Investigating the role of hydrogen sulfide in the regulation of glucagon-like peptide-2 secretion and gut physiology

by

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Abstract

Hydrogen sulfide (H_2S) is an endogenously produced gasotransmitter which regulates a variety of physiological processes including hormone regulation. In individuals with Crohn's disease or colitis, H_2S is elevated. Whether H_2S plays a role in gut disease development or protection is not clear. Glucagon-Like Peptide-2 (GLP-2) is a gut health-promoting peptide hormone produced in the enteroendocrine L-cells (EECs) located primarily in the distal gastrointestinal (GI) tract. In the gut, GLP-2 increases cell proliferation, enhances barrier function, and decreases inflammation. In this study, it is hypothesized that local H_2S produced in the distal GI tract and in EECs can regulate GLP-2 secretion and downstream gut physiology. GLP-2 secretion and gut physiology was examined in mice lacking the H_2S producing enzyme cystathionine gamma-lyase (CSE). Additionally, H_2S production and GLP-2 secretion was examined from mouse enteroendocrine L-cells (GLUTag). CSE knockout (KO) mice had significantly reduced intestinal H_2S production and claudin-7 expression. In cells, H_2S was also produced directly from EECs which could be partially blocked by CSE inhibitors. When EECs were treated with H_2S donors (NaHS and GYY 4137), the effect on GLP-2 secretion was variable with a slight suppression with high dose NaHS and no effect with GYY 4137. Finally, when cells were treated with CSE inhibitor, GLP-2 secretion was significantly reduced. Our work indicates that the EEC L-cell can produce the gasotransmitter H_2S. Adding additional H_2S has a variable effect while inhibiting endogenous production suppresses GLP-2 secretion. These findings suggest that GLP-2 may play a role in the interplay between H_2S and gastric health, which could provide support for the potential use of H_2S drugs in the treatment of GI diseases.

Keywords

Hydrogen sulfide, glucagon-like peptide-2, hormone regulation, gastrointestinal physiology

Abbreviations

AOAA: aminooxyacetic acid

AVG: L-aminoethoxyvinylglycine

BCA: β-cyanoalanine

cAMP: cyclic adenosine monophosphate

CAT: cysteine aminotransferase

CBS: cystathionine beta-synthase

CCK: cholecystokinin

CD: Crohn's Disease

CSE: cystathionine gamma-lyase

dH2O: deionized water

DMEM: Dulbecco's Modified Eagle Medium

DPPIV: dipeptyl peptidase IV

EEC: enteroendocrine cell

ELISA: enzyme-linked immunosorbent assay

FBS: Fetal bovine serum

GABA: gamma-amino butyric acid

GAM: Goat-anti-mouse antibody

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GAR: Goat-anti-rabbit antibody

GI: gastrointestinal

GIP: Glucose-dependent insulinotropic polypeptide

GLP-1: Glucagon-like peptide-1

GLP-2: Glucagon-like peptide-2

GLP-2R: Glucagon-like peptide-2 receptor

GRPP: Glicentin-related pancreatic polypeptide

GYY 4137: morpholin-4-ium 4-methoxyphenyl(morpholino)phosphinodithioate

H_2S: Hydrogen sulfide

H_2O_2: hydrogen peroxide

HA: hydroxylamine

HBSS: Hank's Balanced Salt Solution

HRP: horseradish peroxidase

IBD: inflammatory bowel disorder

IFNy: interferon gamma

IGF: insulin-like growth factor

IP1: intervening peptide 1

IP2: intervening peptide 2

KO: Knockout

MAPK: mitogen-activated protein-kinase

MST: 3-mercaptopyruvate sulfurtransferase

NaHS: Soduim hydrosulfide

P/S: Penicillin/Streptomycin

P5P: pyridoxal-5-phosphate

PBS: phosphate-buffered saline

PN: Parenteral nutrition

PYY: Peptide-yy

SBS: Short bowel syndrome

SRB: Sulfate-reducing bacteria

TBS: Tris-buffered saline

TJP: Tight junction protein

TNF-α: Tumour-necrosis factor alpha

UC: Ulcerative colitis

VOCs: Volatile organic compounds

WT: Wild-type

ZO: Zonula-occludens

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1 Introduction

1.1 Malabsorption disorders

The GI tract is involved in the process of digesting and absorbing nutrients such as carbohydrates, fats, proteins, vitamins and minerals (1). Some people are not able to absorb and digest some of these nutrients, leading to malabsorption. In fact, millions of people worldwide suffer from some sort of malabsorption disorder or deficiency (1). Malabsorption is described as the impairment of absorption of nutrients within any of the areas present in the GI tract such as the stomach, small intestines or large intestine (1). There are a wide range of deficiencies and disorders that fall within this category, along with a wide range of symptoms. Some symptoms can range from minor (such as flatulence, diarrhea and bloating), to intermediate (such as vitamin deficiencies, malnutrition and weight loss), to severe (inflammation, vascular disorders and GI damage leading to surgery) (1). Lactose intolerance and Celiac's disease are two fairly common and relatable examples, however there are three malabsorption disorders that will be highlighted: Short bowel syndrome (SBS), Crohn's disease (CD) and ulcerative colitis (UC).

1.1.1 Short Bowel Syndrome

SBS is a rare condition affecting roughly 1.5 to 13 out of every million people (2). It is a GI disorder characterized by a loss in intestinal capacity to absorb nutrients, due to intestinal damage or surgical removal, which then leads to nutritional deficiencies (3). The specific deficiencies may depend on which areas are affected (4). The damage or removal of intestinal tissue is often due to other diseases or conditions such as GI trauma due to injury, CD, vascular issues, tumours or cancer radiation (3). Current treatments for SBS target these nutritional deficiencies and the threat of malnutrition. Parenteral nutrition (PN) is often used to deliver

nutritional support and prevent malnutrition (3,5). PN is essentially a process in which an intravenous tube is inserted into an individual's vein and delivers electrolytes, vitamins and any other required nutrients (3, 5). Depending on the severity of malnutrition caused by SBS, this process can be lengthy, time-consuming and strenuous, ranging from weeks, to months, to years. This process is often daily or multiple times a week (5). Thankfully, there is another method to improve absorptive functions and limit/reduce PN. Teduglutide is a Health Canada approved treatment for individuals with SBS that require PN (6). This medication is prescribed as a subcutaneous injection and is an analogue to a naturally produced hormone GLP-2, which will be further discussed later (7). This GLP-2 analogue has an extended half-life compared to natural GLP-2. The half-life of teduglutide is 1-2 hours, whereas natural GLP-2 is 5-7 minutes (7). As will be discussed in the subsequent chapter, it is evident as to why this hormone is used to improve SBS and GI functions. Research has shown its effectiveness on GI functions and its efficacy on reducing PN in individuals with SBS (8). Although SBS is an important disorder for this manuscript, there are two other disorders that are also worth mentioning.

1.1.2 Crohn's disease

CD is a condition that falls under the classification of inflammatory bowel disorders (IBD) (9). CD is characterized as inflammation to any part of the GI tract, however in 80% of cases the inflammation occurs within the small intestines (9). This inflammation often leads to malnutrition, which is also paired with other symptoms such as abdominal pain, diarrhea and bowel obstruction (10). The primary treatment for CD is medication; however surgery is often required (11). Surgery is performed to improve the patient's quality of life, or sometimes as a life-saving procedure due to obstruction or medical complications surrounding CD (11). Unfortunately, about 70% of people with CD will eventually require surgery (12). Surgical

removal of damaged or obstructed regions lead to SBS and malnutrition, which then follows the corresponding treatments mentioned previously (3).

1.1.3 Ulcerative Colitis

UC also falls under the chronic IBD classification (9). UC is characterized as inflammation and ulcers in the GI tract, primarily in the large intestine (13). Symptoms for UC are similar to CD, in which abdominal pain, diarrhea and bloody stool are present (13). The main difference is that UC is localized within the colon along with the presence of ulcers. They also differ by the GI wall layer affected. CD however can be present in any region within the GI tract. There is no cure for UC, however treatments target symptom management. Mild symptoms are often treated with medications for inflammation, pain or diarrhea (14). However, depending on severity, surgery may be required, especially in the case of obstruction (12). Around 33% of individuals who suffer from chronic UC undergo surgery, typically by removing portions of the inflamed colon (12). The pathology surrounding all of these disorders has one thing in common; they all occur within the digestive system. Therefore, it is important to dive into the GI tract and its vital components.

1.2 Gastrointestinal enteroendocrine physiology:

The digestive system is comprised of the GI tract, alongside accessory organs such as the gall bladder, liver and pancreas. The GI tract stems from the mouth, followed by the esophagus, stomach, small intestine (subdivided into the duodenum, jejunum and ileum), large intestine and rectum (15). The primary function of the GI tract is to breakdown food, absorb its nutrients and provide our bodies with energy. The breakdown of food and absorption is possible due to the digestive processes from each of these organs. These digestive processes include chemical

digestion from enzymes, mechanical digestion from muscle contractions and the help of GI hormones (16). Within the GI tract, there are cells capable of sensing nutrient intake and are able to secrete a number of hormones. These cells are known as enteroendocrine cells (EECs), ranging from the stomach to the rectum (17). **Figure 1** demonstrates a schematic model of the GI tract, as well as the localization of one type of EECs found within the gut (17). These cells can be found within the intestinal epithelial layer facing the lumen, where they are able to interact with luminal content and nutrients. The interaction with the quantity of nutrients in the lumen leads to the regulation and release of GI hormone production into the bloodstream, each with their own physiological role (18).

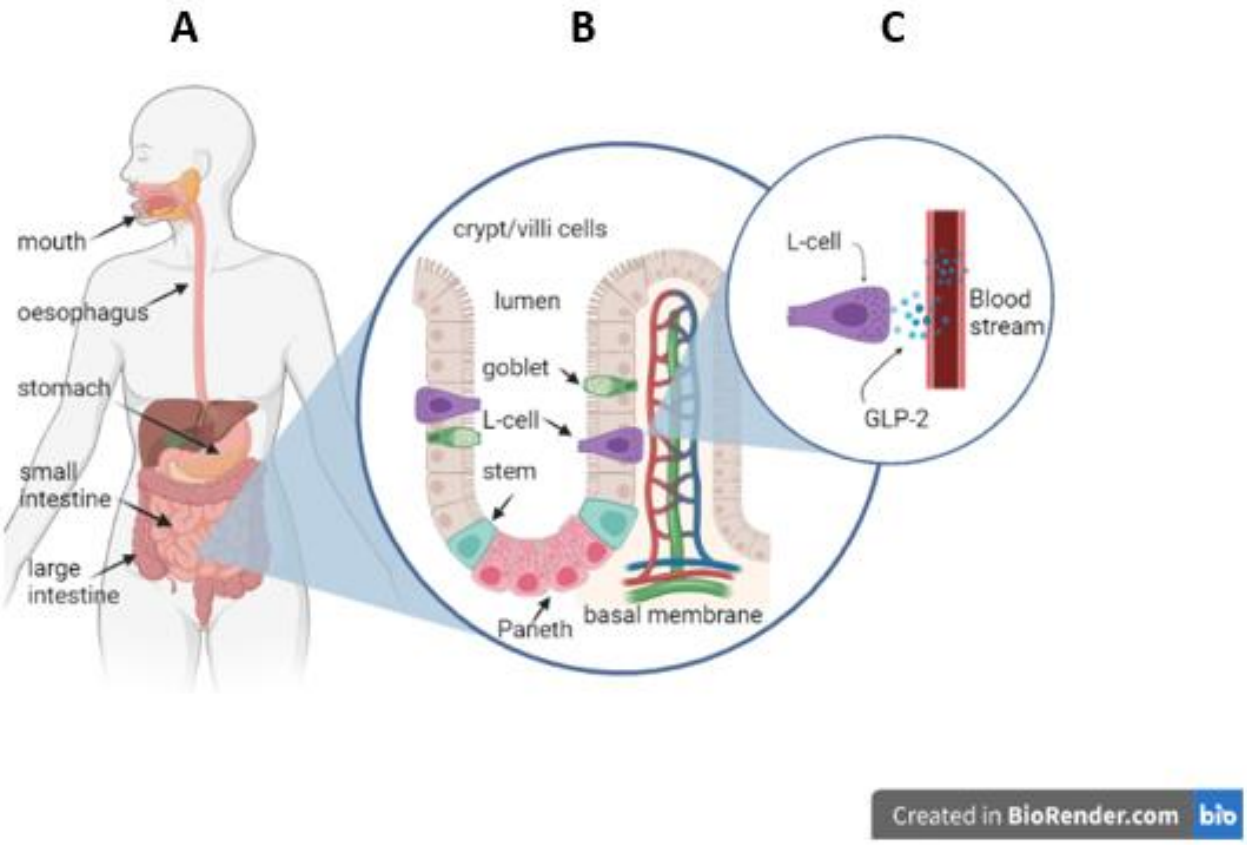

Figure 1: Schematic representation of the physiology of GI tract and intestinal cells. Adapted from (19).

General anatomical representation of the GI tract and its major organs including the intestine (**Figure 1A**), followed by a microscopic view of different types of intestinal cells seen in the crypt/villi (**Figure 1B**), followed by a schematic representation of GLP-2 secretion from the L-cell into the blood stream (**Figure 1C**).

There are a number of EECs that can be found in different locations throughout the GI tract, each with their own roles, functions and hormones produced. These cells constitute about 1% of all intestinal epithelial cells (20). All intestinal epithelial cells are formed from stem cells and differentiate over a period of 2-6 days (20). Table 1 summarizes the type of EECs, where it can be located, the hormones it produces and their functions (17). Of key importance is the L-cell localized in the ileum and colon. This particular EEC is responsible for the production and release of glucagon-like peptide-1 (GLP-1), GLP-2 and peptide-YY (PYY) (17). Although all of these hormones play an important role within the GI tract, this thesis will emphasize the importance of the hormone GLP-2.

Table 1: Summary of EEC type, location, hormones secreted and primary roles. Adapted from (17)

EEC type (localization)	**Hormone(s) secreted**	Primary roles
L-cell (Ileum, colon)	**GLP-2**	- Enhance intestinal function - Enhance blood flow - Promote enterocyte growth
	GLP-1	- Appetite control - Gut motility inhibition - Incretin effect
	PYY	- Appetite, water, electrolyte regulation
K-cell (duodenum, jejunum)	**GIP**	- Incretin effect - Gastric secretion inhibition - Gut motility inhibition
G-cell (stomach, duodenum)	**Gastrin**	- Acid secretion - Gut motility
S-cell (duodenum, jejunum)	**Secretin**	- Bicarbonate secretion - Water regulation
I-cell (duodenum, jejunum)	**CCK**	- Appetite control - Gall bladder secretion - Pancreatic enzyme secretion
P/D1 cell (stomach), **Epsilon cells** (pancreas)	**Ghrelin**	- Appetite control - Energy balance - Growth hormone secretagogue
	Leptin (adipose tissue)	- Appetite control - Energy balance
Alpha cell (pancreas)	**Glucagon**	- Conversion of glycogen to glucose - Inhibition of glycolysis
Beta cell (pancreas)	**Insulin**	- Metabolism and storage of glucose

1.3 GLP-2 synthesis and expression

GLP-2 is a 33 amino-acid peptide hormone derived from the proglucagon gene (21). This gene is capable of producing a number of products, depending on its cleavage site. In the alpha cell, the proglucagon gene is cleaved by prohormone convertase 2, for creating glucagon, glicentin-related pancreatic peptide and the major proglucagon fragment (which contains the GLP-1 and GLP-2 sequence) (22). In the L-cell, the proglucagon gene is cleaved by prohormone convertase 1/3, for creating glicentin, oxyntomodulin, and more importantly GLP-1 and GLP-2 (23, 24). Once cleaved, GLP-2 is co-secreted alongside GLP-1 from L-cells located in the ileum and colon (25). A summary of the cleavage and products can be seen in **Figure 2**.

The apical surface of the L-cell contains microvilli facing the lumen, allowing the cells to have direct contact with luminal content (20). It is considered an open-type EEC that interacts with the luminal content and regulates hormone production (20, 26). GLP-2 is released in response to the luminal content such as sugars, fatty acids and dietary fibers (27). Once L-cells are stimulated by the luminal content, GLP-2 granules undergo exocytosis into the lumen or towards the lamina propria, and then enter the bloodstream through capillaries (28). GLP-2 may have a direct impact on nearby cells and tissue, or will interact with other neuronal receptors and send signals to the nervous system (28). Once GLP-2 enters the bloodstream, it is metabolized and degraded by dipeptidyl peptidase IV (DPPIV) (29). Active GLP-2^{1-33} is modified by DPPIV into inactive GLP-2^{3-33} (30). The inactivation of GLP-2 is fairly rapid as the half-life of this hormone in circulation is around 7 minutes (31).

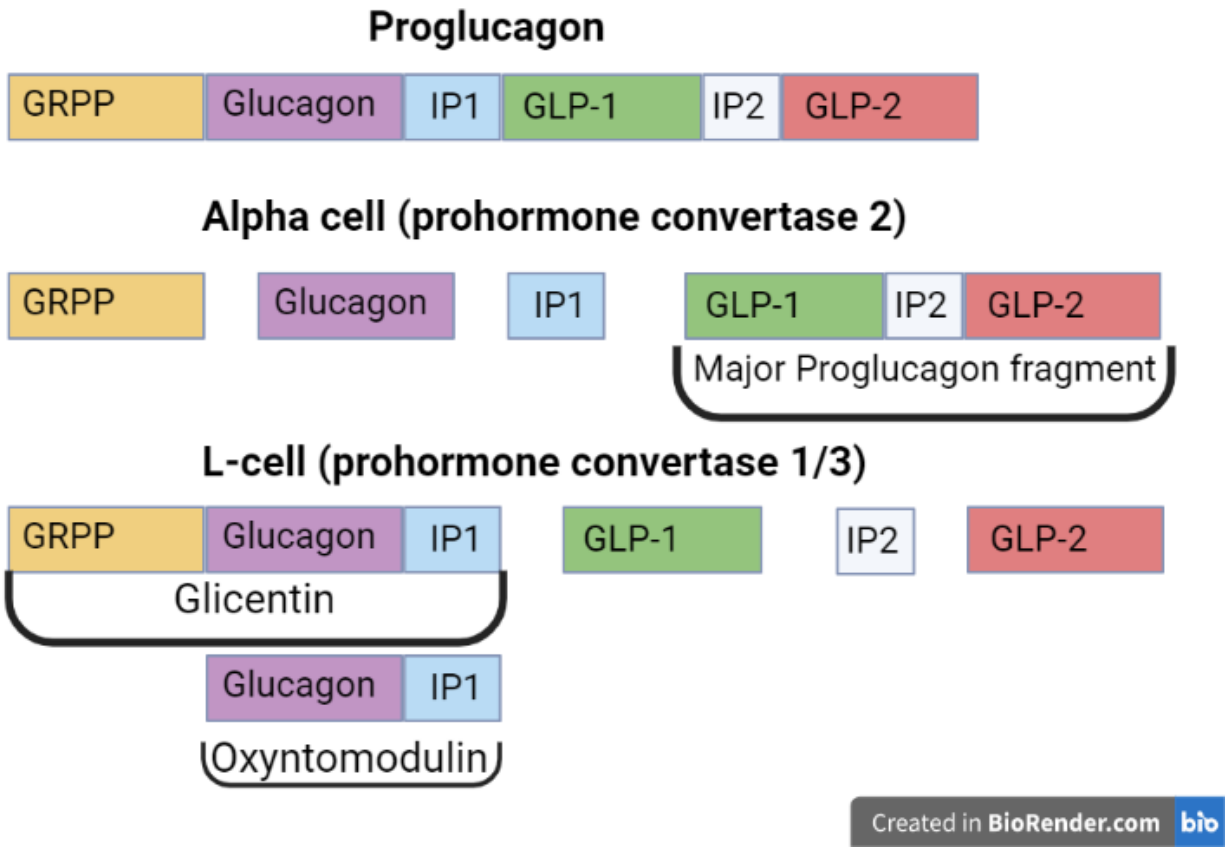

Figure 2: Post-translational processing of proglucagon. Adapted from (22).

Schematic representation of the post-translational processing of proglucagon to GI hormones in the alpha cell using prohormone convertase 2 (with an end result of GRPP, Glucagon, IP1 and major proglucagon fragment containing GLP-1, IP2 and GLP-2) and the L-cell using prohormone convertase 1/3 (with an end result of glicentin, oxyntomodulin, GLP-1, IP2 and GLP-2).

The underlying mechanism surrounding the actions of GLP-2 are mediated by its interaction with the GLP-2 receptor (GLP-2R) (29). GLP-2R falls under the G-protein coupled receptor family (32). The activation of GLP-2R by its GLP-2 ligand affects the cyclic adenosine monophosphate (cAMP) and mitogen-activated protein-kinase (MAPK) pathways, leading to anti-apoptotic and proliferative cellular responses (29, 33). It is expressed in a variety of regions throughout the body, however primarily in the stomach, jejunum, ileum and colon (32, 34). More specifically, GLP-2R is found in a variety of EECs, enteric neurons and stromal cells within the lamina propria (32, 34). GLP-2 is capable of exerting its effects directly on cells containing the GLP-2R, however many of the effects of GLP-2 can be seen indirectly with cells that do not possess the receptor (29). This suggests a direct and indirect interaction with GLP-2 and its functions within the body.

1.4 Functions of GLP-2

1.4.1 Cell proliferation and growth

GLP-2 plays a number of vital roles throughout the body, but more importantly it plays a large role within the GI tract. One of the effects stemming from GLP-2 is cell growth and proliferation. This occurs with the help of insulin-like growth factors (IGF) (35). IGF-I and IGF-II are part of a group of growth factors that are found throughout the body and regulate growth until adulthood (33). GLP-2 has been shown to increase the expression and secretion of IGF-1 within the GI tract, demonstrating that IGF-I may be an important mediator for intestinal growth along with GLP-2 (35). It has been shown that IGF-I plays an important role with small intestinal crypt cell proliferation. This was attained by studies observing IGF-I knockout (KO) mice which indicated a significant difference in intestinal growth in response to GLP-2 in comparison with wild-type (WT) mice (35). Furthermore, the effect of GLP-2 on cell proliferation within the crypt

is completely abolished without IGF-1 (35). In the normal mice, GLP-2 increased the rate of proliferation and cell growth within the upper half of the crypts, demonstrating the clear importance of IGF-I and GLP-2 on cell proliferation (33, 35).

1.4.2 GLP-2 and villi length

GLP-2 plays an important role in increasing the thickness and mass of the mucosal epithelium in the GI tract, most effectively in the jejunum (36, 37). Several studies have demonstrated an increase in villi length and GI mass following GLP-2 treatments (30, 36-39). This increase in GI morphology is also capable of enhancing absorptive functions (40). As the villus height and weight are increased, absorptive capacity is increased. It is suggested that these enhancements could be explained by increases in cell proliferation and decreases in cellular apoptosis (36, 39). The increase in proliferation and the inhibition of apoptosis evidently result in an increased villi-crypt height. Research has shown that GLP-2 increases mesenteric blood flow (41). This increase to the functional surface area and mesenteric blood flow leads to improved nutrient absorption (27, 41, 42).

1.4.3 GLP-2 and intestinal barrier

GLP-2 also plays a role in intestinal barrier function and has been found to enhance the paracellular and transcellular pathway in mice (43). This barrier protects against pathogens and other harmful materials, and also takes part in nutrient, ion and fluid transport (44). This is done by enhancing specific tight junction proteins (TJP) found within the GI system. There are a variety of TJPs that fall within one of the following four categories including claudin, occludin, zonula-occludens (ZO) and junctional adhesion molecules (44,45). Together, these proteins form a complex that provides a structural barrier between cells. A schematic model of the TJP

complex can be seen in **Figure 3**. Various studies have shown that GLP-2 enhances these TJPs and strengthens the barrier. When treated with a GLP-2 analogue (dapiglutide), the expression of claudin-7 is increased and the tight junction leak pathway is tightened (46). Expression of other TJPs can also be increased, such as claudin-2, claudin-10 and claudin-15 (46). The enhanced barrier function and TJPs would lead to an increase in absorptive capacity and a more effective defense system (44).

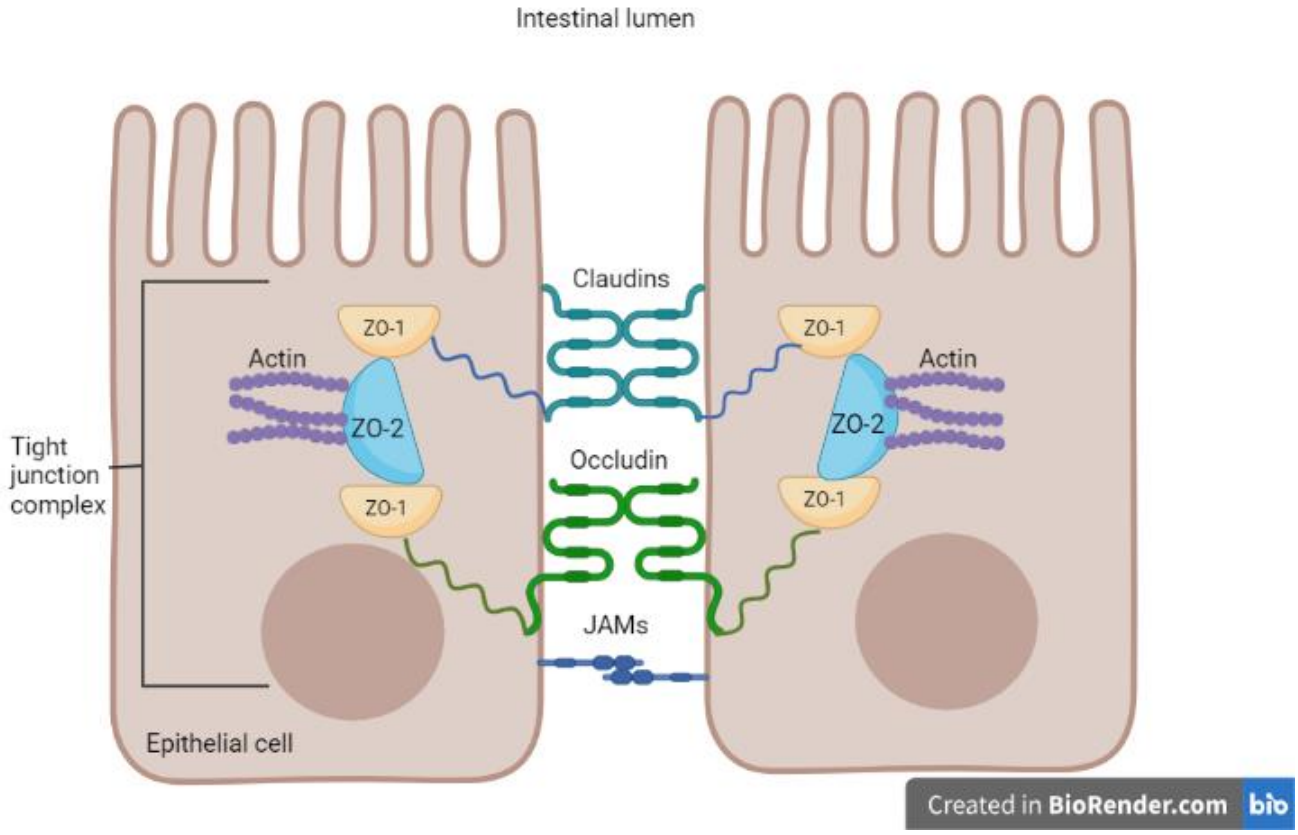

Figure 3: Schematic model of enterocyte TJP complex. Adapted from (45).

Schematic model of the TJP complex of two neighbouring enterocytes, highlighting key TJPs and their structures including claudins, occluding, JAMs and ZO-1/2. Together, these form the TJP complex.

1.4.4 GLP-2, anti-apoptosis and anti-inflammation

GLP-2 has been shown to decrease apoptosis by inhibiting a number of mediators involved in the apoptotic process such as caspase-8, caspase-9, cytochrome-c and caspase-3 cleavage (47). Tumour-necrosis factor alpha (TNF-α) is another mediator involved in cell death and inflammation (48). GLP-2 had a protective, anti-apoptotic, proliferative and anti-oxidant effect on intestinally-damaged mice treated with TNF- α (48). Similar results were seen in a study looking at GI inflammation and damage in rats after receiving human leukocyte antigen B27 and B2-microglobulin genes (49). The results demonstrated that GLP-2 significantly reduced lesions within the gut (49). GLP-2 also reduced gene expression of TNF-α and interferon gamma (IFNy), two inflammatory mediators (49). Not only does GLP-2 play a role in anti-apoptosis, it is also involved in anti-inflammation. Alongside the previously mentioned functions and effects of GLP-2, it is evident that this hormone plays a vital role in GI health and physiology. This hormone has the potential to be used as a treatment method for a number of important reasons.

1.5 Hydrogen sulfide

(H_2S) is a highly flammable and strong colourless gas characterized by its smell resembling rotten eggs. It is present naturally in a variety of places such as hot springs, volcanoes and sewers but also as an environmental pollutant from paper mills and mining (50). It has been considered a toxic gas for many years, and those who work alongside H_2S must be cautious. The effects of exposure to this gas is dependent on the concentration and length of exposure (51). H_2S toxicity can take a variety of forms and symptom severity increases along with exposure. Symptoms include eye irritation, loss of sense of smell, headache, sudden unconsciousness and pulmonary

edema (51). Prolonged exposure to high concentrations of H_2S can lead to lung, brain and heart damage, and can potentially result in death (52). However, one puzzling aspect remains. Although H_2S can be dangerous and highly toxic, it is a gas that is endogenously produced in our body. The concentrations of H_2S in mammalian tissues of humans, rats and cows range between 50-160 μM (53, 54). However, the physiological range of H_2S has also been estimated to be in the nanomolar range in most tissues (55). H_2S has recently emerged as the third gasotransmitter, along with nitric oxide and carbon monoxide (56). These gasotransmitters are endogenously created gases that have been shown to be signaling molecules, similar to neurotransmitters (57). The effects of this novel gasotransmitter have been investigated over the years and continue to be a hot topic of research. It has been shown that H_2S plays a large number of physiological and pathological roles in a variety of systems including cardiovascular, GI, immune, endocrine and nervous (58). The effects of H_2S within these systems will be further discussed and will highlight a potential link between H_2S, GI disorders and GLP-2. The following section will elaborate on endogenous production of H_2S.

1.5.1 H_2S biosynthesis and catabolism

H_2S is produced in our bodies through multiple processes. It can be produced naturally in the cells of an organism, known as endogenous production, or through bacteria in our GI tract, known as the gut microbiome. H_2S production in mammalian cells can be produced through enzymatic and non-enzymatic pathways (58). The key enzymes responsible for the endogenous production of H_2S include cystathionine beta-synthase (CBS), cystathionine gamma-lyase (CSE), 3-mercaptopyruvate sulfurtransferase (MST) and cysteine aminotransferase (CAT) (58). Endogenous production of H_2S relies on amino-acids containing sulfur such as cysteine and methionine (59). Cysteine can be obtained through diet, or synthesized from methionine (59).

Dairy products, meats and nuts are sources of methionine from our diet, which play a role in cysteine synthesis (60). Homocysteine is formed during the transformation of methionine to cysteine and is also important in H_2S production (59). Roughly 70% of endogenous H_2S is produced by cysteine, while the remaining 30% is produced by homocysteine (61). CBS and CSE both use Vitamin B6, also known as pyridoxal-5-phosphate (P5P) in the production of H_2S and have been investigated more thoroughly (58). The precise mechanism and pathway of each enzyme and metabolite in regards to H_2S production are summarized in **Figure 4** (58). The physiological signal that produces and releases H_2S is unknown (62). It is possible that once produced, H_2S is released directly following synthesis from enzymes (62). It can also be stored for later release as bound sulfane sulfur (63), or acid labile sulfur pools in mitochondria (62). Roughly 66% of H_2S is stored as HS-, but it can also be stored as S^{2-} (62). Release can occur as any of the three forms. In peripheral tissues, CSE is more abundant, whereas in the brain CBS is more abundant (58). Endogenous production of H_2S has been established in the GI tract of mice and humans, primarily produced by CSE and CBS enzymes (63). CBS and CSE mRNA were found in the rat ileum (58). Although both of these enzymes have also been found in colon tissue (63), it seems that CSE is the main enzyme producing H_2S in the stomach, while CBS is the main enzyme producing H_2S in the colon (63). However, there is another method in which H_2S is produced in our body.

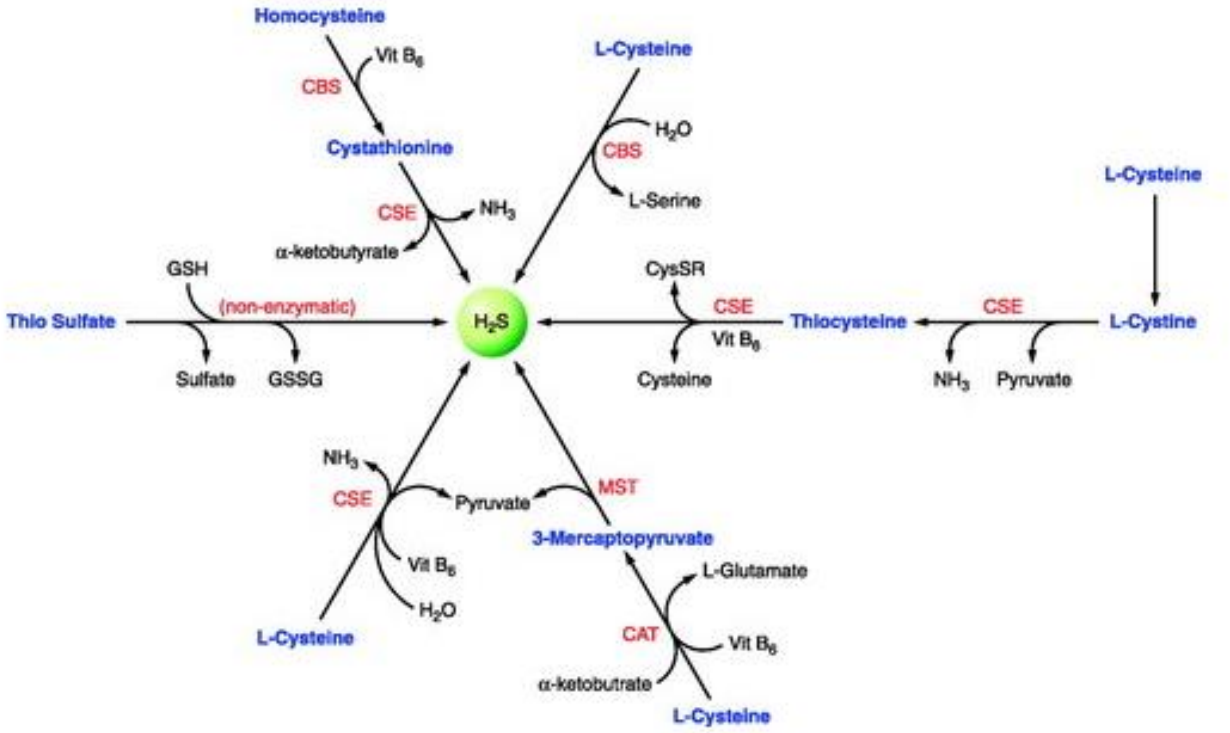

Figure 4: Pathways for endogenous H_2S production. Adapted from 58.

Schematic model for the endogenous production pathways of H_2S. In red are the key enzymes needed for the endogenous production of H_2S including CSE, CBS, MST and CAT, along with essential amino acids containing sulfur compounds in blue. In black are other compounds that are necessary in certain pathways, such as vitamin B6.

1.5.2 H_2S from gut microbiome

H_2S is also known to be produced by bacteria present in the GI tract, known as the gut microbiome. These bacteria play a number of important roles such as helping with digestion, producing vitamins and other nutrients and resisting invasion of pathogens (64). Bacterial fermenters break down proteins and carbohydrates which are used as a source of energy, and also produce and release gas (64). Hydrogen is one of the gases released in the colon during this process, which is then consumed by a group of bacteria known as hydrogenotrophs (64). Among the hydrogenotrophs are methanogens, acetogens, and sulfate-reducing bacteria that produce methane, acetone and H_2S respectively (64). The anaerobic sulfate-reducing bacteria use hydrogen, short-chain fatty acids, alcohols and amino acids as electron donors due to the lack of oxygen (63). Sulfate is used as the terminal electron acceptor, which then leads to the production of H_2S (58). The term used for this process is known as dissimilatory sulfate reduction and is further described by Wang in (58). Sulfate-reducing bacteria and methanogens often compete for hydrogen, and one of the two will predominate in the large intestine. In about 60% of humans, sulfate reduction prevails as dominant over methanogens (64). This bacteria present in the colon is responsible for producing a large portion of H_2S quantities (64). One study revealed that H_2S levels were decreased by 50-80% in germ free mice, indicating the importance of the gut microbiota in terms of H_2S production and generation (65). There are a number of factors that can influence the gut microbiome such as environmental factors, diet and genetics (66). Dysbiosis, which is the number and composition of the gut microbiome, can also be influenced by stress, antibiotics and infection (64). These factors can alter the metabolic properties of the gut microbiome and potentially impact or lead to the emergence of certain diseases (66). Some factors that could influence a potential toxic versus therapeutic effect are the concentration, the

source and length of exposure to H_2S (67). Whether produced endogenously or by the gut microbiome, it is clear that H_2S may play a crucial role in the body.

1.6 Tools used in H_2S research

To determine the impact of H_2S on various systems throughout the body, there are a few methods used. To determine the effect of exogenous H_2S, studies focus on H_2S donors, such as morpholin-4-ium 4-methoxyphenyl(morpholino)phosphinodithioate (GYY 4137) or sodium hydrosulfide (NaHS) (58). NaHS is a fast-releasing H_2S donor that is often used in cell culture studies, which can present challenges. One study demonstrated the H_2S releasing profile of this chemical and determined that the majority of H_2S that is produced is released within a two hour timeframe (68). In comparison, GYY 4137 is a much slower and stable H_2S donor, in which this compound demonstrates a long-term and sustained effect of H_2S, making it favorable for certain studies (68). These H_2S donors can be added to cell culture models. Furthermore, GYY 4137 is often given to animals in the form of injections (58). Protocols in animal models have been developed for the use of this slow and steady H_2S donor in intraperitoneal or intravenous injections (69). These are methods for observing the effects of exogenous delivery of H_2S, however there are endogenous methods as well. As previously mentioned, there are key enzymes produced naturally that are required for the synthesis of endogenous H_2S, which include CBS and CSE. Animal models lacking these enzymes have been developed, such as the CSE-KO mouse. A large body of research has been conducted on these mice to observe the effects of not having an important H_2S-producing enzyme like CSE (70-73). Another method of observing the effects of endogenous H_2S is through the use of H_2S-producing enzyme inhibitors. There are a number of inhibitors that prevent H_2S production by inhibiting CBS and/or CSE. Some of these inhibitors include aminooxyacetic acid (AOAA), L-aminoethoxyvinylglycine (AVG), β-

cyanoalanine (BCA) and hydroxylamine (HA) to name a few (74). These inhibitors target the previously mentioned H_2S-producing enzyme pathways and are effective at different doses ranging from µM to mM (74). There are a number of effective methods to study the effect of H_2S, whether through exogenous donors, or inhibiting endogenous production. The following section will further elucidate some of the effects of H_2S in different systems.

1.7 Signaling effects of H_2S throughout the body

H_2S is capable of exerting a wide range of effects throughout the body. Length of exposure and concentration are two important factors that could guide its trajectory towards having toxic effects or beneficial effects. The following section will describe the effects of H_2S on various systems in the body such as the cardiovascular, endocrine and GI systems.

1.7.1 H_2S effects in the cardiovascular system

The enzymes responsible for H_2S production have been shown to play important roles in the cardiovascular system. These enzymes are located in a number of tissues throughout the body, however a large amount of research has focused on this particular system. H_2S, along with nitric oxide and carbon monoxide are involved in a number of physiological activities (75). These gasotransmitters have been involved with neuromodulation, vasodilation and anti-inflammatory effects (76, 77). The effects on the cardiovascular systems are vast, therefore a brief overview will be provided. H_2S has been shown to have vasodilatory effects in which it can relax smooth muscle cells and increase the diameter of blood vessels in an oxygen-dependent manner (78). A vasorelaxation effect has also been shown (58). It has been reported that H_2S can have a

chronotropic and inotropic effect on the heart, regulating heart rate and contractility (58). H_2S has been shown to reduce hypertensive inflammation, demonstrating anti-hypertensive and anti-inflammatory effects (79). Research has shown that H_2S can inhibit vascular smooth muscle cell proliferation and stimulate vascular endothelial cell proliferation (58). H_2S provides antiplatelet activity, in which it inhibits platelet activation and aggregation (80). Finally, H_2S is involved in cardioprotection, as it attenuates cardiac dysfunction after heart failure via the induction of angiogenesis, reducing oxidative stress and preserving mitochondrial functions (81). Although the effects seen throughout the cardiovascular system are important and interesting, this does not address the systems of interest in our study. Therefore, it is important to discuss the role H_2S plays in the endocrine and GI systems.

1.7.2 H_2S effects in the endocrine system

As previously mentioned, GLP-2 is a hormone that is secreted by EECs, which fall under the endocrine system. Therefore it is important to discuss the effects of H_2S on the endocrine system. One of the organs found within the endocrine system is the pancreas. A large body of research has been done on the pancreas and its affiliation with diabetes. CBS and CSE are both found in the pancreas, resulting in the presence of H_2S (82, 83). H_2S has been shown to be beneficial and to have a protective role for beta cells and regulate insulin secretion (84). H_2S can reduce the production of reactive oxygen species, inhibit the expression of a redox protein (thioredoxin binding protein-2) associated with diabetes that promotes apoptosis, and increase glutathione, which reduces damage from oxidative stress (84-87). Interestingly, although H_2S has been shown to have beneficial effects, a high concentration of H_2S induces apoptosis of beta cells in the pancreas (88). This highlights the importance of concentration and exposure length. H_2S has been shown to inhibit glucose-stimulated insulin secretion from beta cells (89). Within our lab,

we have shown that H_2S can have an effect on hormone secretion and the endocrine system. H_2S has been shown to have an inhibiting effect on ghrelin, an important hormone found in the stomach regarding hunger (90). H_2S has also been shown to have a stimulatory role on GLP-1, the sister hormone of GLP-2 (91).

1.7.3 H_2S effects in the GI tract

A large portion of H_2S is produced in the GI tract through its microbiome. Sulfate-reducing bacteria are key contributors to H_2S production, along with endogenous H_2S-producing enzymes found within cells of the GI tract (92). Evidently, H_2S must have an effect within these regions. H_2S has been shown to have multiple effects, at times contradicting itself (93). For example, H_2S has been shown to play a role in gastric motility. One group found that endogenous H_2S has an excitatory effect on gastric motility (94). However, another group demonstrated that exogenous H_2S has an inhibitory effect on the contractions of the jejunum and colon in humans, mice and rats, and that this was due to sulfhydration of K_{ATP} channels (95).

H_2S has also been shown to play a role in inflammation. In a study using a model of sepsis, administering NaHS subcutaneously improved survivability of the mice before and after the procedure, and decreased a number of inflammatory markers (96). This sepsis model was induced by cecal ligation and puncture, in which the cecum is punctured, releasing contents into the peritoneal cavity for inducing an inflammatory response. However, another study demonstrated that inflammation in response to cecal ligation and perforation was linked to higher circulating levels of H_2S and a higher expression of CSE, in which treating mice with NaHS further increased inflammatory markers (97). Interestingly, the application of a CSE inhibitor

reversed some of these effects. Further experiments demonstrated that the dosage used, as well as the route of administration led to either a pro-inflammatory, or anti-inflammatory response. When NaHS was applied as an intra-peritoneal high dose, the effects were pro-inflammatory, as opposed to an anti-inflammatory response seen with low dose, subcutaneous injections (93). Another important factor influencing directionality of inflammation is the type of H_2S donor used as previously mentioned. Studies have looked into testing the effects of GYY 4137 on inflammation and have found that it has been shown to be anti-inflammatory, and was able to decrease inflammatory markers (98). Similar results have been seen across other studies, demonstrating that the slow releasing GYY 4137 chemical is similar to how H_2S may be released endogenously by enzymes (93). Results using GYY 4137 seem to follow a dose-dependent response, whereas studies using the fast-releasing NaHS donor seem to have a biphasic response, in which certain lower concentration were beneficial, up to a certain point in which it then becomes toxic, producing opposing effects (99). As a result, it is important to consider the type of H_2S donor, method of delivery and exposure length when studying the effects of H_2S in different biological systems.

1.8 H_2S linked to IBD

Interestingly, H_2S has been linked to CD and colitis, which is inflammation of the GI tract or colon respectively, as previously described. Studies have shown that individuals with IBD present higher levels of H_2S compared normal individuals. This has been shown through a variety of testing methods using feces, microbiome and breath. For example, a high number of sulfate-reducing bacteria (SRB) have been found in patients with UC (100). Other H_2S-producing bacteria have also been associated with IBD (101). This highlights one of the potential

dangers of dysbiosis, and a possible contributing factor to the development and pathogenesis of IBD. Furthermore, studies looking at fecal H_2S levels were increased in IBD patients compared to controls (102, 103). In addition, the luminal concentration of H_2S was higher in UC patients compared to normal (102). H_2S production rate has been correlated to the intensity of UC (104, 105). To further expand on H_2S and its link to IBD, breath tests measuring volatile organic compounds (VOCs) have also been studied. A number of VOCs, including H_2S, had significantly different concentration when comparing IBD patients to normal individuals (106, 107). This could be indirectly linked to bacterial composition in the GI tract and feces. Interestingly, this leads to an important question that remains to be unanswered. Are these higher levels of H_2S a cause of IBD, or a response to counteract the illness? If so, are these physiological effects such as inflammation and GI damage somehow linked to GLP-2?

2 Hypothesis and Objectives

H_2S is a gas that is naturally produced throughout the body. Previous research has shown that H_2S can regulate insulin, GLP-1 and ghrelin secretion. This gas has been linked to GI diseases such as CD and UC, in which these individuals have higher levels of H_2S. Symptoms of these diseases include inflammation and reduced gut integrity. One of the hormones that promotes gut integrity is GLP-2. As a result, we set out to determine if there is a link between H_2S, GLP-2 secretion and gut physiology. We hypothesize that intestinal H_2S plays a regulatory role in increasing GLP-2 secretion and that this will lead to enhancements in GI integrity. This will be achieved using *in-vivo* and *in-vitro* models. We will examine the effects of H_2S insufficiency on gut physiology, as well as cellular and circulating GLP-2 levels using WT and CSE-KO mice. GLP-2 levels in plasma and tissue will be measured using an ELISA. Using the same tissue samples, a western blot will be performed for analyzing markers of GI integrity. Furthermore, we will measure the direct effects of H_2S on GLP-2 secretion. This will be done using the GLUTag mouse EEC along with H_2S donors (NaHS and GYY 4137) as well as using an H_2S -producing enzyme inhibitor (AOAA). The following chapter will describe the original research and experiments conducted in this study.

3 Materials and methods

3.1 Cell culture

The cell line used throughout the experiments was murine GLP-2 secreting GLUTag L-cells (a kind contribution from Dr. Drucker, Lunenfeld-Tanenbaum Research Institute, Toronto, ON) ranging from passage 10-25. Dulbecco's Modified Eagle Medium low-glucose (DMEM), Hank's Balanced Salt Solution (HBSS), Fetal Bovine Serum (FBS) and Penicillin/Streptomycin (P/S) were all used during cell culture growth. DMEM supplemented with 10% FBS and 1% P/S was used to maintain cell growth in a humidified incubator at 37°C with 5% CO_2. HBSS was used to rinse and wash cells. Cells were split every 2-3 days into 10cm plates. Cells were used for experimental testing once passages reach 80% confluency.

3.2 GLP-2 secretion experiments

GLUTag cells were seeded into 12-well plates or 24-well plates at 450 000 and 200 000 cells per well respectively and received treatments upon reaching 80% confluency after 48 hours. Growth media was removed and cells were treated for 2 hours with H_2S donors sodium hydrosulfide (NaHS) or GYY 4137 dissolved in 0.5% FBS secretion media (68). The doses of NaHS were 0.1, 1 and 10 mM, whereas the GYY 4137 doses were 1, 10 and 100 µM The 2 hour time frame is representative of an acute GLP-2 production and secretion experiment. Vehicle media served as control for baseline secretion, and 10 µM forskolin served as positive control (91). After 2 hours, the plate was placed on ice to prevent further secretion, and treatment media containing secreted GLP-2 was collected and acidified using 0.1% trifluoroacetic acid. Samples were then stored in freezer at -80°C until further analyzed. GLP-2 content from media was quantified using a multi-

species enzyme-linked immunosorbent assay (ELISA) following the manufacturers protocol (ab222863, Abcam, Cambridge, MA, USA). Results per treatment were presented as secretion relative to vehicle control. A 24 hour secretion experiment using GYY 4137 was also performed following the same methods, however media collection took place 24 hours later, rather than 2 hours to elicit a long-term exposure to H_2S donors. Using the above mentioned methods, a similar secretion experiment was also conducted using an H_2S-producing enzyme inhibitor, AOAA. AOAA was dissolved in methanol and delivered via secretion media for 2 hours and 24 hours. The same procedures were followed using the same GLP-2 ELISA kit.

3.3 Cell viability assays

3.3.1 Neutral Red lysosomal reuptake assay

Cell viability was tested with the neutral red lysosomal reuptake assay as described (108). This assay is based on the capacity of viable cells to bind with the neutral red dye in lysosomes after the cells have been treated with H_2S donors and controls. Cells were seeded in a 24-well plate at 200 000 cells per well and grown for 48 hours until 80% confluency was reached. Neutral red dye stock (Sigma-Aldrich, Oakville, ON, Canada) was prepared at 0.01 g/ml in deionized water. Treatments received a final concentration of 4 µL dye/mL secretion media. Treatments included H_2S donors mentioned previously, vehicle control and 20% DMSO as negative control. A volume of 500 µL of treatment with dye was added to each well. The plate was then incubated for 2 hours at 37°C, 5% CO_2. After 2 hours the wells were aspirated and then 1 mL wash-fixation solution (dH_2O, 1% calcium chloride, 0.37% vol/vol formaldehyde). After aspirating the wash-fixation solution, 1 mL of extraction solution (dH_2O, 50% vol/vol bonded ethanol, 1%

vol/vol glacial acetic acid) was added to each well to extract the dye from viable cells. The plate was then added to an orbital shaker for 5 minutes to enhance dye extraction. Finally, absorbance was measured at 600 nm using the FLUOstar program.

3.3.2 Resazurin reduction assay

Cells were seeded in 24-well plates at 200 000 cells per well in growth media for 48 hours prior to treatment. Cells were then treated when 80% confluency was reached. A resazurin stock solution (Biotium, Inc., Fremont, CA) of 10 mg/mL was diluted to 0.15 mg/mL for the experiment. After the 48 hours, cells were washed and treated with 1:5 resazurin:treatment secretion media in each well. Treatments included H_2S donors as previously mentioned, or AOAA. Vehicle control and a positive control using 0.3% hydrogen peroxide (H_2O_2) was also used. Cells were incubated with dye/treatment for 2 hours and 24 hours at 37°C, 5% CO_2. After 2 hours and 24 hours, fluorescence was measured (Ex/Em 530/590 nm) using fluostar program and compared to control.

3.4 H_2S production rate measurement

A lead acetate paper method was used to quantify H_2S production from tissue and cell lysate (109). Cells were collected from 10cm plates at 80% confluency. Cells were then collected with a cell scrapper and lysed with a needle and lysis buffer. In a 96-well plate, equal amounts of protein from the cell lysate (20 µg) was added to a reaction solution containing 10 µL cysteine (10 mM), 10 µL pyridoxal 5'-phosphate (P5P) (2 mM), and phosphate-buffered saline (PBS) was added to reach a total of 100 µL in each well. When testing H_2S production along with the H_2S-

producing enzyme inhibitor AOAA, 10 µL of AOAA at different concentrations was added to each well. For tissue samples, 4mg of tissue was added to each well along with the same previously mentioned chemicals to form the reaction solution. Then a lead-acetate paper (Sigma-Aldrich) was placed over the wells and the 96-well plate was added to the incubator at 37°C, 5% CO_2 for 2 hours. The intensity of the dark brown precipitate on the paper was measured and quantified using the Biorad Quantity One software and compared to a standard range of H_2S production from NaHS.

3.5 Wild-type and CSE-KO mice protocol

Both WT and CSE-KO mice were maintained on standard rodent chow and had free access to food and water. After 12-week male mice were anesthetized, blood and various tissues were removed and stored in -80°C freezer for further analysis. A section of ileum samples were then cut and weighed. A volume of 10 µL of total lysis buffer (Abcam, ab152163) containing complete protease inhibitor cocktail (Roche, ON, Canada) was added per mg of tissue. Tissues were homogenized on ice using a tissue tearer in a microcentrifuge tube. The homogenates were then centrifuged at 14,000 x g for 10 minutes at 4°C. Supernatants were extracted without disturbing the pellet and stored at -80°C until further use. All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animal care and use protocols were approved by the Animal Care Committees of Laurentian University, Canada.

3.6 Western Blotting

Following tissue extraction from WT and CSE-KO mice, a Bradford Assay was performed to quantify the amount of protein within the tissue lysate samples. A total of 40 ug of protein along with 4x loading buffer and a denaturant (Beta-mercaptoethanol) were heated on a heating block for 10 minutes at 70°C. Samples were then loaded and separated on a precast SDS-page gel (mPAGE® 4-12% Bis-Tris Precast Gel, Millipore Sigma) at 100V and 45A for 1 hour. Protein was then transferred to a PVDF membrane at 100V and 250A for 1 hour. The membrane was then blocked with 5% skim-milk in Tris-buffered saline (TBS) for 1 hour at room temperature and then probed with a primary antibody overnight at 4°C. The antibodies used and their dilution can be seen in table 2. The membrane was then washed and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (GAR or GAM) for 1 hour at room temperature. Protein signal was then visualized using an Immobilon Forte chemiluminescence HRP substrate and luminescence was detected and quantified using the BioRad chemi doc XRS system. Band densitometry results were analyzed and normalized to the intensity of GAPDH loading control.

Table 2: Antibodies used for western blotting

Protein target	Species Raised in; Monoclonal or Polyclonal	Dilution used	Manufacturer	Product number
CSE	Mouse; monoclonal	1:1000	Abnova	H00001491-M01
CBS	Rabbit; polyclonal	1:500	Abcam	ab96252
Claudin-7	Mouse; monoclonal	1:1000	Novus	NBP-2-66784
ZO-1	Rabbit; polyclonal	1:1000	Abcam	ab216880
IGF-1	Rabbit; polyclonal	1:1000	Abcam	ab9572
GAPDH (control)	Rabbit; monoclonal	1:5000	Cell Signalling	14C10
Goat Anti-Rabbit (GAR) Secondary Antibody – HRP Conjugated	Goat polyclonal	1:5000	Biomine Scientific	Bi2M-GAR-AB
Goat Anti-Mouse (GAM) Secondary Antibody – HRP Conjugated	Goat; polyclonal	1:5000	Biomine Scientific	Bi2M-GAM-AB

3.7 GLP-2 ELISA crystal chem

Total GLP-2 levels in plasma and ileum tissue lysates were determined using a mouse-specific GLP-2 ELISA kit (Crystal Chem, 81514) as per the manufacturer's guidelines. For the plasma, 25 µL of plasma was added to each sample well. For tissue lysate, a 1 in 5 dilution in PBS was performed to ensure values are within the curve. Dilution factor was taken into account prior to analysis of data. After following manufacturer's guidelines, absorbance was measured using spectramax plate reader.

3.8 Statistical 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 Bonferri's post hoc test. $P < 0.05$ was considered statistically significant.

4 Results

4.1 Liver tissue lysate from CSE-KO mice does not produce H_2S or express CSE enzyme

To confirm the validity of our CSE-KO model, we performed the lead-acetate paper assay on liver tissue lysate from CSE-KO and WT mice. CSE is the primary H_2S-producing enzyme found in the liver, which makes the liver a suitable control for our tissue samples, as well as assay techniques (58). As a result, H_2S production is evident in WT liver samples, however absent in CSE-KO liver samples (**Figure 5A**). Densitometry was then performed (**Figure 5B**), however the difference between the samples is clear. Additionally, a western blot was performed using the same tissue lysate samples (**Figure 5C**). The membrane was incubated with CSE antibody and demonstrates that CSE expression is present in the WT sample, but absent in CSE-KO samples. This confirms and validates that our CSE-KO model lacks the H_2S-producing enzyme, and further validates our assay techniques. GAPDH antibody was used to determine equal loading of protein.

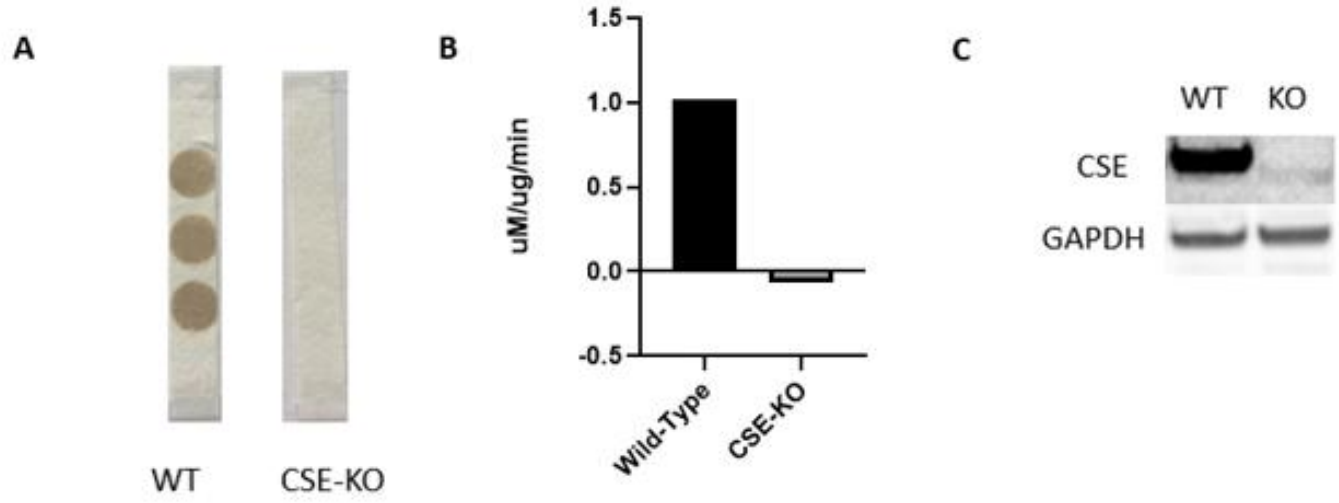

Figure 5: Validation of CSE-KO using liver tissue lysate demonstrates true knockout.

H_2S production rate in Wild-type and CSE-KO liver tissue lysate was measured using the (A) lead-acetate paper method and (B) densitometry was quantified using the BioRad program. Liver was used as control, as CSE is the primary H_2S-producing enzyme in the liver. (C) A Western Blot was also performed using a CSE antibody to validate knockout in liver tissue.

4.2 Intestinal tissue of CSE-KO mouse produces less H_2S

Using the same techniques as previously described, we wanted to determine if there are any differences in H_2S production within the intestinal tissue. CSE-KO mice produced significantly less H_2S when compared to WT mice (t-test $P < 0.05$) (**Figure 6A**). The mean H_2S production in WT mice was 0.62 ± 0.102 µM/µg/min, compared to a mean of 0.33 ± 0.080 µM/µg/min in CSE-KO samples (**Figure 6B**). A western blot using a CSE antibody was also performed to ensure CSE is not expressed in intestinal tissue lysate. CSE expression was absent in CSE-KO intestinal tissue lysate (**Figure 6C**).

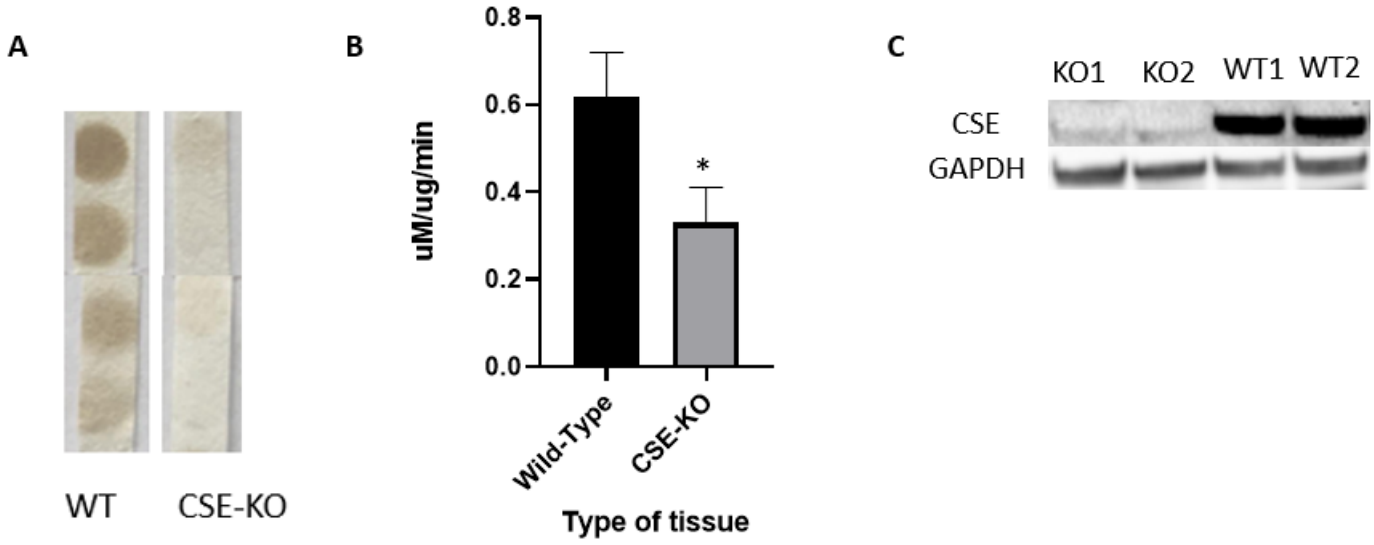

Figure 6: H_2S production in intestinal tissue lysate of CSE-KO mouse is decreased compared to WT controls

H_2S production rate in Wild-type and CSE-KO intestinal tissue (ileum) lysate was measured using the (A) lead-acetate paper method and (B) densitometry was quantified using the quantity one BioRad program. (n=8) (C) A Western Blot was also performed using CSE antibody to validate knockout in intestinal tissue. * (t-test $P < 0.05$)

4.3 CSE deficiency does not affect cellular or circulating GLP-2 in unfasted mice

We then wanted to determine if the difference in H_2S production would play a role in circulating GLP-2 in the plasma of unfasted WT and CSE-KO mice, as well as cellular GLP-2 in intestinal tissue lysate. The concentration of GLP-2 was determined using a GLP-2 ELISA. For the tissue, GLP-2 concentration was compared to total protein from a Bradford assay. Plasma GLP-2 was not significantly different, with a mean concentration in WT mice of 0.515 ± 0.043 ng/mL, and a mean concentration of 0.559 ± 0.066 ng/mL in CSE-KO mice (**Figure 7A**). After normalizing

GLP-2 levels in the intestinal tissue lysate to total protein obtained in the Bradford assay, the mean values were almost identical for WT and CSE-KO at 0.0050 ± 0.0006 ng/µg and 0.0049 ± 0.0004 ng/µg respectively (**Figure 7B**). Unfortunately, GLP-2 is time-sensitive to when the animals previously ate, and therefore could have contributed to high variability between samples. As a result, there were no significant differences between WT and CSE-KO plasma samples or intestinal tissue lysate.

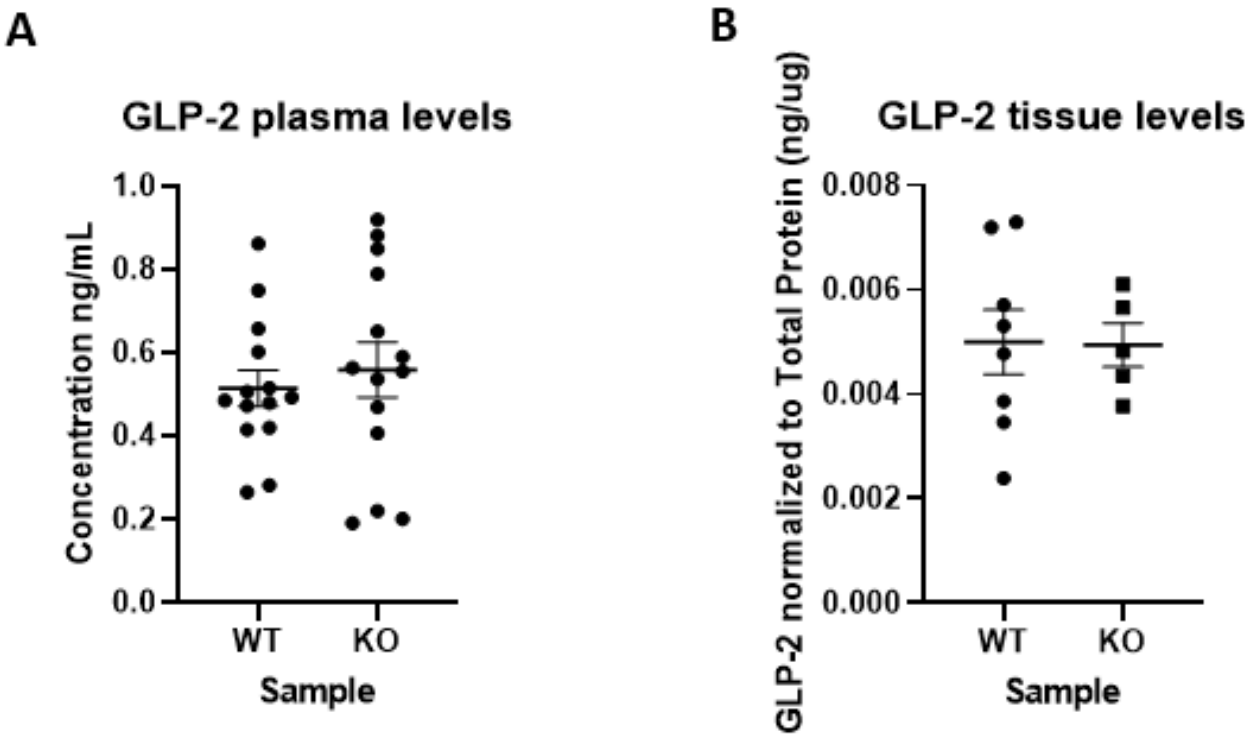

Figure 7: GLP-2 concentration in plasma and intestinal tissue lysate of mice remains unchanged.

GLP-2 concentrations were measured in non-fasting (A) plasma samples from Wild-Type and CSE-KO mice (n=14) and (B) non-fasted distal intestinal tissue (n=5-8) using GLP-2 ELISA kit. No statistical significant differences seen using t-test.

4.4 High amounts of NaHS suppress GLP-2 secretion after 2 hours

Since we did not see any differences in GLP-2 secretion using WT and CSE-KO plasma and intestinal tissue lysate (**Figure 7**), we wanted to use a more direct approach to observe the effects of H_2S on GLP-2 secretion using GLUTag cells. As a result, NaHS significantly suppresses GLP-2 secretion at its highest dose (10 mM) causing a 35% decrease compared to untreated controls (**Figure 8A**). The decrease seen with GYY 4137 and other doses was not significant. To ensure this decrease in GLP-2 secretion was not a result of the higher dose killing the cells, two cell viability assays were performed using the neutral red and resazurin viability assays. In the neutral red assay, cell viability was increased (**Figure 8B**). Cell viability was not affected by treatments using the resazurin viability assay (**Figure 8C**).

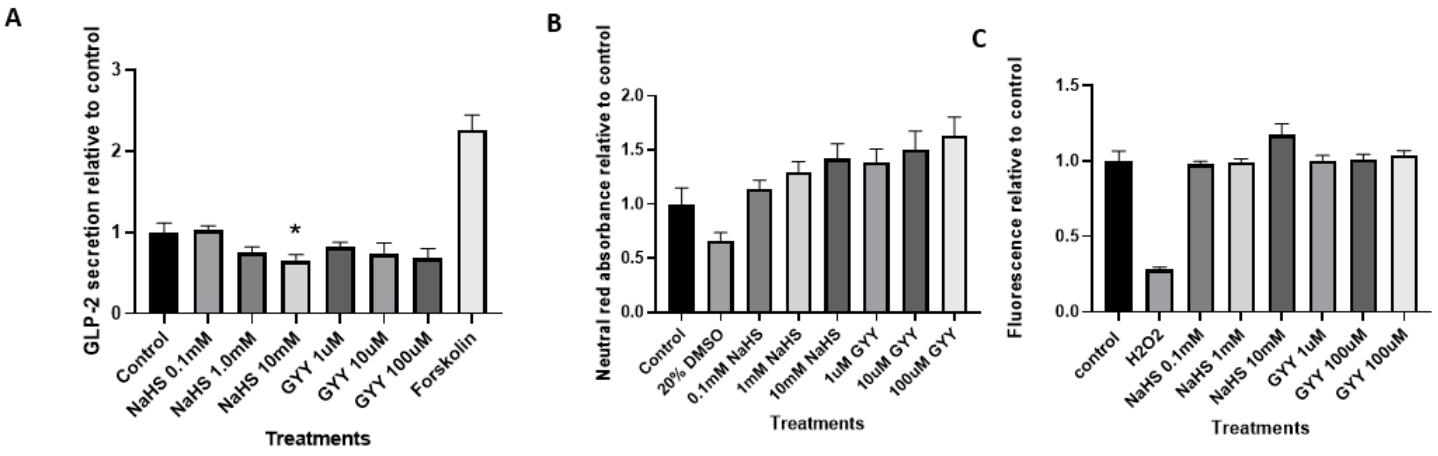

Figure 8: NaHS donor at 10 mM suppresses GLP-2 secretion without affecting cell viability.

(A) GLP-2 secretion was measured in media from GLUTag cells treated with varying concentrations of H_2S donors (NaHS and GYY 4137) for 2 hours and then analyzed by GLP-2 ELISA kit. Data is presented relative to vehicle control with Forskolin as positive control. (n=6) Cell viability was examined under similar conditions as described previously using NaHS or GYY 4137 treatment via (B) Neutral Red Re-uptake Assay for lysosomal stability and (C) Resazurin Reduction Assay for mitochondrial activity. Data is presented relative to vehicle control and measurements taken after 2 hours of incubation. 20% DMSO and 0.3% H_2O_2 served as positive control. (n=6) $*P < 0.05$

4.5 GYY 4137 has no significant effect on GLP-2 secretion after 24hr

Since we did not see any clear difference with GYY after 2 hours (**Figure 8A**) and we know that it is a slow-releasing H_2S donor, we decided follow a chronic approach to treating our cells with this compound for a 24 hour period. GYY seems to decrease GLP-2 secretion as the concentration increases, however no significant result was shown with one-way ANOVA (**Figure 9A**). Therefore, GYY 4137 does not affect GLP-2 secretion. At a higher dose of 1 mM

(1000 µM), a significant suppression of GLP-2 secretion was seen (data not shown). However, after performing a resazurin cell viability assay (**Figure 9B**), the highest dose was toxic to the cells. There was no effect on cell viability with the lower doses.

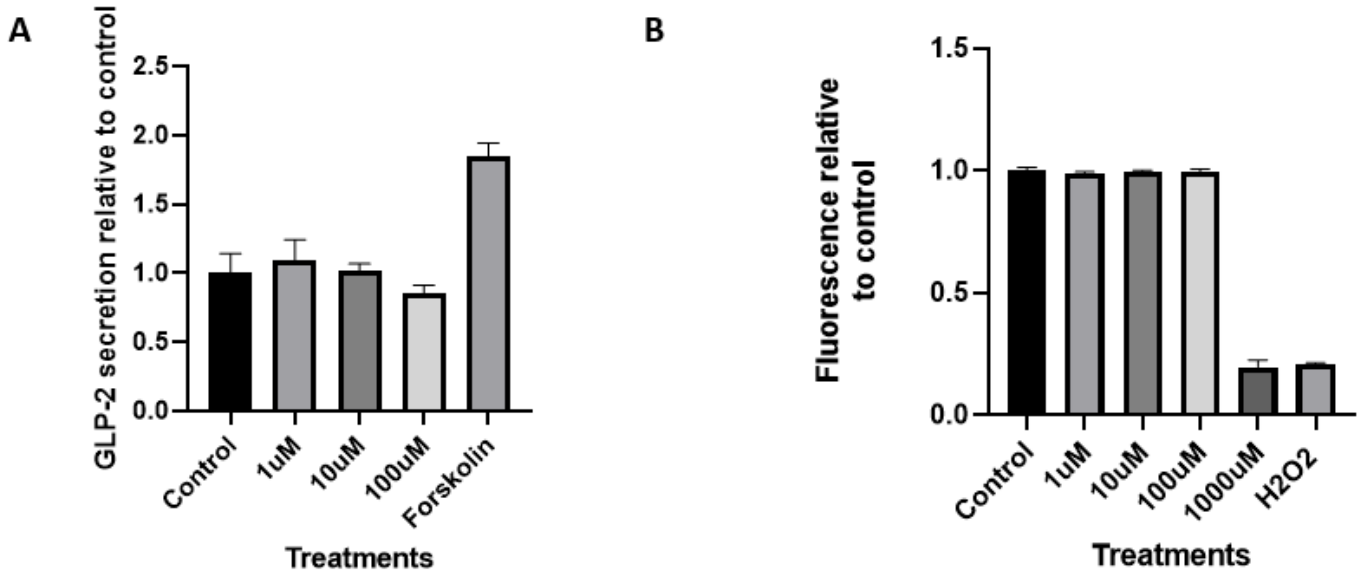

Figure 9: GYY 4137 has no significant effect GLP-2 secretion after 24 hours

(A) GLP-2 secretion was measured in media from GLUTag cells treated with varying concentrations of GYY 4137 for 24 hours and then analyzed by GLP-2 ELISA kit. Data is presented relative to vehicle control with Forskolin as positive control. (n=6-9) No statistically significant difference with one-way ANOVA. Cell viability was examined under similar conditions as described previously GYY treatment via (B) Resazurin Reduction Assay for mitochondrial activity. Data is presented relative to vehicle control and measurements taken after 24 hours of incubation. 0.3% H_2O_2 served as negative control. (n=6-9).

4.6 GLUTag cell lysate produces H_2S

We then wanted to determine the role of endogenous H_2S on GLP-2 secretion. Before doing so, we had to ensure that our GLUTag cells are able to produce H_2S and express H_2S-producing enzymes. Using the lead-acetate paper assay, cells were seeded in a 96-well plate (**Figure 10A**). Cell lysate was also placed into some of the wells. Seeded cells were unable to produce H_2S, however cell lysate is capable of producing H_2S with a mean production rate around 0.2 µM/µg/min (**Figure 10B**). To confirm the production of H_2S was being generated from H_2S-producing enzymes, a western blot was performed with antibodies for CSE and CBS. Expression of CSE was confirmed within our cell line (**Figure 10C**). No expression of CBS was seen (data not shown).

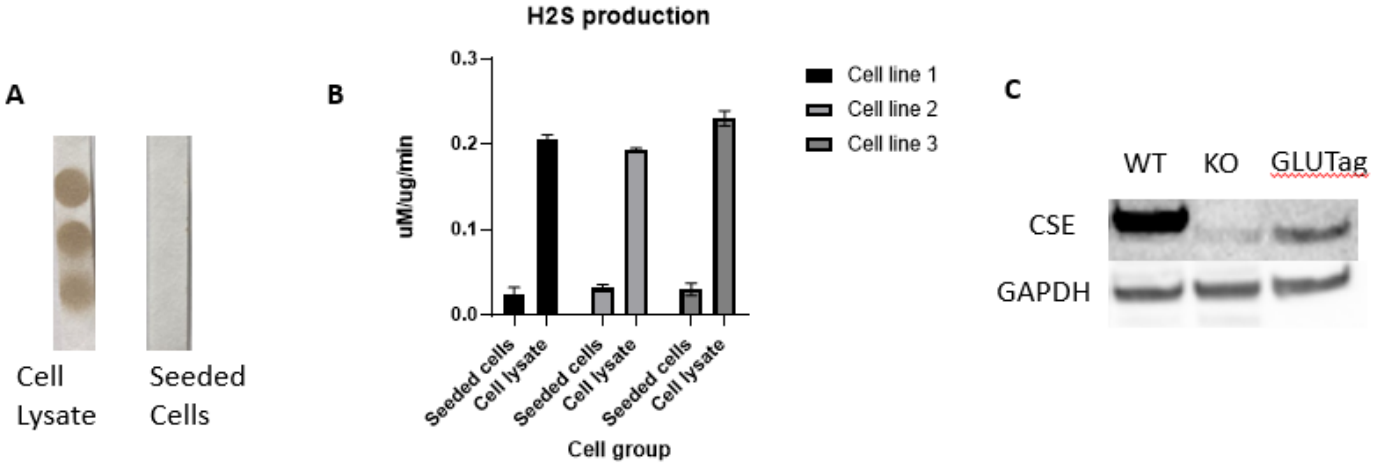

Figure 10: Enteroendocrine L-cell lysate (GLUTag) produces H_2S, seeded cells do not.

H_2S production rate in GLUTag cell lysate and seeded GLUTag cells at 80% confluency was measured using the (A) lead-acetate paper method and (B) densitometry was quantified using the BioRad program. (n=3) (C) A Western blot was performed using CSE antibody, GAPDH as loading control.

4.7 AOAA inhibits H_2S production in GLUTag cell

Now that we confirmed that GLUTag cells produce H_2S, and that the H_2S-producing enzyme CSE is also present, we then wanted to determine the impact of inhibiting H_2S production with AOAA; a dual CSE and CBS inhibitor (74). Using GLUTag cell lysate, we used a panel of 1-25 mM to determine at what concentration H_2S production is inhibited (**Figure 11A**). H_2S production decreased as AOAA concentration increased, with maximal inhibition seen at 25 mM (**Figure 11B**).

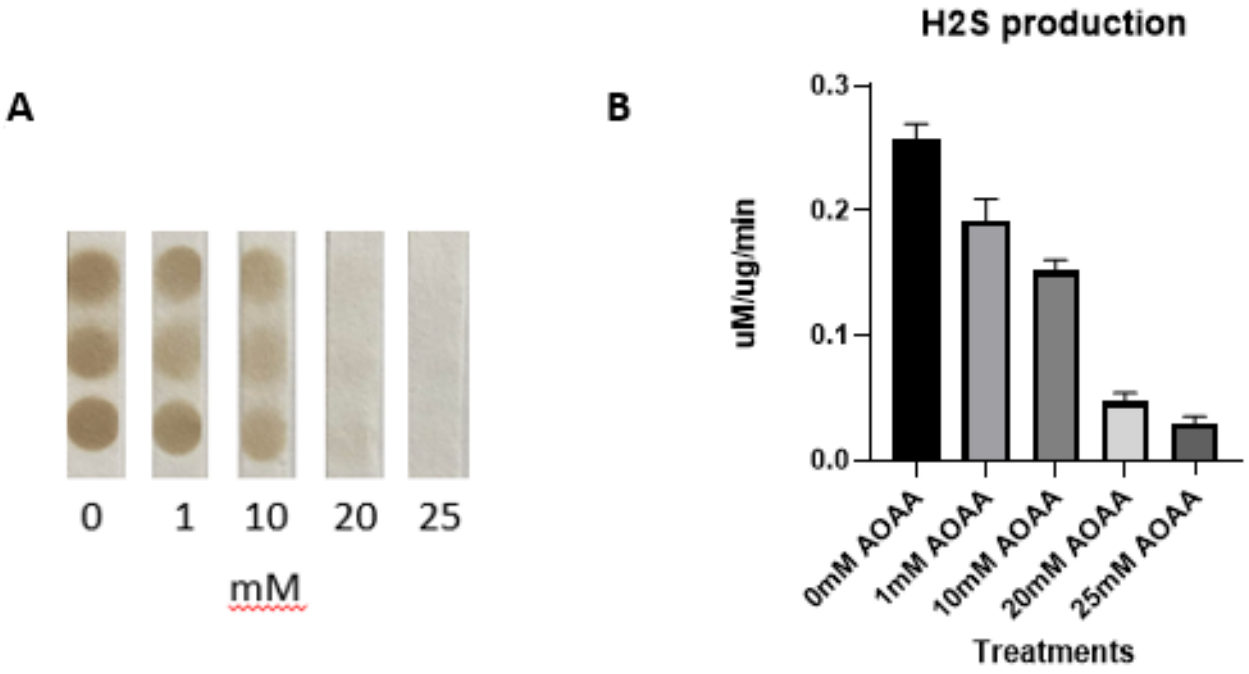

Figure 11: H_2S-producing enzyme inhibitor inhibits H_2S production in GLUTag cells

H_2S production rate in GLUTag cell lysate with varying concentrations of AOAA for 2 hours was measured using the (A) lead-acetate paper method and (B) densitometry was quantified using the Quantity One BioRad program. (n=3)

4.8 AOAA alters GLP-2 secretion and cell viability after 2 hours

Now that we determined a range of concentrations for H_2S inhibition (**Figure 11**), we wanted to determine the impact of inhibiting endogenous H_2S production on GLP-2 secretion. Cells were treated with AOAA at 0, 1, 10 or 25 mM for 2 hours. Although no clear significance was shown, AOAA was able to alter GLP-2 secretion (**Figure 12A**). Treatment with AOAA significantly increased cell viability with the highest increase almost two-fold seen at 25 mM (**Figure 12B**).

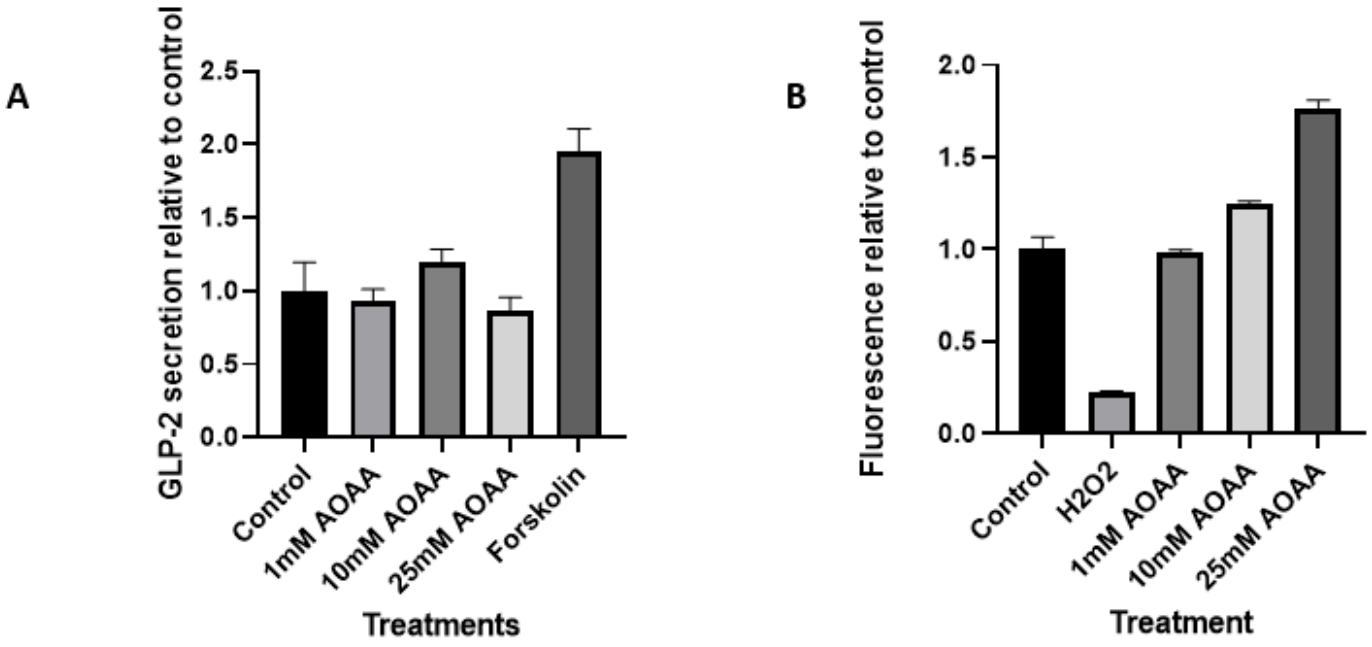

Figure 12: Inhibiting H_2S production in GLUTag cell alters GLP-2 secretion and cell viability

(A) GLP-2 secretion was measured in media from GLUTag cells treated with varying concentrations of H_2S-producing enzyme inhibitor AOAA (CSE+CBS) for 2 hours and then analyzed by GLP-2 ELISA kit. Data is presented relative to control with Forskolin as positive control. (n=9) No statistically significant difference with one-way ANOVA. Cell viability was measured using (B) Resazurin Reduction Assay for mitochondrial activity. Fluorescence was taken 2 hours post-incubation. (n=6).

4.9 AOAA dose-dependently suppresses GLP-2 secretion after 24 hours without killing cells

Since we determined that AOAA can alter GLP-2 secretion in an acute setting of 2 hours (**Figure 12**), we were unsure if this time frame was long enough to invoke an effect on GLP-2 secretion. We decided to examine the effect of a longer-term incubation of AOAA for 24 hours on GLP-2 secretion. AOAA suppressed GLP-2 secretion dose-dependently, with the largest inhibition seen at the highest dose (**Figure 13A**). Compared to the control, the mean GLP-2 concentration secreted at 1 mM, 10 mM and 25 mM was 0.60 ± 0.034, 0.45 ± 0.032 and 0.25 ± 0.035 respectively. To ensure no cell death was occurring and impacting the results, a resazurin cell viability assay was conducted (**Figure 13B**). Treatment with AOAA had no effect on cell viability.

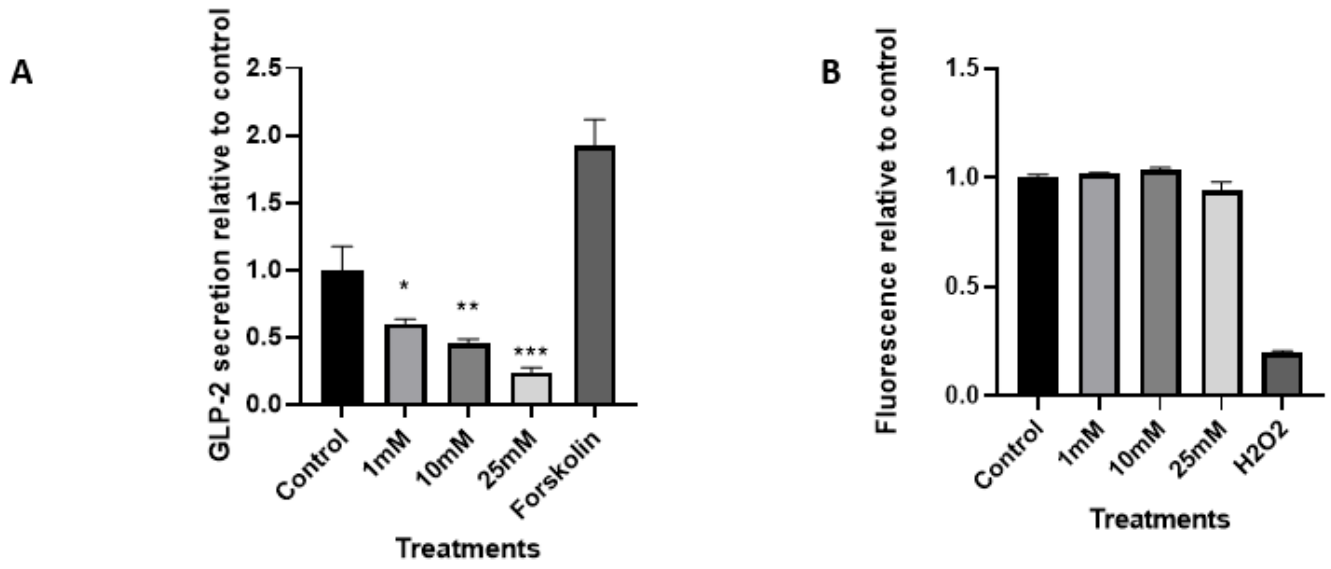

Figure 13: AOAA suppresses GLP-2 secretion after 24 hours while maintaining cell viability

(A) GLP-2 secretion was measured in media from GLUTag cells treated with varying concentrations of H_2S-producing enzyme inhibitor AOAA (CSE+CBS) for 24 hours and then analyzed by GLP-2 ELISA kit. Data is presented relative to control with Forskolin as positive control. (n=6). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ Cell viability was measured using (B) Resazurin Reduction Assay for mitochondrial activity. Fluorescence was taken 24 hours post-incubation. (n=6)

4.10 Expression of Claudin-7 is decreased in CSE-KO mouse intestinal tissue lysate

Although cellular and circulating GLP-2 had no differences between WT and CSE-KO mice (**Figure 7**), we wanted to determine if there were any differences in GI physiology. One of the targets for measuring gut integrity is through TJPs. Previous research has shown that GLP-2 injections can influence claudin-7 expression (46). Using intestinal tissue lysate from WT and CSE-KO mice, a western blot was conducted using a claudin-7 antibody (**Figure 14A**). Expression of intestinal claudin-7 was decreased in CSE-KO mice relative to the GAPDH control, with a mean densitometry of roughly 0.034 ± 0.003, compared to WT mice with a mean of roughly 0.081 ± 0.015 (**Figure 14B**). Claudin-7 expression was significantly reduced in the CSE-KO mouse, suggesting an impairment to GI barrier.

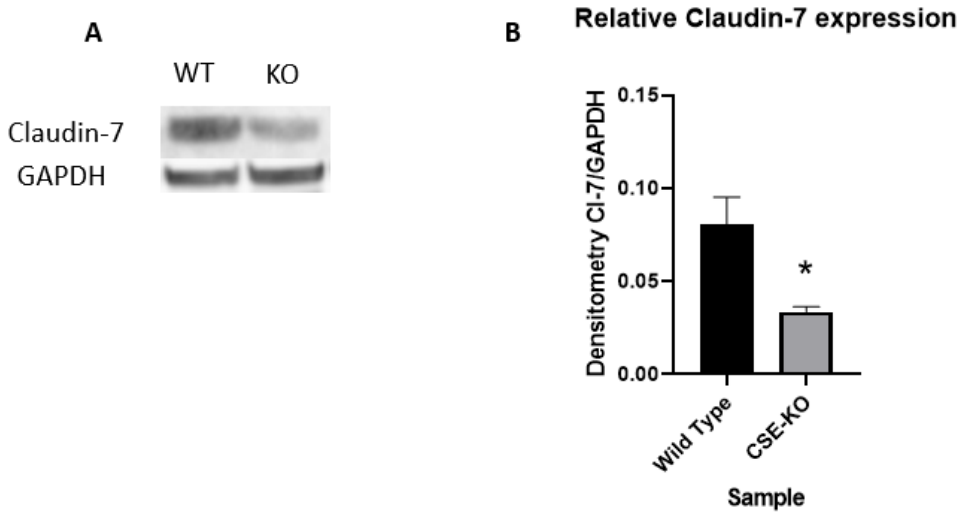

Figure 14: Tight junction protein claudin-7 expression is decreased in CSE-KO mouse

(A)Western Blot was performed using anti-claudin 7 antibody to determine difference in tight junction protein expression between WT and CSE-KO mice. (B) Densitometry was then performed using the quantity one BioRad program and compared to loading control GAPDH antibody. (n=5-7) (t-test $*P < 0.05$)

4.11 Expression of ZO-1 is unchanged in CSE-KO mouse intestinal tissue lysate

To further investigate the effects of removing H_2S with CSE-KO mice on GI physiology, we wanted to study a panel of TJPs. One common TJP often studied is ZO-1. We wanted to determine if TJP expression in CSE-KO mice is decreased for all TJPs. Using intestinal tissue lysate from our WT and CSE-KO mice, a western blot was performed using the ZO-1 antibody (**Figure 15A**). Ultimately, CSE-KO mice had slightly increased ZO-1 expression (insignificant). WT mice had a mean densitometry of roughly 0.91 ± 0.178, compared to a mean of roughly 1.4 ± 0.300 for CSE-KO mice (**Figure 15B**). This further suggests a difference in GI integrity between our two types of mice. However, there were no statistical differences using t-test.

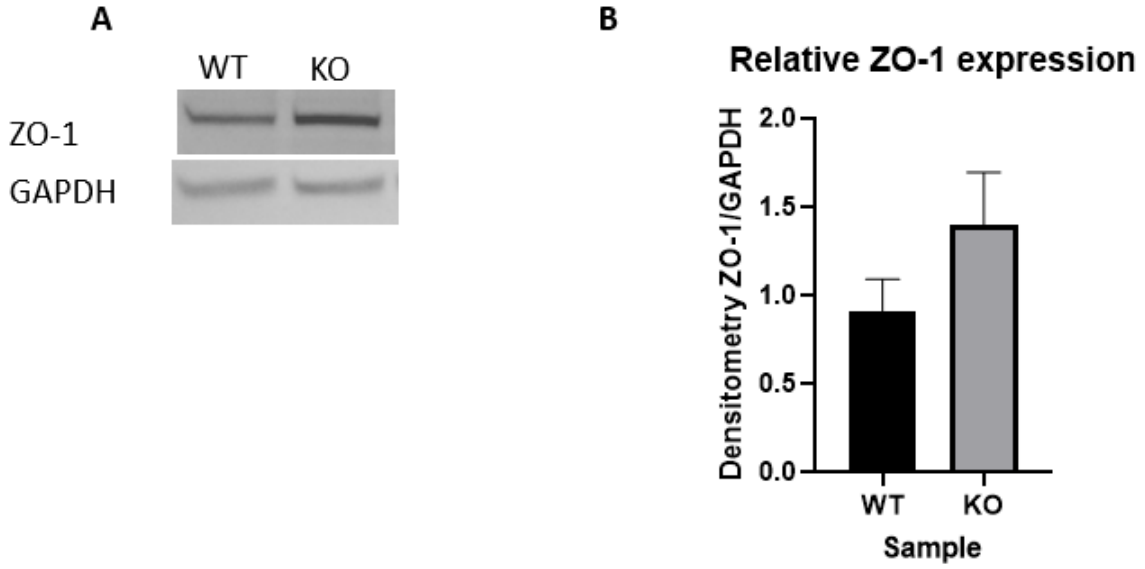

Figure 15: Tight junction protein ZO-1 expression has no statistical difference

(A)Western Blot was performed using anti-ZO-1 antibody to determine difference in tight junction protein expression between WT and CSE-KO mice. (B) Densitometry was then performed using the quantity one BioRad program and compared to loading control GAPDH antibody. (n=5-7)

4.12 Expression of IGF-1 is unchanged in CSE-KO mouse intestinal tissue lysate

Although TJPs are plausible for measuring GI integrity, we wanted to ensure that the differences seen were a downstream result of GLP-2. There were no differences in cellular or circulating GLP-2 (**Figure 7**), however it is possible that we may observe differences in a downstream target of the GLP-2R such as IGF-1. Using intestinal tissue lysate from WT and CSE-KO mice, a western blot using IGF-1 antibody was performed (**Figure 16A**). Expression of IGF-1 was unchanged in the mouse samples. The mean densitometry for WT and CSE-KO was 1.29 ± 0.293 and 1.28 ± 0.243 respectively (**Figure 16B**). No statistical difference seen using t-test. The same was done with one sample of liver tissue lysate from each animal (**Figure 16C**). The relative expression of IGF-1 in the WT mouse was 3.57, and 1.62 for the CSE-KO mouse (**Figure 16D**).

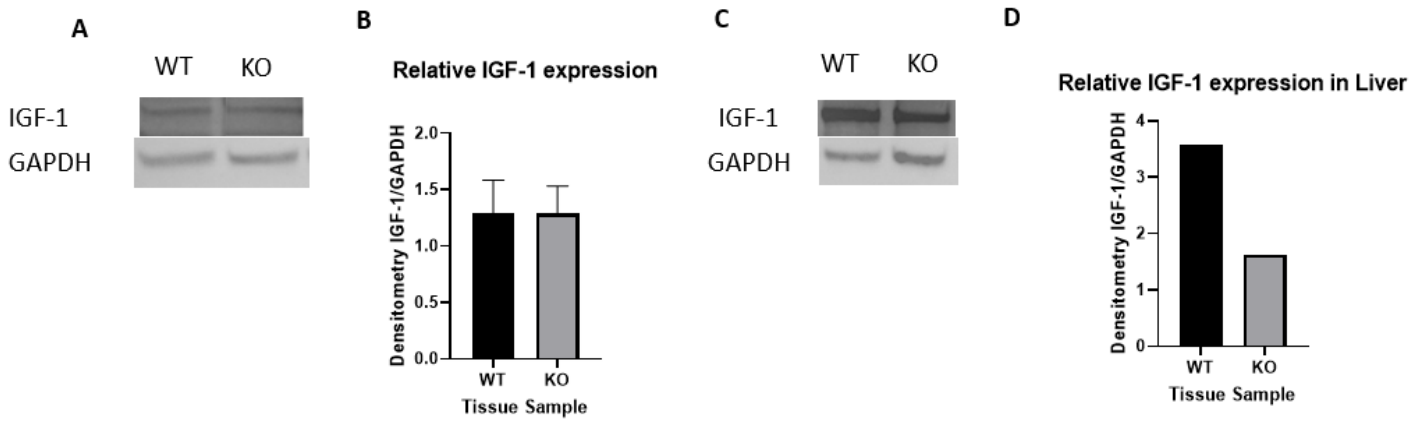

Figure 16: IGF-1 expression is unchanged in CSE-KO mouse intestinal tissue but possibly decreased in liver tissue.

(A)Western Blot was performed using anti-IGF-1 antibody to determine difference in downstream target of GLP-2R between WT and CSE-KO mice intestinal tissue lysate. (B) Densitometry was then performed using the quantity one BioRad program and compared to loading control GAPDH antibody. (n=5-7) (C) Western blot was also performed on liver tissue lysate for one WT and CSE-KO and followed-up with (D) Densitometry. (n=1)

5 Discussion

In this study, we examined the role of H_2S in the regulation of GLP-2 secretion and GI physiology. This was investigated through a combination of animal models, using WT and CSE-KO mouse plasma and tissue samples, as well as cell models, using GLUTag cells. Within the cell model, we applied H_2S donors as exogenous delivery of H_2S, or we applied the H_2S-producing enzyme inhibitor AOAA to investigate the impact of endogenous H_2S.

Using tissue liver lysate from our WT and CSE-KO mice, we were able to validate that CSE-KO mice has lacked CSE expression and as a result does not produce H_2S in the liver. This confirmed the method was sufficient to perform the same procedures with intestinal tissue lysate. Ultimately, CSE-KO mice produced less H_2S in comparison to WT mice. Interestingly, CSE-KO mice were still able to produce some H_2S, although the amount was much lower than WT mice. Perhaps other enzymes are responsible for this H_2S production in the GI tract, as CBS was shown to be present in the GI tract (110). CBS and CSE seem to be the main H_2S-producing enzymes within the GI tract, however the CAT-MST pathway are also involved in the endogenous production of H_2S (111). Finally, the gut microbiome is another source of H_2S production, particularly SRB (58). However, these bacteria thrive under anaerobic conditions and most likely do not contribute to the H_2S production seen with the lead-acetate paper assay (58). These are potential sources that may contribute to the remaining H_2S production seen within the GI tract of CSE-KO mice. However, the significant reduction in H_2S production suggests a crucial role for CSE in the GI tract. The reduction of H_2S through the CSE-KO model has been shown to alter physiological processes including hepatic regulation of cholesterol, regulation of beta-cell functioning, ameliorating colitis disease progression and proliferation of smooth muscle

cells (70-73). We thus wanted to explore the impact on GI physiology including GLP-2 regulation.

Our findings indicate that within this experimental model and methods, there were no significant differences between the concentration of unfasted circulating or cellular GLP-2 in WT and CSE-KO mice. This lack of difference between the two types of mice may be due to experimental design. One important factor in terms of GLP-2 is that it is released in response to nutrient ingestion (112), and therefore animals must be fasted and given a timed nutrient ingestion before blood collection. Unfortunately, the samples graciously donated for this study were harvested from the animals in an unfasted condition and would have contributed to the variability seen. Additional experiments with fasted CSE-KO mice would be optimal for further demonstrating the effects of diminished H_2S on circulating GLP-2. Alternatively, injection of an H_2S donor could be used to show the impact of exogenous H_2S on GLP-2.

Using GLUTag L-cells, we demonstrated that the delivery of the H_2S donor NaHS at a high dose can suppress GLP-2 secretion after 2 hours. NaHS is an effective quick-releasing H_2S donor, in which gas is released as soon as it is in solution (68). Furthermore, almost all of the gas is released within a 2 hour timeframe. We anticipated some toxicity with this 10 mM dose, however our cell viability assays did not show any impaired lysosome integrity or mitochondrial function. GYY 4137 is another H_2S donor that was used, and no effect after 2 or 24 hour treatment was observed. In fact, at the highest dose of 1000 µM (1 mM), GLP-2 secretion was suppressed by about half compared to the control. However, after performing the resazurin cell viability assay, it was shown that the higher dose was toxic to our cells. This would explain the significant decrease in GLP-2 secretion. The lower doses did not impact cell viability. Previous studies within our lab used a range of doses of GYY 4137 of 10 µM to 500 µM (90). Perhaps it

may have been beneficial to have a high dose at 500 µM rather than 1000 µM (1 mM). Further research within this area would be beneficial. Although these H_2S donors exhibit different methods of delivery (quick vs slow release), it is important to note that the concentration ranges used were within the physiological range as luminal concentrations of H_2S range from µM to mM (58). Interestingly, these results are in contrast to what we have previously discovered within our lab (91). In previous studies we observed an enhancement of GLP-1 secreted when treated with similar doses of H_2S donors over 2 hours. As GLP-1 and GLP-2 are co-secreted in a 1:1 ratio, this contrasting result is perplexing (112). The opposite effect (NaHS) or lack of effect (GYY 4137) we observed here could have been due to methodology differences as a different ELISA manufacturer was used for the GLP-2 assay. Further studies could look into this discrepancy and ensure validity of these assay results. Cell viability was assessed to ensure that cell death was not occurring or causing suppression in our testing protocol. The neutral red reuptake assay is based on the capacity of viable cells to bind with the neutral red dye in lysosomes. We demonstrated that these H_2S donors do not kill the cells, however may increase cell viability based on this assay. Interestingly, H_2S plays a role with lysosomal activity. H_2S was shown to promote autophagy, a process involved in adaptation to stress, homeostasis and removal of damaged materials (113). Another study demonstrated a protective role of H_2S on cell viability of endothelial cells through autophagy (114). Since H_2S can impact lysosomes, the resazurin cell viability was used as an alternative and no differences were seen with H_2S donor treatments.

Now that we determined that exogenous H_2S can play a role in GLP-2 secretion, we wanted to determine the importance of endogenous H_2S production. Prior to examining this effect, we had to ensure that our cell line is capable of producing H_2S and has the enzymes responsible for this.

Western blot confirmed the presence of CSE but not CBS in the GLUTag cell. Using the lead-acetate paper assay, we have shown that the GLUTag cell lysate is capable of producing H_2S. We were unable to show any expression of CBS within the L-cells. We also attempted to measure H_2S from intact cells, however we were unable to successfully detect H_2S. It is possible that our protocol was not suitable for seeded cells, as previous studies underwent different methodologies to elicit H_2S release in live cells (115). However, it is clear that H_2S can be produced from cell lysate with the help from the CSE enzyme.

To further expand on the role of endogenous H_2S, the CBS/CSE inhibitor AOAA was applied to the cell lysate to determine a range of concentrations that will inhibit H_2S production. A previous study investigated the concentrations of various inhibitors and determined AOAA had an IC_{50} of 1.09 µM ± 0.12 and 8.52 µM ± 0.71 for CSE and CBS, respectively (74). Originally, we used these concentrations with our cell lysate, however there no changes in H_2S production. This could be attributed to different models being used, as in their study, purified protein samples of CSE and CBS were used, rather than extracted cell lysates in our study (74). Therefore, we incubated the cell lysate with a dosage range between 1-25 mM. Inhibition of H_2S was demonstrated as the inhibitor concentration increased, with total inhibition seen around 25 mM.

After administering AOAA to GLUTag cells for 2 hours, there was no significant change in GLP-2 secretion. Interestingly, there were large changes in cell viability in this acute treatment. At the highest dose, cell viability increased near twofold. This could suggests some kind of adaptation to cellular stress in a short-term model. There are many cell stress pathways that could initiate some compensatory mechanism to cellular stress (116). Although there were changes in cell viability, it is possible that the 2 hour timeframe was not long enough for the inhibitor to fully take into effect. Previous studies have described the use of AOAA in cells for a 24h time

period (115). Therefore, we investigated the impact of administering AOAA to GLUTag cells for 24 hours prior to analyzing GLP-2 secretion. Ultimately, GLP-2 secretion decreased dose-dependently as AOAA increased. This suggests that a certain amount of H_2S is necessary for GLP-2 secretion and confirms an important role for H_2S. Although AOAA is specific for CSE and CBS inhibition, we cannot ignore the possibility that this inhibitor may inhibit other targets within the cell, especially when considering the concentrations used and length of incubation. For example, studies have shown that AOAA can also inhibit gamma-amino butyric acid (GABA), as well as other P5P-dependent transaminases (117, 118). Fortunately, cell viability was not affected after 24 hours, suggesting that the decline in GLP-2 secretion is not a result of cell death. Future cell studies with CSE or CBS knockdown models will help resolve this.

It seems as though there may be a relationship between H_2S and GLP-2 secretion. Although our animal conditions were not optimal for measuring GLP-2 in the WT and CSE-KO samples, we decided to investigate if there are any changes in the downstream effects of GLP-2, such as TJP expression, as well as a downstream target of the GLP-2R IGF-1. In previous studies, when mice had been injected with teduglutide, a GLP-2 analogue, claudin-7 expression was increased (46). Interestingly in our study, CSE-KO mice had reduced claudin-7 expression. This could indicate that the low H_2S environment in the CSE-KO mice leads to lower GLP-2 and downstream reduced gut integrity. However, further studies would need to be done with fasting samples to validate this theory. Consequently, expression of ZO-1 in CSE-KO mice was not significantly changed. This is in line with another study observing the effects of TJPs from teduglutide in which they demonstrated no significant difference in ZO-1 expression (119). Further studies observing changes in ZO-1 expression and GLP-2 should be explored. This slight (not significant) increase we saw could be a result of compensating for the lack of claudin-7, or other

potential TJPs not studied. It would be ideal to gather a panel of TJPs and observe their expression in both models to fully understand the impact of reduced H_2S on TJP expression. Another downstream target of the GLP-2R is IGF-1. In our study, there were no differences in IGF-1 expression within the intestinal tissue. One possible explanation could be attributed to the animals not being in a fasted condition. Food consumption leads to GLP-2 secretion, which then leads to GLP-2R activation, which is then followed-up with IGF-1 production. In addition, fasting has been shown to influence IGF-1 (120). Therefore, not knowing if the animals had recently ate could lead to this cascade of events, further leading to varying levels of IGF-1 within the animal samples used. Obtaining fasting samples would be optimal to further investigate this relationship. Interestingly, it seems as though within our liver samples (n=1), IGF-1 is decreased in the CSE-KO mouse. This is the primary area of IGF-1 production, which is then transported to other tissues (121). It may be beneficial to explore IGF-1 production in the liver alongside the previously mentioned fasting samples.

6 Conclusion

In this proof of concept study we investigated the role of both exogenous and endogenous H_2S in GLP-2 secretion and gut physiology. We were able to demonstrate that CSE-KO mice produce less H_2S compared to WT, however there were no differences in cellular or circulating GLP-2, nor any differences in IGF-1 expression. TJP expression was altered, as claudin-7 expression was significantly reduced and ZO-1 was slightly higher without any significance. H_2S donors had an effect on GLP-2 secretion, in which a high dose of NaHS suppressed GLP-2 after 2 hours, and GYY 4137 pointed towards a suppressive trend after 24 hours. GLUTag cells were capable of producing H_2S, as CSE expression was found within these cells, which was further inhibited with AOAA. Finally, inhibiting endogenous H_2S with AOAA lead to reduced GLP-2 secretion. Together, these findings support a potential role in the interplay between H_2S and GI health. Furthermore, these findings could provide support for the potential use of H_2S drugs in the treatment of GI disorders/diseases. As the current understanding of the role of H_2S continues to be discovered, further studies may need to be further explored to maximize its full potential in the world of GI health.

7 Limitations

While this work provides some initial evidence for an interaction between H_2S, GLP-2 and GI health, a number of questions still remain unanswered along with some limitations to our study. One limitation previously mentioned is that our plasma and tissue samples were collected without the animals being in an optimal fasting condition. As discussed, GLP-2 levels can vary, depending on when the animal previously had a meal and could greatly impact the results. It would be vital to repeat this experiment after obtaining fasting samples to get a more accurate representation of circulating and cellular GLP-2 in the CSE-KO mice, as well as IGF-1 levels. This could support the results seen in TJP expression. Another limitation is that this study only tested on the GLUTag murine enteroendocrine L-cell. Other cell lines could have provided insight into the interaction between H_2S and GLP-2. The colon contains large sources of H_2S, as well as GLP-2 (58, 17). Therefore, it would be important to look at cell lines specific to the colon such as the human Caco-2 cells (122). Other cell lines of equal importance for this study could have been looked at such as the human GLP-2 secreting NCI-H716 cell line (123). Another intestinal neuroendocrine cell model that could be studied is the STC-1 cell line (124). It would be beneficial to get a whole understanding of the entirety of these cell lines to further expand on the interaction of GLP-2 and H_2S. Finally, we had intended to look at the differences in villi length and compare any GI damage if present. However, we were unable to obtain paraffin-fixed tissue from the animals. Future studies should look into acquiring paraffin-fixed tissue slides to observe under microscope.

8 Future work

There are still numerous information gaps that are needed to fully understand the complex interaction of H_2S, GLP-2 and GI health. To begin, this study was able to demonstrate a clear reduction in GLP-2 and H_2S using AOAA (an H_2S-producing enzyme inhibitor), however there are a number of other inhibitors as well. It would be interesting to see the efficacy of inhibition from other donors to ensure that the results obtained are not strictly from incubation with AOAA. For example, BCA, AVG and HA could have also been applied (74). Some of these inhibitors are specific to CBS and/or CSE. A follow-up study could look at the impact of inhibiting CSE or CBS alone and comparing the results to determine the impact of these different endogenous enzymes in GLP-2 secretion. Additionally, different doses and incubation periods may impact results.

The primary focus of using CSE-KO mice and comparing to WT mice was to determine any differences in GLP-2 and GI health as a response to decreased H_2S production, as CSE-KO mice lack one of the important H_2S-producing enzymes. However, there are other sources of H_2S that were not explored. In our lab, we demonstrated that mice given a diet rich in H_2S (chondroitin sulfate) had an increase in SRB (91). This demonstrated an alteration in the gut microbiome due to diet. It would be interesting to compare the bacterial composition between WT and CSE-KO mice. Perhaps there would be some differences, or an increase in SRB to compensate for the lack of H_2S production responsible from CSE.

Although this study observed a number of targets for GI integrity in WT and CSE-KO mice, it did not study an exhaustive list. For example, the TJPs studied included ZO-1 and claudin-7. A larger panel of TJPs would be crucial to a better understanding of the downstream effects of

reduced H_2S and GLP-2. Other important TJPs could include other claudins, ZOs and occludin. Another target to study GI health and integrity could be to look at inflammatory markers. As previously mentioned, GLP-2 and H_2S are involved in inflammation and apoptosis. Markers for inflammation, such as TNF-α, IFNy and a panel if interleukins could be explored. Caspases and cytochrome-c could also be explored to compare apoptotic markers. It would also be beneficial to explore the effects of H_2S donors after cells have undergone treatment. For example, if there is an increase to markers of apoptosis or inflammation in the cells that could be measured after exogenous delivery of H_2S donors.

One important aspect that has yet to be established is the method and pathway in which H_2S can suppress GLP-2 secretion. In our study we were able to demonstrate a role between the two, but were unable to explain how they interact in terms of cellular mechanisms. This would be ideal to establish an understanding of the relationship between how H_2S enters the cell, to the end point of suppressing GLP-2 release. Interestingly, our findings are contrasted by another study performed in our lab, demonstrating that GLP-1 is enhanced by H_2S donors. The underlying mechanism of enhanced secretion is also a mystery. A follow-up study observing the cellular mechanism of how GLP-1 and GLP-2 secretion differs in response to H_2S is necessary. Additionally, as both hormones are co-secreted, it would be vital to study both GLP-1 and GLP-2 simultaneously to resolve the discrepancies possibly seen between both studies.

Finally, there are a few animal study protocols that could enhance our study and our understanding of H_2S, GLP-2 and GI health. First, there has been research previously done in which GYY 4137 was injected into mice, followed by observing its effect on different hormones such as ghrelin (90). Injecting mice with this donor would further support studies seen in cell culture models. From this model, additional supporting tests can be performed. Some of these

tests could include measuring GLP-2 levels post-injection, measuring targets of gut integrity after long-term injections such as TJPs or studying GI physiology such as villi length. An additional animal model would be to deliver DSS to induce colitis in mice. This could eliminate the “middle-man” in which we are making inferences rather than measuring directly. Rather than assuming individuals with colitis have increased H_2S and then studying GLP-2, we could harvest tissue and measure H_2S directly. We could take fasting plasma samples, measure GLP-2, GI integrity and combine these results with cell culture data. Additionally, this study could complement our studies and future studies seen in CSE-KO mice.

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