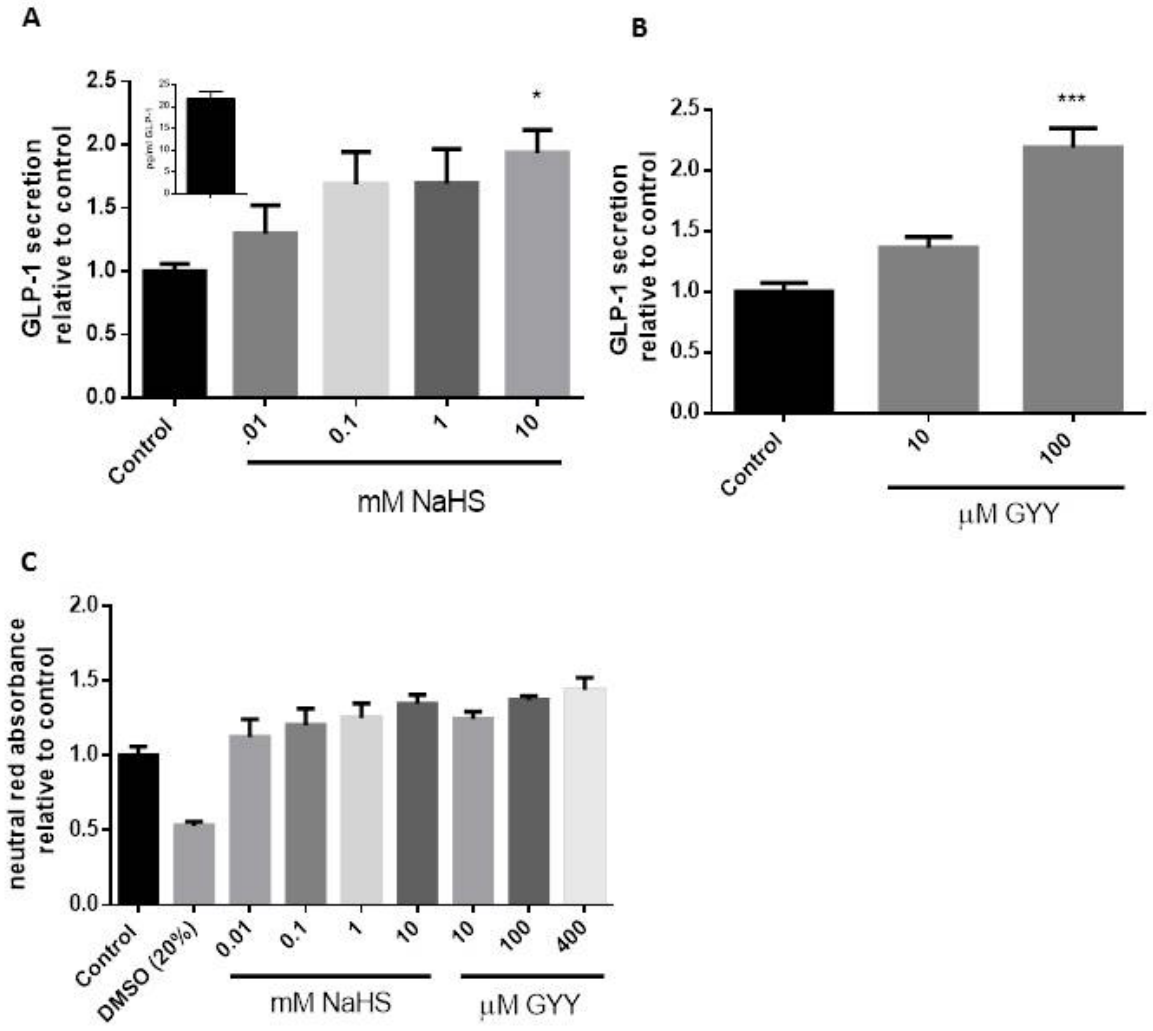

Figure 3. Effects of H_2S on GLP-1 secretion and cell viability in GLUTag cells.

GLP-1 secretion was analyzed in media from cells treated with NaHS (A) or GYY4137 (B) for 2 hours. Cell viability was examined under similar conditions (C). Data is presented relative to vehicle control with the absolute values from control in inset 3A. n=6-9; * = $P < 0.05$ vs. control cells; *** = $P < 0.001$ vs control cells.

P38 MAPK is involved in H_2S-stimulated GLP-1 secretion *in vitro*

To determine potential intracellular mechanisms involved in the enhanced GLP-1 secretion by H_2S, we treated GLUTag cells with the most effective dose of NaHS (10 mM) for 10 min, and examined the phosphorylation of p38 MAPK and AKT by Western blot. P38 MAPK phosphorylation was significantly increased by 1.61±0.18 fold after treatment ($P < 0.05$; Fig. 4B). We did not observe an increase in phosphorylated AKT (Fig. 4A). To confirm the importance of p38 MAPK in H_2S-stimulated GLP-1 secretion, we co-incubated the p38 MAPK inhibitor IV with NaHS. NaHS-stimulated GLP-1 secretion was completely blocked by the addition of the kinase inhibitor (Fig. 4C).

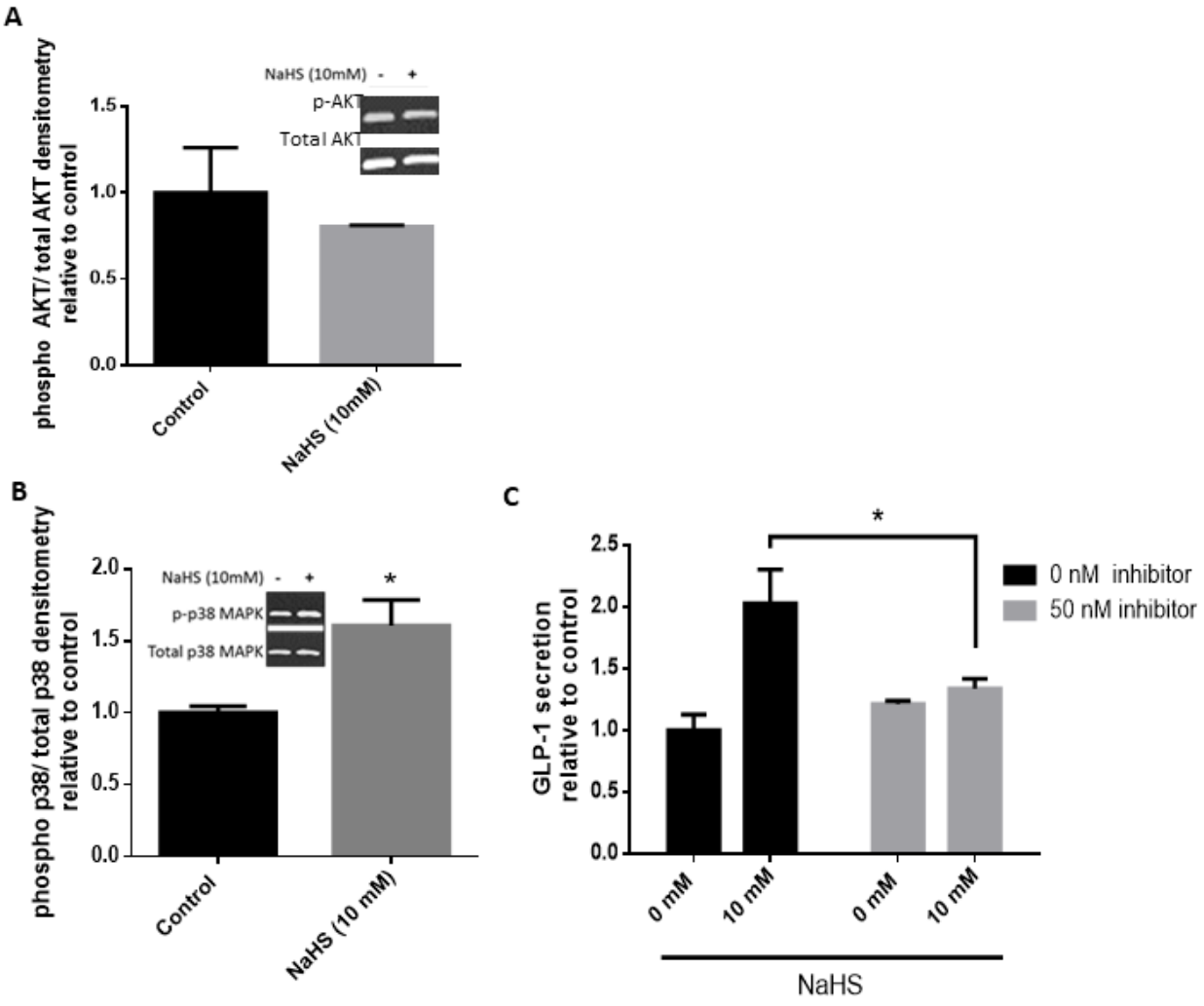

Figure 4. Effect of H_2S donor (NaHS) on p38 MAPK and AKT phosphorylation in GLUTag cells.

AKT phosphorylation/total AKT was examined in GLUTag cells treated with NaHS (10 mM) for 10 min by western blot (A). P38 MAPK phosphorylation/total p38 MAPK was examined in GLUTag cells treated with NaHS (10 mM) for 10 min by western blot (B). GLP-1 secretion was examined in NaHS (0-10mM) treated cells co-incubated with and without the p38 MAPK inhibitor IV (50 nM) (C). Data is presented relative to vehicle control. n = 6; *, $P < 0.05$ vs. control cells.

Chondroitin sulfate prebiotic diet increases H_2S and SRB levels

To increase the amount of SRB and microbial H_2S *in vivo*, mice were provided with a prebiotic diet consisting of a 3% chondroitin sulfate in the presence of a diet low in fermentable carbohydrates. Fecal DNA was collected throughout the study and analyzed for the abundance of *D. piger* using targeted qPCR. In comparison to their respective baseline levels, the control group had a 3.4 ± 0.33 fold increase of *D. piger*, and the treatment group had a 4.4 ± 0.26 fold increase. Importantly, there was a 1.29 ± 0.07 fold change in treatment vs control *D. piger* levels following the four week diet ($P < 0.05$ t = Day 28, $P < 0.05$ for chondroitin sulfate interaction in 2-way ANOVA; Fig. 5A). Therefore, the prebiotic chondroitin sulfate significantly increased the abundance of *D. piger*.

To verify that this increase in fecal *D. piger* levels corresponded with increased levels of H_2S, the amount of fecal and terminal colon H_2S were determined using the methylene blue method. The pre-diet intervention levels (in feces) were similar in both control and treatment groups (0.49 umol/g, Fig. 5B*)*. After the four-week prebiotic diet, fecal and colonic contents were examined for H_2S. Colonic material was less exposed to oxygen (a factor that causes loss of H_2S) and was therefore used in the post diet comparison. The treatment group had a 1.50 ± 0.03 fold increase of colonic H_2S (0.81 μmol/g) compared to control ($P < 0.05$, Fig. 5C).

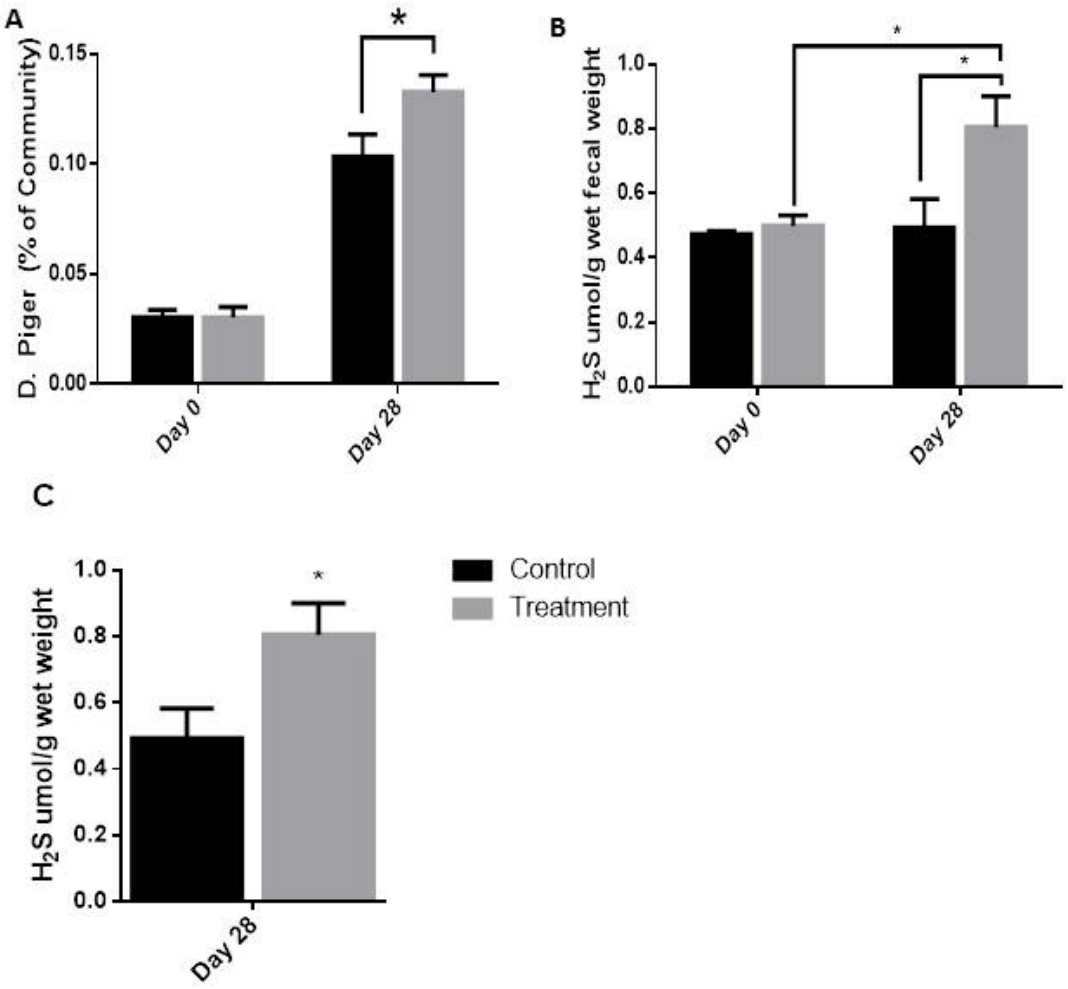

Figure 5. The effect of dietary chondroitin sulfate intervention on SRB and H_2S levels in mice.

Fecal *D. piger* levels were examined by qPCR at day 0 and following the 4-week chondroitin sulfate diet (A). H_2S levels were examined in both groups at the start of the study in feces (B) and at the end of the study from colonic contents (C). n = 13 per group; *, $P < 0.05$ vs control animals.

Chondroitin sulfate prebiotic diet enhances GLP-1 response, improves oral glucose clearance, and reduces feeding

To determine the effect of the increased SRB and H_2S levels on GLP-1 secretion and downstream metabolic function, control and prebiotic treated mice were examined after 4 weeks of diet intervention. Chondroitin sulfate treated mice had a significant increase in glucose stimulated GLP-1 secretion (overall treatment effect compared to control $P < 0.05$ with post-hoc significance at 10 min, $P < 0.001$; Fig. 6A*).* Area under the curve for GLP-1 secretion was significantly elevated in the prebiotic treated group (1.21 ±0.06 of control, $P < 0.05$; Fig. 6B). Oral glucose tolerance was examined pre- and post- prebiotic diet intervention. Both groups began the study with similar glucose tolerance (Data not shown). Following the four week diet the chondroitin sulfate group had significantly improved glucose tolerance (overall treatment effect compared to control $P < 0.01$ with post hoc significance at 60 min, $P < 0.01$; Fig. 6C). AUC of glucose was significantly lower in the prebiotic group (0.87 ± 0.05 of control, $P < 0.05$; Fig. 6D). Insulin response was also significantly improved in the chondroitin sulfate group (overall treatment effect compared to control $P < 0.01$; Fig. 6E & 6F). Finally, while differences in weight gain were not observed in the time course of this study (data not shown), significant reduction in food consumption began to emerge in the treatment group after day 18 (AUC day 18-28; 0.87±0.06 fold, $P < 0.05$, Fig. 4G & 4H).

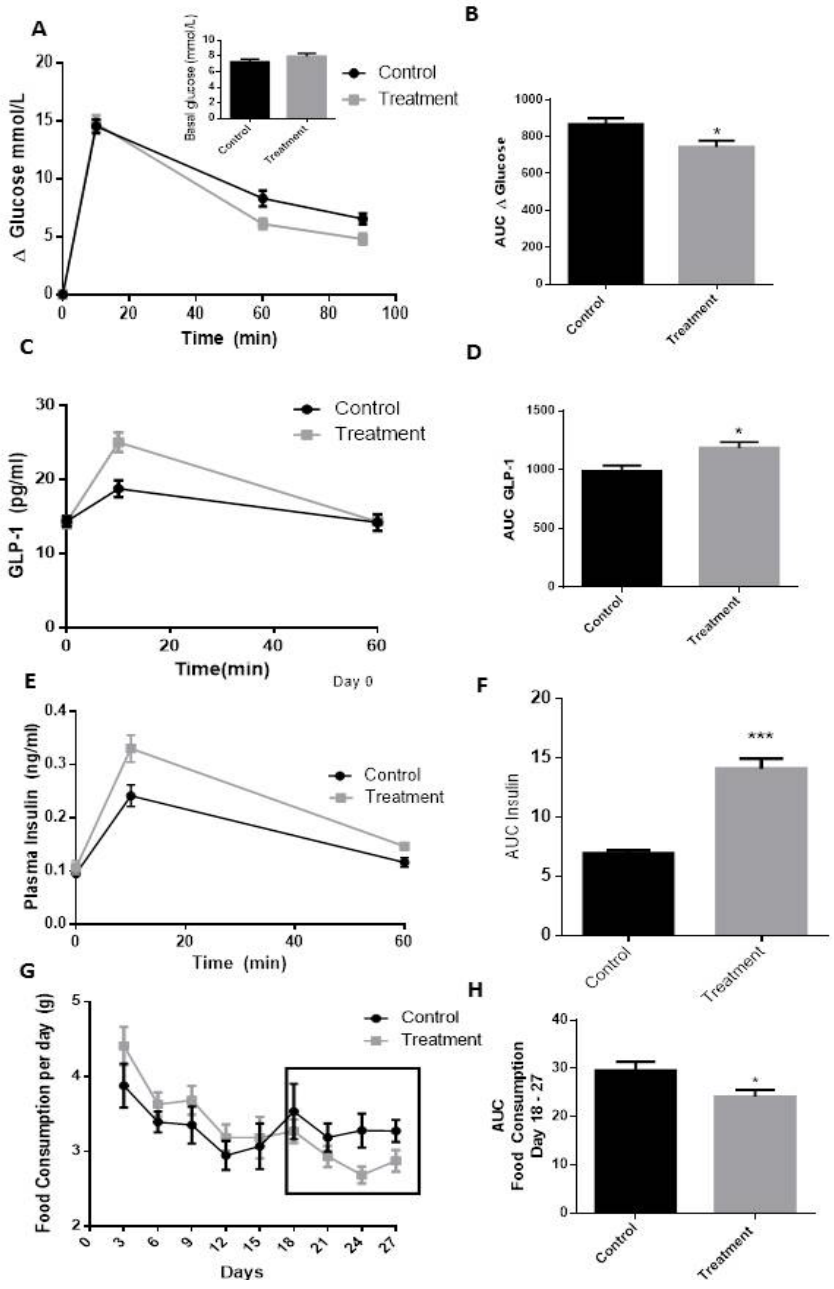

Figure 6. GLP-1 and metabolic profiles of mice after 4-week prebiotic diet.

The GLP-1 response to oral glucose was examined in control and chondroitin sulfate treated animals (A and B AUC). Delta glucose was examined after OGTT (C and D AUC). Plasma insulin was examined after OGTT (E and F AUC). Food consumption was examined in the last 9 days of the study (G and H AUC). n = 13 per group; *, $P < 0.05$ vs control animals.

Discussion

The incretin hormone GLP-1 has become an important therapeutic target in the treatment of T2DM and obesity, and novel strategies to increase GLP-1 remain a major research focus. In addition, the GI microbiome has recently emerged as a potential player in the regulation of metabolic health in part through the production of microbial metabolites. These metabolites include H_2S, which is an established regulatory gas (gasotransmitter) in several systems. Since H_2S is already known to regulate certain metabolic hormones, and H_2S producing SRB reside in the same environment of the GLP-1 secreting L-cells, we aimed to determine if H_2S plays a regulatory role in GLP-1 secretion and its downstream metabolism. Our results indicate that H_2S directly stimulated GLP-1 secretion, and that increasing SRB in mice using a prebiotic diet led to enhanced GLP-1 and insulin secretion, improved glucose tolerance, and reduced feeding.

When examining the direct effect of a secretagogue on GLP-1 secretion, it is important to demonstrate the effect using a treatment concentration within the physiological range. In our *in vitro* work, H_2S donors were used in the µM to mM range. While these H_2S donors liberate the gas in different manners, the concentration of gas produced over the 2 hours are expected to be within the range of luminal concentrations of H_2S produced through microbial respiration (99,116,166). This is in contrast to the nM concentrations of H_2S that are produced endogenously from the colonic epithelium (167). To estimate the quantity of H_2S that is present within the colon, mouse colonic content H_2S was determined. Once again, these concentrations were found to be within the uM range (umol/gram wet weight) and are in line with other work examining fecal H_2S concentrations (between 0.29 umol/g – 1.6 umol/g (168–171)).

We also found that the H_2S stimulation of GLP-1 secretion occurred through the P38 mitogen-activated protein kinase (p38 MAPK). This is of particular importance since H_2S is not known to

have a cell based receptor, and the mechanism of H_2S action remains an active area of research. However, our finding that p38 MAPK is mechanistically involved is in agreement with other studies that have examined the mechanism of H_2S action in other systems (105,172,173). Importantly, Reimer and colleagues have demonstrated a role for p38 MAPK phosphorylation in the secretion of GLP-1 (174). Combined, these works support a role for H_2S mediated activation of p38 MAPK as the signaling mechanism for enhanced GLP-1 secretion.

Our finding that H_2S stimulates GLP-1 secretion appears to be in contrast to a recent study by Bala and colleagues (175). They demonstrated that H_2S inhibits oleanolic-acid stimulated GLP-1 secretion in the STC-1 cell line. There are however several important differences between our and Bala *et al.* cell culture experiments. Mainly, their study uses the secretin tumor cell line, STC-1. While this cell line produces and secretes GLP-1, it is derived from the small intestine (176), and may have alternate regulation compared to the colonic derived GLUTag cell line. Since SRB require the anaerobic environment of the colon, microbial H_2S concentrations (in the uM-mM range) would likely not be present in the upper small intestine. Therefore, it is possible that the STC-1 from the small intestine, and the GLUTag cells from the colon, use different pathways and react differently to H_2S stimuli. Nevertheless, findings from cell culture studies in tumor cell lines should be confirmed with an *in vivo* model as was completed here.

Supplementing the diet with the sulfated glycosaminoglycan, chondroitin sulfate, led to an increase in SRB compared to control. This compound was selected because unlike other sulfate molecules that are quickly absorbed by the gut, glycan-bound sulfate (such as chondroitin sulfate) is poorly absorbed in the small intestine (177), and is more likely to liberate the sulfate in the distal GI tract where it can be used by SRB for H_2S production. Similar to Rey and colleagues, we observed an increase in the abundance of *D. piger* and H_2S levels in mice on

chondroitin sulfate diet (157). However, our levels of *D. piger* community abundance were lower than in Rey's study. This is likely due to their mice being germ free and inoculated with an 8 species defined microbiome community including *D. piger.* Interestingly, control mice in our study also had an increase in SRB after 4 weeks. This is likely due to the low fermentable carbohydrate diet, which enhances the use of host derived sulfated glycans in the lumen, to be natural substrate used by SRB (157). Nevertheless, supplementing additional sulfate in the treatment group with chondroitin sulfate further enhanced the SRB levels. Interestingly, only the chondroitin sulfate treated mice had increased levels of H_2S, which suggests additional sulfur substrates are required to increase H_2S production.

In agreement with our *in vitro* work, the chondroitin sulfate treated mice (which exhibited higher levels of colonic H_2S) had a significant improvement in their GLP-1 response. However, this difference was observed only after oral glucose administration and not at the baseline (time 0). What may be happening in the H_2S enriched *in vivo* environment is a "priming" of the L-cell in preparation for subsequent glucose-stimulated GLP-1 secretion. Indeed, endocrine cell priming has been previously described in other cells such as β-cells and ghrelin cells (178,179). Along with increased GLP-1 secretion, we observed an improved glucose tolerance and reduced food consumption in the chondroitin sulfate animals. The latter became apparent in last 9 days of the study, indicating that there was no initial taste aversion to the chondroitin sulfate. These findings are in agreement with GLP-1's established role in mediating increased insulin stimulation and satiety (180). Surprisingly, we did not observe a significant weight difference during the course of this study. However, this may be due to the short duration of this study (4 weeks). Future studies may examine long term weight changes or weight loss interventions to fully elucidate the role of H_2S in body weight regulation. In addition, future use of germ free mice given a SRB

probiotic, and a diet-reversal period, would strengthen the relationship between microbial H_2S production and enhanced GLP-1 secretion.

In summary, we have demonstrated a role for H_2S in the stimulation of GLP-1 secretion. This effect was shown directly in L-cells and indirectly through prebiotic enhancement of the SRB community. These results provide some of the first evidence of how microbial gases are able to influence gut endocrine system and downstream metabolism.

3. Extended Discussion

3.1 Clinical implications of prebiotics, probiotics, and H_2S-releasing agents

As summarized by (181), there are many studies that confirm the beneficial use of probiotics and prebiotics in humans and rodents. Probiotics contain a live microbial supplement, and are orally ingested to alter the gut microflora of the host (Reviewed in (182)). On the other hand, prebiotics are non-living supplements, containing non-digestible dietary components which selectively help the proliferation and/or activity of certain gut bacteria (Reviewed in (183)). These studies demonstrate that the beneficial effects from prebiotics or probiotics can occur as early as 2 weeks to 8 weeks (181). In the context of glucose homeostasis, a few yogurt-based probiotics containing *Lactobacillus* and *Bifidobacteria* (carbohydrate fermenters) have been shown to reduce fasting glucose, and delay the progression of glucose intolerance and hyperglycemia in diabetic rats (184) and humans (185). Prebiotics containing non-digestible carbohydrates have also been shown to improve glycaemia and insulimia of patients with T2DM (186–188). Therefore, prebiotics and probiotics are possible tools to modulate the gut microbiota for the management of T2DM.

The use of chondroitin sulfate has been seen mainly as a supplement for patients with osteoarthritis (OA), atherosclerosis, IBD, degenerative diseases, and other autoimmune diseases, and has been shown to have many anti-inflammatory properties, as reviewed in (189). Chondroitin sulfate was used in this study to increase the growth of SRB, and thus the levels of colonic H_2S (189). H_2S also has anti-inflammatory and cytoprotective properties as previously discussed. Clinical trials are underway to examine H_2S-releasing agents in combination with non-steroidal anti-inflammatory drugs (NSAIDs), used to reduce pain and inflammation (Reviewed in (190)). In these trials, H_2S is added to reduce the gastric injury that occurs as a side

effect of the NSAID (191–194). Therefore, the beneficial use of H_2S in drugs is well established in that category.

The rationale behind using chondroitin sulfate as opposed to another sulfur-donor is that many H_2S donors are partially, if not fully, absorbed in the stomach and intestine before it reaches the colon. The fermenting and sulfate-reducing bacteria that work together to create H_2S from chondroitin sulfate are more abundant in the distal small intestine and colon. Therefore, we suggest this could be a novel method to produce H_2S at a steady rate in the lower GI tract without it being absorbed in other regions of the GI tract. The established safety profile and other health benefits of chondroitin sulfate, along with the established anti-inflammatory/cytoprotective properties of H_2S, make chondroitin sulfate an attractive compound to explore in future research regarding colon health.

As shown in Chapter 2, we have established that H_2S production and a marker species of SRB are increased with the chondroitin sulfate prebiotic diet. This resulted in an increase of GLP-1 secretion from the colonic L-cells and subsequent glycemic benefits. The mechanism of H_2S's action on GLP-1 secretion and the L-cell will now be discussed.

3.2 Molecular mechanisms of H_2S action on GLP-1 secretory pathway

H_2S remains a mystery as to what molecular pathways are being activated, due to the lack of a known cellular receptor. In various tissues where H_2S's action was studied, a few mechanisms have been proposed. Some reports have suggested that H_2S modifies proteins, including ion channels and enzymes by interacting with the cysteine residues through a process called persulfhydration (126). Persulfhydration occurs when a sulfur group from H_2S is added to a thiol group of a cysteine residue. This reaction yields a hydropersulfide (-SSH), which has been shown to increase the biological activity and reactivity of a protein or enzyme. For example, the

activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an enzyme involved in glycolysis and other cellular processes, is greatly increased when sulfhydrated (126). Whether sulfhydration has a similar effect in other enzymes is an active field of research.

In my work I demonstrated that NaHS causes an increase in the phosphorylation of the p38 mitogen activated protein kinase (MAPK) and that blocking this kinase prevents the action of NaHS. The MAPK family is known to be involved in cell proliferation, differentiation, and apoptosis (195). Importantly, the p38 MAPK is also known to play a role in GLP-1 regulation. Several groups have demonstrated the activation of this kinase during GLP-1 secretion (174,196). The literature surrounding the effect of H_2S in p38 MAPK activation is conflicting, likely due to different cell models and methodology. NaHS upregulates p38 MAPK in human cultured umbilical vein endothelial cells (197) and gastric mucosal epithelial cells (198), however inhibits this kinase in human polymorphonuclear leukocytes (199), and cultured rat aortic vascular smooth muscle cells (200). P38 MAPK has several downstream targets, including many transcription factors and protein kinases. Changes in gene expression of proglucagon or proprotein convertases are likely not to have occurred during the 2 hour incubation of our *in vitro* experiments. P38 MAPK has been previously shown to be involved in the exocytosis of secretory vesicles of human neutrophils and microglial cells, although the direct targets and downstream mechanisms are unknown (201,202). It is possible that the enhancement (or inhibition of by p38 MAPK inhibitor IV) of p38 MAPK is involved in mediating the exocytosis of the GLP-1 vesicles. The area of research involving p38 MAPK and vesicle exocytosis, and the exocytotic mechanisms of GLP-1 release are understudied and require further examination to conclude the role of this kinase.

We also examined the activation of protein kinase B (AKT) *in vitro*. This is another kinase involved in proliferation, differentiation, and apoptosis (203). Interestingly, the AKT pathway is a known target of H_2S. H_2S stimulates AKT phosphorylation in cultured endothelial cells (204), ischemic hind-limb muscles in rats (205), and colon cancer cells (206) . In the context of the GLP-1 secretory pathway, AKT is also known to be phosphorylated in insulin-induced GLP-1 secretion (207). However, in this study we did not see an activation of AKT by H_2S. It is possible that AKT is phosphorylated at a time point we have not examined, or that simply H_2S does not induce the phosphorylation of the AKT pathway in this cell line.

Another interesting kinase to examine could be the extracellular signal-regulated kinase (ERK) 1/2. This kinase is an established H_2S target in a few cell models (206,208,209). ERK 1/2 is also involved in GLP-1 secretion, as seen in its activation during meat hydrosylate-stimulated (174) and insulin-induced GLP-1 secretion (207).

As described in the review in chapter 1, H_2S has been shown to open K_{ATP} channels and close calcium channels. However, the closure of the K_{ATP} channel and the opening of the calcium channel are involved in potentiating the release of GLP-1 vesicles. Since we observed an enhanced stimulation of GLP-1, it would be interesting to observe how these channels are altered by H_2S. Therefore these pathways and other H_2S targets could be subject to future examination in the L-cell to map out the detailed mechanism as to how H_2S stimulates GLP-1 secretion.

3.4 Microbial H_2S: Future perspectives

The goal of this study was to investigate the role of colonic microbial H_2S on the neighbouring GLP-1 secreting cells. Although we have demonstrated an important correlation between the increases of SRB, fecal and colonic H_2S, and increases in GLP-1, there is still further

investigation warranted between SRB and GLP-1. To add depth to this study and significantly highlight the role of SRB/microbial H_2S production and GLP-1, it would be interesting to examine our research question through manipulating the SRB microbiota in several manners. The use of antibiotics to neutralize the SRB could be cost-effective and simple design, however multiple studies have noted that SRB are extremely resistant to most antibiotics (210,211), and antibiotics would most likely not eliminate all traces of SRB. GF mice are also frequently used as models to study the importance of the microbiota. Microbial H_2S studies have used GF mice before to differentiate microbial vs endogenous production of H_2S (117). However, GF mice also have higher levels of GLP-1 due to undigested bile acids arriving to the colon and stimulating the L-cells (212). Therefore, this would also not be the ideal model to observe a marked stimulation of GLP-1. An ideal system for future work would be the use of gnotobiotic mice. Gnotobiotic mice are GF mice inoculated with a defined assemblage of bacterial species. This gnotobiotic microflora could be with or in absence of *Desulfovibrio piger*, to study the direct relationship between SRB/ H_2S and GLP-1. In the absence of *D. piger*, chondroitin sulfate should still be metabolized into simple sugars and sulfate; however the production of hydrogen sulfide would be very limited in the absence of any bacteria capable of using the dissimilatory sulfate reduction pathway. It is also possible to inoculate the mice with modified bacteria lacking sulfatase enzymes to completely rule out the involvement of sulfate. Taken together, these additional studies would strengthen our conclusions that it is indeed microbial H_2S that is responsible for evoking changes in GLP-1 secretion.

4. Conclusions

H_2S is a gasotransmitter that has been studied extensively in several biological systems; however, an important missing link is the enteroendocrine system. GLP-1, among the many peptides secreted from the enteroendocrine system, is responsible for glucose-dependent insulin secretion, delayed gastric emptying, and inducing satiety. In this body of work, we were able to show a potential regulatory role for H_2S (a microbial metabolite from SRB) on GLP-1 secretion. We've demonstrated that H_2S donors can directly stimulate GLP-1 secretion without reducing cell viability *in vitro*, and that p38 MAPK is an important pathway mitigating this effect. *In vivo*, we were able to successfully elevate SRB and colonic H_2S levels using a chondroitin sulfate prebiotic. These animals exhibited a reduction in food intake, and an improvement of GLP-1/insulin response and glucose clearance after a glucose challenge. A greater understanding of the crosstalk between microbial metabolites and GI hormones like GLP-1 will enable and foster new research into potential therapies using H_2S for the management of T2DM.

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