A Gut Reaction: Effects of an *E. coli*-Produced Toxin on Gut Hormone Regulation and Gut Physiology

by

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Abstract

Enterotoxigenic Escherichia coli (ETEC) produces the heat-stable enterotoxin b (STb), which is responsible for secretory diarrhea in humans and animals. STb disrupts intestinal fluid homeostasis, epithelial barrier function, and promotes apoptosis. Glucagon-like peptide-2 (GLP-2) is an intestinotrophic growth hormone secreted by enteroendocrine L-cells which enhances cellular proliferation, epithelial barrier function, and inhibits apoptosis. As many similarities are clear between GLP-2 production and secretion pathways and STb cell signaling mechanisms, this study aims to investigate the effects STb has on the physiology of *in-vitro* L-cells and the secretion of GLP-2. Enteroendocrine cells which secreted the highest amounts of growth hormone were found to be more resistant to STb intoxication. STb was found to enhance the secretion of GLP-2, potentially due to similar cellular mechanisms of hormone production and secretion and STb cell signaling. GLP-2 was found to inhibit the toxigenic effects of STb. Future work will elucidate the mechanism of this interaction. This work will support the use of GLP-2 therapies in enterotoxigenic related diseases.

Keywords

Escherichia coli, ETEC, toxins, proteins, hormones, glucagon-like peptide-2, hormone secretion, enterotoxins, gastrointestinal, enterotoxigenic, enteroendocrine

Abbreviations

5-HT: 5-hydroxytryptamine

AJ: Adhesion junction

CaCC: Calcium-activated chloride channel

CaMKII: Calcium/calmodulin-dependent protein kinase-2

CCK: Cholecystokinin

CFTR: Cystic fibrosis transmembrane conductance regulator

ETEC: Enterotoxigenic *Escherichia coli*

Gαi3: Pertussis toxin-sensitive GTP binding regulatory protein

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 polypeptide

IBD: Inflammatory bowel disease

IP1: Intervening peptide 1

IP2: Intervening peptide 2

JAM: Junctional adhesion molecule

LTs: Heat-labile enterotoxins

MAPK: Mitogen-activated protein kinase

NHE3: Sodium-hydrogen exchanger 3

PC1/3: Prohormone convertase 1/3

PGE_2: Prostaglandin E_2

PKC: Protein kinase C

PYY: Peptide tyrosine tyrosine

SBS: Short bowel syndrome

STb: Heat-stable enterotoxin b

STs: Heat-stable enterotoxins

T2DM: Type 2 diabetes mellitus

TER: Transepithelial electrical resistance

TJ: Tight junction

ZO: Zonula occludens

Co-Authorship Statement

The published review paper shown in section 1.3 was written and completed by myself with the guidance of Dr. Gagnon and Dr. Saleh.

The research project shown in Chapter 2 was conducted and completed by myself. The protein purifications, cell culture work, viability assays and hormone ELISA were all completed by myself. Dr. Gagnon and Dr. Saleh provided the general goal of the project, crucial training on all methods and experiments listed in section 2.3, and full guidance on manuscript writing.

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I also wish to acknowledge all of my lab colleagues, lab technicians, and the faculty and staff of Laurentian University.

Table of Contents

List of Figures

Chapter 1

This thesis will follow a manuscript-based format. Chapter 1 will encompass a background introduction, first by briefly discussing the link between pathogenic *Escherichia coli* (*E.coli*) and at-risk populations, shedding light on a new population which can be greatly affected. Following this, a published review article will outline the toxigenic mechanisms of the heat-stable enterotoxin b (STb), produced and secreted by the enterotoxigenic *E.coli* (ETEC) during an enteric infection. Ending remarks within this section focuses on the potential link between enterotoxins and enteroendocrine L-cells, followed by a review on the glucagon-like peptide-2 (GLP-2) intestinal growth hormone secreted by these L-cells. Chapter 2 will comprise the original research of the effects of STb on L-cell hormone regulation and physiology. This will include a brief introduction, methodology, experimental results and a concise discussion and conclusion. Finally, Chapter 3 will consist of a detailed extended discussion on all of the ideas and results discussed within this thesis, followed by concluding remarks and the potential implications of the study.

1.1 Pathogenic *E.coli*, ETEC and At-Risk Populations

Continuously studied for over two centuries, *E. coli* has become one of the most extensively researched species of bacteria to date [1,2]. *E. coli* has been clearly linked to the causation of diarrheal illness, as well as other intestinal disorders such as inflammatory bowel disease (IBD) [1,3]. Most *E.coli* strains which inhabit the normal intestinal flora of humans and animals are harmless, causing no disease or illness [4]. However, there are six known pathotypes of *E.coli* which possess disease-eliciting mechanisms which greatly affect the host digestive tract [5]. These include enteropathogenic *E.coli* (EPEC), enterohaemorrhagic *E.coli* (EHEC),

enteroaggregative *E.coli* (EAEC), enteroinvasive *E.coli* (EIEC), diffusely adherent *E.coli* (DAEC) and enterotoxigenic *E.coli* (ETEC) [6]. Among these six pathotypes, ETEC is the most common, notably within the developing world [7]. As such, this chapter will focus on the mechanisms and effects of ETEC. Currently, there are no approved or practical vaccines against ETEC infection available, and is a major priority for the World Health Organization [8]. However, utilizing both antitoxic and anti-ETEC fimbrial immunity may be the most effective and safe way to do so [9].

There are three major groups of populations that are at high risk of ETEC infection. The first group includes individuals of developing countries, where food and water contamination is prevailing and medical resources are scarce [7]. Acute infectious diarrhea has been deemed the second most common cause of death in children within these developing populations [10]. Unlike *V. Cholerae*, *Shigella* spp., and rotavirus, which can be efficiently detected using readily available assays, ETEC is more difficult to detect and often not acknowledged as the cause [11]. Preexisting malnutrition has been linked to more severe enteric ETEC infections due to the host's immunocompromised state, predisposing them to a greater bacterial load on the intestinal mucosal surfaces [12]. Studies within these areas have shown that ETEC is a frequent cause of watery diarrhea in infants 2 years of age or younger, with these incidence rates decreasing as age increases [13]. This susceptibility of infection in these young children has also been noted in other areas of poor hygiene and public health conditions [14].

The second group of at-risk individuals includes travelers. ETEC is a major cause of traveler's diarrhea in individuals who travel to areas of high contamination and infection rates [7]. These individuals also regularly import the organism back to the developed world [7]. Several studies

reported the seasonality of ETEC infection rates showing higher rates during warmer seasons, suggesting travelers are more at-risk of ETEC infections during these periods [7,15,16].

The third major at-risk population includes farm animals. In particular, neonatal and post weaning pigs due to several factors such as hygiene, stress of weaning, absence of antibodies originally received from the sow's milk, and dietary changes [17,18]. ETEC has major financial implications within farming and agricultural industries due to its pathogenic nature [19]. Diarrhea due to ETEC has been a major cause of morbidity and mortality in pigs according to the National Swine Surveys in the USA [20]. ETEC is a common agent of porcine diarrhea and is frequently diagnosed in neonatal and post weaning piglets that have died from diarrhea [21]. Also, ETEC is a major clinical disease found in captive wild boars, commonly caused by a gut microbiome imbalance [22]. This suggests a role of captive and aggregated conditions which may cause this susceptibility. ETEC is also commonly associated with newborn calves, dogs and sheep, however mostly non-existent in other farm animals such as horses, rabbits and poultry [23,24].

In addition to these three at-risk groups, an emerging population of potential risk of ETEC infection are individuals with Type-2 diabetes mellitus (T2DM) and obesity. Obesity is widely known to increase morbidity and mortality through several effects on almost every human system and organ [25]. In particular, obesity impairs the immune response through multiple immune mediators, leading to high infection susceptibility [25]. Also, dysbiosis of the gut microbiome can occur in obese conditions, which in turn can dysregulate the protective barrier typically provided by the microbiome from intestinal pathogens [26]. Individuals with diabetes mellitus have also been associated with increased risk of traveler's diarrhea, indicated infection susceptibility [27]. Obese and/or diabetic individuals may also be at-risk due their decreased

ability to produce and utilize curial intestinal metabolic hormones [28]. Of these hormones, the glucagon-like peptide hormones, produced and secreted by enteroendocrine L-cells, are one such group which can be greatly affected in obesity [29]. This group of hormones includes glucagon-like peptide-1 (GLP-1), which increases glycemic control and promotes satiety [30], and glucagon-like peptide-2 (GLP-2), which is co-produced and secreted with GLP-1 by L-cells and regulates intestinal integrity and growth. GLP-2, through its interaction with the GLP-2 receptor (GLP-2R), can enhance intestinal barrier function, increase absorption of fluids and nutrients and inhibit cell death [31].

1.2 ETEC Enterotoxins

ETEC can induce watery diarrhea in the host, resulting from the ingestion of contaminated food or drink. These symptoms range from a mild illness to a severe purging disease. [6,7]. ETEC colonizes the surface of the intestinal mucosa, producing enterotoxins which induce intestinal fluid and ion secretion, disrupt intestinal barrier integrity and promote cell death [5,6]. ETEC enterotoxins include the heat-labile enterotoxins (LTs) and the heat-stable enterotoxins (STs) [17]. These toxins are labelled as such due to their inactivation temperatures, as LTs become inactive by heating at 60°C for 15 min, however STs remain stable up to 100°C for 15 min [32]. During an infection, ETEC might only express an ST, only an LT, or both STs and LTs [6]. LTs consist of two subsets, LT-I and LT-II. Intoxication by LTs rely on the activation of adenylate cyclase, increasing intracellular cyclic adenosine monophosphate (cAMP) levels followed by the eventual secretion of chloride ions through a transmembrane conductance regulator (CTFR) channel from secretory crypt cells [33].

STs include two subsets STa and STb, both of which differ in structure and mechanism of intoxication [34]. STa stimulates guanylate cyclase activity, in turn increasing intracellular

cGMP levels and ultimately increases unwanted ion secretion [6,35]. STb is known to elevate intracellular Ca^{2+} levels and stimulate several secretagogues, including calmodulin-dependent protein kinase II (CaMKII), prostaglandin E_2 (PGE_2), protein kinase C (PKC) and others, all of which contribute the secretion of crucial fluids and ions from intestinal cells into the lumen [5]. All four subsets of ETEC enterotoxins can induce severe watery diarrhea through these intoxication mechanisms [17]. This paper will focus on STb and its known mechanisms and effects on the gut, as they share several similarities to GLP-2 production and secretion pathways, as well as to the actions and effects of the hormone. The following introduction section is a published review on the mechanism of interaction between ETEC and the gut epithelium, including GLP-2.

1.3 Impact of the *Escherichia coli* Heat-Stable Enterotoxin b (STb) on Gut Health and Function

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1.3.1 Abstract

Enterotoxigenic *Escherichia coli* (ETEC) produces the heat-stable enterotoxin b (STb) which is responsible for secretory diarrhea in humans and animals. This toxin is secreted within the intestinal lumen of animals and humans following ETEC colonization, becoming active on enterocytes and altering fluid homeostasis. Several studies have outlined the nature of this toxin and its effects on gut health and the integrity of the intestinal epithelium. This review summarizes the mechanisms of how STb alters the gastrointestinal tract. These include the manipulation of mucosal tight junction protein integrity, the formation of enterocyte cellular pores and toxin internalization, and the stimulation of programmed cell death. We conclude with insights into the potential link between STb intoxication and altered gut hormone regulation and downstream physiology.

1.3.2 Introduction

Infectious diarrhea is one of the leading causes of death in children of developing countries [36]. Enterotoxigenic *Escherichia coli* (ETEC) is known to cause travelers' diarrhea and diarrhea in farm animals [17]. The global burden of ETEC infection is extensive and remains a major public health issue. In 2006, the World Health Organization estimated annually 400-500 million diarrheal episodes in newborns and children and 400 million in individuals 15 years or older, all ETEC related [37]. ETEC can be characterized by its capability to produce and secrete several virulence factors, including colonization factors (adhesins) and enterotoxins [38]. Adhesins allow for the attachment of the bacteria to intestinal epithelial cells. These can be characterized as filamentous appendages on the surface of the bacteria called fimbriae, on which adhesins can be found [5]. The loss of these colonization factors may render the bacteria unable to colonize and cause infection [39]. Colonization of the intestinal tract allows for specific delivery of

enterotoxins which disrupt normal intestinal fluid homeostasis [40]. ETEC enterotoxins are differentiated by their heat stability. They are classified into heat-liable (LT-I and LT-II) and heat-stable (STa and STb) enterotoxins [5].

The heat-stable enterotoxin b (STb) is one of several toxins secreted by ETEC responsible for inducing diarrhea [41]. STb is synthesized by ETEC as a pre-polypeptide consisting of 71 amino acids. The signal peptide is then cleaved within the periplasmic space, yielding a mature 48 amino acid peptide of 5.2 kDa [42]. This mature STb peptide is comprised of two α-helices affixed by two disulfide bridges (Figure 1). STb is heat-stable, possessing stable toxigenic properties up to 100°C [17]. It is a fast acting toxin, inducing a diarrheal response of moderate extent. Within mouse intestinal loops, it was found that STb can trigger a fluid response within just 30 minutes, with fluid accumulation reaching its maximum at 3 hours and negative after 16 hours [43].

Upon ETEC colonization and enterotoxin secretion, STb-mediated intoxication begins by STb binding to its receptor, sulfatide. This is an acidic glycosphingolipid located on the surface of intestinal epithelial cells [38]. The internalization of STb by the cells results in the activation of a pertussis toxin-sensitive GTP-binding regulatory protein (Gαi3). This leads to an influx of extracellular calcium ions into the cell by means of a receptor-dependent ligand-gated calcium channel [39]. This increase in intracellular calcium activates CaMKII through a calmodulin-calcium2+ complex. CaMKII activation leads to the opening of a calcium-activated chloride channel, allowing for the secretion of chloride from the cell into the lumen [40]. PKC and downstream cystic fibrosis transmembrane conductance regulator (CFTR) are also activated,

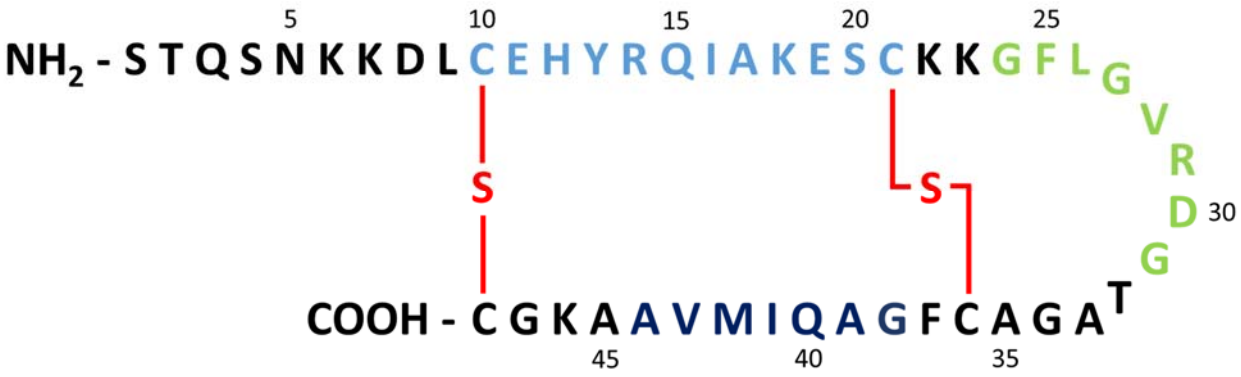

Figure 1. Amino Acid Skeleton of Mature STb Toxin.

The toxin is comprised of 48 amino acid residues, starting at the N-terminal. Disulfide bridges are shown in red. The integrity of the disulfide bond at Cys 21 and Cys 36 is necessary for the toxigenic region to be active. Light blue residues 10-21: amphipathic alpha-helix portion; Residues 18-30: Receptor binding/toxigenic region; Green residues 24-31: tight junction manipulation region; Residues 37-42: oligomerization region; Dark blue residues 38-44: hydrophobic alpha-helix region [44,45]

resulting in the secretion of H_2O, HCO_3^+ and various electrolytes from the cell. PKC also inhibits sodium uptake by acting upon an unidentified sodium channel deemed NHE3, increasing luminal sodium concentrations [46,47]. Increased intracellular calcium levels may also stimulate phospholipases A2 and C, releasing arachidonic acids from phospholipids present within the cell membrane. This step permits the formation of PGE_2 and 5-hydroxytryptamine. These secretagogues also mediate the transport of water and electrolytes form the cell into the lumen by an unknown mechanism (Figure 2) [48].

There are many gut functions which can become negatively altered by STb during an ETEC infection. This toxin has been observed to effect numerous animals as well as humans. This review addresses the impacts of STb on overall gut function, outlining various physiological changes which can occur and the resulting alterations on overall gut health. Also, the effects of various gut hormones on intestinal function will be discussed and how the regulation of these hormones may become altered during STb intoxication. This realm of bacterial toxin-induced gut hormone dysregulation has not yet been discussed in literature. This is imperative as gut hormone regulation is essential for overall gut health and bodily functions.

1.3.3 STb Alters Barrier Function through Tight Junction Manipulation

Within the intestine, the luminal surface consists of a dynamic layer of epithelial cells. It acts as a barrier between luminal materials and the underlying neuronal and immune systems, also sustaining nutrient, fluid and ion transport [49,50]. This barrier is upheld by conjunctions between adjacent enterocytes. These are intercellular junctions are known as adherens junctions (AJs) and tight junctions (TJs). TJs are multi-protein complexes which form a continuous loop-like ring around cells at the apical and lateral membrane domains. These proteins function as a

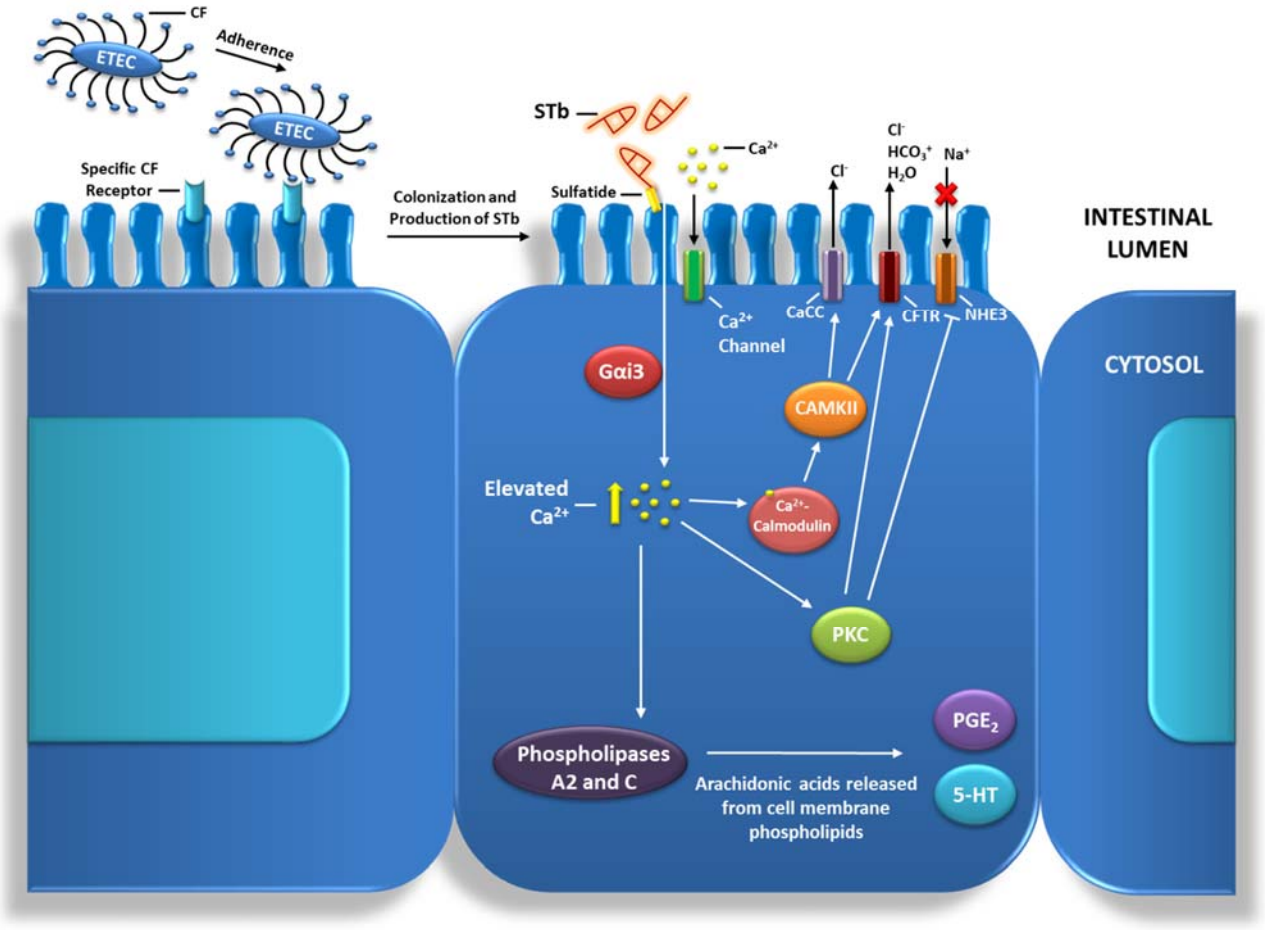

Figure 2. Pathogenesis of ETEC and Mechanism of Action of STb.

ETEC adherence and signaling pathway of STb leading to water and electrolyte secretion. CF: colonization factor; Gαi3: pertussis toxin-sensitive GTP-binding regulatory protein; CaMKII: calcium/calmodulin-dependent protein kinase II; CaCC: calcium-activated chloride channel; PKC: Protein kinase C; CFTR: cystic fibrosis transmembrane conductance regulator; NHE3: Na^+/H^+-exchanger 3; PGE_2: prostaglandin E_2; 5-HT: 5-hydroxytryptamine.

selective paracellular barrier, facilitating the flow of solutes and ions through the lateral intercellular space. They prevent entrance and transfer of intestinal antigens, microorganisms and their potentially harmful products [51]. TJs are comprised of four unique groups of transmembrane proteins: claudins, 12ccluding, zonula occludens (Zos) and junctional adhesion molecules (JAMs) [52]. Transmembrane claudins and 12ccluding are supported by scaffolding ZO proteins. The ZO complex provides intracellular structural support for the claudin and 12ccluding multiprotein complex at the cytoplasmic surface. (Figure 3a) [53].

Enteric pathogenic bacteria have crafted various methods in order to disrupt TJ complexes, either by cellular cytoskeleton alteration or by altering specific proteins within the TJ complex. Alteration of specific TJ proteins occur either through degradation by bacterial proteases or through biochemical phosphorylation/dephosphorylation pathways [49]. Transepithelial electrical resistance (TER) functions by establishing electrochemical gradients and can be a measurement for the degree of tightness of these TJs. Paracellular flux is a measure for transport efficiency over time and can be a measurement for paracellular permeability [40,54]. With regards to STb, Ngendahayo & Dubreuil (2013) [55] demonstrated how purified STb triggered a significant reduction of TER alongside an increase in paracellular permeability in a human colonic cell line. Alterations in F-actin stress fibers were accompanied by this increase in paracellular permeability. Condensation and dissolution of these F-actin filaments were accompanied by a redistribution and/or fragmentation of specific TJ proteins 12ccluding, claudin-1 and ZO-1 [40].

Additional studies show the re-distribution of claudin-1 between membrane and cytoplasmic locations when the same cell line was treated with STb, showing 40% more claudin-1 present within the cytoplasm compared to controls. This loss of membrane claudin-1 was attributed to a

dephosphorylation of this TJ protein from the complex (Figure 3b) [56]. All of these combined TJ alterations during an ETEC infection by STb are involved with disrupting intestinal barrier function, decreasing TER and increasing permeability of the epithelium. These changes all contribute to the observed diarrhea within the host. The degradation of TJ proteins can have downstream effects to the host, leading to other forms of enteric pathology. Long-term effects of TJ disruption and degradation in the host needs to be further investigated.

1.3.4 STb Pore Formation and Internalization by Enterocytes

As mentioned earlier, the luminal surface of the intestine consists of a dynamic barrier of epithelial cells which impede the transport of materials through TER and paracellular flux mechanisms [42,47]. This epithelial barrier must maintain efficient uptake of nutrients while also preventing the absorption of unwanted luminal antigens, microorganism and toxins [50]. Bacteria and their subsequent products have derived methods in order to penetrate this barrier such as TJ alteration, known as paracellular translocation. This is when the bacteria or its products traverse between adjacent cells through TJ protein manipulation [51]. TJ proteins normally prevent paracellular passage of bacteria, however in some cases their toxins, such as STb, have been shown to dislodge crucial TJ proteins, allowing for paracellular passage [49,51]. Alternative to this, products may pass through the enterocytes themselves. This is known as transcellular passage, either through endocytosis, internalization or pore formation within the plasma membrane. Studies have shown the presence of bacteria and their toxins within the cytoplasm of enterocytes, measuring translocation *in-vivo* where the enterocyte monolayers remained unimpaired [57].

There are a number of toxins which undergo oligomerization in order to form pores within the plasma membrane of target cells. This process is crucial for the internalization of the toxin and

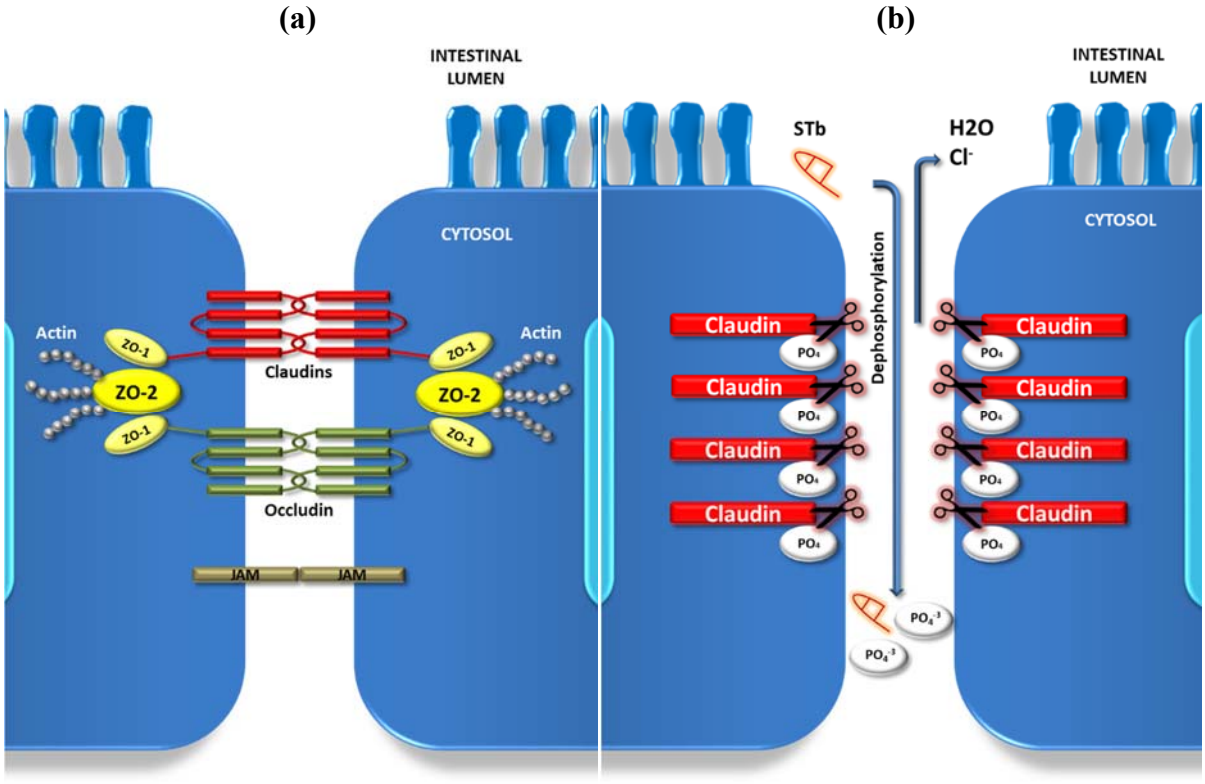

Figure 3. Enterocyte TJ Complex and the Dephosphorylation of Claudin-1.

(a) Schematic of the TJ complex between adjoining cells showing JAM proteins and the claudin and 14ccluding transmembrane complexes supported by ZO complexes and the cytoskeleton. ZO-1/2: zonula occluden 1/2; JAM: junctional adhesion molecules. (b) Mechanism of action of STb-induced claudin-1 dephosphorylation. Claudin-1 moves from the transmembrane position into the cytoplasm following STb dephosphorylation [44].

subsequent intoxication of the cell. These oligomeric channels can either form prior to membrane insertion or once its monomeric peptides are inserted into the membrane [58]. This process can also occur for smaller peptides of 20-50 amino acid residues, forming at least tetramers depending on the size of the bacterial peptide [59]. The first STb oligomerization characteristic was reported by Labrie et al. (2000) [59]. STb peptides formed hexamers and heptamers independent of temperature and sulfatide receptor binding. STb structural integrity was essential for its oligomerization, as reduced conditions in the presence of β-mercaptoethanol and detergent prevented oligomerization. Site-directed mutations lowering STb hydrophobicity within its hydrophobic α-helix portion rendered the toxin unable to form oligomers. This suggests that the C-terminal hydrophobic α-helix portion of STb is associated with the domain responsible for STb-STb inter-binding [59].

A later study demonstrated the internalization of STb utilizing an anti-toxin gold labeling assay and electron microscopy [60]. The fusion STb protein was internalized within rat enterocytes, while a mutant STb with reduced hydrophobicity did not become internalized. The gold particles did not aim at any particular subcellular compartments within the enterocytes, showing a random distribution of STb within the cell after internalization [60]. Alternative to this, a later study revealed STb clusters within an NIH-3T3 murine cell line which matched with mitochondria labelling. Mitochondria hyperpolarization, an initial event of intoxication, was observed after STb cell treatment, increasing dose dependently, also permeabilizing the plasma membrane [61]. Moreover, electrophysiological studies utilizing artificial planar lipid bilayers demonstrated the ability of STb to form voltage-dependent ionic pores independent of sulfatide receptor binding [62]. The ability of STb to form pores has not been further investigated since these early studies. This step of oligomerization and pore formation permits cell intoxication, and as such needs to

be further elucidated. Various therapies may need to be generated in order to reduce STb pore formation, either by enhancing the enterocyte plasma membrane or by mitigating STb oligomerization before pore formation. Knowing whether or not the oligomerization of STb occurs prior to insertion or once the monomer peptides are inserted would also be an asset to future related research.

1.3.5 STb Triggers Enterocyte Programmed Cell Death

Intestinal cellular proliferation must be counterbalanced by programmed cell death in order to maintain a stable growth and density of enterocytes [63]. Apoptosis occurs involuntarily, maintaining a balance between surviving cells and newly proliferated ones. This processes is characterized by morphological and biochemical alterations such as cell shrinking, DNA fragmentation and membrane blebbing [63]. The death of these cells has little effect on intestinal barrier function, as a balance of live and dead cells are constantly maintained [64]. Key regulators for apoptosis are a family of cysteine proteases known as caspases, acting as common death effector molecules [65].

Apoptosis is activated either through extrinsic or intrinsic pathways. Extrinsic apoptosis is activated by the binding of extracellular death ligands to their respective cell death receptors. The binding of these ligands to their receptors triggers a certain death domain binding. A downstream target then associates with procaspase-8, leading to its activation into caspase-8, the extrinsic apoptosis executioner caspase. This then cleaves procaspase-3 into active caspase-3 by activating a downstream cascade leading to cell death [57–61]. However, intrinsic apoptosis activates independently of ligand and transmembrane receptor binding. BH3-only apoptotic activating proteins and downstream targets activate Mitochondrial Outer Membrane Permeabilization (MOMP), releasing mitochondrial cytochrome C into the cytoplasm. This heme

protein and downstream Apoptosis Protease Activating Factor -1 (APAF-1) then oligomerize into an apoptosome through procaspase-9 interaction. Procaspase-9 is then cleaved into caspase-9, leading to caspase-3 initiation and endonuclease DNA degradation and cell death [63–68].

The intestinal mucosa is the main harbor and initial interaction site for pathogenic microorganisms [65]. Intrinsic and extrinsic pathways can both be triggered by pathogens themselves, such as ETEC, or by their derived products, such as STb. It was found that caspase-9, the mitochondrion-mediated intrinsic initiator, and its effector caspase-3 were both triggered by STb within a mouse and human cell line. Caspase-8, the extrinsic initiator, was not activated however, suggesting that STb activates intrinsic, caspase-dependent apoptosis [41]. The death of these cells may be responsible for the loss of mucosal surface area and luminal fluid accumulation, suggesting that STb-mediated apoptosis and subsequent diarrhea are linked.

With regards to ETEC infection as a whole, Xia et al. (2018) demonstrated how ETEC infection *in-vivo* actually inhibited the activation of caspase-9 mitochondrial-mediated, intrinsic apoptosis. ETEC was found to utilize and activate Caspase-8 extrinsic apoptosis instead. This suggests that during an ETEC infection, intrinsic apoptosis becomes inhibited, while extrinsic apoptosis becomes activated and utilized to cause cell death [69]. The reasoning for this discrepancy is still unknown. A number of factors can be attributed to this, such as the various other toxins secreted during ETEC infection, as well as the effect the bacteria itself may have on intestinal epithelial cells. Also, internal signals initiating intrinsic apoptosis can be attributed to the internalization of STb specifically. Cell death by STb intoxication can be a great threat to host intestinal health. The death of these cells and their long-term effects must be examined.

1.3.6 STb Causes Deterioration of the Intestinal Absorptive Mucosa

Absorption and secretion is a simultaneous physiological process within the mammalian intestinal tract. The recirculation of water and ions from the intestinal lumen into the enterocyte can occur either trans-or-paracellularly. Water influx can occur through diffusion directed by osmotic gradients during ion exchange, or by the hydration of solutes which then enter absorptive cells through various transporters [70,71]. This will ensure isotonicity of luminal contents prior to absorption [72]. However, if water efflux surpasses water influx, a net secretory state occurs within the intestine, leading to malabsorption and most often diarrhea [70]. The majority of nutrient transport occurs within the small intestine, while the large intestine is generally responsible for water and ion exchange [73]. The small intestinal surface area is dramatically enlarged by miniature projections of villi and microvilli. They are covered with absorptive columnar epithelial cells at the tip, while crypt cells are most often secretory. Nearly all ingested nutrients are absorbed into the blood stream through this exceptionally polarized layer of epithelial cells which form the intestinal mucosa [70,71,73].

The deterioration of the intestinal mucosa, such as the loss of absorptive cells and decreased villus height, can be attributed to alterations in the flux of water and ions within the lumen. The first report of cellular alterations caused by STb was conducted by Whipp et al. (1985) [74]. They demonstrated alterations of villous length by STb within pig intestinal loops, however having no effect on crypt depth [74]. STb significantly lowered the rate of net fluid and ion absorption. The loss of the intestinal villi epithelium through virus manipulation experiments (transmissible gastroenteritis virus) substantially decreased the response to STb. This suggests that differentiated villous epithelium must be present in order for a maximal STb response to occur [74]. Following this study, Whipp et al. (1986) [75] showed that STb exposure to pig

intestinal loops brought about microscopic alterations of the intestinal mucosa and its structural integrity. This included decreased villous height (11-19% shrinkage, including loss and atrophy of villous absorptive cells) and an increased amount of sloughed epithelial cells within the lumen. Also, a shift in the morphology of the cells from columnar to cuboidal epithelium upon the affected villi was observed. This was accompanied by an increased incidence of disrupted epithelium upon the affected villous, accordant with a compromised absorptive capability [75].

In a follow-up study by Whipp et al. (1987) (Figure 4), STb was attributed to morphological alterations and lesions correlated to a loss and atrophy of villous absorptive cells. Crypt surface area showed no significant difference when treated with STb. The intestinal mucosal surface area was 20% less when treated with STb compared to controls. STb exposure also lowered sucrase activity by 15%, with this observation being correlated with villous atrophy [76]. These combined observations by Whipp and colleagues make it apparent that STb induces lesions within the intestinal epithelium, suggesting a loss of absorptive mucosal cells. The deterioration of the abortive mucosa can have many direct and indirect effects on gut health. STb alteration of the mucosa can debilitate epithelial absorption of luminal nutrients and can induce a net secretion, contributing to malabsorption and diarrhea.

1.3.7 Fate of Enteroendocrine Cells and Gut Hormone Regulation

There is a great deal of knowledge on the effects of STb on the intestinal epithelium. This epithelium consists of absorptive enterocytes, making up the mucosal barrier. Dispersed among these mucosal cells, specialized enteroendocrine epithelial cells can also be found. These cells represent approximately 1% of the entire gastrointestinal epithelium [77], however when counted throughout the GI tract, make up the largest endocrine organ in the body. These cells produce

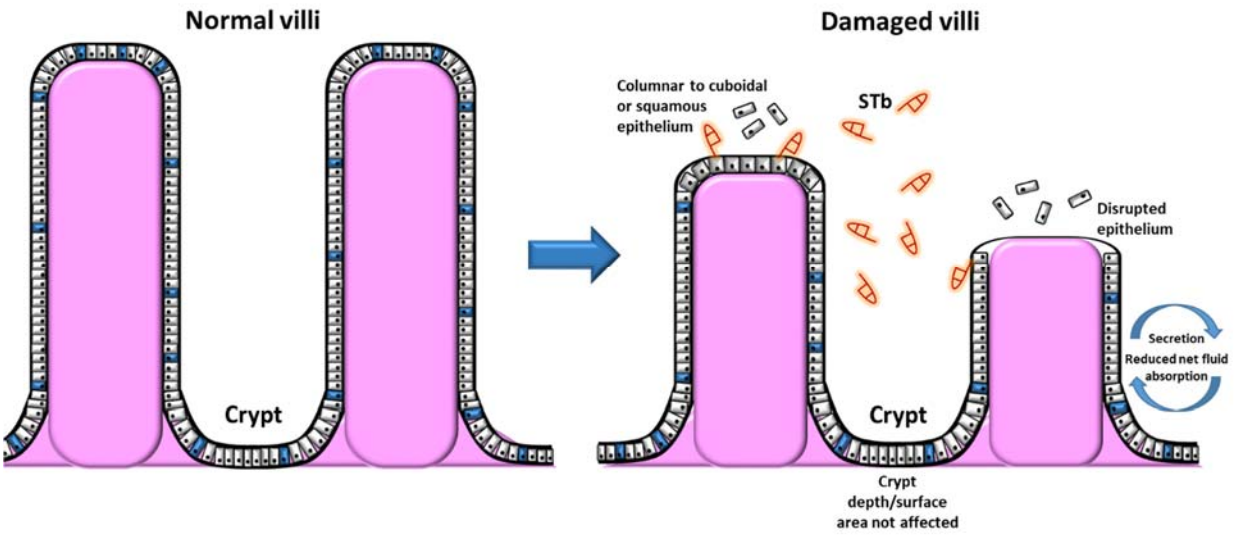

Figure 4. Normal Intestinal Villi Compared to STb-damaged Villi.

Morphological alterations and villus disruption is apparent, however no alteration in crypt depth or surface area is observed [44,76].

and secrete a wide variety of regulatory hormones and signaling molecules which function to complete different tasks [78]. They regulate functions such as appetite, contraction and motility of various organs, intestinal growth and barrier enhancement as well as glucose homeostasis. Within the small and large intestines, enteroendocrine L cells make up the majority of endocrine cells within the gastrointestinal tract [77,78]. These cells secrete glucagon-like peptide 1 (GLP-1), a 30 amino acid peptide hormone produced through differential processing of proglucagon by prohormone convertase 1/3 (PC1/3) [79]. The secretion of GLP-1 is stimulated by the ingestion of glucose and various other nutrients. GLP-1 enhances glucose dependent insulin secretion from β-pancreatic cells [79,80]. With the additional effect of inhibiting glucagon secretion from the pancreas, GLP-1 functions to lower blood glucose levels after a meal and inhibits the conversion of glycogen reserves into glucose [81]. GLP-1 is an anorexigenic hormone, promoting satiety and slowing gastric emptying, allowing for nutrients to be fully absorbed within the proximal gut [82]. Interestingly, GLP-1 receptor agonists are used for the treatment of Type 2 diabetes mellitus (T2DM) and obesity due to its ability to reduce blood glucose levels and lower body weight [79]. GLP-1 has also been noted to improve inflammatory macrophage-derived insulin resistance through the inhibition of the NF-κB pathway and the secretion of inflammatory cytokines in macrophages [83].

Co-secreted with GLP-1 is Glucagon-like peptide 2 (GLP-2), a peptide hormone of 33 amino acid residues, also processed from proglucagon [84]. GLP-2 is a potent intestinotrophic growth factor, inducing crypt cell proliferation and inhibiting apoptosis of intestinal epithelial cells. This results in increased villous height as well as greater absorptive mucosal surface area within the gut [84]. GLP-2 has been observed to significantly reduce intestinal fluxes of Na^+, bacterial epithelial penetration, and inflammatory colonic cells [85]. With these combined effects under

stress conditions, GLP-2 can significantly enhance intestinal barrier function and reduce the penetration of unwanted luminal antigens [86]. The enhancement of the intestinal barrier by GLP-2 can be attributed to an increased expression of TJ proteins. Yu et al. (2014) revealed how GLP-2 significantly increased the mRNA and protein expressions of ZO-1, occludin and claudin-1. A mitogen-activated protein kinase (MAPK) pathway inhibitor in conjunction with GLP-2 mitigated the same mRNA and protein expressions. This suggests that GLP-2 improves the expression of crucial TJ proteins potentially through the MAPK signaling pathway [87]. GLP-2 has also been noted to act as a protective factor against the deregulation of glucose metabolism which occurs in obese conditions [84]. Importantly, GLP-2 is approved for use in intestinal malabsorption diseases including Crohn's disease and short bowel syndrome (SBS) [88]. Teduglutide is a potent analogue of GLP-2, having a half-life of around 25 times that of natural GLP-2 [89]. Newly emerging brands such as Gattex employ this analogue to treat SBS symptoms like nausea and severe diarrhea [88], similar to the symptoms caused by STb.

As described throughout, STb has numerous harmful effects on intestinal cells and the absorptive mucosa, impairing many gut functions. Intestinal endocrine cells and intestinal epithelial cells share similar morphologies, such as the STb receptor, sulfatide, dispersed on the cell surface, as well as downstream targets. Once STb is bound, there is no known mechanisms which would mitigate STb from becoming internalized, inducing apoptosis or fluid secretion. Enteroendocrine L cells would be at most risk during an ETEC infection, as the bacteria tends to harbor within the distal gut. The effect of STb on the secretion of GLP-1 and GLP-2, as well as the physiological alterations to these endocrine cells must be elucidated. These cells are vulnerable to intoxication, so one might question the fate of these cells, as well as the fate of gut hormone regulation as a whole during an ETEC infection. Research has yet to shine its light on this area of study, as

intestinal hormone deregulation could have underlying effects on top of the observed effects of an ETEC infection.

1.3.8 Conclusion

From the data discussed thus far, it is evident that new areas and routes for STb are emerging and future research must occur to uncover the exact mode of action of STb and its effects. Purification techniques, cell culturing and many other methodologies have improved within the past decades, allowing for a clearer and deeper understanding of new STb-related actions. There are several STb-induced effects which can be attributed to alternative STb-related mechanisms. For example, fluid and ion loss can be attributed to a number of mechanisms other than ionic transporter activation. One example of this is the paracellular loss of fluids and ions through tight junction manipulation. Another is the transcellular loss of fluids and ions through cellular permeabilization or plasma membrane pore formation. The significance of each effect relative to its proposed mechanism needs to be further determined in order to understand their contribution within the process. The ability of STb to conduct such actions in a non-specific, almost mechanistic manner is dangerous, as almost all gut cells from a wide variety of species are vulnerable to STb intoxication. However, the ability of STb to form pores and manipulate tight junctions non-specifically can be harnessed for example in drug delivery. Gut hormone dysregulation from bacterial toxins is a novel research area which needs greater attention. GLP-2 possesses properties opposite to that of STb. Hormone therapy following ETEC infection may become a useful addition to the existing ETEC related therapies. All of these different research areas must be collectively studied in order to finally take control of global ETEC infection.

1.4 The role of GLP-2 in Gut Health and Function

1.4.1 Enteroendocrine Cells and GLP-2

Various cells within the gastrointestinal (GI) tract secrete over 20 regulatory hormones which influence several physiological processes. [90]. The intestinal epithelium not only acts as a point of nutrient absorption and processing, but also as an efficient barrier against the extensive bulk of commensal and pathogenic bacteria and luminal antigens [91]. Within this barrier, various cells function to complete different tasks. Among these, enteroendocrine cells (EECs) can be found dispersed throughout the epithelium of the GI tract [91,92]. EECs are highly specialized signal transduction conduits which regulate numerous metabolic processes [91]. They respond to luminal nutrients by producing and secreting peptide hormones which regulate GI enzyme secretion, GI motility, GI replenishment, insulin release and appetite [92,93]. These imperative sensory cells amount to only 1% of the GI epithelium, however collectively form the largest endocrine system within the human body [94].

Of these crucial hormones, GLP-2 is a 33 amino-acid peptide hormone possessing a molecular weight of 3.76 kDa, and is secreted by a specific group of EECs known as enteroendocrine L-cells (Fig. 5) [95]. The apical surface of these L-cells are covered with microvilli which have immediate contact with luminal contents [96]. Once differentiated, L cells can be found distributed along the length of the intestinal epithelium, mainly localized within the distal gut and colon [94]. Once L-cells become stimulated, granules containing GLP-2, along with other L-cell specific hormones, are exocytosed through the basolateral surface into the luminal space or the network of the lamina propria [97]. GLP-2 may act directly upon tissues and surrounding cells, or they can bind to specific neuronal receptors which send metabolic signals to the peripheral and central nervous system [97].

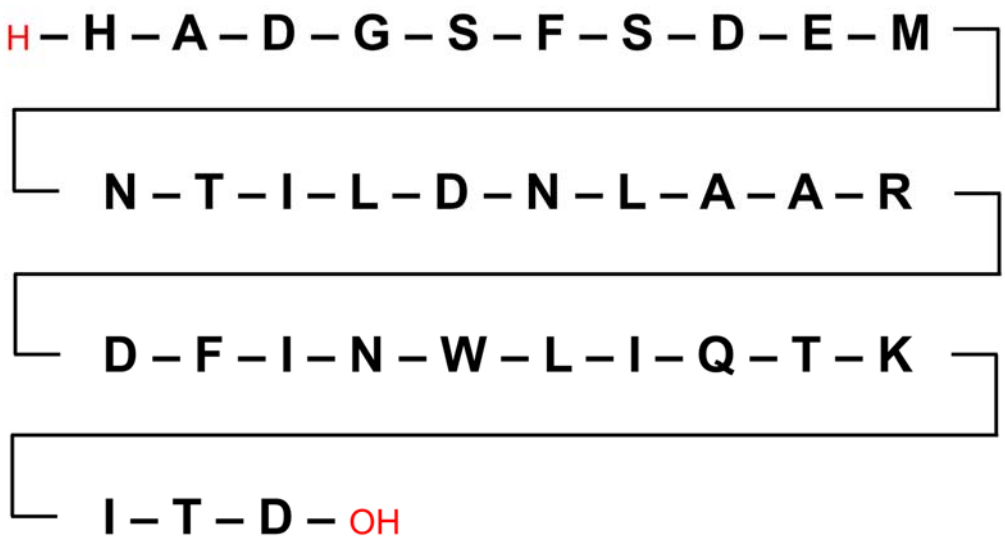

Figure 5. Amino Acid Skeleton of the GLP-2 Hormone Secreted by L-cells.

Adapted from [98].

Within L-cells, GLP-2 is derived from the cleavage of a pro-hormone, proglucagon, and abundantly secreted in the distal gut where the majority of L-cells can be found [78,98]. Proglucagon is synthesized within the ER, transported through the Golgi, and processed within secretory granules through cleavage by prohormone convertase 1/3 (PC1/3). Proglucagon cleavage yields glicentin and major proglucagon fragment (MPGF), which are further cleaved and processed into oxyntomodulin, glicentin-related pancreatic polypeptide (GRPP), GLP-1 and GLP-2 through various prohormone convertase enzymes (Fig. 6) [99–101]. Once L-cells become stimulated, GLP-2 is secreted into the interstitial space [102]. Once in circulation, GLP-$2^{1\text{-}33}$ is rapidly metabolized in humans by the enzyme dipeptidylpeptidase IV, yielding the inactive peptide GLP-$2^{3\text{-}33}$ [101].

The cellular actions of GLP-2 are initially mediated by its binding to the GLP-2 receptor (GLP-2R) [99]. The GLP-2R is a 7-transmambrane-spanning G protein-coupled receptor within the class II glucagon/GIP family of receptors [101]. Within the gut, the GLP-2R was found to localize on enteroendocrine cells, enteric neurons and lamina propria stromal cells, employing multiple mediators from several gut endocrine and epithelial cell populations [99,100]. However, studies have demonstrated GLP-2 actions and effects on various enterocyte epithelial cell lines *in-vitro*, suggesting that these epithelial cells also express functional GLP-2 receptors [103]. Jasleen *et al*., (2002) also suggests that multiple GLP-2R subtypes may exist which directly transduce GLP-2 actions independent of traditional GLP-2R signaling [103].

Indirect peripheral GLP-2 response from the brain is imperative in regulating appetite, gut homeostasis, enhancing blood flow and the absorption of nutrients and fluids, as well as strengthening immune defense [97]. Guan et al., (2012) showed how hypothalamic *GLP-2R* KO mice displayed increased food intake and meal frequency, rapid gastric emptying and late-onset

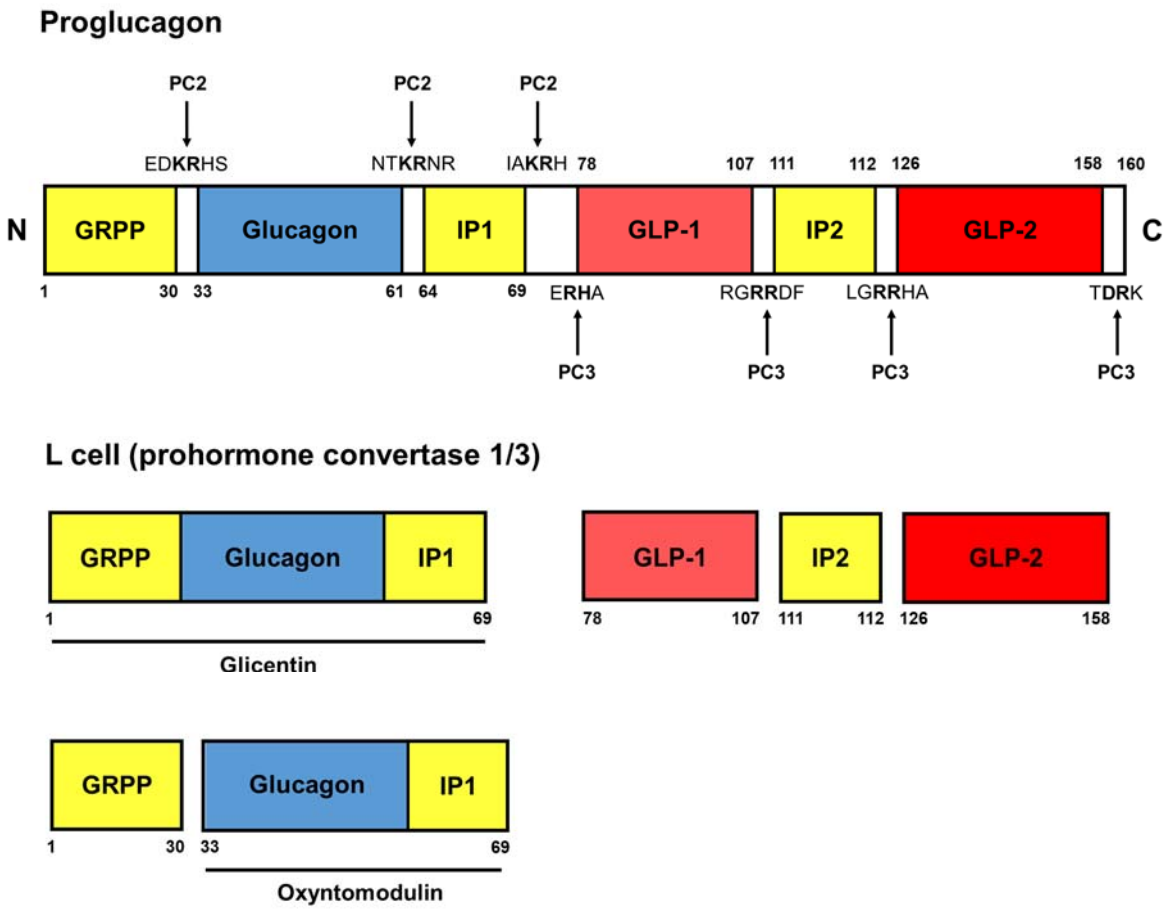

Figure 6. Posttranslational Processing of Proglucagon.

Adapted from [104,105] . GRPP: Glicentin-related pancreatic polypeptide; IP1: Intervening peptide 1; IP2: Intervening peptide 2; PC2/3: Prohormone Convertase Enzyme 2/3.

obesity [106]. The GLP-2R is also known to localize on enteric neurons and can mediate a number of secondary actions [107]. Cinci *et al*., (2011) revealed how the GLP-2R was found to be expressed by both inhibitory and excitatory enteric neurons within the mouse duodenum. GLP-2 administration caused a decrease in spontaneous contractions mediated by nitric oxide release as well as a reduction in provoked cholinergic contractions [107]. Thus, the distinction between hormones and neurotransmitters is generally definitive, however GLP-2 may act as a neurotransmitter within regions of the brain while also serving as a peptide hormone within the gut [108,109]

1.4.2 Gut Replenishing Actions of GLP-2

GLP-2 has been shown to exert numerous and direct anabolic actions within the GI tract. The insulin-like growth factor (IGF) and the mitogen-activated protein kinase (MAPK) pathways both play a major role in the gastrointestinal actions of GLP-2 [110,111]. IGF-1 and IGF-2 are primarily expressed by myofibroblasts and smooth muscle cells within the intestine [112]. Potent growth can be observed in response to both circulating and locally produced IGF-1 and IGF-2, correlated with the expression of the type 1 IGF receptor within the gut [113–116]. GLP-2 has been shown to enhance the expression and secretion of IGF-1 required for intestinal growth, identifying IGF-1 as an imperative mediator for the trophic effects of the GLP-2 hormone [110].

GLP-2 is potent in its trophic effects, mainly characterized by increased GI mass and mucosal thickness/density [84]. Morphologically, GLP-2 has been shown to increase intestinal villous height/length and the depth of intestinal villous crypts [85]. The proliferative force of GLP-2 is most clear following experimental gut injury [117]. GLP-2 has been shown to significantly elevate morbidity and enhance intestinal epithelial repair in several gut related maladies, including mucositis, enteritis and SBS [117,118]. Along with enhancing intestinal epithelial

mass, GLP-2 also enhances intestinal barrier function of both transcellular and paracellular pathways [97]. The regulation and enhancement of the intestinal barrier can be attributed to the hormone's ability to enhance the expression of crucial tight junction proteins [80]. GLP-2 was shown to increase the mRNA and protein of zonula occludens-1, occludin and claudin-1 [80], in turn increasing tight junction integrity and decreasing unwanted permeability of luminal contents. In ruminants, Walker *et al*., (2015) demonstrated how *Eimeria* bovis (*E.bovis*) administration resulted in decreased mRNA and protein expression of specific tight junction proteins such as claudins 1, 2 and 4, JAM2, occludin and ZO-1 [117]. However, GLP-2 administration was shown to significantly attenuate the infection by increasing the mRNA expression of said tight junction proteins [117]. Thus *in-vivo* and *in-vitro*, GLP-2 has consistently been shown to enhance the intestinal epithelial barrier though the enhancement of cell-to-cell junctions. This not only decreases unwanted immune reactions and inflammation, but also protects against further infection.

Alongside enhancing intestinal epithelial proliferation and mucosal mass [103], GLP-2 has been shown to inhibit cellular apoptosis by decreasing caspase-8 and-3 activity [119]. With regards to chemotherapy agents which induce unwanted induction of apoptosis and cell-cycle rest, Boushey *et al*., (2001) revealed how in mice, GLP-2 treatment significantly improved cell survival, reduced bacteremia, decreased epithelial injury and inhibited crypt cell death [119]. GLP-2 treatment also improved overall survival and reduced weight loss in these mice while having no impact on the effectiveness of the chemotherapy [119]. Arda-Pirincci & Sehnaz Bolkent (2011) also demonstrated the antiapoptotic effects of GLP-2, revealing proliferative, protective and antioxidant effects against tumour necrosis factor-alpha (TNF-α)-induced intestinal injury [120]. Burrin *et al*., (2007) demonstrated that the GLP-2R-activated intracellular signals involved in

both cell survival and proliferation is rapid in neonatal pigs, preceding trophic actions on the intestinal epithelium [102]. Therefore, the ability of the GLP-2 hormone to rapidly inhibit apoptotic signaling pathways plays a major role in the subsequent proliferative growth and increased mass of the gut epithelium.

1.4.3 The Putative Overlapping Mechanisms of STb Signaling and GLP-2 Secretion

There are several signaling mechanisms which have been elucidated with regards to the production and secretion of GLP-2. GLP-2 secretion occurs first by conversion of energy in the form of adenosine triphosphate (ATP) into cAMP via adenylate cyclase [121]. cAMP is also known to regulate the expression of the proglucagon gene, playing a crucial regulatory role in the production and secretion of GLP-2 from L-cells [122]. Increased intracellular cAMP can then activate protein kinase A (PKA) and PKC, mediating the release of Ca^{2+} from endoplasmic reticulum (ER) stores through IP_3 receptor (IP_3R) activation, as well as the ryanodine receptor (RyR) activation, on the ER membrane [123,124]. PKC activation has also been shown to activate CaMKII in glucagon-like peptide secretion mechanisms, which also promotes the release of Ca^{2+} from ER stores through the same channel receptors [125]. Apart from the adenylate cyclase pathway, a secondary G protein-coupled pathway also leads to the stimulation of phospholipase C, also releasing Ca^{2+} from IP_3-sensitive intracellular ER Ca^{2+} stores [124]. These increased intracellular Ca^{2+} levels then play a crucial role in the production, packaging and secretion of GLP-2 from the Golgi, to the ER, and into secretory vesicles to be exocytosed [126,127].

1.5 Rational and Hypothesis

As GLP2 is a well-established gut-protective hormone and STb is a known intestinal destabilizing toxin, a potential link between the two is likely. Furthermore, many signaling pathways with regards to GLP-2 secretion share resemblance with STb-cell signaling pathways. STb-mediated extracellular Ca^{2+} influx activates mediators such as PKC, CaMKII and Phospholipase C; whereas GLP-2 secretion mechanisms also involve these mediators, ultimately leading to intracellular ER Ca^{2+} release and hormone secretion (Fig. 7). There may be some underlying relationship between this apoptotic STb toxin and the secretion of this antiapoptotic GLP-2 growth hormone.

We hypothesize that the secretion of the GLP-2 growth hormone will be enhanced by L-cells treated with STb. We also hypothesize that the toxin will have a limited impact on the viability of these L-cells due to the antiapoptotic nature of the GLP-2 growth hormone. Finally, we hypothesize that GLP-2 hormone supplementation will rescue a STb-susceptible cell model from intoxication.

The next chapter will present the original research in a manuscript format.

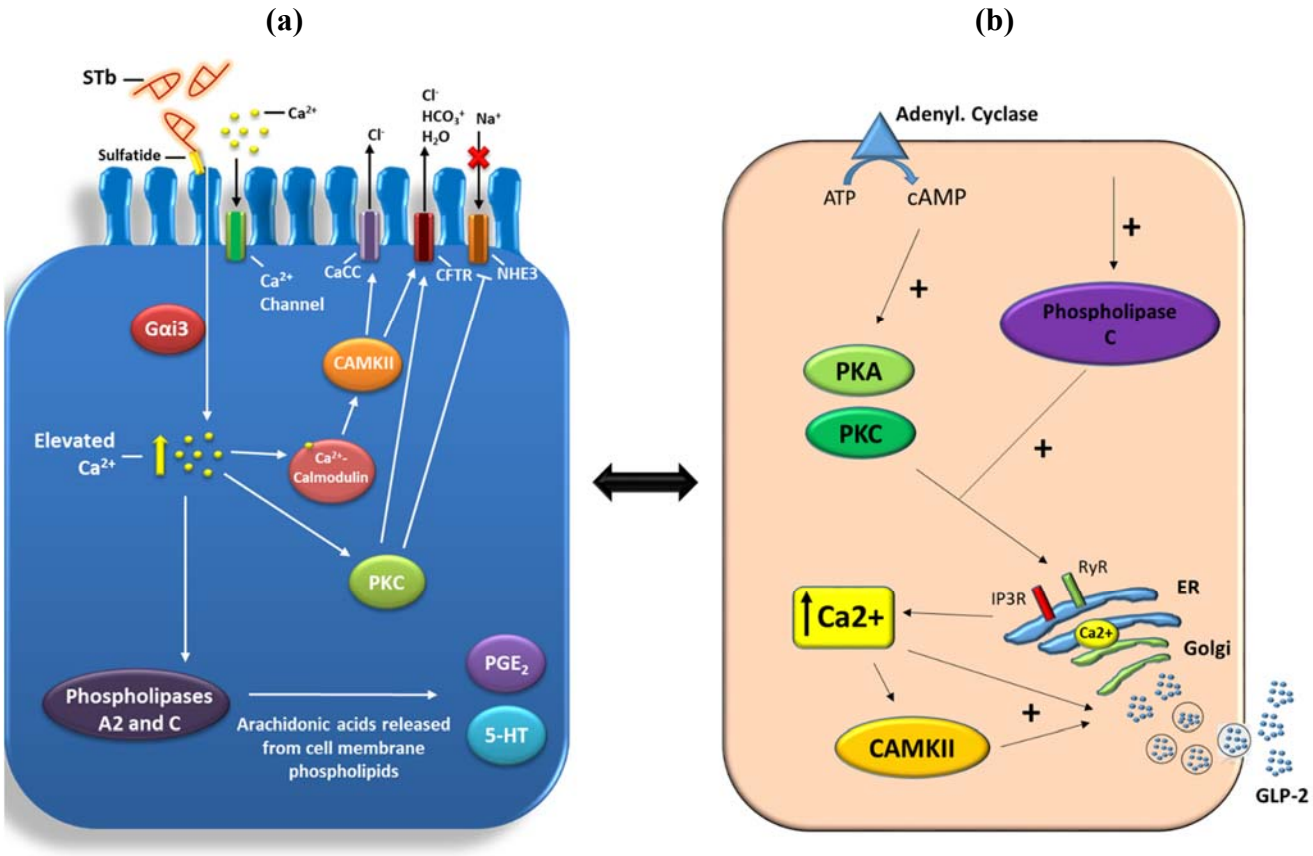

Figure 7. Comparison of STb-mediated Cell Signaling and GLP-2 Secretion from L-cells.

(A) STb intoxication cell-signaling schematic and (B) GLP-2 production and secretion pathways

Chapter 2

2 Investigation of the *E. coli* STb on Enteroendocrine Cell Function

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2.1 Abstract

ETEC derived STb is responsible for secretory diarrhea in animals and humans. STb disrupts intestinal fluid homeostasis, epithelial barrier function, and promotes cell death. GLP-2 is a potent intestinotrophic growth hormone secreted by enteroendocrine L-cells. GLP-2 enhances crypt cell proliferation and epithelial barrier function, and inhibits enterocyte apoptosis. As many similarities have been outlined between cellular pathways of GLP-2 production and secretion and STb cell signaling mechanisms, this study aims to investigate the effects STb has on the physiology of *in-vitro* L-cells and the secretion of GLP-2. After purifying HIS-labelled STb from bacterial culture media; L-cell interaction, cell viability and GLP-2 secretion from the GLUTag murine and NCI-H716 human L-cell line, and the STC-1 murine secretin cell line, were assessed after STb treatment. Immunofluorescence revealed a difference in STb localization correlated to cellular division within the L-cells. In ell viability experiments, the GLUTag L-cell line, which produced and secreted the most GLP-2 growth hormone, was found to show the most resistance to STb intoxication. The STC-1 secretin cell line, which secreted the lowest amount of GLP-2, was the most susceptible to intoxication. STb was found to enhance GLP-2 secretion from the endocrine cells after just 2 hrs of treatment. GLP-2-rich cell culture media was shown to significantly protect from STb-induced mitochondrial suppression from the STC-1 cells. These results suggest a protective role of GLP-2 during STb intoxication. Future work will elucidate the mechanism of this interaction. This work will support the use of GLP-2 therapies in enterotoxigenic related diseases.

2.2 Introduction

ETEC is a major cause of infectious watery diarrhea in animals and humans, resulting from the ingestion of contaminated food or drink [6,36]. STb is a subset of the heat-stable ETEC

enterotoxins known to trigger enterocyte fluid secretion [5]. STb-mediated intoxication occurs once the toxin binds to its receptor, sulfatide [46]. The internalization of STb then activates a GTP-binding regulatory protein, opening a receptor-dependent calcium channel, increasing intracellular Ca^{2+} levels [47]. Activation of CaMKII prompts the secretion of Cl^- ions through a calcium-activated chloride channel [128]. Increased Ca^{2+} levels also activates protein kinase C (PKC) and downstream cystic fibrosis transmembrane conductance regulator (CFTR), leading to the secretion of water, bicarbonate and crucial electrolytes [46,47]. Increased Ca^{2+} levels can also activate phospholipases A2 and C, forming PGE_2 and 5-hydroxytryptamine from phospholipid arachidonic acids which also mediate the secretion of water and ions from enterocytes into the lumen [48]. Apart from disrupting intestinal fluid homeostasis; STb has been shown to alter intestinal barrier function through the manipulation of tight junction proteins [55,56]. STb also triggers enterocyte apoptosis through intrinsic caspase-9 and -3-mediated pathways, resulting in a reduction of the absorptive mucosal surface area, contributing to the observed diarrheal disease.

The glucagon-like peptide hormones, produced and secreted by enteroendocrine L-cells within the distal gut, are a group of hormones that play crucial roles in metabolism and gut integrity [29]. GLP-2 is known for its impactful role in the regulation of intestinal integrity [30,31]. Through its interaction with the GLP-2 receptor (GLP-2R) on L-cells, enteric neurons and other cells, GLP-2 is known to bestow numerous beneficial actions which replenish the gut [31]. GLP-2 actions are characterized by increased intestinal mass and mucosal density, and have been shown to significantly reduce morbidity and enhance intestinal repair following gut related diseases [117,118]. This is accompanied by specific morphological enhancements such as increasing intestinal villous height and crypt depth [85]. GLP-2 also enhances intestinal barrier

function by upregulating the expression of tight junction proteins [86]. GLP-2 has also been shown to inhibit enterocyte apoptosis through caspase-8 and-3 inhibition, improving cell survival and decreasing epithelial injury [119]. Importantly, the signaling pathways related to STb and GLP-2 secretion from L-cells show considerable overlap, suggesting there may be a role for STb in GLP-2 secretion.

Due to the contrasting effects of GLP-2 and STb, and the significant resemblance of GLP-2 secretion/STb cell signaling pathways, we hypothesized that STb plays a direct role in L-cell regulation. We hypothesize that STb will not only bind and interact with L-cells, but the secretion of the GLP-2 growth hormone will also become enhanced as a protective measure against STb intoxication. This study aims to determine the effects STb may have on L-cells, uncovering a potential underlying relationship between the apoptotic STb toxin and the secretion of the antiapoptotic GLP-2 growth hormone.

2.3 Materials and Methods

2.3.1 Culture Media, Reagents and Cell Lines

Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) medium, Hank's Balanced Salt Solution (HBSS), Fetal Bovine Serum and Penicillin/Streptomycin (Pen/Strep) were all purchased from MilliporeSigma (St. Louis, Missouri, United States). The cell models chosen for this study were the mouse GLP-2 secreting GLUTag L-cells (passage 10-25; kind contribution from Dr. Drucker, Lunenfeld-Tanenbaum Research Institute, Toronto, ON, Canada), human GLP-2 secreting NCI-H716 (ATCC, Manassas, VA, USA) and the murine STC-1 secretin cell line (ATCC, Manassas, VA, USA). DMEM supplemented with 10% FBS and 1% Pen/Strep was used to maintain the murine cell

lines, whereas RPMI-1640 supplemented the same was used for the human cell line. All cells were maintained within 10 cm plates and grown at 37°C, 5% CO_2 in a humidified incubator. Passages were taken once 85% cell confluency was reached.

2.3.2 Production and Purification of STb Toxin

Established by Kinkar (2020) [129], a modified *stB* (Invitrogen by Thermofisher Scientific Inc., Toronto, Canada) was inserted into a plasmid and injected into a colony of *E.coli* Bacteria. A His-tag was genetically introduced into the wild-type sequence in order to easily purify and quantify the toxin. Proper STb sequence and presence of the His-tag and glycine linker peptide was confirmed through N-terminal protein sequencing. The recombinant *E. coli* HB101 was mass cultured at 35°C, 150 RPM on an orbital shaker until the optimal OD (0.5 AU, ~2 hrs) of the growth medium had been reached (ABS at 600 nm). The bacteria was induced to secrete large amounts of the toxin by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG 1 M, final conc. 1 mM) to the growth medium, allowing for an additional 5 hours of growth and STb secretion, totaling 7 hours of growth. Bacterial cells were separated from culture media containing STb through centrifugation (16,000 G, 30 min), and filter sterilized to remove any residual cells (0.2 µm filter paper, Sartorius 11307, Goettingen, Germany). The media was further condensed using filter paper apparatus to remove residual liquids and impurities under 1 kDa (Amicon™ Stirred Cells, Millipore Sigma). After condensing 3 L of growth medium to ~50 mL, STb was purified using fast protein liquid chromatography (FPLC) methodology. A nickel column was used to purify the toxin due to its high binding affinity to the His-tag. Once the column was saturated with STb and cleaned with condition buffer (25 mM phosphate buffer (P) 25 mM imidazole, 500 mM NaCl, pH 7.45) the sample was eluted and collected in fractions using elution buffer (20 mM Na(P), 500 mM imidazole, pH 7.52). Purity and initial quantity was

visualized through tricine-SDS-PAGE. To desalt the samples we diluted and condensed using 1x PBS at 1:100 sample-diluent (~5 mL:500mL) until the sample condensed back to 5 mL. STb protein concentration was quantified using spectrophotometry and Bradford methodology. A neutralized STb vehicle control was also generated for experiments where 400 ng/mL of STb was neutralized via trypsin digestion (1:100 trypsin:sample), followed by DTT (10 mM) and heat incubation (5 min at 80°C).

2.3.3 Fluorescent Immunocytochemistry

Cells were grown and incubated on coverslips within 6-well plates for 48 hours in 10% serum media. Wells containing NCI-H716 cells were coated with Matrigel matrix (Sigma-Aldrich, Oakville, ON, Canada) for proper adherence. Cells were then washed twice with phosphate buffer saline (1X PBS), and treated with 400 ng/mL STb in serum-free growth medium for 30 min at 37°C, 5% CO_2. After, 10% neutral buffered formalin (NBF) (4% formaldehyde, 37% dH_2O, 1X PBS) was used to fix the cells for 20 min at room temperature. Coverslips were then washed twice and blocked with blocking solution (2% milk in PBST, 25% Tween 20) for 1 hr. After washing five times, coverslips were blocked with primary anti-His antibody solution (2% milk in PBST, 0.25 µg/mL Mouse anti-His, GenScript®) for 2 hrs, followed by washing and blocking with secondary antibody solution (2% milk in PBST, 1:1000 stock Anti-Mouse IgG, Alexa Fluor® 488 Conjugate, Cell Signaling Technology, MA, USA) for 1 hr. Coverslips were then washed for 1 min five times, mounted onto microscope slides with Fluoroshield Mounting Medium with DAPI (Abcam, Toronto, ON, Canada) and fixed onto the slides using clear nail polish. Cells were observed in the dark using an inverted Zeiss Axioplan fluorescent microscope and images were taken using Zeiss AxioVison software (Zeiss, Oberkochen, Germany).

2.3.4 Neutral Red Lysosomal uptake Assay

The Neutral Red assay was conducted using the protocol outlined by Repetto (2008) [130], with slight modifications. Cells were seeded at 200,000 cells/well and grown in 24-well plates in 10% serum media for 48 hrs prior to treatment. Neutral Red dye (Sigma-Aldrich, Oakville, ON, Canada) stock was made at 0.01 g/mL in dH_2O. Treatments received a final concentration of 4 uL dye/mL serum-free media. Treatments included either 100 or 400 ng/mL STb, neutralized STb vehicle control or a 0.3% H_2O_2 positive control. 1 mL of treatment with dye was added to each well and incubated for 2 hrs at 37°C, 5% CO_2. Treatments and dye were aspirated after and cells were washed with wash fixative solution (dH_2O, 1% calcium chloride, 0.37% vol/vol formaldehyde). An extraction solution (dH_2O, 50% vol/vol bonded ethanol, 1% vol/vol glacial acetic acid) was then added to the wells for dye extraction from viable cells. Cells were gently agitated on a platform orbital shaker for 5 min for complete dye extraction and the absorbance was measured at 600 nm.

2.3.5 Resazurin Reduction Assay

Cells were seeded at 200,000 cells/well and grown in 24-well plates in 10% serum media for 48 hrs prior to treatment. A Resazurin stock solution (Biotium, Inc., Fremont, CA) of 10 mg/mL was diluted to 0.15 mg/mL and used for experiments. After 48 hrs, cells were washed and growth media was replaced with serum-free treatments including 1:5 Resazurin:treatment media were added within the wells. Treatments included either 100 or 400 ng/mL STb, neutralized STb vehicle control or a 0.3% H_2O_2 positive control. Cells and dye were allowed to adjust for 1 hr and the fluorescence (Ex/Em 530/590 nm) was then taken for the first reading. Fluorescence was measured at 1, 2, 4, 6, 24 and 48 hrs. Treatment plates were incubated at 37°C, 5% CO_2 throughout the experiment and were kept away from light when conducting measurements.

Fluorescence values for the positive H_2O_2 control were subtracted from treatment fluorescence to correct for background reduction.

2.3.6 GLP-2 Secretion Analysis

Cells were seeded at 200,000 cells/well and grown in 24-well plates in 10% serum media for 48 hrs prior to treatment. Once cells reached proper confluency, growth media was replaced with secretion media (FBS reduced to 0.5%) containing STb treatments or controls. Cells were then washed and treatments were added to wells and incubated for 2 hrs at 37°C, 5% CO_2, allowing for sufficient time for acute GLP-2 production and secretion. Treatment media containing secreted GLP-2 was then collected and acidified using trifluoroacetic acid (TFA, final conc. 0.1%) and stored at -20°C until GLP-2 detection. GLP-2 content was quantified using a multi-species enzyme-linked immunosorbent assay (ELISA) (ab222863, Abcam, Cambridge, MA, USA) as per the manufacturer's guidelines. Results for treatments are presented as percent secretion relative to vehicle control.

2.3.7 Statistical Analysis

All of the data presented are expressed as mean ± SEM. All measures and groups were tested for normality using the Shapiro-Wilk or Kolmogorov-Smirnov normality tests, depending on the sample size. Sets which passed the normality testing were then assessed using parametric statistical analyses. Studies with multiple treatments or doses were analyzed by one-way ANOVA and further analyzed by a Tukey's post-hoc test. Studies with 2 or more independent variables were analyzed by two-way ANOVA, followed by a Tukey's post hoc test to measure the significance of each treatment at specific time points. $P < 0.05$ was considered significant.

2.4 Results

2.4.1 STb Purification from Recombinant *E. coli* HB101 Growth Media

In order to observe the effects of STb on the endocrine cell models, the toxin needed to be generated and purified for use in experiments. The recombinant *E. coli* HB101 showed consistent growth over the 7 hr period. The bacteria entered the log phase of growth after 2 hours, which is when STb secretion was activated through IPTG (Figure 8a). The purified toxin was visualized through gel electrophoresis, revealing a molecular weight of ~6.6 kDa with the His tag and linker peptide (Figure 8b) with no other bands present.

2.4.2 STb Interacts with Endocrine Cell Models, Localization Differs in L-cells

To determine if the purified STb was binding and interacting with the cell models, cells were treated with the toxin and immunofluorescence methodology was utilized to determine binding and localization. Initial results showed a positive interaction of the toxin to both L-cell models and the secretin cell model. STb was found to primarily localize within the nucleus of the cells within all endocrine models. A color shift from the initial blue (nuclei) and green signal (STb) to a cyan signal can be observed, indicating co-localization. However, GLUTag L-cells which were in a state of cell division did not show this nuclear co-localization (Figure 9a). The NCI-H716 L-cell model (Figure 9b) and STC-1 secretin model (Figure 9c) showed this same pattern, with most cells up-taking STb within the nucleus. Certain cells however displayed a clear separation of signals, indicated by a strong zone of relief from where the nuclear signal is seen. These results indicate that STb does interact with all of the endocrine cell models through similar nuclear localization patterns, dependent on cellular division with regards to the GLUTag L-cell model.

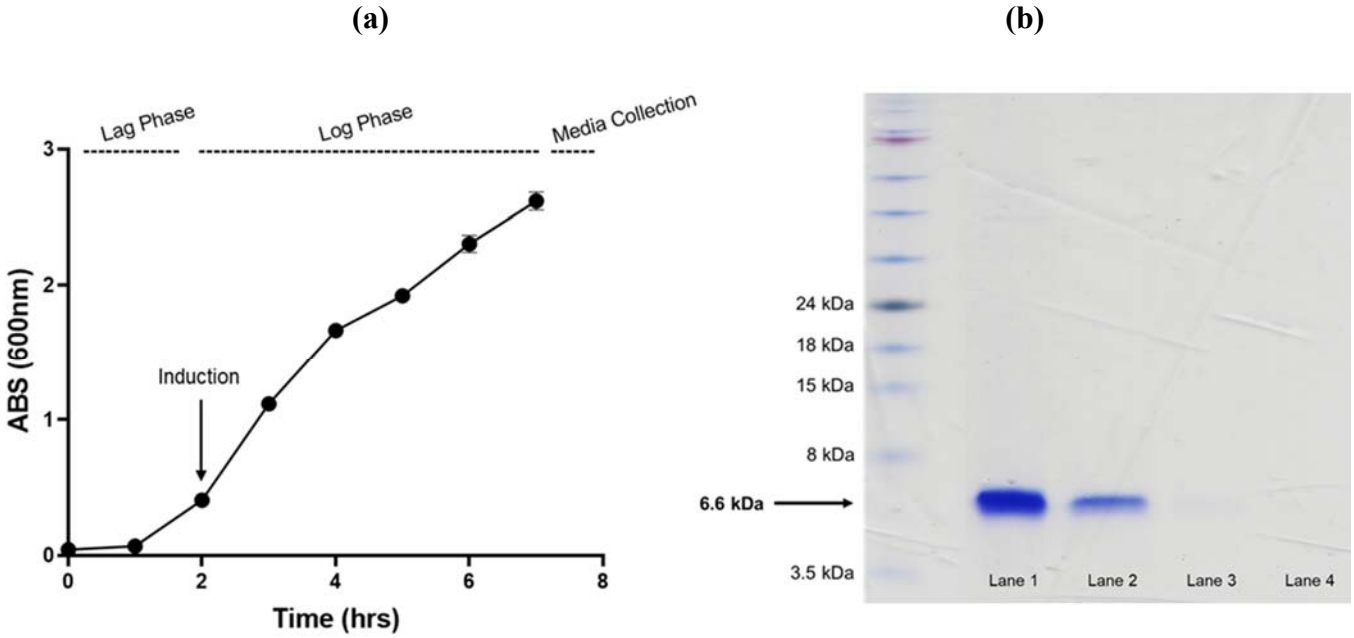

Figure 8. Production and Purification of STb.

(A) Absorbance readings (600 nm) were taken during ETEC growth and represented over time (hr). Growth phase can be seen above dotted lines. (B) Purified STb toxin was visualized via electrophoresis. Well lanes are outlines below bands. Molecular weight markers can be seen on the left. The purified STb toxin was visualized at ~6.6 kDa.

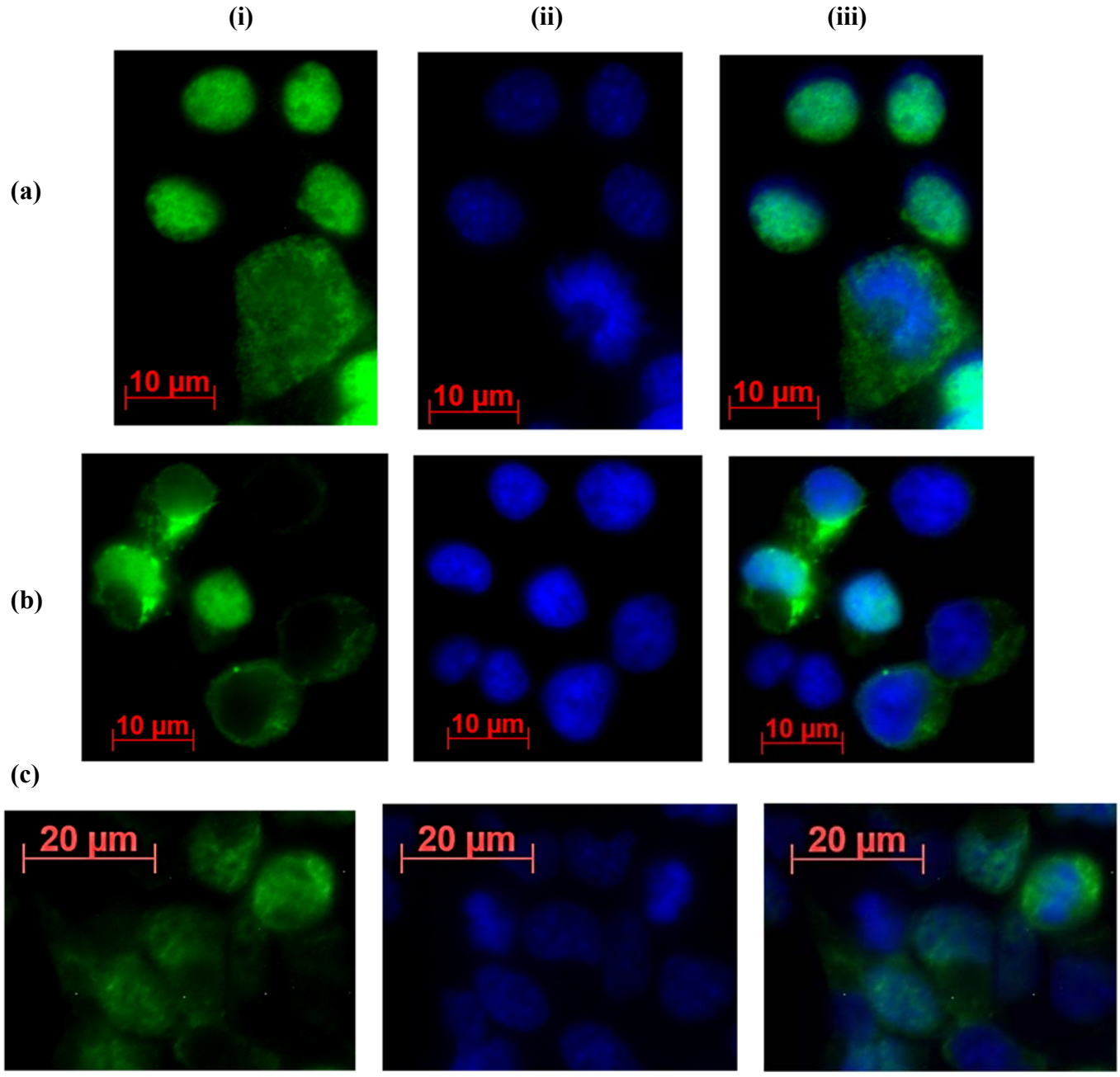

Figure 9. STb Interacts with Endocrine Cell Models.

STb binding and localization was visualized by immunocytochemistry for the GLUTag murine L-cell model (A), NCI-H716 human L-cell model (b) and the STC-1 murine secretin cell line (c). Field (i) shows STb binding (FITC), field (ii) shows the nuclear signal (DAPI) and field (ii) shows the two fields combined.

2.4.3 L-cells are Resistant to STb Intoxication, STC-1 Cells Show Susceptibility

Once STb binding and interaction had been established, cell viability after treatment with STb was measured. This was done through the Neutral Red uptake assay, which measures the cells ability to uptake the neutral red dye within their lysosomes. The GLUTag mouse L-cell line did not show any significant susceptibility to STb intoxication, indicated by no loss in dye uptake ability relative to control, after an acute 2 hr treatment with 100 and 400 ng/mL STb ($p > 0.05$ one-way ANOVA). A 24 hr chronic STb treatment at the same dosage had no significant effect on the L-cell line as well ($p > 0.05$, Figure 10a). The NCI-H716 L-cell line showed slight susceptibility after 2 hrs of toxin treatment at both 100 ng/mL (8.5% reduction ± 2.1, $p < 0.05$) and 400 ng/mL (9.2% ± 2.3%, $p < 0.05$). After 24 hrs of treatment, the cell model showed significant susceptibility and reduced dye uptake with the 400 ng/mL treatment (10.7% ± 1.8%, $p < 0.001$, Figure 10b). The STC-1 secretin cell line showed a slight decrease in cellular viability and dye uptake ability after 2 hrs of STb treatment at 400 ng/mL dosage (5.3% ± 1.1%, $p < 0.05$). After the 24 hr treatment however at the same dosage, the STC-1 model showed significant susceptibility to STb intoxication and decreased dye uptake ability (20.4 % ± 2.8%, $p < 0.001$, Figure 10c). These results indicate that STb causes toxicity in all but the GLUTag L-cell model.

2.4.4 GLUTag L-cells Show No Decrease in Mitochondrial Activity from STb Treatment, NCI-H716 and STC-1 Cell Models Show Susceptibility

To complement our Neutral Red uptake results, the Resazurin Reduction cell viability assay was utilized in order to quantify the effects of STb. Resazurin reduces into resorufin through the mitochondrial respiration of active cells. Similar to the Neutral Red uptake assay results, the GLUTag L-cells were found to be resistant to STb intoxication after 2, 4, 6, 24 and 48 hrs of 100

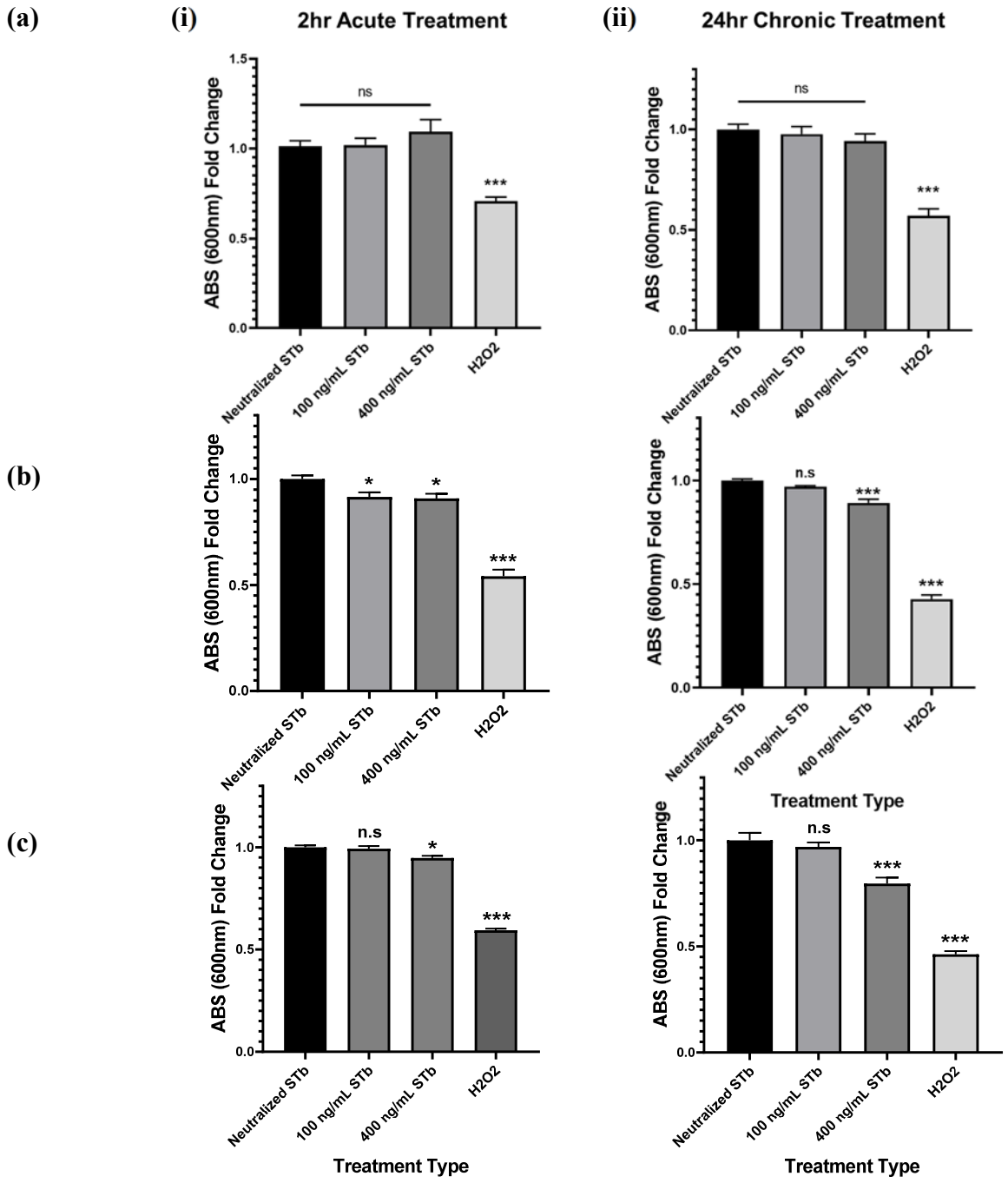

Figure 10. L-cells are Resistant to STb-induced Cellular Toxicity

Neutral red uptake was measured after 2 (i) and 24 hours (ii) at 100 and 400 ng/mL STb dosage for the GLUTag L-cell line (A), NCI-H716 L-cell line (B) and STC-1 secretin cell line (C). n = 6. Results are shown as mean ± SEM and analyzed by one-way ANOVA and Tukey's post hoc test. *, $p < 0.05$, ***, $p < 0.001$ vs. control (neutralized STb vehicle control).

and 400 ng/mL STb treatment ($p > 0.05$ two-way ANOVA, Figure 11a), showing no susceptibility to mitochondrial activity suppression. For the NCI-H716 L-cell model however, mitochondrial activity was reduced significantly after 24 hrs with the 400 ng/mL dose (36.5% reduction ± 6.9%, $p < 0.001$) and at 48 hrs (32.3% ± 8.7%, $p < 0.01$). The 100 ng/mL STb treatment only showed a slightly significant reduction in mitochondrial activity at 4 hrs (21.7% ± 5.4%, $p < 0.05$) and at 6 hrs (23.4% ± 5.9%, $p < 0.05$), however recovering after 24 hrs (Figure 11b). The STC-1 secretin cell model also showed significant susceptibility to mitochondrial activity suppression with the 100 ng/mL STb treatment at 4 hrs (24.6% ± 4.5 %, $p < 0.001$), 6 hrs (17.1% ± 2.6%, $p < 0.05$), and 48 hrs (31.5% ± 6.9%, $p < 0.001$), and the 400 ng/mL treatment at 4 hrs (21.7% ± 3.2%, $p < 0.01$), 6 hrs (16.1% ± 3.4%, $p < 0.05$), 24 hrs (31.8% ± 3.6%, $p < 0.001$) and 48 hrs (31.4% ± 4.1%, $p < 0.001$, Figure 11c). These results combined indicated significant susceptibility of the STC-1 cell model to STb intoxication, slight susceptibility within the NCI-H716 L-cells and a resistance to intoxication from the GLUTag L-cell model.

2.4.5 STb Enhances GLP-2 Secretion from Cell Models

In order to determine the effect STb has on the secretion of GLP-2 from these hormone secreting cell models, a GLP-2 ELISA was utilized to measure the amount of GLP-2 secreted in 2 hrs from culture media in the presence or absence of STb. STb was found to significantly cause a fold-increase in GLP-2 secretion from the GLUTag L-cell model after 2 hrs of treatment with 100 ng/mL STb (2.0 fold ± 0.2, $p < 0.01$, one-way ANOVA) and 400 ng/mL STb (2.4 ± 0.1, $p < 0.001$, Figure 12a). Treatment with STb did not show a significant impact on the secretion of GLP-2 within the NCI-H716 L-cell model at 100 ng/mL ($p > 0.05$) and only slightly significant at 400 ng/mL (1.2 ± 0.03, $p < 0.05$, Figure 12b). For the STC-1 cell model, a slightly significant fold-increase in GLP-2 secretion was seen after treating cells with 100 ng/mL STb

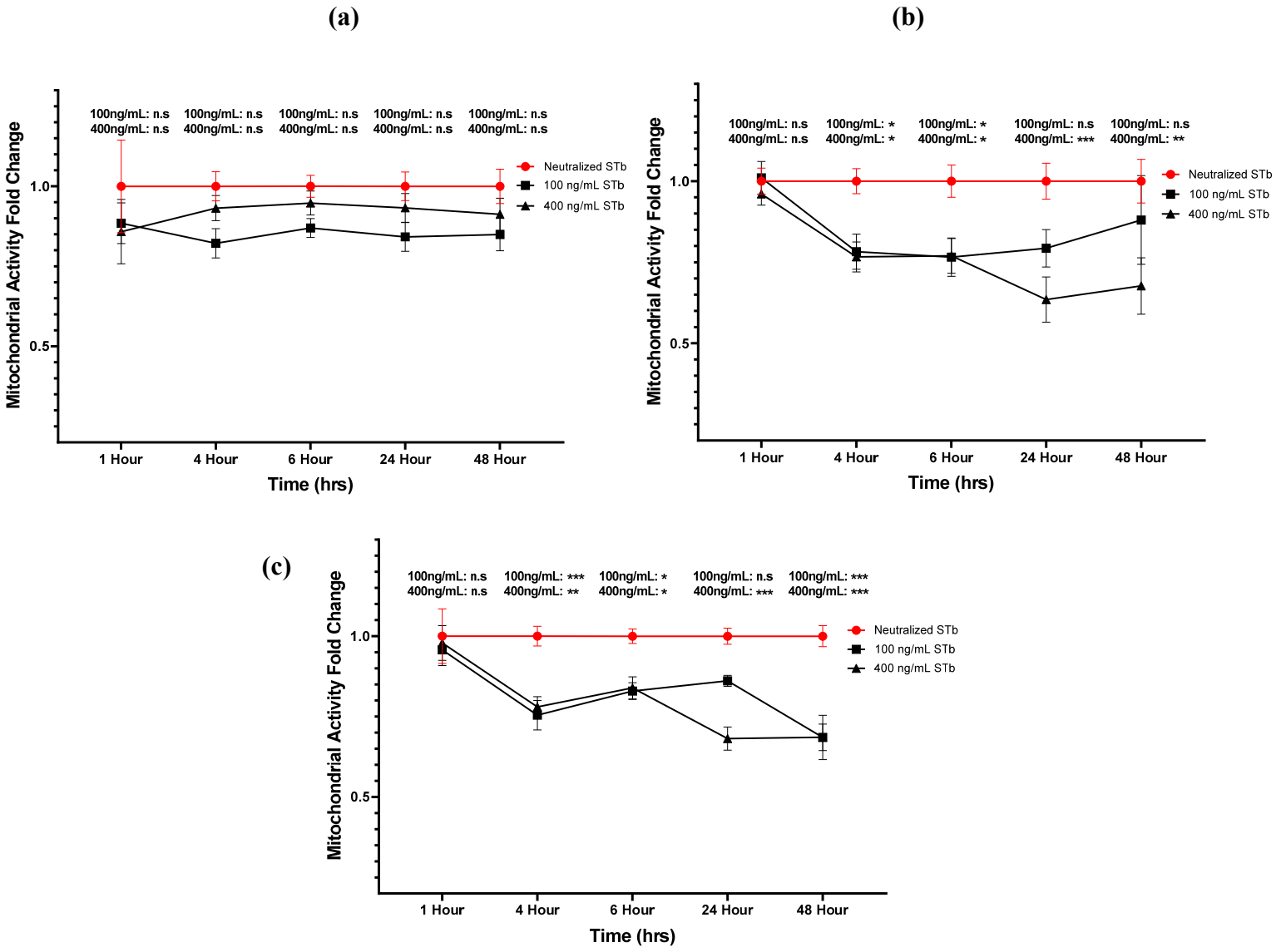

Figure 11. L-cells are Resistant to STb-induced Mitochondrial Activity

Resazurin reduction and mitochondrial activity was measured at 1, 4, 6, 24 and 48 hrs during the 48 hr STb treatment with 100 and 400 ng/mL STb for the GLUTag L-cell line (A), NCI-H716 human L-cell line (B) and the STC-1 secretin cell model (C). n = 12. Results are shown as mean ± SEM and analyzed by two-way ANOVA and Tukey's post hoc test. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ vs. control (red, neutralized STb vehicle control).

(1.7 ± 0.1, $p < 0.05$), however more significant with 400 ng/mL (1.9 ± 0.2, $p < 0.01$, Figure 12c). These results indicate that treatment with STb significantly enhanced the secretion of the GLP-2 hormone from the GLUTag L-cells and the STC-1 model, however having little impact on the secretion of GLP-2 from the NCI-H716 human L-cells. The 400 ng/mL was more significant in its impact on GLP-2 secretion compared to the 100 ng/mL treatment, indicating a potential dose response.

2.4.6 GLUTag L-cell Model has the Highest GLP-2 Secretion Output

To compare the secretion output of the GLP-2 hormone between cell models at baseline secretion (no treatment), as well as STb-enhanced secretion (400 ng/mL treatment), the GLP-2 ELISA was utilized in order to measure secreted hormone concentrations (ng/mL) from culture media after 2 hrs of incubation. The GLUTag L-cell line was found to secrete the highest baseline concentration of GLP-2 within the 2 hr time frame with no treatment (2.5 ng/mL ± 0.25 ng/mL), followed by the NCI-H716 human L-cell model (1.4 ng/mL ± 0.09 ng/mL). The STC-1 secretin cell model was found to secrete the lowest concentration of the GLP-2 hormone (0.7 ng/mL ± 0.06 ng/mL). GLP-2 output from all three cell models were found to be significantly different from one another ($p < 0.001$, one-way ANOVA, Figure 13a). Comparing STb-enhanced GLP-2 output, the toxin was found to have the most impact on GLP-2 secretion within the GLUTag L-cell model (6.1 ng/mL ± 0.2 ng/mL, $p < 0.001$). STb-enhanced GLP-2 secretion was not significantly different between the NCI-H716 L-cell model (1.7 ng/mL ± 0.1 ng/mL) and the STC-1 model (1.2 ng/mL ± 0.03 ng/mL) ($p > 0.05$, Figure 13b). These results imply that the toxin had the greatest impact on GLP-2 secretion on the GLUTag L-cell model, followed by the STC-1 model as the baseline secretion for the NCI-H716 L-cell model was higher than the STC-1 model, however not significantly different with regards to toxin-enhanced secretion.

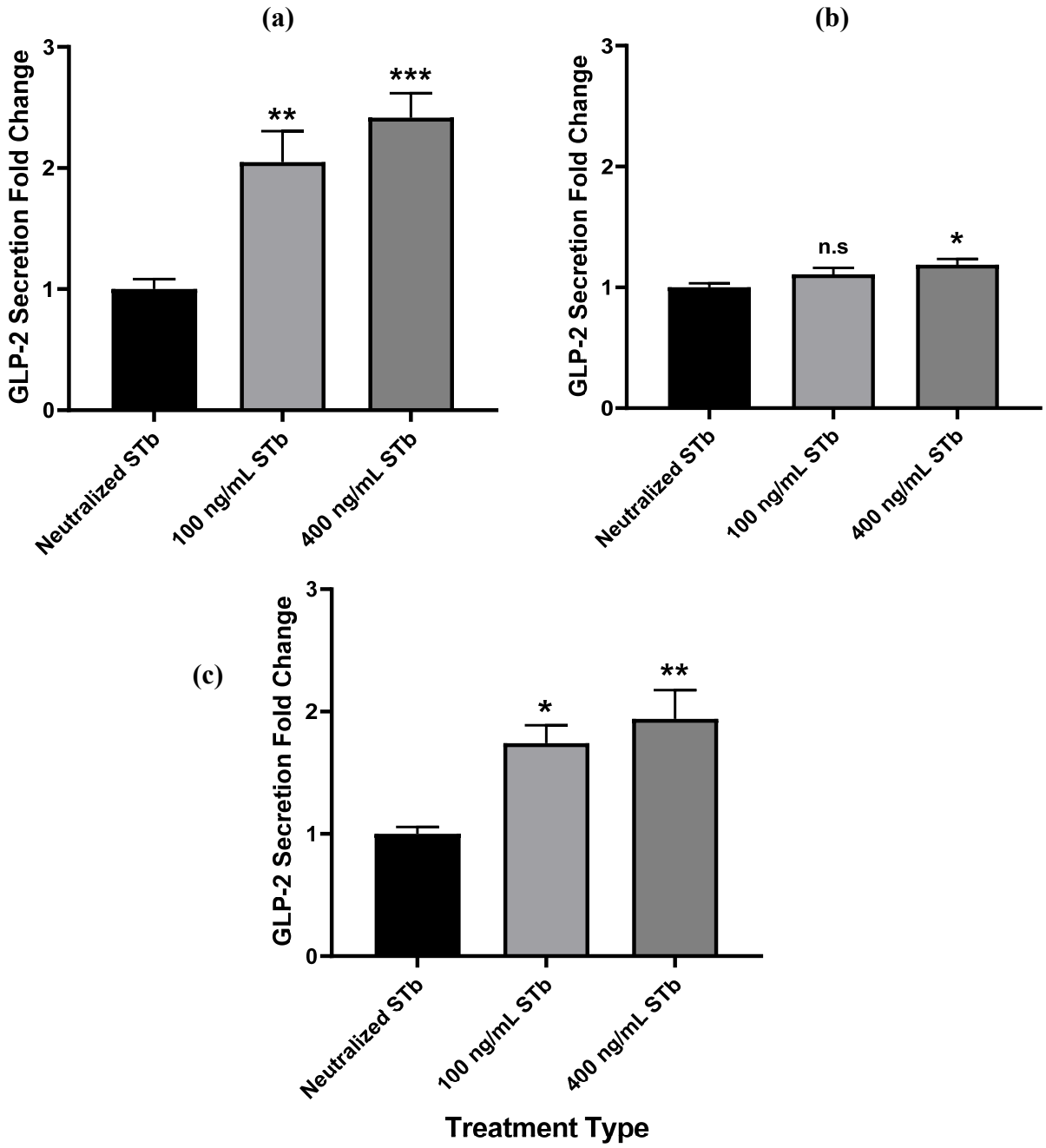

Figure 12. STb Stimulates GLP-2 Secretion Differentially in Gut Endocrine Cells.

GLP-2 secretion was measured from culture media via GLP-2 ELISA after 2 hrs of treatment with 100 and 400 ng/mL STb or control for the GLUTag L-cells (A), NCI-H716 human L-cells (B) and the STC-1 cell model (C). n = 6. Results are shown as mean ± SEM and analyzed by one-way ANOVA and Tukey's post hoc test. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ vs. control (neutralized STb vehicle control).

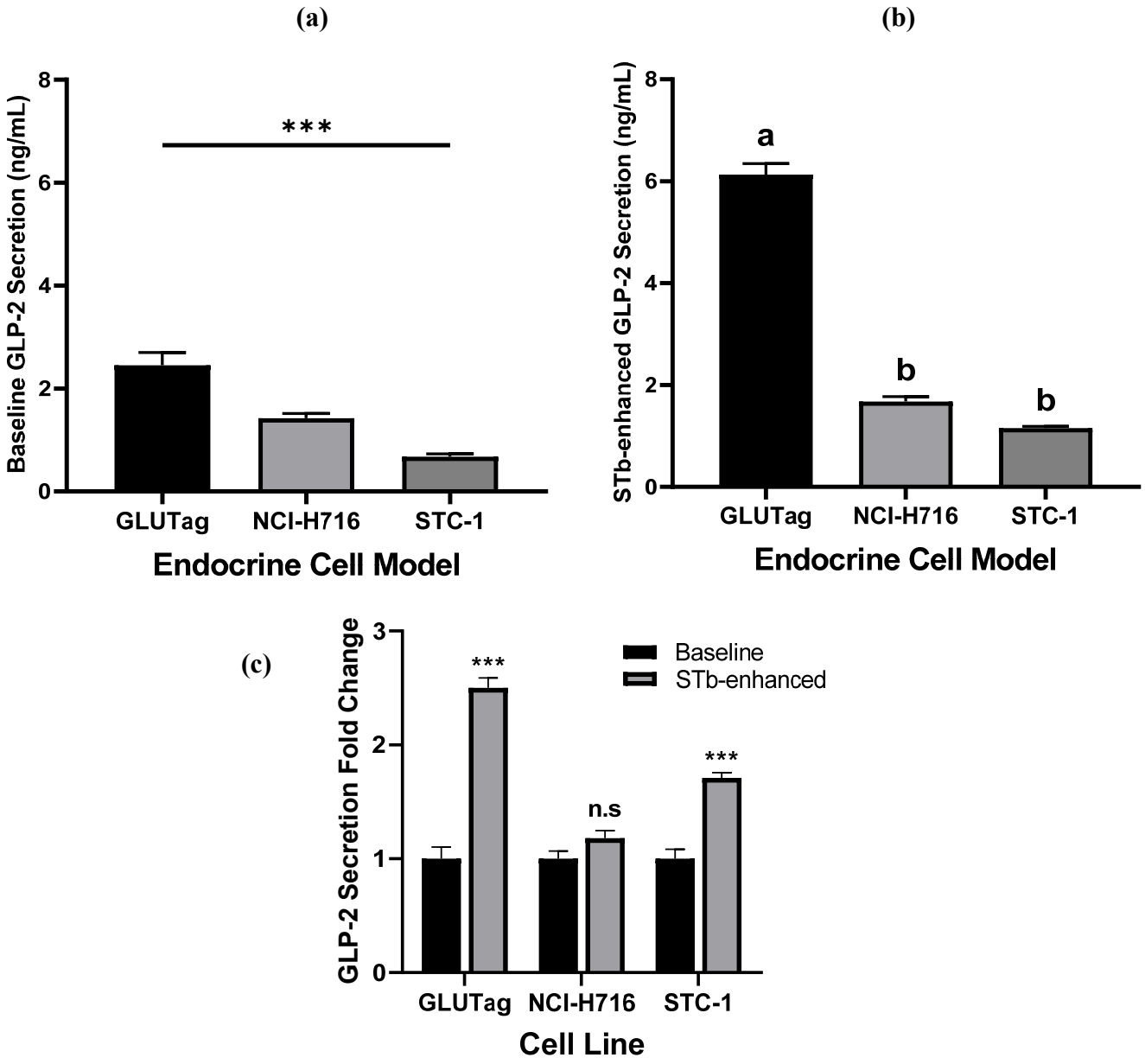

Figure 13. GLUTag L-cell Model has the Highest GLP-2 Secretion Output.

GLP-2 secretion was measured from culture media via GLP-2 ELISA after 2 hrs of incubation with no treatment (baseline secretion) (A), as well as 400 ng/mL STb (B) to compare baseline and STb-enhanced GLP-2 secretion. GLP-2 concentrations are displayed in ng/mL for the GLUTag murine L-cell model, the NCI-H716 human L-cells and the STC-1 murine secretin cell line. Figure (C) shows the effect of STb expressed as a ratio of GLP-2 with/without toxin treatment for each cell model. n = 3. Results are shown as mean ± SEM and analyzed by one-way ANOVA and Tukey's post hoc test. ***, $p < 0.001$.

2.4.7 GLP-2 Supplementation Recovered STb-induced Mitochondrial Activity Suppression

After confirming that the STC-1 cell model was most susceptible to STb intoxication and secreted the lowest concentration of GLP-2, we wanted to establish whether or not it was the lack of GLP-2 which was causing this susceptibility. Or alternatively, whether GLP-2 could rescue this STb-induced toxicity. Knowing that the GLUTag L-cells secreted a significantly higher amount of the growth hormone, we collected media incubated with GLUTag cells and used this as the incubation media in the subsequent experiment. Media containing the secreted hormone (~2.5 ng/mL) was supplemented onto STC-1 cells along with 400 ng/mL of the toxin or control, and the experiment was conducted as per section 2.4.4. GLUTag conditioned media significantly inhibited the toxigenic effects of STb in STC-1 cells, especially after 24 and 48 hours of incubation. After 4 hrs, STb reduced mitochondrial activity by 15.6% ± 4.1% ($p < 0.05$, two-way ANOVA), whereas the toxin treatment supplemented with growth hormone was not significantly different from the control (5.4% ± 3.9% reduction, $p > 0.05$). After 24 hrs of treatment, STb significantly reduced mitochondrial activity as seen previously (26.3% ± 2.2%, $p < 0.001$), however when supplemented with GLP-2, mitochondrial activity was not significantly different from the control ($p > 0.05$). Finally after 48 hrs, the toxin treatment significantly reduced mitochondrial activity (29.4% ± 1.7%, $p < 0.001$), whereas the toxin treatment supplemented with GLP-2 was not significantly different from the control ($p > 0.05$). With regards to the GLP-2 supplementation alone with no toxin, there was no significant enhancement of growth or mitochondrial activity for the majority of the experiment. Mitochondrial activity was significantly enhanced however after 6 hrs of treatment (13.3% ± 3.5% enhancement, $p < 0.05$, Figure 14). These results indicate that GLP-2 supplementation significantly suppressed STb intoxication in STC-1 cells, however had no effect on growth or mitochondrial activity alone.

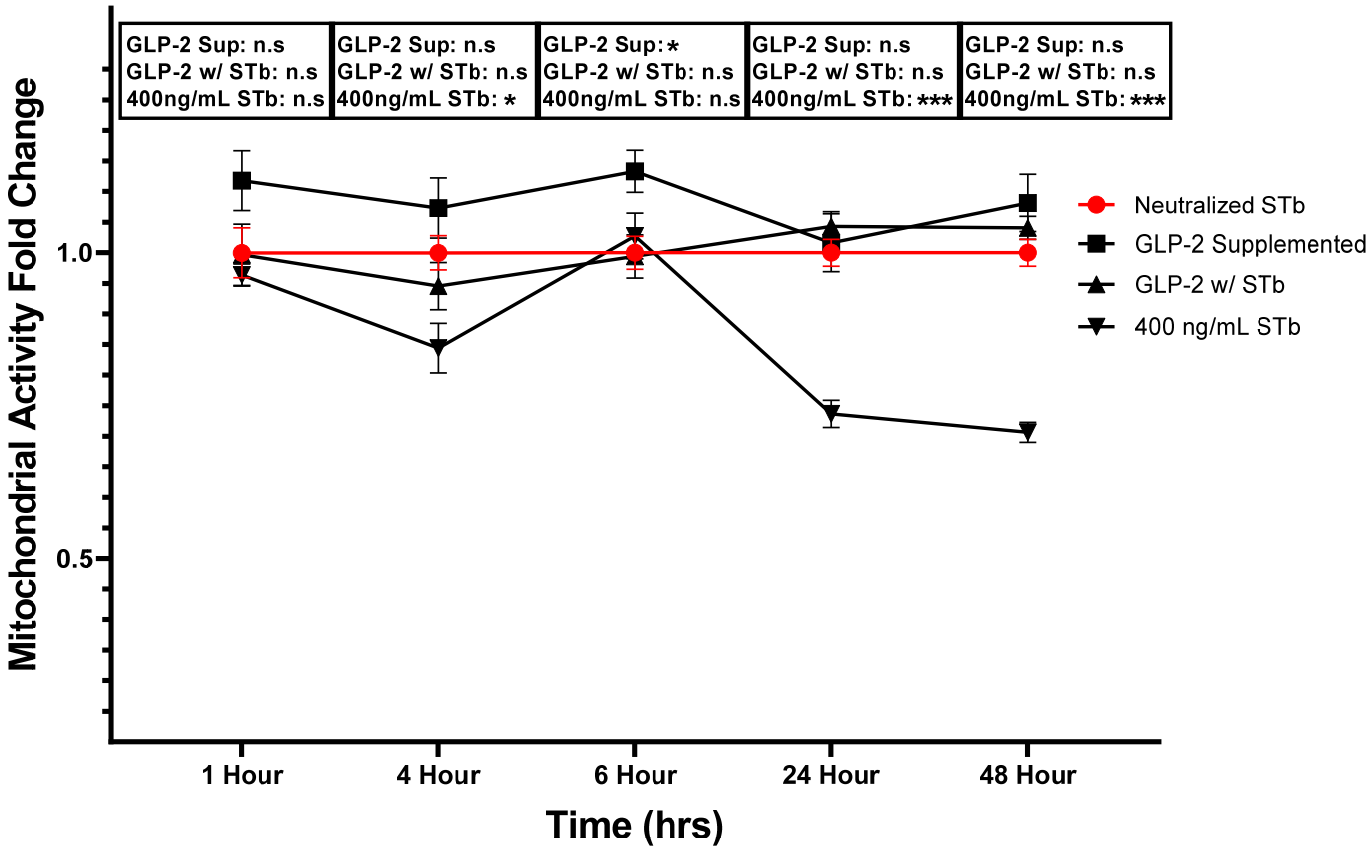

Figure 14. GLP-2 Supplementation Recovered STb-induced Mitochondrial Activity Suppression.

Resazurin reduction and mitochondrial activity was measured at 1, 4, 6, 24 and 48 hrs during the 48 hr STb treatment with 400 ng/mL STb, 400 ng/mL STb with ~2.5 ng/mL GLP-2 supplementation and the same GLP-2 supplementation alone with no toxin. n = 12. Results are shown as mean ± SEM and analyzed by two-way ANOVA and Tukey's post hoc test. *, $p < 0.05$, ***, $p < 0.001$ vs. control (red, neutralized STb vehicle control).

2.5 Discussion

Within our STb cellular localization experiments, we found the bulk of the STb was localized in the nucleus of non-dividing cells. However, this is not the only *E.coli* toxin which has been observed to localize within the nucleus of cells. The *E.coli* cytolethal distending toxin (Cdt), belonging to the AB toxin superfamily along with STb, was also observed to partially localize within the nucleus of Hela cells within just 2 hours, with full localization occurring after just 3 hours [131]. Also, in most cases but not all, proteins such as STb which possess a molecular mass less than 40 kDa (6.6 kDa) may diffuse freely through the nuclear membrane pore complex with higher efficiency at lower molecular weights [132]. The question remains, what is the significance of this nuclear localization of STb by the enteroendocrine cell models. As the same trend was observed between all three cell models, the GLUTag L-cells and STC-1 cells shared more resemblance with regards to STb localization compared to the NCI-H716 L-cells. This may be due to various factors, one being that the GLUTag and STC-1 cells are both that of murine origin, whereas the NCI-H716 L-cells are that of human origin.

In the toxicity experiments, both the Neutral Red uptake assay and Rezasurin Reduction assay demonstrated variable toxicity between the cell models studied. In these experiments, only the GLUTag L-cell line was not affected by STb incubation. The toxicity observed in STC-1 and NCI-H716 cells is in agreement with literature on STb's known toxicity on epithelial enterocytes [41], and extends these prior findings to enteroendocrine cell models. 24 hours of toxin incubation was required to cause these toxicity effects. Previous literature on various other cell models has shown a requirement of a 24 hr incubation with the toxin *in-vitro* to show significant cell stress [33]. Also, there was no significant dose response with regards to the 100 and 400 ng/mL toxin treatments, which could be explained by a full saturation of the toxin on the cells.

Future work could employ several lower doses of the toxin to demonstrate a potential dose response of toxicity. In contrast to NCI-H716 and STC-1 cells, no toxicity was seen in the GLUTag L-cell line. This lack of toxicity may be linked to the production and actions of GLP-2. Indeed, GLP-2 has clearly been shown to prevent apoptosis [120] and prevent toxicity from bacterial toxins [111,133]. As the GLUTag L-cell line has been shown to express the GLP-2R, however not expressed within the NCI-H716 human L-cell line [134], a potential autocrine pathway where GLP-2 is both secreted and then activating the GLP-2R pathway could be occurring. Why this resistance to toxicity is occurring in only the GLUTag cells and not the other enteroendocrine cell lines may also be due to the degree of GLP-2 production, or the expression of the GLP2-R. The former was explored in the next experiments.

The ability of this apoptotic toxin to proficiently enhance the secretion of this potent growth hormone from the GLUTag L-cells, while not having any toxigenic effect on the cell, is both eccentric and plausible. A potential link between the GLUTag L-cell's ability to secrete the highest amount of GLP-2 compared to the other cell models in this study, and its unresponsiveness to STb intoxication, may be apparent. Also, the lack of efficiency in GLP-2 section enhancement observed within the NCI-H716 human L-cells may also play a role in its susceptibility. These L-cells were seen to secrete a greater amount of GLP-2 compared to the STC-1 secretin model, however STb enhanced the secretion of GLP-2 from the STC-1 cells at a greater level. The reasoning for this lack of hormone secretion enhancement from the NCI-H716 L-cells may be due to various mechanistic differences which needs further exploration. This apparent relationship needs extensive consideration in order to pin-point the exact target signal mediator responsible for this secretion enhancement.

GLP-2 is known for its antiapoptotic effects [120]. This is distinct from its proliferative effects however, which occurs much later after caspase inhibition signaling [117]. This was apparent once we had recovered the STC-1 cell model from STb intoxication, as cells supplemented with GLP-2 alone with no toxin treatment did not show significant proliferation relative to control. Previous research showing the proliferative effects of the hormone *in-vitro* through GLP-2 supplementation were conducted over a three day span with daily hormone supplementation at a much higher dosage [103]. Our experiment entailed a much shorter time frame with a single initial GLP-2 dosage to a much higher cell count. Further experimentation is essential in order to understand the potential antitoxigenic effects of this potent antiapoptotic growth hormone.

STb receptor presence and characteristics between cell lines can also be an important factor to take into consideration when interpreting these outcomes. The STb receptor, sulfatide, is physiologically abundant within the GI tract and especially in several cancer cell lines and primary colorectal cancer tissues [135]. However, the presence, abundance and role of sulfatide in these endocrine models has not yet been established. However, the presence of sulfatide on other endocrine cells such as beta cells of the pancreases is well established [136]. The differences of abundance of the STb receptor sulfatide between the endocrine models selected for this study may play a crucial role in the differences in effects with regards to toxicity. With regards to GLP-2 secretion, the significance of the sulfatide receptor in this secretion enhancement needs further elucidation.

2.6 Conclusion

In conclusion, STb was able to bind and interact with both L-cell models and the secretin cell line. The GLUTag L-cell line was resistant to STb intoxication even after chronic treatment, while the NCI-H716 human L-cell model and the STC-1 cell lines showed significant

susceptibility. STb was able to significantly enhance the secretion of the GLP-2 growth hormone within the GLUTag L-cells and the STC-1 cells, but only slightly within the human cells. The GLUTag L-cells secreted the highest concentration of growth hormone which could be seen as a potential cause of this strong resistance to intoxication. The NCI-H716 also secreted a significantly higher concentration of the growth hormone compared to the STC-1 cells, however the inability of STb to enhance growth hormone secretion within the human cells could potentially be a cause of their susceptibility. GLP-2 growth hormone supplementation significantly decreased STb-induced mitochondrial activity suppression within STC-1 cells, however had no effect on overall cellular proliferation. We suggest a protective role of these enteroencodrine L-cells which may physiologically enhance their secretion of growth hormone in the presence of bacterial toxins. This study sheds light on the potential of GLP-2 in treating bacterial induced infections such as ETEC infection and STb intoxication.

Chapter 3

3 Extended Discussion and Conclusion

3.1 Potential Experiments

While this work provides some initial evidence for the interaction between STb and GLP-2-producing gut endocrine cells, much of the mechanistic work remains to be done. The immunofluorescent experiments enabled the visualization of this nuclear localization, however these experiments provide a "snapshot" of how STb interacts within the cell. The internalization of the toxin and its migration into the nucleus could have been explored through alternative methods. A live cell imaging approach would have been useful to track its migration over time, accurately demonstrated by McSweeney & Dreyfus *et al* (2004) with regards to the Cdt *E.coli* toxin [131]. Live cell imaging would not only allow us to track the movement of STb into the cell as well as through the nuclear membrane, it would provide a specific time frame for this translocation. A nuclear extraction of STb treated cells is an additional experiment which could compliment these results, confirming the presence of the toxin within the nucleus at various time points. Observing the potential migration and translocation of STb on non-cancerous cell models, such as normal epithelial cell lines or primary culture models, would provide an indication on whether or not this nuclear localization phenomena is distinct only in enteroendocrine cells.

The apoptotic effects of STb, revealed by the Neutral red assay and Resazurin reduction assay, was apparent after 24 hrs of treatment. However, these assays alone could have been accompanied by alternative verification methods, such as measuring intrinsic or extrinsic caspase activity, detecting cell death using mitochondrial markers, or by identifying DNA fragmentation. All of these potential experiments would greatly reinforce the premise of the observed apoptotic actions of the toxin on these cell models. Additionally, non-immortalized epithelial cell lines or

primary intestinal cultures, instead of the immortalized cancer cell models, would have potentially shown more physiology accurate results with regards to the toxins true apoptotic potential. With regards to the GLP-2-STb recovery experiment, additional experiments could have been implemented in order to measure caspase activity on GLP-2 supplemented cells treated with STb compared to cells treated with STb alone. All of these suggested experiments in theory could be very useful in order to fully understand this apparent relationship between STb and GLP-2 secreting L-cells.

Our GLP-2 ELISA revealed a significant enhancement of GLP-2 secretion from GLUTag and STC-1 murine cells were treated with STb. However, this was not the case for the NCI-H716 human L-cell line. Only a very slight enhancement of GLP-2 secretion was observed with the highest dose of STb (400 ng/mL). These results could suggest a species specific effect of hormone secretion enhancement, however additional research is necessary to confirm this. As the NCI-H716 human L-cell model is only human GLP-2 secreting cell line available, it would be difficult to test this hypothesis. However, studies have implemented the use of pig models with regards to human enteroendocrine research, as it has been proposed they are a more applicable model for humans due to more similar hormone expressions compared to rodents [137]. Also, the efficiency of GLP-2 secretion at longer incubation times would have been beneficial to measure, as any decrease in cell viability was mostly observed after 4 hrs of incubation with STb. The capability of cells to secrete GLP-2 may decrease over time, which could play a role in the increased susceptibility over time. This was suggested from our GLP-2-STb recovery experiment, as supplementation with GLP-2 allowed the STC-1 cells to remain viable compared to the control.

3.2 *In-vivo* Implementation

The implementation of theories and results obtained from *in-vitro* experimentation into *in-vivo* conditions is a crucial step in any scientific study. This allows for any *in-vitro* experimental results to be solidified in a physiologically significant condition. With regards to this study, observing the effect of STb on gut health and GLP-2 secretion *in-vivo* would be imperative in order to fully understand the physiological significance of this relationship. Replicating these results within *in-vivo* mouse models would provide good indication of physiological relevance as the *in-vitro* models used were murine as well. *In-vitro*, STb has a clear effect on the STC-1 cell model, as well as on the L-cell models with regards to enhancing GLP-2 secretion. *In-vivo*, these outcomes may differ, potentially revealing entirely new reactions which are more physiologically significant and relevant.

The nuclear uptake of STb observed within the *in-vitro* L-cell models, for example, may be significant only within *in-vitro* conditions within these immortalized enteroendocrine cells. The significance of STb *in-vitro* nuclear localization, and the lack of impact on viability as seen with the GLUTag L-cells, may change *in-vivo*. L-cells within the GI tract are greatly dispersed from one another [120], decreasing free-floating GLP-2 concentrations and allowing for a more significant intoxication from STb. Apart from enteroendocrine cells, the apoptotic effect of STb *in-vivo* on the intestinal epithelium is apparent [75]. Recovering STb-induced degradation of the intestinal epithelium and intestinal villi *in-vivo* through GLP-2 supplementation would be beneficial in order to verify our overall theory of STb-induced apoptosis and GLP-2 recovery. These experiments would also further solidify the use of the GLP-2 growth hormone as a therapeutic measure for ETEC infection as a whole.

With regards to STb-induced GLP-2 secretion enhancement, quantifying this *in-vivo* would be challenging, as mentioned before L-cells are significantly dispersed from one-another in physiological conditions [120]. Measuring free-floating GLP-2 *in-vivo* after STb treatment would not entirely justify enhanced secretion, as increased GLP-2 concentrations may result from L-cell rupture from STb-induced apoptosis. This was not the case within our study, as the toxin had little to no effect on cell viability *in-vitro* on L-cells. However, quantifying gene expression of the proglucagon precursor from harvested gut samples could potentially resolve this issue. Also, measuring GLP-2 from plasma may also be a more reliable consideration. Thus, the implementation of *in-vivo* models for this study is imperative in order to justify the use of this intestinal replenishing hormone for ETEC related therapies.

3.3 Experimental Limitations

There were several limitations which arose during the course of this research. Using immortalized cancer cell models can always be a limitation when measuring metabolic actions such as GLP-2 secretion after treatment. Cancer cell models can undergo significant genetic changes under culture conditions, associated with differential gene expression activation, resulting in marked alterations in proliferation and cellular morphology [138]. These changes can significantly alter the response to various treatments [138], or in our case with STb, potentially skewing results. Thus it is crucial to follow proper cell culturing methodology in order to reduce the occurrence of genetic mutations. With regards to the production and secretion of the hormone, we had noted discrepancies in the efficiency of hormone secretion as higher passages were reached. Also, the responsiveness to STb with regards to enhancing GLP-2 secretion shared displayed some inconsistency at higher passages. It is known that cell models which reach high passages can experience alterations in protein expression as well as responses to stimuli

compared to cells at a lower passage [139]. Replicating results may become an issue due to this, thus it was crucial within our experiments to maintain healthy cell passages in order to obtain the most reproducible results.

Another limitation with regards to this study is the lack of validated GLP-2R antibodies for immunofluorescent visualization. Studies have been able to detect the presence of the GLP-2R so far only though means of gene expression, with no validated immunofluorescent images. Observing the localization and density of the GLP-2R through immunofluorescence would greatly benefit research within this area. As it would not only confirm the presence of the receptor on cells in a timely manner, but would also allow a plethora of additional research to be conducted such as receptor presence and density after treatment with various substances. With regards to our experiments, studies have shown that the GLUTag and NCI-H716 do express the GLP-2R receptor [134], however it has not yet been confirmed within the STC-1 cells. Possessing a valid GLP-2R would allow for efficient observation and comparison of GLP-2R expression, localization and density between the three cell models and would further validate our results with regards to GLP-2's replenishing effects.

3.4 Unanswered Questions

Several questions had arisen throughout this study which remain unanswered. Without actually conducting the experiments, one is left to only speculate based on previous literature within the particular area of study. First, the ability of STb to localize within the nucleus of the enteroendocrine models within a considerably short time frame gives rise to numerous questions. One may ask how does a toxin, which does not possess a nuclear localization sequence (NLS) within its amino acid sequence, become internalized and translocate within the nucleus with such proficiency. Establishing whether or not this is a true toxin nuclear localization, and not from

external factors such as experimental manipulation, is crucial to solidify these observations. Experimental manipulation may include fixation and washing of the cells, which may have had an effect on the permeability of the cells and allowed for a more accessible translocation.

Another compelling area which lacked clarification was the lack of proliferation of enteroendocrine cells in response to GLP-2 growth hormone supplementation. There has been plentiful evidence of the proliferative actions of the GLP-2 hormone *in-vivo* and *in-vitro* either by enhancing cell proliferation, enhancing tissue mass and density and inhibiting cellular apoptosis [103,110,111,117–120]. However, the idea that GLP-2 can have a proliferative effect on L-cells themselves is an area which has not yet been researched. It is clear that GLP-2 bestows its proliferative effects through GLP-2R binding on L-cells, the lamina propria and enteric neurons, which then initiate secondary mediators to apply indirect proliferative actions [99,100]. It is also clear that GLP-2 has been shown to directly act upon various enterocytes *in-vitro* to enhance proliferation independent of L-cell, lamina propria and enteric neuronal initiation and secondary mediation [103]. In this particular study, Jasleen *et al*., (2002) however did not determine whether or not the chosen enterocyte cell lines possessed the GLP-2R, but proposed a multiple GLP-2R subtype theory which would allow for direct GLP-2 actions on enterocytes [103]. In the case of our cellular models, our data suggested a more anti-apoptotic or anti-toxigenic effect by GLP-2, rather than potent proliferation. However, with the use of a modified GLP-2 analog such as the medical grade Teduglutide, which possesses a half-life 25x that of natural GLP-2 [89], we may have seen proliferative characteristics from L-cells. Also, increasing concentrations of the hormone and increasing dosage frequency and incubation times may have also been beneficial in order to gain a proliferative effect from the hormone.

Finally, the significance of the apparent similarities between the cellular pathways of GLP-2 production and secretion and known STb cell signaling pathways is a wide-ranging area of the research which needs greater attention. Also, the significance of each pathway alone with regards to L-cell physiology and functionality needs further examination. The fact that STb was able to enhance the secretion of the GLP-2 hormone from the GLUTag L-cell line as well as the STC-1 secretin cell line suggests a homologous action of STb between cell types. This also may suggest the use of a secondary mediator, whether it is Ca^{2+} influx or one of the activated downstream targets shared between the STb signaling and GLP-2 production and secretion, which may also be similar between the different cell types. However, signaling mediators may also be different between the secretin and L-cell types, which would indicate similar actions of secretion enhancement, however different secondary mediators. These potential differences in mediator selection by STb signaling between cell types may be influenced by several factors such as differences in protein expression of mediators and substrate protein expression. For example, PKC has been shown to possess numerous functions throughout several cell types. This can include mediating contractions of smooth muscle cells within the GI tract, or neuronal excitation in neurons of the CNS, all of which are mediated by specific ligands binding to specific receptors within the specific cell types [140,141]. This suggests there may also be potential differences between the expression and significance of similar mediators in both pathways. The lack of specific ligand and receptor expression needed in one cell type could initiate other similar mediators within the pathways to enhance GLP-2 secretion, giving the same end result in both cell types. Answering all of these questions would greatly advance the spectrum of knowledge on bacterial toxins and their effects on L-cell physiology and growth hormone secretion.

3.5 Conclusion

To conclude, this paper detailed the literature on the known toxigenic effects of STb. Its potential relation and risk to individuals with T2DM and obesity was discussed, as well as the intestinal GLP-2 growth hormone and its opposing effects to STb intoxication. From our study, it was found that endocrine cells which secreted the highest amounts of GLP-2 were more resistant to STb intoxication. STb was found to enhance the secretion of GLP-2 in a non-cell specific manner which may be linked to numerous similarities in cellular mechanisms of GLP-2 production and secretion and STb cell signaling pathways. GLP-2 supplementation inhibited STb intoxication, however had no effect on cell proliferation. Many areas of this research were discussed thoroughly, ultimately leading to several questions which need elucidation. Future work within this field of research must be extensive, as many potential outcomes and differences were apparent after the fact. This work will shed light on the intricate nature of this bacterial toxin and its inter-species effects in several areas of action. Eventually, this research may collectively support the idea and use of GLP-2 hormone therapies in enterotoxigenic disease related endeavors.

3.6 References

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