BUTYRATE-INDUCED EXPRESSION OF PROGLUCAGON: IMPLICATIONS FOR ENTEROENDOCRINE SIGNALING AND INTESTINAL GROWTH

BY

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DISSERTATION

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ABSTRACT

While the gastrointestinal tract is primarily recognized for its digestive and absorptive capacity, it is also the largest endocrine organ in the body. There are a number of different types of endocrine cells in the gut which have the ability to mediate nutrient-gene interactions. Of interest for this research is the L cell of the distal ileum and colon which expresses the proglucagon gene. Proglucagon encodes a number of important hormones, namely glucagon-like peptide 1 (GLP-1), glucagon-like peptide 2 (GLP-2), glicentin, and oxyntomodulin and thus may impact intestinal development and dysfunction, type 2 diabetes, and obesity. Evidence suggests that fibers and short-chain fatty acids (SCFAs), particularly butyrate, are classes of nutrients capable of regulating the proglucagon gene. To examine this, we treated enteroendocrine L cells with butyrate and found increased proglucagon mRNA abundance. However, the mechanism by which butyrate affects proglucagon was less understood. Therefore, we isolated the human proglucagon gene promoter and treated with butyrate, revealing that butyrate regulates proglucagon transcription by activating the promoter. In addition, it has been suggested that butyrate may be interacting with receptors or transporters to stimulate its effects. To examine this, we silenced the SCFA receptor GPR43 and the bitter taste receptor T2R38 and revealed that butyrate no longer had the capacity to stimulate increased proglucagon abundance. Additionally, we also examined the abundance of SCFA transporters and noted increases in SLC5A8 in the ileum and MCT-1 in the colon following supplementation of fermentable fibers into the diet. Further interaction between butyrate and proglucagon was explored by infusing SCFAs and butyrate into the lumen of the ileum and colon. The greatest effects were noted in aspects of intestinal structure, with changes noted in crypt-villus

architecture, DNA, RNA, and protein concentrations throughout the intestine. These improved structural parameters corresponded with increased proglucagon abundance, suggesting GLP-2 was likely mediating these adaptations to the nutrient. Thus, the provision of the SCFA butyrate may be particularly important and beneficial to patients suffering with intestinal dysfunction, type 2 diabetes, and obesity by upregulating proglucagon expression and ultimately stimulating greater hormone release.

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LIST OF ABBREVIATIONS

AE	Anion exchanger
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
BW	Body weight
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CD	Crohn's disease
cDNA	Complimentary deoxyribonucleic acid
CRE	Cyclic adenosine monophosphate response element
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPP-IV	Dipeptidyl peptidase IV
DSS	Dextran sodium sulfate
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
FOS	Fructooligosaccharide
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide 1
GLP-1R	Glucagon-like peptide 1 receptor
GLP-2	Glucagon-like peptide 2
GLP-2R	Glucagon-like peptide 2 receptor
GLUT2	Basolateral sodium independent glucose transporter
GRP	Gastrin-releasing peptide
GRPP	Glicentin-related pancreatic peptide
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate

IBD	Inflammatory bowel disease
IF	Intestinal failure
IFN-γ	Interferon-gamma
IL	Interleukin
KO	Knockout
MAPK	Mitogen-activated protein kinase
MCT	Monocarboxylate transporter
MPGF	Major proglucagon fragment
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MSH	Melanocyte-stimulating hormone
NCBI	National Center for Biotechnology Information
NHE	Sodium-hydrogen exchanger
NS	Not significant
PBS	Phosphate-buffered saline
PC	Prohormone convertase
PCR	Polymerase chain reaction
PGDP	Proglucagon-derived peptide
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PN	Parenteral nutrition
POMC	Pro-opiomelanocortin
PYY	Peptide YY
RLU	Relative light unit
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SBS	Short bowel syndrome
SCFA	Short-chain fatty acid

SEM	Standard error of the mean
SGLT-1	Brush border sodium dependent glucose transporter
siRNA	Small interfering RNA
SMCT	Sodium-coupled monocarboxylate transporter
SNP	Single nucleotide polymorphism
SPS	Soy polysaccharides
TNBS	Trinitrobenzene sulfonic acid
TNF-α	Tumor necrosis factor-alpha
TPN	Total parenteral nutrition
UC	Ulcerative colitis
VEGA	Vertebrate Genome Annotation database
VIP	Vasoactive intestinal peptide
5-HT	5-hydroxytryptamine

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

While the gastrointestinal tract is primarily recognized for its ability to digest and absorb nutrients, there are actually numerous endocrine cells located throughout, together comprising the largest endocrine organ in the body. Specifically, this includes the D, enterochromaffin, and G cells of the stomach, the K, I, S, and M cells in the duodenum and jejunum, and the N and L cells in the ileum and colon. Not only do these cells secrete hormones that are essential for stimulating digestive enzymes such as gastrin, cholecystokinin, and secretin, but they also produce hormones such as GLP-1 that interacts with the pancreas to stimulate insulin secretion and the brain to increase satiety, motilin which activates the migrating motor complex in the gut, and PYY that acts as a part of the ileal brake mechanism to slow transit time and maximize absorption. Within the intestine, the majority of these cells are "open" endocrine cells; that is, they are in direct contact with the lumen. Thus, they are releasing hormones in response to the nutrient load present, both proximally and distally in the gut. The overall goal of this literature review is to examine the endocrine events mediated by the distally derived proglucagon-derived peptides (PGDPs) and short-chain fatty acids (SCFAs).

PROGLUCAGON

The human proglucagon gene, also called the glucagon or preproglucagon gene, consists of 9.4 kilobases, with 6 exons and 5 introns (White and Saunders, 1986) and is located on chromosome 2 (Tricoli et al., 1984). Initially studied due to its encoding of glucagon, much has

been learned regarding a number of other biologically active peptides the gene codes for including glucagon-like peptide 1 (GLP-1), glucagon-like peptide 2 (GLP-2), glicentin, and oxyntomodulin.

Proglucagon Gene Structure

The human proglucagon gene was first isolated and sequenced in 1983 by Bell and associates (Bell et al., 1983). It is comprised of 6 exons encoding the following: Exon 1 is the 5' untranslated region, Exon 2 contains a portion of glicentin related pancreatic peptide (GRPP), Exon 3 is the remaining GRPP sequence and glucagon, Exon 4 encodes GLP-1, Exon 5 makes up GLP-2, and Exon 6 is the 3' untranslated region (Bell et al., 1983; Heinrich et al., 1984; White and Saunders, 1986).

Research revealed that these peptides appear in a number of locations, including the pancreas, intestine, and brain. Through the use of antisera and complementary peptides, glucagon was isolated primarily to the pancreas, GLP-2 to the intestine, and GLP-1 in both the pancreas and intestine (Mojsov et al., 1986). With differing peptide originations, it suggests one of the following possibilities: either that there are, in fact, two similar genes being activated with specific peptides produced from each gene or that a single gene encodes for all of the peptides and that liberation of specific proteins was based on the location in the body in which it was occurring. Evidence supported the idea of one gene, with studies showing that all six exons were identically expressed in both islet and intestinal cells (Mojsov et al., 1986). Additionally, the same transcriptional start site was used in both the pancreas and colon (Novak et al., 1987). Thus, it appeared that there is a tissue-specific response to physiological factors, specifically post-translational processing.

Post-translational Processing

Early studies found that the glucagon, GLP-1, and GLP-2 exons code for basic amino acids pairs located at both ends of the peptide, which commonly serve as digestion sites in prohormone processing (Mojsov et al., 1986). The human prohormone proglucagon peptide consists of 160 amino acids and is cleaved as the prohormone moves through the secretory pathway, releasing peptides which are then packaged into vesicles and released upon stimulation (Heinrich et al., 1984). Initial studies using the pancreatic α cell line αTC1-6 revealed the role of the prohormone convertase PC2 in the accumulation of the major proglucagon fragment (MPGF) and ultimately glucagon (Rouille et al., 1994). Additionally, the use of GH3 and AtT-20 endocrine cells transfected with PC1/3-containing vectors resulted in release of glicentin, oxyntomodulin, GLP-1, and GLP-2, but not glucagon (Dhanvantari et al., 1996). *In vivo* data were consistent with these findings as PC2 null mice suffer with glucagon deficiency while PC1/3 null mice show little expression of intestinal PGDPs (Furuta et al., 2001; Ugleholdt et al., 2004). Together, these results indicate that the same proglucagon prohormone peptide is present in both the intestine and pancreas and that tissue-specific posttranslational processing occurs, with PC1/3 releasing glicentin, oxyntomodulin, GLP-1, and GLP-2 within the intestinal L cells and PC2 liberating MPGF and glucagon within the pancreas (**Figure 1.1**).

Peptide Secretion

After undergoing post-translational processing, the PGDPs are secreted from the intestinal L cells through both direct interactions with nutrients and indirect neural mechanisms. Circulating levels of PGDPs have been reported to increase following consumption of a meal in healthy humans (Vilsboll et al., 2001). This increase occurs in a biphasic manner, with initial

release beginning as early as 15 to 30 minutes after nutrient consumption and a second release occurring after 60 to 90 minutes (Jeppesen et al., 1999; Xiao et al., 1999). Examination of secretion patterns indicates the peptides are secreted from the L cell simultaneously, as both luminal glucose and arterial GRP administration resulted in significant elevations in GLP-1, GLP-2, and glicentin release (Orskov et al., 1986). Therefore, the rate of degradation becomes a significant factor in determining the levels of the PGDPs circulating in the blood and thus their biological activities.

As the L cell is an open enteroendocrine cell type, it has direct access to the luminal contents of the intestine and therefore will respond appropriately to the nutrients present. A number of classes of nutrients have been shown to stimulate PGDP secretion directly, including ileal administration of emulsified fats (Roberge and Brubaker, 1991), glucose (Dumoulin et al., 1998), and hydrolyzed polypeptides (Cordier-Bussat et al., 1998). However, many of the responses were elicited using high concentrations of substrates, and thus secretion in response to these nutrients may be a part of a feedback mechanism during periods of rapid transit and malabsorption. As expected, the L cell also samples and responds to nutrients typically present in the distal ileum and colon such as SCFAs and fermentable fibers, which have also been shown to stimulate secretion of the PGDPs (Plaisancie et al., 1995; Reimer and McBurney, 1996; Dumoulin et al., 1998; Cani et al., 2004).

Luminal administration of nutrients into the duodenum also stimulated rapid release of PGDPs despite the low number of L cells present in this segment of the intestine, indicating a neural mechanism of secretion may also be at work (Roberge and Brubaker, 1991). This effect is likely mediated using the intestinal peptides glucose-dependent insulinotropic peptide (GIP) and gastrin-releasing peptide (GRP). Duodenal fat administration increased plasma GIP as quickly

as 10 minutes following treatment and was associated with 2-fold increases in plasma PGDPs (Roberge and Brubaker, 1993). Similarly, GRP infusion increased plasma PDGP levels (250 ± 18 pg/mL to 321 ± 9 pg/mL), which was repressed in the presence of the GRP antagonist BW10 (Roberge et al., 1996). These results indicate there may be a feed-forward mechanism occurring, stimulating release of the peptides in response to ingested nutrients, despite the fact that they have yet to reach the ileum and colon.

Proglucagon-Derived Peptides

Glucagon-like peptide-1

Produced as GLP-1 (1-37), GLP-1 is quickly truncated to a 30-amino acid peptide which exists as 2 active forms in the body, GLP-1 (7-37) and GLP-1 (7-36 amide) (Drucker, 2007). With a half-life of only 1-2 minutes (Meier et al., 2004), GLP-1 will be quickly metabolized and degraded by the enzyme dipeptidyl peptidase IV (DPP-IV) located in the endothelial cells, releasing the inactive peptides GLP-1 (9-36 amide) and GLP-1 (9-37). Despite the fact that only 10 – 15% of the GLP-1 released from the L cells actually survives degradation and reaches systemic circulation (Holst, 2007), it is well established that GLP-1 has incretin effects and may also play a role in appetite control. Thus, GLP-1 has considerable implications for both type 2 diabetes and obesity.

GLP-1 Receptor

GLP-1 is known to have numerous effects on the body, which are mediated via signaling induced by the GLP-1 receptor (GLP-1R). First isolated in 1992, it is a member of the glucagon family of G-protein coupled membrane receptors and consists of 463 amino acids arranged into

seven hydrophobic segments (Thorens, 1992). The GLP-1R has been localized to various tissues throughout the body including the pancreas, brain, stomach, intestine, heart, lungs, kidneys, and skin of various species including humans, rats, and mice (Wei and Mojsov, 1995; Bullock et al., 1996; Dunphy et al., 1998; List et al., 2006; Tornehave et al., 2008), suggesting a role for GLP-1 beyond the known incretin and appetite effects.

Binding of GLP-1 to its receptor will stimulate an interaction with G proteins, initiating the signaling cascade. The GLP-1R acts through multiple G proteins, including $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{1,2}$ (Montrose-Rafizadeh et al., 1999), G_o, G_{11} (Bavec et al., 2003), and $G\alpha_i/G\alpha_o$ (Hallbrink et al., 2001), ultimately activating adenylate cyclase. Using the RIN 1046-38 rat islet insulinoma cell line, 5 nM GLP-1 (7-37), but not GLP-1 (1-37), increased cAMP levels by 4-fold (Drucker et al., 1987). While cAMP is known to activate PKA, GLP-1 is also able to act independently of the kinase, as suggested by the cAMP-dependent influx of calcium into mouse islets despite the use of numerous PKA inhibitors (Bode et al., 1999). In turn, a number of other secondary messengers are activated, including MAPK, PKC, Wnt signaling, Akt, and GSK3, which ultimately impact insulin secretion and satiety signals in the body.

Incretin Effect of GLP-1

Incretins are hormones produced within the gastrointestinal tract that stimulate insulin secretion from the pancreas. Although known to be produced in the intestine, the precursor GLP-1 (1-37), the active hormone GLP-1 (7-37), and the GLP-1 receptor can be localized to the pancreas as well, suggesting a potential role there. Upon binding its receptor in the pancreas, GLP-1 is believed to stimulate increased cAMP levels, depolarization of the cell, calcium influx, and ultimately insulin secretion via exocytosis. Indeed, when GLP-1 was perfused into a rat

pancreas at physiological concentrations for 6 minutes, insulin release quickly increased by 20-fold (Mojsov et al., 1987). In isolated pancreatic islets, use of the GLP-1R antagonist exendin (9-39) amide reduced promotion of glucose-stimulated insulin secretion more than 2-fold (Flamez et al., 1999). Furthermore, in GLP-1R KO models, plasma insulin levels were reduced by 34 - 43% following a glucose challenge, a result of decreased insulin release in both the first and second phases of secretion (Preitner et al., 2004). In addition, studies have also discovered that islets from GLP-1R KO mice display reduced intracellular cAMP levels (Flamez et al., 1999), thus revealing the importance of changes in intracellular signaling induced by GLP-1 binding in insulin secretion.

However, GLP-1 not only stimulates insulin secretion, but also affects the beta cell, ultimately increasing insulin production. Numerous studies have revealed GLP-1 to increase pancreatic beta cell mass through both hypertrophic and hyperplastic mechanisms (Xu et al., 1999; Perfetti et al., 2000; Stoffers et al., 2000). Additionally, GLP-1 is also capable of inducing differentiation of stem cells into beta cells. Using primate embryonic stem cells, treatment with GLP-1 transformed the cells into insulin-producing cells (Yue et al., 2006). Along with increased proliferation by 1.4-fold, GLP-1 has also been shown to reduce the number of pancreatic apoptotic cell bodies by 3.6-fold in a diabetic rat model (Farilla et al., 2002). More specifically, *in vitro* analysis revealed GLP-1 to prevent apoptosis by activating anti-apoptotic proteins, including a near doubling in both Bcl-2 and Bcl-xL expression via a cAMP-dependent signaling pathway (Hui et al., 2003). With a greater number of functional beta cells, insulin production would be expected to increase. Indeed, *in vitro* studies have revealed that transfection of the portion of the proglucagon gene encoding for GLP-1 into MIN6 cells significantly increased insulin mRNA transcripts as well as insulin protein secreted from the

cells in a glucose concentration-dependent manner (Hui et al., 2002). These findings are likely the result of increased PDX-1 expression (Stoffers et al., 2000; Hui et al., 2002), a transcription factor important for insulin promoter activation, as well as increased GLUT2 transporter and glucokinase enzyme expression (Wang et al., 1997), both of which are important in glucose sensing and insulin secretion mechanisms within the beta cell. Taken together, these studies reveal a definite incretin role of GLP-1 and thus provide a therapeutic target for type 2 diabetes treatment.

GLP-1 and Appetite Control

As GLP-1 is a gastrointestinal hormone produced in response to nutrient stimulation, it is not surprising that the hormone would also respond to the nutrient load by affecting appetite and energy intake. Within the gastrointestinal tract, GLP-1 is believed to be a part of the ileal break mechanism as it decreases gastric acid secretion (Schjoldager et al., 1989b), gastric emptying (Naslund et al., 1999), and gastrointestinal motility in the stomach, duodenum, and jejunum (Hellstrom et al., 2008), ultimately slowing nutrient transport through the gut to maximize absorption. These effects are thought to be mediated through neural mechanisms as vagal afferent denervation prevented GLP-1 from decreasing gastric emptying (Imeryuz et al., 1997), raising the possibility that GLP-1 is involved in a gut-brain interaction.

Indeed, while intestinally-derived GLP-1 can enter into the brain (Orskov et al., 1996), the proglucagon gene is also expressed and processed in the brain as well (Drucker and Asa, 1988). Recent studies using brain imaging have revealed that elevated GLP-1 plasma concentrations were associated with increased neuronal activity in the hypothalamus and the prefrontal cortex, areas that are known to play a role in eating behavior (Pannacciulli et al.,

2007). Intravenous administration of GLP-1 (50 pmol/kg/hour) into humans significantly increased satiety and fullness and decreased hunger following consumption of a meal while also reducing food intake by 12% at the next meal (Flint et al., 1998). Furthermore, when participants consumed a meal following a galactose and guar gum challenge, GLP-1 concentrations were significantly elevated (13.7 vs. 7.3 pmol/l) and were associated with increased satiety in patients (Adam and Westerterp-Plantenga, 2005), which may be due to the ability of fiber to both slow gastric emptying and be fermented to SCFAs, stimulating GLP-1 release and interaction with the brain. Likely as a result of the brain-mediated reduction in food intake and increased satiety, numerous studies have reported weight loss with GLP-1 administration (Rodriquez de Fonseca et al., 2000; Larsen et al., 2001; Raun et al., 2007). Thus, these findings indicate a potential role for GLP-1 in obesity and weight loss therapy.

Clinical Applications of GLP-1

With such promising incretin and energy intake effects seen both *in vivo* and *in vitro,* GLP-1 and its analogs have great therapeutic potential in the treatment of both type 2 diabetes and obesity. When native GLP-1 (7-36) amide was continuously infused for 6 weeks in type 2 diabetes patients, plasma blood glucose levels dropped, corresponding to a decrease in hemoglobin A_{1C} levels at the end of the study (GLP-1 group started at 9.2% and dropped to 7.9% compared to the saline group that started at 8.9% and remained unchanged at 9.1%). Additionally, GLP-1 patients decreased body weight throughout the study, resulting in a 1.9 kg weight loss whereas saline had no effect on body weight (Zander et al., 2002).

However, due to the short half-life of native GLP-1, numerous GLP-1 analogs are currently being investigated, such as exenatide, which have homology to the endogenous GLP-1

peptide and thus bind to the GLP-1R, but with substitutions in the amino acid sequence resulting in DPP-IV resistance. Exenatide is a synthetic form of exendin-4, a naturally occurring GLP-1 homolog isolated from the saliva of the Gila monster (Raufman, 1996). When combined with metformin treatment in obese type 2 diabetics for 30 weeks, injections of exenatide twice daily reduced the hemoglobin A_{1C} to less than 7.0% in 46% of patients and significantly reduced body weight (DeFronzo et al., 2005). Similar results were reported when exenatide was combined with sulfonylureas or with both metformin and sulfonylureas (Buse et al., 2004; Kendall et al., 2005). In addition to development of GLP-1 analogs, DPP-IV inhibitors are also being investigated, such as sitagliptin. By competitively inhibiting the degradation enzyme, circulating GLP-1 remains active longer, stimulating greater incretin effects. Indeed, patients treated with sitagliptin once daily for 24 weeks reported improved fasting and postprandial blood glucose levels and a significant reduction in hemoglobin A_{1C} levels as well as improved beta cell function and insulin levels (Aschner et al., 2006).

Clearly, GLP-1 may have great therapeutic potential and thus further research is warranted into its functions and capabilities in the body. While many of these studies have focused on exogenous administration of GLP-1, we also know that GLP-1 is normally produced in the body and is likely regulated by nutrient content in the intestine. Our research into the regulation of the proglucagon gene will expand our knowledge of PGDP production and secretion and may provide new insight into regulating GLP-1 concentrations using dietary interventions.

Glucagon-like peptide 2

GLP-2 is a 33-amino acid peptide that has intestinotrophic effects, which were first reported by Drucker and colleagues as tumors secreting PGDPs stimulated intestinal growth in mice (Drucker et al., 1996). With a half-life of only 6-7 minutes (Hartmann et al., 2000), GLP-2 will be quickly metabolized and degraded by the enzyme DPP-IV, releasing the inactive peptide, GLP-2 (3-33). While the majority of circulating peptide will be in its inactive form, it is well established from *in vivo*, *in vitro*, and human clinical trials that GLP-2 can stimulate both structural and functional adaptations in models of intestinal injury. Thus, GLP-2 has considerable implications for individuals with intestinal compromise, such as intestinal failure and inflammatory bowel disease.

GLP-2 Receptor

While GLP-2 is known to have trophic effects, it is not well understood how exactly GLP-2 induces these effects within the body. However in 1999, Munroe and colleagues isolated rat and human GLP-2 receptors (GLP-2R), noting the presence of higher RNA levels located within the duodenum, jejunum, ileum, colon, and stomach (Munroe et al., 1999). These were the primary locations in which GLP-2 effects were seen, suggesting a receptor-mediated response.

The GLP-2R belongs to family B of the glucagon G-protein coupled receptor superfamily, consisting of a 7-transmembrane domain with a hydrophobic N-terminal region (Munroe et al., 1999). Upon detection of the receptor throughout the segments of intestine, numerous studies have attempted to identify where exactly these receptors are located and thus how it relates to the intestinotrophic properties of the peptide. In rats and humans, early work determined that GLP-2R mRNA tended to be localized in the lower portion of the pyloric antrum

in the stomach and at the base of the villus epithelium and along the crypt-villus axis in the intestine (Yusta et al., 2000b). This pattern resembled that of endocrine cells in the GI tract, which was confirmed through cell staining. However, studies in other species have questioned these findings. In mice, the GLP-2R was found in the in the neurons of the small and large intestine, but not within the epithelial tissue as suggested previously (Bjerknes and Cheng, 2001). Further research in the area by Guan and associates may have resolved the discrepancies, though, as receptor mRNA was located in both enteroendocrine cells of the villus and crypt epithelium and enteric neurons of the submucosal and myenteric plexus in pigs and humans (Guan et al., 2006).

With receptors localized in enteroendocrine and neuronal cells, it raises the question of direct versus indirect signaling involved in GLP-2 action. With enteroendocrine cells playing a role, GLP-2 effects could be indirect to a certain degree, with other factor(s) being released and stimulating the intestinal responses reported in the literature. However, hormones such as gastrin, secretin, and somatostatin could not be positively linked to GLP-2 receptor-containing cells (Yusta et al., 2000b). Further examination did link PYY, GLP-1, GLP-2, 5-HT, VIP, and eNOS to receptors, though, suggesting a specific role for the compounds in GLP-2-mediated events (Yusta et al., 2000b; Guan et al., 2006). Thus, the effects could be, at least partially, due to activation of other endocrine and neuronal cells and the subsequent release of the above mentioned compounds.

Much work has also been done in an attempt to determine the signaling and regulation involved with activation of the GLP-2R, particularly with apoptotic pathways. Early work has shown that activation of GLP-2R resulted in increased levels of cAMP (Munroe et al., 1999). Therefore, the role of cAMP-dependent pathways in GLP-2 responses has been further explored.

When cell injury and apoptosis were chemically-induced with cycloheximide, GLP-2 again increased cAMP levels and improved cell survival in a protein kinase A (PKA), mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K)-independent manner (Yusta et al., 2000a). Moreover, when glycogen synthase kinase-3 (GSK3), a downstream target of PI3K/Akt and cAMP/PKA pathways, was examined by inhibiting the PI3K pathway, inhibition of GSK3 prevented apoptosis in a PKA-dependent mechanism (Yusta et al., 2002). These two studies from Yusta and associates provide conflicting evidence, as a chemically-induced apoptosis is prevented in a PKA-independent mechanism while the PI3K inhibition reduces apoptosis in a PKA-dependent manner. This establishes the possibility that there are multiple GLP-2R-mediated signaling pathways involved in preventing apoptosis, which are dependent on the apoptosis stimulus itself.

In addition to inhibition of apoptosis, cellular proliferation mechanisms induced by GLP-2 have also been examined. GLP-2 has been shown to enhance thymidine uptake and thus DNA synthesis with increasing levels of cAMP in a PKA-dependent manner (Walsh et al., 2003). However, Rocha and colleagues reported cAMP levels decreased with GLP-2 treatment resulting in increased cellular proliferation (Rocha et al., 2004). Again, these results are in contrast with one another, particularly with the decreased cAMP levels. In the majority of the GLP-2 receptor signaling studies, including both apoptosis and proliferation effects, cAMP levels rose in the cells upon treatment with GLP-2, suggesting that the actions of GLP-2 are dependent on stimulating increases in cAMP within the cell. As with the apoptotic pathways discussed previously, this raises the possibility that there are multiple pathways in which GLP-2 is trophic to the intestine. Further research is needed to determine the exact role of these factors in order to gain a greater insight into GLP-2-mediated responses.

GLP-2 and Intestinal Development

Given the intestinotrophic nature of GLP-2, it is not surprising that it may also play a role in fetal and neonatal intestinal development. Lovshin and colleagues detected GLP-2 in both fetal and neonatal rat intestine, with plasma GLP-2 levels 8-fold higher in 12-day old rats than in adult rats, which gradually decreased as the animals aged; additionally, the GLP-2 receptor was also found in the fetal and neonatal intestines (Lovshin et al., 2000). Similarly, GLP-2 was identified in the plasma of fetal and neonatal piglets; however, exogenous GLP-2 administration had trophic effects in neonates, but not the fetuses (Petersen et al., 2001). Indeed, in humans, GLP-2 has been found in the umbilical cord blood from healthy full term infants while both fasting and feeding plasma GLP-2 levels were significantly higher in premature infants as compared to adults (Bode et al., 2007; Amin et al., 2008). While the relationship between GLP-2 and intestinal development has not been directly established, these findings indicate a potential role for GLP-2 in the normal developmental processes occurring prior to and shortly after birth.

GLP-2 and Intestinal Injury

Along with intestinal maturation, GLP-2 has shown to have reparative functions in multiple models of intestinal injury including short bowel syndrome (SBS), total parenteral nutrition (TPN) -induced mucosal atrophy, and inflammatory bowel disease (IBD). These intestinotrophic effects are both structural and functional in nature, with changes in morphology and functional capacity reported. Indeed, early research revealed that the presence of proglucagon-producing tumors stimulated increases in intestinal weight due to increased villus height throughout the small bowel (Drucker et al., 1996). Moreover, rats infused with GLP-2 improved intestinal functionality, displaying a 2-fold increase in GLUT2 abundance, a 53%

increase in fructose uptake (Au et al., 2002), and improved glucose uptake via SGLT-1 by increasing its expression in the brush border membrane (Cheeseman, 1997). With enhancement of both structural and functional parameters in the intact intestine, it raises the possibility that GLP-2 administration may be particularly important in adaptation following intestinal compromise.

In adults, SBS is defined as having less than 200 cm of intact small intestine (Buchman, 2006), which oftentimes results in nutrient malabsorption and deficiencies due to a reduction in functional absorptive surface area. However, intestinal adaptation can occur, particularly when driven by luminal nutrients and various humoral factors, including GLP-2. Indeed, human infants with intestinal dysfunction due to necrotizing enterocolitis, atresias, and volvulus display a correlation between remaining intestine and GLP-2 concentrations, with GLP-2 concentrations being a predictor of weaning from TPN (Sigalet et al., 2004), indicating a potential role for GLP-2 in the adaptive response.

In animal models of SBS, GLP-2 administration has shown to be advantageous after resection. Following a 90% resection in rats, 7 days of intravenous GLP-2 increased villus height and crypt cell proliferation and reduced intestinal permeability (Martin et al., 2004). Similarly, GLP-2 administration with a 60% jejunoileal resection in rats increased duodenum and jejunum mucosal mass, DNA, and protein, with synergistic effects in each when combined with enteral nutrition (Liu et al., 2006). These adaptive processes may be mediated through the ability of exogenous GLP-2 to stimulate further increases in proglucagon mRNA and the GLP-2 receptor following resection (Koopmann et al., 2008). Thus, these results indicate that GLP-2 will enhance structural adaptation, ultimately increasing the absorptive surface area of the residual bowel.

While the bowel is capable of adapting following resection, management of SBS oftentimes requires parenteral nutrition (PN) to meet the patients' nutritional needs. However, while it prevents nutritional deficiencies, PN becomes problematic as it further atrophies the intestine due to a lack of luminal stimulation through the intestine. Despite this, GLP-2 therapy may be beneficial in reversing PN-induced mucosal atrophy. In piglets receiving both PN and enteral nutrients, improvements in intestinal architecture were reported and correlated with increased endogenous plasma GLP-2 levels as well as proglucagon mRNA (Burrin et al., 2000; Dahly et al., 2003), indicating a therapeutic potential for GLP-2 during PN treatment. Similarly, in neonatal piglets receiving TPN, intravenous GLP-2 increased villus height and crypt depth by suppressing apoptosis and stimulating cell proliferation, resulting in an overall increased small intestinal mass by approximately 50% compared to controls (Burrin et al., 2005).

In addition to increasing the epithelial architecture, nutrient absorption will also be improved with GLP-2 despite the lack of enteral nutrition. Following 7 days of TPN, piglets receiving GLP-2 had improved absorptive capacity, as demonstrated by greater glucose (58 vs. 31% intake) and galactose (52 vs. 27 % intake) absorption compared to controls (Cottrell et al., 2006). Similarly, the absorptive capacities for glucose, leucine, proline, and lysine were 50% higher along the small intestine in GLP-2-treated piglets compared to their TPN controls (Sangild et al., 2006). These improvements in functional capacity may also be accompanied by improvements in circulation, as GLP-2 treatment increased duodenal and jejunal blood flow in TPN-fed piglets (Stephens et al., 2006). Therefore, GLP-2 appears to counteract the atrophy effects of TPN by stimulating structural changes that are translated into improved nutrient absorption.

One of the conditions oftentimes resulting in SBS is IBD, which consists of 2 related gastrointestinal disorders, ulcerative colitis (UC) and Crohn's disease (CD). While the underlying causes of IBD are poorly understood, these mediators stimulate increased pro-inflammatory cytokines, ultimately resulting in tissue injury and decreased function. However, GLP-2 may exert its trophic effects as well as anti-inflammatory properties, improving patients' outcomes. Indeed, in human patients suffering with IBD, circulating GLP-2 concentrations are significantly elevated as compared to healthy individuals, with a 229% and a 317% increase in GLP-2 concentration reported in UC and CD patients, respectively (Xiao et al., 2000). In DSS-treated mice, administration of a human GLP-2 analog, [Gly^2]GLP-2, improved colitis, with elevated crypt cell proliferation leading to significant increases in intact mucosal surface area (Drucker et al., 1999).

Along with its trophic properties, GLP-2 may also be responsible for anti-inflammatory responses in the intestine. Exogenous GLP-2 administration in a rat IBD model reduced mucosal lesions by 90% and was associated with decreased TNF-α and IFN-γ expression to negligible levels (Alavi et al., 2000). Additional studies suggest GLP-2 activates vasoactive intestinal polypeptide (VIP), which may be responsible for mediating the anti-inflammatory events. Inhibition of the VIP receptor hindered GLP-2-mediated effects, with increased body weight loss, myeloperoxidase (MPO) activity, and crypt cell apoptosis reported (Sigalet et al., 2007). Furthermore, in the presence of active VIP, GLP-2 will reduce mucosal cytokine production including TNF-α, INF-γ, IL-1β, and IL-10 in a colitis model (Sigalet et al., 2007). Together, these results indicate that GLP-2 administration reduces pro-inflammatory mediators of IBD and stimulates cell proliferation, ultimately resulting in mucosal healing.

Clinical Applications of GLP-2

With such promising results *in vivo*, there is much hope that GLP-2 therapy can be applied to patients suffering with intestinal dysfunction. Indeed, a human GLP-2 analog called teduglutide is currently in Phase III clinical trials in SBS patients. Teduglutide, also known as [Gly^2]GLP-2 or ALX-0600, is identical to endogenous GLP-2 except that a glycine has replaced the alanine residue at position 2 in the protein, resulting a molecule that is resistant to DPP-IV degradation (Ferrone and Scolapio, 2006). In healthy individuals, subcutaneous teduglutide injections were shown to be safe and well tolerated, with only mild adverse effects reported (Marier et al., 2008). Importantly, when administered subcutaneously to patients with PN-dependent intestinal failure for 21 days, teduglutide increased wet weight absorption, decreased fecal energy excretion, and improved intestinal structure, with increased villus height and crypt depth reported (Jeppesen et al., 2005). Clearly, these positive results warrant further studies to gain a better understanding of teduglutide's effects and application to gastrointestinal disorders.

Together, the *in vitro*, *in vivo*, and human clinical data indicate GLP-2 may have great potential for modulating intestinal development and adaptation. While exogenous administration of the peptide has stimulated improvements in structure and functional capacity, we also believe that the body uses endogenous GLP-2 to stimulate intestinal repair as evidenced by increased circulating concentrations during intestinal compromise. Thus, further research into the therapeutic potential of GLP-2 is necessary, including regulation of endogenous GLP-2. In particular, our research exploring the proglucagon gene will further our understanding GLP-2 regulation and potentially provide nutritional strategies for activating its production in the L cells.

Glicentin

Glicentin is a 69-amino acid peptide believed to have limited trophic effects within the intestine. Although the half-life of glicentin is currently unknown, it is believed to have a longer survival time than other proglucagon-derived peptides, as glucagon-like immunoreactive materials display a half-life of 15.9 minutes (Tanaka et al., 1979). Glicentin may be capable of stimulating intestinal adaptation, but its effects seem to be specific to the proximal intestine. In rats receiving a 70% resection, glicentin administration for up to 28 days stimulated increased duodenal weight, protein content, and mucosa weight (Hirotani et al., 1998; Hirotani et al., 2000). When maintained on an elemental diet, comparable effects were reported in the rat jejunum following 2 weeks of glicentin treatment, but no effects were seen in the the ileum (Myojo et al., 1997). Similarly, in a rat model employing jejunal and ileal Thiry-Vella fistulas, 7 days of glicentin treatment increased jejunal, but not ileal, mucosal weight and protein content within the fistula (Hashimoto et al., 2003). Models of TPN-induced mucosal atrophy demonstrated similar trends, again with glicentin administration stimulating crypt cell proliferation in the small intestine, but not the colon (Sasaki et al., 2001). However, it is important to note though that while glicentin is stimulating adaptation in these models, the degree of adaptation is much less than that seen with either GLP-2 or oral nutrients (Hirotani et al., 2000; Sasaki et al., 2001). Thus, while it is capable of stimulating a limited trophic response within the proximal intestine, glicentin likely is not the intestinotrophic peptide encoded by proglucagon.

Despite the fact that current research suggests glicentin may be of limited benefit to the intestine, further research is necessary to determine other potential functions of the peptide.

Indeed, the true importance of glicentin may be in its ability to be converted to the satiety signal oxyntomodulin, which will be discussed below. Given this, our research into the dietary regulation of proglucagon activation may be particularly important for gaining a better understanding of the events ultimately leading to greater glicentin production and release.

Oxyntomodulin

Oxyntomodulin is a 37-amino acid peptide derived from cleavage of the glicentin protein that acts as a satiety signal. Similar to the other PGDPs, oxyntomodulin also has a relatively short half-life of approximately 6 minutes (Kervran et al., 1990). Oxyntomodulin has been shown to inhibit gastric emptying, gastric acid secretion, and gastroduodenal motility as stimulated by a meal in rats and humans (Schjoldager et al., 1989a; Jarrousse et al., 1994). Similarly, the hormone will also inhibit sodium, bicarbonate, and protein pancreatic secretions by inhibiting CCK (Anini et al., 2000). Furthermore, oxyntomodulin administered either intracerebroventricularly or intraperitoneally will inhibit food intake in rats for up to 24 hours by interacting with the anorectic pro-opiomelanocortin (POMC) neurons of the brain and stimulating release of melanocyte-stimulating hormone (α-MSH; Dakin et al., 2001; Dakin et al., 2004).

With increased satiety and decreased food intake, it is not surprising then that rats treated with oxyntomodulin would have a reduction in body weight, with decreases in both white and brown adipose tissue reported (Dakin et al., 2004). Indeed, similar results have been reported in human trials. When oxyntomodulin was administered subcutaneously in a randomized double-blind crossover study to overweight and obese patients for 4 days, there was a reduction in energy intake by 128 kcal and an increase in energy expenditure by 143 kcal/day due to

increased physical activity, ultimately resulting in decreased body weight by 0.5% (Wynne et al., 2006).

Thus, these studies indicate oxyntomodulin may be important for stimulating satiety and potentially a useful therapy for treating obesity. Similar to the other PGDPs, oxytomodulin has been explored through exogenous administration to both animals and humans. Given its role in satiety, it is not surprising that the peptide is also produced in response to the luminal environment. With conversion of proglucagon into oxyntomodulin, our research into the regulation of the gene may be particularly important in providing additional rationale for including certain nutrients in the diet and their ability to stimulate satiety.

Together, these results indicate PGDPs are actively mediating a number of processes throughout the body including insulin secretion from the pancreas, activation of satiety signals in the brain, and growth of the intestine during times of development and dysfunction. Given the number of health concerns that are impacted by the PGDPs, understanding the mechanisms by which nutrients stimulate their production and release from the L cells becomes increasingly important. We believe our research into proglucagon will further our knowledge of the regulation of the gene and may have implications for uses of the PGDPs.

SHORT-CHAIN FATTY ACIDS

Known for their ability to increase fecal bulk and intestinal transit time, dietary fibers will also undergo fermentation by the commensal microflora, releasing numerous end products,

including short-chain fatty acids (SCFAs). While there are many SCFAs generated, the primary end products are the 2-carbon acetate (CH_3COOH), 3-carbon propionate (CH_3CH_2COOH), and 4-carbon butyrate ($CH_3CH_2CH_2COOH$), which together make up 83% of all SCFAs produced in the intestine (Cummings, 1984). Indeed, due to our symbiotic relationship with the microbiota, we are able to utilize SCFAs, for numerous physiological functions including energy, maintaining intestinal homeostasis and integrity, stimulating trophic effects, and nutrient absorption. Moreover, given the location of their production, there is great potential for an interaction with enteroendcrine L cells and proglucagon, which will be explored below.

Production of Short-Chain Fatty Acids

Short-chain fatty acids are the primary end products of fermentation, or anaerobic breakdown, of substrates that escape digestion and absorption in the proximal intestine, such as undigestible carbohydrates and protein as well as a number of endogenous substrates including GI secretions, mucus, and sloughed epithelial cells (Cummings and Englyst, 1987). In addition to SCFAs, fermentation reactions by the microbiota of the intestine result in production of gases such as methane, carbon dioxide, and hydrogen gas (Cummings and Macfarlane, 1991). Based on the molar ratios of SCFAs produced, the fermentation reaction for a carbohydrate is 59 $C_6H_{12}O_6 + 38\ H_2O \rightarrow 60\ CH_3COOH + 22\ CH_3CH_2COOH + 18\ CH_3(CH_2)_2COOH + 96\ CO_2 + 268\ H^+$ (Roy et al., 2006). The primary SCFAs produced are acetate, propionate, and butyrate, which are produced at a fairly constant ratio of 60:25:15 acetate:propionate:butyrate (Cummings, 1984). However, other SCFAs are also produced including formate, valerate, caproate, isobutyrate, 2-methyl-butyrate, and isovalerate, although to a much lesser extent (Macfarlane and Macfarlane, 2003). Depending on the substrate, fermentation can begin in the ileum; however,

most occurs in the colon. Concentrations of SCFAs produced will vary based on diet, but can range from 70 -140 mM in the proximal colon, dropping to 20 – 70 mM in the distal colon, as substrate availability drops and SCFAs produced are absorbed by the host (Topping and Clifton, 2001).

Substrates for Short-Chain Fatty Acid Production

Nutrients surviving digestion in the small intestine, either due to the lack of endogenous enzymes, physical binding, or malabsorption resulting from a non-functional intestine, as well as numerous endogenous secretions will be fermented by the resident bacteria for energy, releasing SCFAs that will be used by the host. However, the primary substrates for SCFA production are dietary fibers and resistant starches. According to the American Association of Cereal Chemists, dietary fiber is defined as "the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation" (Marlett et al., 2002). Thus, this definition encompasses several types of fibers including cellulose, hemicellulose, lignin, gums, mucilages, pectin, fructans, and modified celluloses.

But, in terms of fermentability, these fibers are not equivalent to one another; in fact, the amount of SCFAs produced will vary a great deal. Early *in vivo* studies revealed that insoluble fibers, such as wheat bran and the cellulose component of sugar beet fiber, were more resistant to fermentation and recovered in rat fecal samples as compared to the more soluble fibers guar gum and pectin which were highly fermentable in the rat intestine (Nyman and Asp, 1982). *In vitro*

fermentation using canine fecal bacteria cultures revealed sugar beet pectin, fructans, and guar gum to be rapidly fermented (5.1, 4.8 – 5.3, and 8.2 – 8.8 hours, respectively, to reach maximal rate of gas production), sugar beet pulp (11.3 hours) and soy fiber (13.0 hours) moderately fermented, and wheat fiber (undetermined time) poorly fermentable (Bosch et al., 2008). Additionally, of the rapidly fermented fibers, pectin produced the most SCFAs (9.69 mmol/g total SCFA) with particularly high levels of acetate (7.57 mmol/g) measured whereas the 3 fructans, which differed by degree of polymerization, produced much higher levels of propionate (2.58 – 3.07 mmol/g) and butyrate (0.48 - .051 mmol/g) following 8 hours of fermentation (Bosch et al., 2008). Similarly, when human fecal bacteria were used, citrus pectin resulted in significantly higher acetate levels (5765 μmol/g substrate) as compared to other substrates whereas soy fiber, arabic gum, and an arabic-guar gum mixture had higher proportions of propionate (400, 1758, and 2140 μmol/g substrate, respectively) and butyrate (158, 585, and 713 μmol/g substrate, respectively) produced at 24 hours (Titgemeyer et al., 1991). However, it is important to note that people consume mixed diets with varying types and amounts of dietary fiber. These combinations of fibers in the diet will also affect the SCFA profile. Indeed, when guar gum, pectin, and wheat bran were fed either alone or in combination to rats, pectin alone produced high acetate (73.0 μmol/g wet contents) and low butyrate levels (73.0. 8.4, and 9.0 μmol/g wet contents for acetate, propionate, and butyrate, respectively) whereas guar gum resulted in increased levels of propionate and low concentrations of butyrate (73.8, 38.0, and 6.6 μmol/g wet contents for acetate, propionate, and butyrate, respectively). However, when the two fibers were combined, significantly higher proportions of butyrate were produced (68.0, 11.9, and 16.6 μmol/g wet contents for acetate, propionate, and butyrate, respectively), indicating possible synergistic effects of the combination on the microbiota (Henningsson et al., 2002).

Absorption of Short-Chain Fatty Acids

Upon production, SCFAs will be rapidly and efficiently absorbed by the host; in fact, less than 5% of total SCFAs produced will be recovered in feces (Topping and Clifton, 2001). SCFAs are believed to be absorbed via multiple routes including passive diffusion, carrier-mediated mechanisms and active transport. Due to their lipophilic nature, SCFAs are capable of passively diffusing across the cell membrane, but only if in the protonated form. However, this can be accomplished by lowering the pH close to the epithelium, which may involve Na^+/H^+ exchangers, K^+/H^+ pumps, and carbonic anhydrase moving H^+ ions out into the lumen. Indeed, when mucosal pH levels were progressively reduced in Ussing chambers, SCFA flux across rat colonic tissue significantly increased, which could be reversed by either increasing mucosal pH or reducing serosal pH, indicating a passive mechanism (Charney et al., 1998). Furthermore, SCFA absorption rates increased linearly in rat cecum when increasing concentrations of SCFAs were present and there appeared to be no competition between SCFAs for absorption when all were incubated together (Fleming et al., 1991). Together, these results suggest that passive diffusion may account for the majority of SCFAs absorbed (Roy et al., 2006).

However, there are other SCFA absorption mechanisms at work as well. Evidence indicates they may be absorbed using a carrier-mediated mechanism by exchanging the SCFA in anionic form for HCO_3^-. Von Engelhardt and colleagues showed that when guinea pig colon was incubated in Ringer's solution lacking HCO_3^- and CO_2, mucosal to serosal transport of propionate was significantly reduced; furthermore, when carbonic anhydrase was inhibited thus reducing HCO_3^- levels, transport was again reduced (von Engelhardt et al., 1994). These anionic exchangers are believed to exist in both the apical and basolateral membranes (Wachtershauser and Stein, 2000).

Moreover, research has also shown that there are multiple H^+- and Na^+-coupled monocarboxylate transporters present to move SCFAs across the epithelium. Initially known to be involved in lactate uptake, the H^+-coupled electroneutral transporter MCT-1 was localized in colonic luminal membrane vesicles of pigs and humans and shown to be important for SCFA transport as butyrate transport was 2.4-fold higher in oocytes containing MCT-1 (Ritzhaupt et al., 1998). Similarly, the Na^+-coupled electrogenic transporter SLC5A8, also called SMCT1, was also localized to the apical membrane (Iwanaga et al., 2006) and shown to transport SCFAs when human SLC5A8 cDNA was injected into oocytes, with uptake increased 2 to 45-fold depending on the fatty acid tested (Miyauchi et al., 2004). Other MCT isoforms also appear to be highly expressed in human colon, including MCT4 and MCT5, which have been localized to the basolateral membrane, indicating they may be particularly important for shuttling SCFAs out of colonocytes and into circulation (Gill et al., 2005). Thus, it appears that all three mechanisms are important for absorption and together, absorb 95% of all SCFAs produced in the gut (**Figure 1.2**).

Metabolism of Short-Chain Fatty Acids

Upon absorption, SCFAs will be quickly metabolized, either serving as energy substrates or quickly entering portal circulation to the liver. The majority of acetate will be taken up by the liver and used primarily for fatty acid and cholesterol synthesis (Demigne et al., 1995). Acetate that remains within the colonocytes will be used in phospholipid production, indicating a role in cell membrane synthesis (Zambell et al., 2003). Like acetate, the majority of propionate will be taken up by the liver. Particularly in ruminants, propionate will be used as a substrate for gluconeogenesis (Brockman, 1990). However, the actual significance of this conversion in

nonruminants is not completely understood. Unlike acetate and propionate, butyrate will be extensively metabolized within the colonocytes, serving as the primary energy source for the cells. Through its oxidation, butyrate provides 60-70% of the energy needs for the colonocytes (Topping and Clifton, 2001), thus sparing glucose and glutamine for other tissues (Wong et al., 2006).

Physiological Effects of Short-Chain Fatty Acids

The symbiotic relationship between the microbiota and the host allow for utilization of the SCFAs by the host as the bacteria break down fermentable substrates for energy. Indeed, absorption of SCFAs alone by the host will trigger the uptake of other nutrients in the distal intestine. Moreover, upon metabolism, SCFAs have even greater effects on the host, providing energy and stimulating trophic effects well beyond their site of production.

Short-Chain Fatty Acids and Nutrient Absorption

While the colon was once thought to only play a role in waste storage and elimination, it is now evident that it is also capable or absorbing nutrients including water, vitamins, and minerals, which can be stimulated by SCFAs. As previously discussed, there are multiple SCFA transport mechanisms coupled to sodium absorption, including Na^+/H^+ exchangers and SLC5A8, which transport 1 and 2 sodium ions across the epithelium, respectively. Thus, it is not surprising then that SCFA absorption would stimulate sodium absorption in the colon as well. Using Ussing chambers, Zaharia and colleagues found that 50 mM acetate, propionate, and butyrate individually increased net sodium flux across rat proximal colon by nearly 300% (Zaharia et al., 2001). SCFAs have also shown to be important in reversing colonic secretion of

water and ions in cholera toxin models. Indeed, when cholera toxin was given to rabbits, colonic perfusion with either 30 mM acetate, 30 mM propionate, or 30 mM butyrate significantly inhibited sodium secretion induced by the toxin (butyrate 95.5% reduction, propionate 92.2% reduction, acetate 76.4% reduction; Rabbani et al., 1999). Similarly, when intragastric tube feedings were used in healthy humans, cecal infusion of a SCFA mixture of 50 mM acetate, 20 mM propionate, and 20 mM butyrate significantly improved sodium absorption in the ascending colon, with 0.30 mmol/min absorbed with SCFAs compared to 0.20 mmol/min secreted in the controls (Bowling et al., 1993). These models also revealed improvements in water absorption as well. The water secretory rate was reduced from 208.6 µl/min/cm in cholera toxin control rabbits to 8.2 µl/min/cm for butyrate, 20.8 µl/min/cm for propionate, and 62.6 µl/min/cm for acetate while cecal SCFA infusion in humans reversed colonic water secretion of 1.0 ml/min in controls to absorption of 1.6 ml/min (Bowling et al., 1993; Rabbani et al., 1999).

Along with sodium, chloride, potassium, and water, SCFAs are also believed to affect the absorption of divalent cations such as calcium and magnesium. While the exact mechanisms are not known, it is believed that SCFAs may improve calcium and magnesium absorption through either their trophic effects, thus increasing the absorptive surface area in the colon, or by decreasing colonic pH and thereby solubilizing calcium and magnesium, which makes it easier to absorb. In rats fed either a control or fructooligosaccharide (FOS)-containing diet for 14 days, fecal calcium and magnesium levels were significantly lower with FOS, indicating enhanced absorption (calcium: 839 vs. 471 µmol/g dry contents; magnesium: 105.3 vs. 30.5 µmol/g dry contents; Ohta et al., 1995). Similarly, when rat tissue was examined using Ussing chambers, the addition of 130 mM SCFAs (80 mM acetate, 40 mM propionate, and 10 mM butyrate) to the mucosal side of cecal and colonic tissue increased the calcium concentration on the serosal side

by more than 300% (Mineo et al., 2001). Magnesium absorption has also shown to improve in post-menopausal women with fiber supplementation, as the addition of short-chain FOS for 5 weeks increased intestinal absorption by 12.3% and retention by 11.4% (Tahiri et al., 2001).

Short-Chain Fatty Acids and Intestinal Injury

Along with improving nutrient absorption, SCFAs are also known to be trophic to the intestinal epithelium, affecting not only structure but also functional aspects of the intestine. In the normal rat intestine, TPN supplemented with SCFAs either intravenously or intracecally improved jejunal and ileal mucosa compared to the mucosal atrophy present in control animals, with increases in weight, RNA, DNA, and protein content reported (Koruda et al., 1990). While no differences in villus height or crypt depth were reported, when piglets received cecal infusions of butyrate in addition to sow's milk replacer formula, cell proliferation significantly increased by 111% in the jejunum, 78% in the ileum, 119% in the cecum, and 89% in the colon as compared to piglets receiving formula alone (Kien et al., 2007), indicating both local and distant effects of butyrate.

Additionally, SCFAs can enhance gene expression, with improvements in GLUT2 mRNA and protein as well as c-myc, c-jun, and c-fos mRNA abundance, potentially increasing glucose transport and cell proliferation and differentiation in normal rats receiving TPN (Tappenden et al., 1998; Tappenden and McBurney, 1998; Drozdowski et al., 2002). Short-chain fatty acids also modulated intestinal, but not systemic, cytokine abundance in piglets with increases in IL-1β and IL-6 protein reported, indicative of immune system activation and potential protection of the host (Milo et al., 2002). With such promising results in healthy intact

intestine, it is not surprising then that SCFAs would also be beneficial in several disease states, such as SBS and IBD.

In models of SBS, administration of SCFAs, either through TPN or through fermentable substrates, has shown to be quite beneficial in stimulating intestinal adaptation following resection. When rats underwent an 80% jejunoileal resection and received either standard TPN or TPN supplemented with SCFAs, mucosal and submucosal weights, ileal DNA and RNA were significantly improved with the addition of SCFAs as early as 3 days and continued to be improved following 7 days of treatment (Tappenden et al., 1996). Similarly, when piglets received an 80% jejunoileal resection and received either TPN or TPN supplemented with butyrate specifically, butyrate increased structure along the small intestine, with increases in villus height in the jejunum (485 vs. 663 μm in control and butyrate groups, respectively) and ileum (593 vs. 754 μm in control and butyrate groups, respectively), which were associated with increased proliferation and decreased apoptosis (Bartholome et al., 2004).

The addition of SCFAs either intravenously or orally will also improve nutrient absorption in models of SBS. Rats receiving an 80% jejunoileal resection and then randomized to either standard TPN or TPN supplemented with SCFA showed significantly increased GLUT2 expression (3.7 vs. 10.1 densitometry units in TPN and TPN + SCFA groups, respectively) and tended to improve SGLT-1 expression (6.3 vs. 11.8 densitometry units in TPN and TPN + SCFA groups, respectively) following 3 days of treatment, which was associated with significantly elevated ileal glucose uptake (Tappenden et al., 1997). Moreover, when butyrate was supplemented into an enteral formula provided to piglets receiving a 75% intestinal resection, butyrate significantly increased the portal efflux of amino acids (19 vs. 35 μmol/kg bodyweight/min for enteral and enteral + butyrate groups, respectively) and tended to increase

the efflux of glucose (12 vs 49 μmol/kg bodyweight/min for enteral and enteral + butyrate groups, respectively), indicative of improved absorptive capacity of the gut (Welters et al., 1996). Together, these data indicate significant improvements in SBS with SCFA treatment, which warrants further studies in the clinical applicability of the therapy to humans.

Additionally, as they are important for colonic homeostasis and trophic to the intestine, SCFAs may be significant mediators in IBD prognosis. In a rat model of TNBS-induced colitis, addition of fermentable dietary fiber reduces colonic damage as well as TNF-α (600 vs. 223 pg/g) and NOS activity (2000 vs. 1250 pmol L-citrulline/mg protein 30 min) as compared to controls (Rodriguez-Cabezas et al., 2002). More specifically, butyrate enemas improve colonic lesions and significantly reduce MPO activity (20 vs. 4 U/mg tissue in butyrate and saline treatment; Butzner et al., 1996). Similar studies with SCFAs have also been conducted in humans with either oral butyrate or increased dietary fiber administered. With oat bran administration to UC patients, butyrate concentration increased by 36% (11 vs. 15 μmol/g feces at 0 and 4 weeks treatment) and prevented relapse in all the patients enrolled while oral butyrate treatment in CD patients showed marked improvement, with 69% of patients responding to treatment including decreased CD activity scores, IL-1β (10.4 vs. 2.4 pg/mg protein at 0 and 8 weeks treatment), and NF-κB (39.2 vs. 26.7 pg/mg protein at 0 and 8 weeks treatment; Hallert et al., 2003; Di Sabatino et al., 2005). Collectively, these data demonstrate SCFAs can modulate the inflammatory response and improve outcomes in a clinical setting, and therefore may be key factors in promoting recovery in IBD patients.

Together, these results indicate SCFAs are inducing numerous processes throughout the body including nutrient absorption, metabolic processes such as lipogenesis and

gluconeogenesis, and intestinal adaptation. Yet, the mechanisms by which SCFAs are stimulating these effects in the body are not fully understood. There is potential for SCFAs to directly stimulate effects by serving as a local energy source. However, SCFAs may also be interacting with specific receptors and transporters located within the intestine, stimulating cell signaling cascades that may activate other mediators, such as proglucagon and GLP-2. We believe our research will be beneficial in elucidating the specific effects of SCFAs on the intestine and the potential mechanisms by which they may be stimulating the changes.

SUMMARY

As previously discussed, the presence of PGDPs and SCFAs in the intestine stimulate a number of outcomes throughout the body, including insulin secretion, satiety signaling, structural improvements in the gut, nutrient absorption, and energy production. Thus, additional research examining the nutritional regulation and effects of PGDPs and SCFAs becomes increasingly important. The information gained would provide a greater understanding of the relationship between PGDPs and SCFAs and may provide new strategies for treating disorders such as Type 2 diabetes, obesity, and intestinal dysfunction.

AIMS AND SCOPE OF RESEARCH

Due to the focus of our laboratory, our research will encompass the intestinal effects of SCFAs and PGDPs, namely GLP-2. It is clear that both GLP-2 and SCFAs will stimulate intestinal maintenance and adaptation at the gross structural and microarchitecture levels as well as improvements in nutrient uptake by enhancing transporter expression and activity. However, the interaction between the trophic factors is less understood. The addition of 30% fiber to an

elemental diet for 14 days increased ileal proglucagon mRNA levels in rats (11.47 vs. 6.52 densitometry units in fiber vs. control groups, respectively) as well as the concentration of the PGDP GLP-1 in the plasma (19.8 vs. 15.4 pg/ml in fiber vs. control groups, respectively; Reimer and McBurney, 1996). Similar effects were reported with resistant starch in rats, increasing GLP-1 and PYY secretion from the L cells (Zhou et al., 2008). As these are fermentable substrates, the production of SCFAs in the distal gut may be driving increases in PGDPs. Indeed, in both intact and resected intestine, the addition of SCFAs to standard TPN significantly increases proglucagon mRNA abundance as well as plasma GLP-2 levels (Tappenden et al., 1996; Tappenden and McBurney, 1998).

Of the SCFAs, butyrate is believed to be the specific one responsible for the trophic effects, particularly as it remains in the intestinal cells as a fuel source. In fact, butyrate has great potential be the mediator of GLP-2 release, as SCFAs are produced primarily in the distal ileum and colon, the exact location of the enteroendocrine L cells which produce and release GLP-2. Similar to the rat studies, trophic effects were reported in resected piglets receiving TPN supplemented with butyrate alone, which were associated with increases in plasma GLP-2 concentrations (Bartholome et al., 2004). Moreover, examination of the interaction between butyrate and GLP-2 revealed that butyrate increased proglucagon mRNA abundance, but had no effect on L cell density, PC1/3, DPP-IV, or the GLP-2 receptor, indicating butyrate is driving production of GLP-2 via transcriptional regulation (Bartholome, 2004).

Yet, little is known regarding the mechanism by which butyrate stimulates GLP-2. Elucidation of the mechanism will provide knowledge of the pathway by which intestinal adaptation occurs and therefore may reveal new strategies for stimulating adaptation using a modification of the diet rather than exogenous administration of hormones. Therefore, it is our

overall goal to gain a better understanding of the molecular mechanisms by which butyrate induces GLP-2, thereby stimulating intestinal adaptation.

The **specific aim** of this thesis research is to examine the effects of SCFAs, specifically butyrate, on proglucagon gene expression in the intestine in both *in vitro* and *in vivo* models. This specific aim will be investigated using the following hypotheses:

Hypothesis 1: Butyrate upregulates proglucagon mRNA abundance in enteroendocrine L cells. **Chapter 2** establishes that NCI-H716 cells, a model of the enteroendocrine L cell, respond similarly to *in vivo* models, increasing proglucagon mRNA abundance following treatment with butyrate. Furthermore, it demonstrates that butyrate increases proglucagon mRNA abundance by activation of the gene promoter.

Hypothesis 2: Butyrate activates the proglucagon promoter by interacting with receptors and transporters on the enteroendocrine L cell and these receptors and transporters are upregulated in the presence of SCFAs. **Chapter 3** investigates the interaction between SCFAs and the L cell. Using an *in vitro* model, it establishes that butyrate increases proglucagon mRNA abundance by acting through GPR43 and T2R38 receptors present on the L cell. It further uses *in vivo* models to reveal luminal, but not systemic, SCFAs to upregulate SCFA transporter abundance, including MCT-1 and SLC5A8.

Hypothesis 3: Luminal butyrate provided into the distal intestine increases structural and functional indices of adaptation by increasing proglucagon and GLP-2 levels in the adult rat. **Chapter 4** explores the effects of site of SCFA production on proglucagon expression and markers of adaptation. It establishes that luminal SCFAs, specifically butyrate alone, will increases structural markers of intestinal growth and adaptation and that proglucagon and GLP-2

are likely mediating some of these effects. Furthermore, it also demonstrates the importance of ileal L cells specifically in stimulating trophic effects throughout the intestine.

Chapter 5 summarizes the results of these studies and discusses the insights gained from the research. It also explores future directions of the research both *in vitro* and *in vivo* including further proglucagon promoter analysis, examination of receptors and transporters on L cells and the subsequent intracellular events occurring, and luminal butyrate administration in different *in vivo* models including resection and various growth factor knock out models.

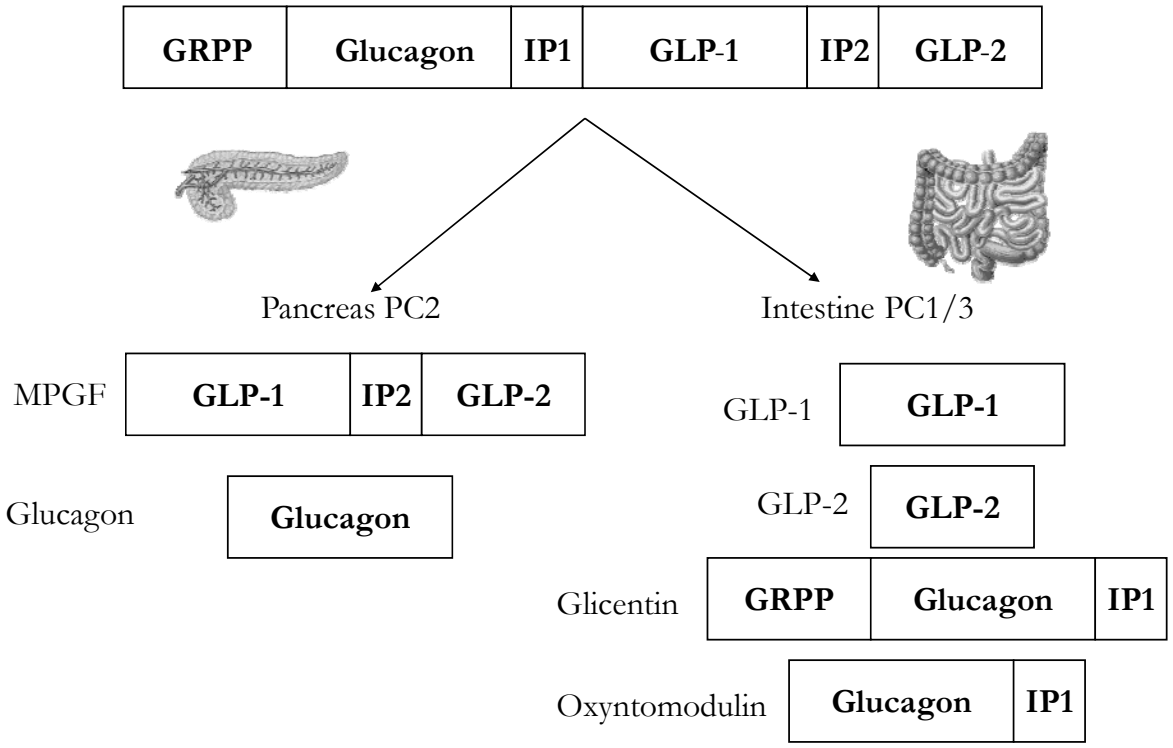


Figure 1.1 Proglucagon post-translational processing in the pancreas and small intestine. Within the pancreas, prohormone convertase 2 (PC2) will liberate the major proglucagon fragment (MPGF) and glucagon whereas prohormone 1/3 (PC1/3) is active in the intestine, releasing glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), glicentin, and oxyntomodulin. GRPP, glicentin-related pancreatic polypeptide; IP, intervening peptide. Figure adapted from (Drucker, 2005).

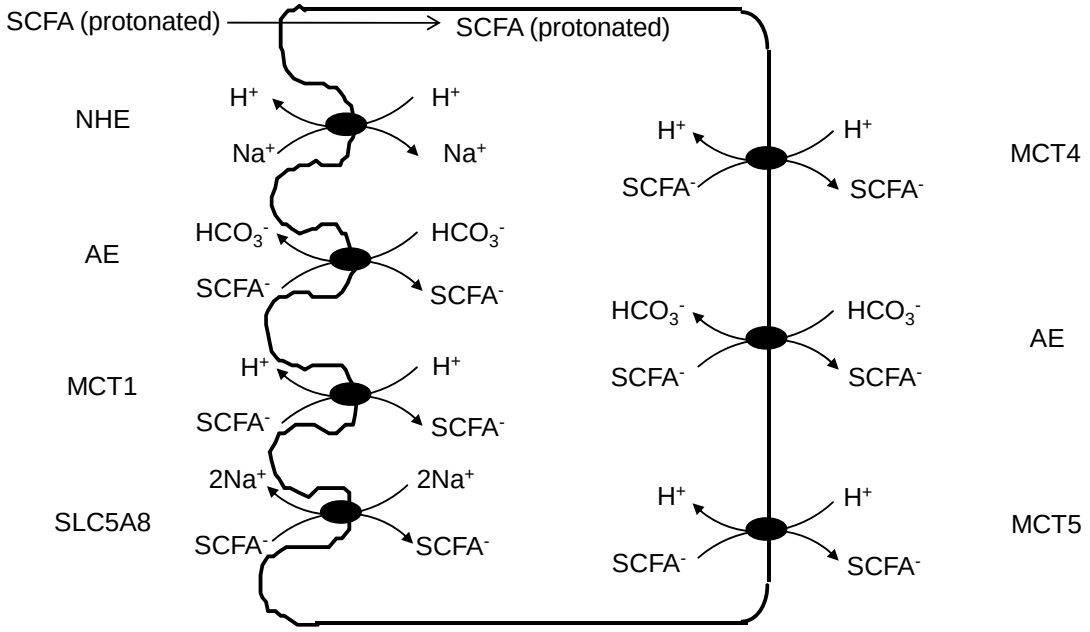


Figure 1.2 Routes of SCFA absorption. Mechanisms for SCFA absorption across the apical membrane include: passive diffusion of the protonated form, which requires luminal hydrogen ions obtained through sodium-hydrogen exchangers (NHE); carrier-mediated anion exchangers (AE), which exchange SCFAs for bicarbonate ions; hydrogen-dependent electroneutral transporters (MCT-1), which transport SCFA and hydrogen ions; and sodium-dependent electrochemical transporters (SLC5A8), which transport SCFA and sodium ions. SCFAs can then be transported across the basolateral membrane using carrier-mediated anion exchangers or hydrogen-dependent electroneutral transporters (MCT4 or MCT5).

CHAPTER 2

BUTYRATE INCREASES PROGLUCAGON mRNA ABUNDANCE THROUGH ACTIVATION OF THE PROGLUCAGON GENE PROMOTER IN NCI-H716 CELLS

ABSTRACT

Previous *in vivo* models of intestinal failure have shown butyrate to stimulate improvements in structural indices and functional capacity of the intestine which were associated with increased plasma GLP-2 levels, a hormone believed to induce trophic effects. However, the mechanism by which butyrate stimulates GLP-2 is poorly understood. The aim of this study was to examine the transcriptional regulation of proglucagon by butyrate. Using NCI-H716 cells, we hypothesized that proglucagon mRNA abundance increases in response to butyrate due to activation of the proglucagon promoter. NCI-H716 cells were treated with 1 of 6 doses of butyrate (0, 2.5, 5, 7.5, 10, or 20 mM) and were harvested at 4 time points (0.5, 1, 2, and 4 hours following treatment) using repeated measures. Semi-quantitative real-time PCR revealed proglucagon mRNA abundance tended to increase with butyrate treatment, particularly at the 2 and 4 hour time points, reaching significance with 7.5 mM butyrate following 2 hours of treatment ($p = 0.039$). The human proglucagon gene promoter was subcloned into a pGL4 reporter vector expressing firefly luciferase and transiently transfected into NCI-H716 cells. Following treatment with 7.5 mM butyrate, luciferase activity, as driven by the proglucagon promoter, was increased compared to 0 mM butyrate controls ($p < 0.0001$). The results of these experiments have shown butyrate to upregulate proglucagon mRNA abundance through an activation of the proglucagon gene promoter, thereby furthering our understanding of the molecular mechanisms wherein butyrate and GLP-2 interact during intestinal adaptation.

INTRODUCTION

Intestinal failure is defined as "the critical reduction of functional gut mass below the minimal amount necessary for adequate digestion and absorption to satisfy body nutrient and fluid requirements for maintenance in adults or growth in children" (Goulet and Ruemmele, 2006). There are a number of underlying causes of intestinal failure including pseudo-obstructions, cancer, Crohn's disease, short bowel syndrome, atresias, volvulus, and necrotizing enterocolitis. Management oftentimes requires parenteral nutrition, which while beneficial in that it provides proper nutrition, can also very detrimental as mucosal atrophy ensues (Buchman et al., 1995). However, the residual bowel is capable of intestinal adaptation, particularly when stimulated by luminal nutrients, pancreatic secretions, and numerous humoral factors.

Of interest, the proglucagon gene encodes for five biologically active peptides including glucagon, GLP-1, GLP-2, glicentin, and oxyntomodulin and is expressed in the enteroendocrine L cells of the intestine, the alpha cells of the pancreas, and neuronal cells within the brain (Lund et al., 1981; Bell et al., 1983; White and Saunders, 1986; Drucker and Asa, 1988). Initially discovered to stimulate proliferation of the small intestinal epithelium by implantation of proglucagon-producing tumors into mice (Drucker et al., 1996), GLP-2 specifically is a trophic hormone that stimulates both structural and functional adaptations within the intestine. Early studies revealed proglucagon mRNA levels to be elevated following intestinal resection (Rountree et al., 1992), and more recent reports indicate that these elevated proglucagon and GLP-2 levels are associated with a number of intestinal improvements. Intestinal indices shown to be impacted in models of intestinal failure include increased residual bowel length and weight, crypt depth and villus height, blood flow within the mesentary, improved nutrient transport and intestinal permeability as well as improved wet weight absorption and decreased fecal energy

excretion (Cheeseman, 1997; Au et al., 2002; Martin et al., 2004; Jeppesen et al., 2005; Martin et al., 2005; Liu et al., 2006; Sangild et al., 2006; Sigalet et al., 2006; Deniz et al., 2007).

Proglucagon synthesis and secretion within the intestine can be stimulated by a number of factors including neural mechanisms and direct interactions with nutrients. Of interest are dietary fibers and their fermentation end products, short-chain fatty acids. Indeed, when rats received a diet supplemented with 30% fiber (5% cellulose and 25% fermentable fiber mix) for 14 days, short-chain fatty acids, particularly acetate and butyrate, were significantly elevated and associated with increased ileal proglucagon mRNA levels (Reimer and McBurney, 1996). Similarly, when rats were fed a diet containing 100 g/kg oligofructose for 3 weeks, colonic proglucagon mRNA levels were elevated compared to controls (Cani et al., 2004). More specifically, short-chain fatty acids, including butyrate supplementation alone, can stimulate a number of structural adaptations within the intestine including improved gross measurements of weight and length, increases in cellular proliferation and decreased apoptosis at the microarchitecture level resulting in increased villus height and crypt depth as well as functional improvements with changes such as increases in GLUT2 at both the mRNA and protein levels, glucose uptake, and enhanced amino acid absorption which are associated with elevated proglucagon and GLP-2 levels (Welters et al., 1996; Tappenden et al., 1997; Tappenden et al., 1998; Tappenden and McBurney, 1998; Bartholome et al., 2004).

Thus, it is apparent that published studies have now established that short-chain fatty acids, particularly butyrate, stimulate intestinal adaptation as well as increases in plasma GLP-2 concentration. Previous work from our lab suggested butyrate may be transcriptionally regulating proglucagon as butyrate was shown to increase proglucagon mRNA levels, but have no effect on L cell density, PC 1/3 or DPP-IV levels, or GLP-2 receptor abundance (Bartholome,

2004). Yet, the transcriptional regulation of the gene is not completely understood. Therefore, the aim of this study was to examine the role of the transcriptional activation of proglucagon, ultimately leading to elevated GLP-2 levels and an increase in the adaptive response.

MATERIALS AND METHODS

Culturing NCI-H716 Cells

NCI-H716 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained as described. Briefly, cells were maintained in 75 cm^2 tissue culture flasks (TPP, St. Louis, MO) in RPMI-1640 media containing 2.0 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10 mM HEPES buffer, 1.0 mM sodium pyruvate, and 4.5 g/L glucose and supplemented with 10% fetal bovine serum (all media reagents obtained from Hyclone, Logan, UT) in a 37° C incubator injected with 5% CO_2.

Determination of Proglucagon mRNA Abundance with Real-Time PCR

NCI-H716 cells were seeded into T-75 flasks at a concentration of 2 x 10^6 cells/flask. In experiment 1, the effect of NCI-H716 cell aggregation on proglucagon mRNA abundance was explored using a repeated measures design in which samples were taken from each flask (n=10) every 24 hours starting with a baseline measurement immediately following seeding of flasks. In experiment 2, the effect of butyrate treatment on proglucagon mRNA abundance was explored again with a repeated measures design (n=6 for each treatment-time combination). Briefly, flasks were randomized to receive one of six doses of butyric acid (0, 2.5, 5, 7.5, 10, or 20 mM butyrate; Sigma, St. Louis, MO) and samples taken from each flask at four time points (30 minutes, 1 hour, 2 hours, and 4 hours following treatment). Upon harvesting, cells were pelleted

by centrifugation at speed 7 (Centrific Centrifuge; Fisher, Pittsburgh, PA) and washed in 1x PBS (Hyclone, Logan, UT).

Total cellular RNA was isolated from the cell pellets using Trizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol and RNA concentration quantified using a spectrophotometer (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE). Reverse transcription reactions were performed in a Gene Mate Genius thermocycler (ISC Bioexpress, Kaysville, UT) in a total volume of 20 µl containing 1 µg RNA, 250 ng Random Primers, 0.5 mM dNTP mix, 1x First-Strand Buffer, 0.01 M DTT, 40 units RNase Out, and 200 units Superscript II reverse transcriptase (all reverse transcription reagents were obtained from Invitrogen, Carlsbad, CA). RNA, random primers, and dNTPs were heated to 65° C for 5 minutes after which the remaining reagents were added and reactions incubated at 25° C for 10 minutes followed by 42° C for 50 minutes and then finally inactivated at 70° C for 15 minutes.

Resultant cDNA was diluted 1:10 and used for semi-quantitative real-time PCR reactions. Reactions were performed in a total volume of 10 µl containing 2 µl cDNA, 1x TaqMan Universal Master Mix, and 1x of either the FAM-labeled human proglucagon mRNA (Assay ID Hs00174967_m1) or the FAM-labeled human 18S rRNA (Assay ID Hs99999901_s1) TaqMan Gene Expression Assay primer-probe set (all real-time PCR reagents were obtained from Applied Biosystems, Foster City, CA). Reactions were run in a 384-well plate in a TaqMan ABI 7900 real-time PCR machine and analyzed with Sequence Detection Systems 7900HT version 2.2.1 software (Applied Biosystems, Foster City, CA) Briefly, samples were heated to 50° C for 2 minutes and then 95° C for 10 minutes to allow for DNA polymerase activation followed by 40 cycles of 95° C for 15 seconds and 60° C for 1 minute to allow for denaturing, annealing, and

extension. Data were analyzed using the standard curve method with pooled cDNA serving as the standard. Proglucagon levels were normalized to 18S rRNA.

Proglucagon Promoter Isolation and Construct Preparation

Based on homologies to previously used rodent proglucagon promoters, 2.5 kb of the proglucagon promoter was isolated from human genomic DNA (Promega, Madison, WI). The oligonucleotide primers used for the amplification of the human proglucagon promoter were designed using the OligoPerfect Designer program (Invitrogen; http://www.invitrogen.com/ site/us/en/home/Products-and-Services/Applications/ Nucleic-Acid-Amplification-and-Expression-Profiling/Oligonucleotide-Design/Primer-Design-Tools.html) and the National Center for Biotechnology Information (NCBI) databases (http://www.ncbi.nlm.nih.gov/). Primers were designed with KpnI and XhoI restriction sites built in as well as extra bases added as the enzymes are endonucleases. The primer sequences were: (forward) 5'-GAGAGAGGTACCTTATAATCATCTTACCTTCTGGTAGTGTG-3' and (reverse) 5'-GAGAGACTCGAGTAAATCTGTGGAAATTGTCCTTAGGACTA-3' (Invitrogen, Carlsbad, CA). PCR reactions were carried out in a Gene Mate Genius thermocycler (ISC Bioexpress, Kaysville, UT) in a 50 µl volume including 1 µl genomic DNA, 1x Pfx Amplification Buffer, 0.3 mM dNTP mix, 1.0 mM $MgSO_4$, 3 µM of each the forward and reverse primers, and 1 unit Platinum Pfx DNA polymerase (all PCR reagents were obtained from Invitrogen, Carlsbad, CA). Reactions were denatured for 2 minutes at 94° C followed by 35 cycles of denaturing at 94° C for 15 seconds, annealing at 55° C for 30 seconds, and extending at 68° C for 2.5 minutes.

Following amplification, samples were prepared for insertion into the Firefly luciferase-expressing reporter vector pGL4 (Promega, Madison, WI) by digesting the samples with Xho I

and Kpn I. Briefly, 15 µl of DNA were combined with 1x REact 2 Buffer and 20 units Xho I (Invitrogen, Carlsbad, CA) and heated to 37° C for 1 hour. Following cleavage, DNA was precipitated with isopropanol (Sigma, St. Louis, MO) and resuspended in 16 µl DEPC water. DNA was then combined with 1x REact 4 Buffer and 20 units Kpn I (Invitrogen, Carlsbad, CA) and again heated to 37° C for 1 hour. Resultant proglucagon and pGL4 DNA underwent ligation reactions using 1x DNA Ligase Reaction Buffer and 5 units of T4 DNA Ligase (Invitrogen, Carlsbad, CA).

Transfection of NCI-H716 Cells

On the day of transfection, NCI-H716 cells were pelleted and washed in PBS (Hyclone, Logan, UT) and then resuspended in serum-free media. Using a hemocytometer to determine cell concentration, 2.5 x 10^5 cells in 0.5 mL were seeded into 24-well plates. Transfection complexes were prepared by diluting 2000 ng of the proglucagon construct as well as 20 ng of the TK-Renilla control vector in 100 µl of serum-free media. In order to enhance transfection efficiency, DNA was incubated with 0.5 µl of PLUS reagent (Invitrogen, Carlsbad, CA) for 5 minutes at room temperature. Following incubation, 5 µl of Lipofectamine LTX (Invitrogen, Carlsbad, CA) were added to each reaction and allowed to incubate at room temperature for 30 minutes to allow for complex formation after which the DNA-Lipofectamine LTX complexes were added to the wells. The plate was incubated at 37° C in a CO_2 incubator for 4 hours to allow for transfection after which the media was changed, cells were treated with either 0 or 7.5 mM butyrate, and incubated for 24 hours prior to testing gene expression. This procedure was repeated on 5 separate occasions.

Luciferase Assays

Proglucagon promoter activation was determined by measuring the Firefly luciferase expression using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to manufacturer's protocol. Briefly, following completion of transfection and treatment, cells were pelleted, rinsed with PBS (Hyclone, Logan, UT), and then lysed in 500 µl 1x Passive Lysis Buffer on a Belly Dancer rocking platform (Stovall Life Sciences, Greensboro, NC) for 15 minutes. Luciferase activity was measured in a Zylux FB12 luminometer (Zylux, Oak Ridge, TN). Firefly luciferase activity was determined by combining 100 µl Luciferase Assay Reagent II and 20 µl cell lysate. Following measurement, 100 µl 1x Stop and Glo Reagent was added and Renilla luciferase activity measured. Activity was normalized to DNA concentration.

DNA Content

DNA content of the cell lysates was determined using the Hoechst microplate method. Briefly, 2 µl of sample was combined with 200 µl of a buffer solution containing 10 mM Tris, 1mM EDTA, 200 mM NaCl, and 1.6 nmol 33258 bisBenzimide Hoechst dye at pH 7.4. Samples were read at an excitation of 360 nm and emission of 450 nm using the SpectraMax Gemini XS fluorometer (Molecular Devices, Sunnyvale, CA). Cell lysate DNA concentration was calculated using a Herring sperm DNA (Promega, Madison, WI) standard curve.

Statistical Analysis

In the proglucagon mRNA abundance experiments, statistical differences between treatments were determined using a repeated measures analysis of variance (ANOVA) design in which time served as the repeated variable and the effect of the experimental variation between

flasks was blocked for (block = flask; main effects = dose and time; interaction = dose x time). In the luciferase assays, the effect of plate was blocked for (block = plate; main effect = treatment) and a one-way ANOVA was used to determine significance. Analysis was carried out using a mixed model in SAS (Version 9.1; SAS Institute, Cary, NC). All values are reported as fold changes normalized to 0 mM butyrate and statistical significance was defined as $p \leq 0.05$.

RESULTS

Proglucagon mRNA abundance

Semi-quantitative real-time PCR was performed to determine proglucagon mRNA abundance. In order to examine the effects of NCI-H716 cell aggregation on proglucagon mRNA abundance, cells were harvested over 5 days. Proglucagon mRNA abundance did not differ over time ($p = 0.41$), indicating that cell aggregation was not affecting proglucagon gene expression (**Figure 2.1**). However, when cells were treated with butyrate, proglucagon expression did change. Proglucagon expression tended to increase with 2.5, 5, 7.5, or 10 mM butyrate, particularly at the 2 and 4 hour timepoints. Moreover, the mRNA abundance reached significance following 7.5 mM butyrate treatment for 2 hours (2.16-fold increase as compared to controls; $p = 0.039$, **Figure 2.2**).

Proglucagon promoter activation

In order to determine if proglucagon mRNA abundance increases due to transcriptional regulation, we measured luciferase activity following butyrate treatment of the promoter construct. Thus, if the proglucagon promoter was activated, it would increase luciferase activity. When the construct was transiently transfected into NCI-H716 cells, luciferase activity, as driven

by the proglucagon promoter, was significantly increased when treated with 7.5 mM butyrate as compared to controls (1.8-fold increase; $p < 0.0001$, **Figure 2.3**).

DISCUSSION

Following intestinal injury, the intestine will undergo remarkable adaptations in order to maximize its digestive and absorptive capacity, a process which is stimulated by numerous factors within the body but can also be enhanced through exogenous administration of growth factors and specific nutrients. Previous research has well established GLP-2 and butyrate to be trophic to the gut; additionally, butyrate appears to be interacting with the proglucagon gene to stimulate GLP-2. Therefore, in the present study, we investigated the effects of butyrate on the proglucagon gene promoter and mRNA abundance in human L cells.

Due to the molecular nature of the study, a cell culture system would be the most appropriate model for examining the transcriptional activation of the proglucagon gene. In order to examine this regulation, we chose NCI-H716 cells as a model of the enteroendocrine L cell. The NCI-H716 cell line is a human colon cancer cell line isolated from a poorly differentiated adenocarcinoma of the cecum that contains dense granules within the cytoplasm (Park et al., 1987) and expresses chromogranin A (de Bruine et al., 1993), both characteristic traits of endocrine cells; additionally, the cells also secrete GLP-1, another peptide produced by the proglucagon gene (Reimer et al., 2001). While the proglucagon gene is oftentimes studied using pancreatic cell lines or the mouse cell lines STC-1 or GLUTag as a model of the enteroendocrine cell, we chose to use NCI-H716 cells for the following reasons. First, previous research has revealed that when transgenic mice were used that expressed human growth hormone under the control of the proglucagon gene, expression of human growth hormone was not significantly

changed following a fasting-refeeding period or receiving a 30% fiber diet for 14 days. However, wild-type mice exhibited elevated proglucagon levels in both situations, indicative of potential differences in proglucagon regulation between species (Nian et al., 2002). Furthermore, while 1604 basepairs of the human proglucagon gene were adequate to see expression within enteroendocrine cells, 5775 basepairs were required to for expression within islet cells, demonstrating differences in regulation between cell types in the body (Nian et al., 1999). Therefore, in order to examine the more relevant human proglucagon gene, it was necessary not only to use an enteroendocrine cell line but also for it to be human-derived.

Using NCI-H716 cells as a model of the L cell, we have determined that proglucagon mRNA levels are significantly elevated following butyrate treatment due to transcriptional activation of the gene. Similar to *in vivo* findings, we saw a near doubling in proglucagon mRNA abundance and promoter activation as measured through luciferase activity. Previous work from our lab has shown a significant 56% increase in ileal proglucagon and a 16% increase in colonic proglucagon mRNA levels following butyrate supplementation, which were associated with elevated plasma GLP-2 levels (Bartholome et al., 2004). Additionally, these increases appeared to be transcriptionally regulated as there were no differences in L cell density, PC 1/3 protein levels, DPP-IV activity, or GLP-2 receptor abundance that could account for the significant increase in proglucagon mRNA or GLP-2 levels (Bartholome, 2004).

Conversely, our findings are in contrast to those of Cao and colleagues in which treatment of NCI-H716 cells with sodium butyrate actually decreased proglucagon mRNA abundance (Cao et al., 2003). However, we believe this to be due to the dosage of butyrate used (2 mM) and time points tested (24 and 48 hours). In preliminary experiments designed to determine an appropriate timeframe for measuring proglucagon, we found comparable results at

24 hours, with similar or slightly lower proglucagon expression following butyrate treatment. Instead, proglucagon levels tended to increase when butyrate treatment ranged from 2.5 – 10 mM at more acute time points. Possibly the decrease in expression at longer time points could be due to the volatile nature of short chain fatty acids and thus the lack of stimulus to maintain the elevated proglucagon levels within the cells.

Much work has been done to uncover factors contributing to transcription of the proglucagon gene and various transcription factors have been identified that may play a role in intestinal gene expression. For example, when plasmids containing Cdx-2/3 were co-transfected into BHK cells, proglucagon promoter activation was increased 6- to 15-fold (Jin and Drucker, 1996). Transfection of either adenovirus system-derived Pax-6 or Cdx-2/3 increased proglucagon promoter activation; additionally, Pax-6 actually increased mRNA abundance in GLUTag cells (Trinh et al., 2003). Importantly, both of these transcription factors have been identified in NCI-H716 cells (Cao et al., 2003), and thus could be involved in the increased proglucagon mRNA abundance we have reported. Moreover, the interaction of specific signaling pathways with the gene have also been examined. Using islet cells, a cAMP response element (CRE) had been identified in the proglucagon promoter (Knepel et al., 1990). In intestinal cells, the importance of this CRE has been established as proglucagon transcription is promoted in a cAMP-dependent manner, using both protein kinase A (PKA; Drucker et al., 1994) and exchange protein directly activated by cAMP (Epac; Islam et al., 2008). Additionally, the Wnt pathway has also been implicated in proglucagon transcription as the addition of lithium increased proglucagon mRNA abundance, which is believed to be the result of elevated β-catenin that combines to form the transcription factor cat/TCF (Ni et al., 2003).

Yet, little is known about the exact mechanism by which butyrate interacts with the gene, ultimately resulting in elevated GLP-2 levels. It is possible that butyrate interacts with one of the transcription factors or signaling pathways previously described. However, it is important to note that the majority of the work had been carried out using rodent proglucagon sequences and cell lines and thus due to the potential for species differences, it may not all apply to transcriptional regulation of the human proglucagon gene. Conversely, butyrate may interact directly with the human proglucagon promoter through a potential butyrate response element yet to be identified. Thus, in summary, while we have shown butyrate to significantly stimulate the human proglucagon promoter ultimately resulting in increased proglucagon mRNA abundance, additional research is needed to further elucidate the mechanisms by which butyrate achieves these actions. Elucidation of the mechanism would not only provide further information about the regulation of intestinal adaptation process in general but may also reveal new strategies for stimulating it through a modification of the diet.

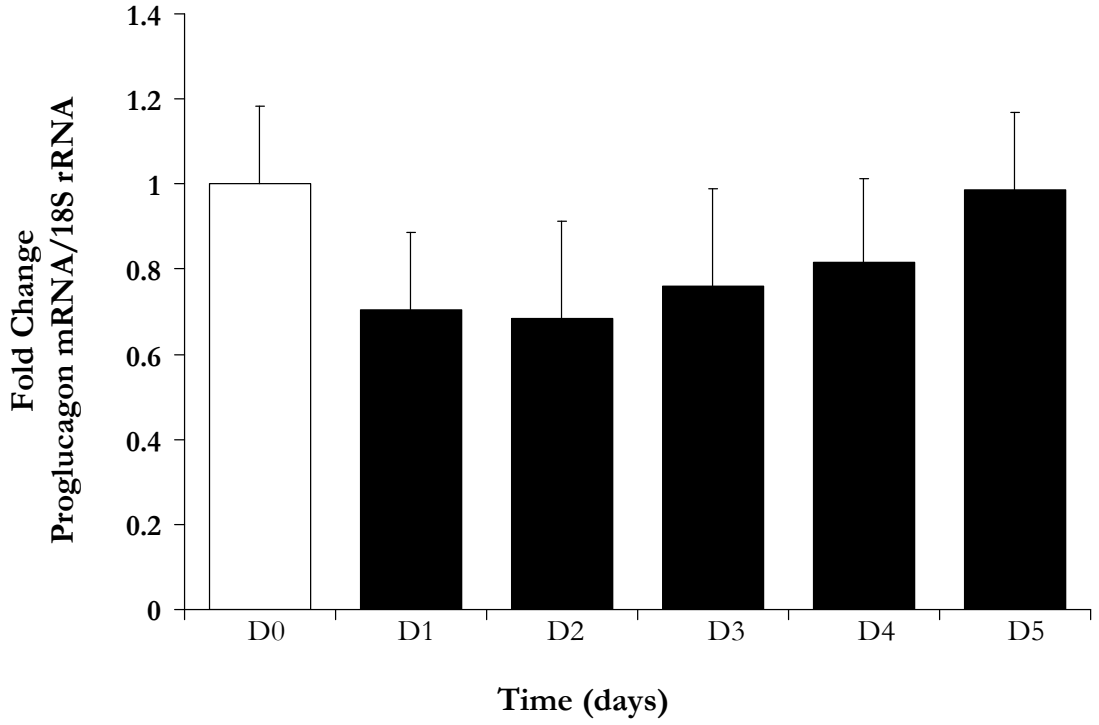
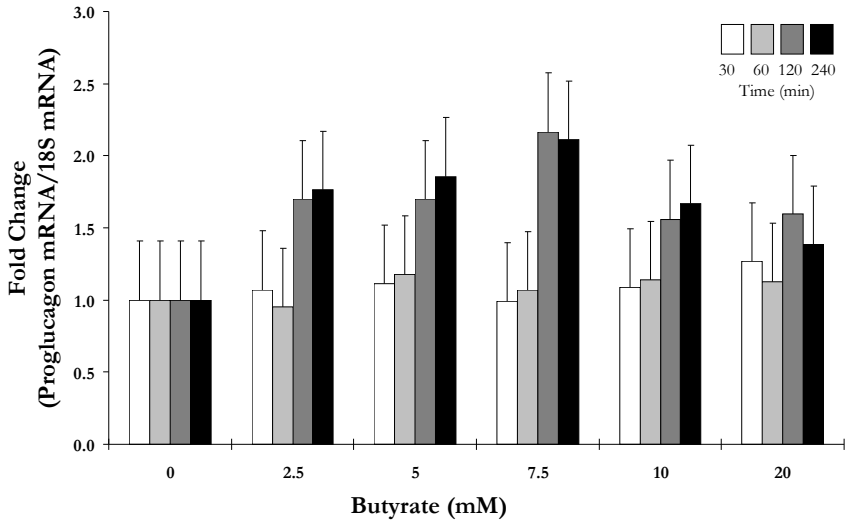


Figure 2.1 The effect of NCI-H716 cell aggregation on proglucagon mRNA abundance. Proglucagon gene expression did not significantly differ over the 5 days tested ($p = 0.41$).

A



B

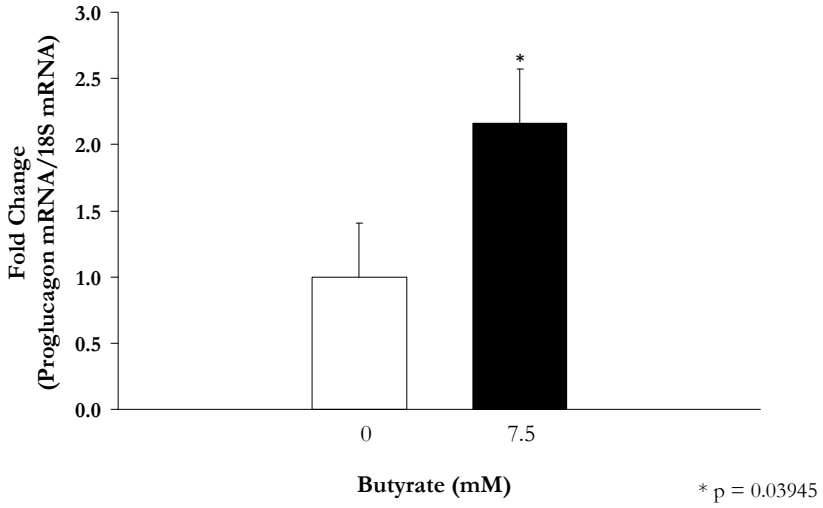


Figure 2.2 The effect of butyrate treatment on proglucagon mRNA abundance in NCI-H716 cells. **A** Proglucagon mRNA abundance tended to increase following butyrate treatment, particularly at the 2 and 4 hour time points. **B** Proglucagon mRNA abundance was significantly elevated in the presence of 7.5 mM butyrate following 2 hours of treatment ($p = 0.039$).

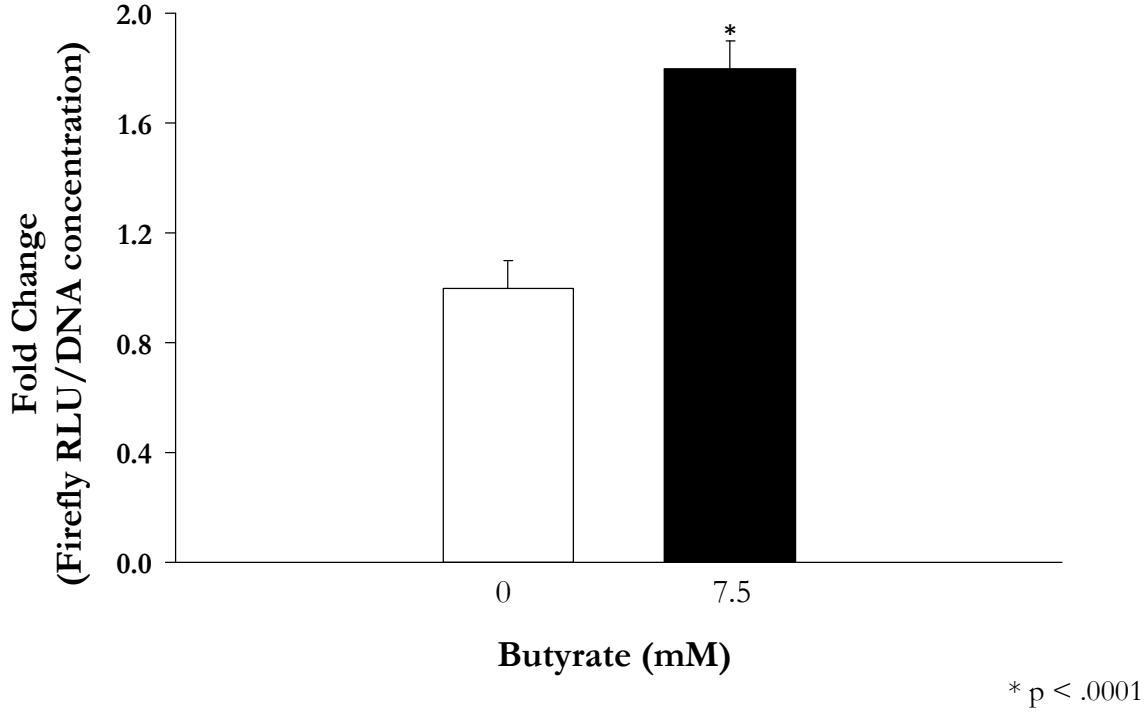


Figure 2.3 The effect of butyrate treatment on proglucagon promoter activation following transient transfection into NCI-H716 cells. Luciferase activity, as driven by the proglucagon promoter, was significantly elevated in the presence of 7.5 mM butyrate, indicative of proglucagon promoter activation ($p < 0.0001$).

CHAPTER 3

SHORT-CHAIN FATTY ACIDS INCREASE BUTYRATE TRANSPORTERS AND PROGLUCAGON ACTIVATION *IN VIVO* AND *IN VITRO*

ABSTRACT

Previous studies have established that short-chain fatty acids can interact with enteroendocrine L cells in the distal intestine to stimulate production and secretion of proglucagon-derived peptides such as GLP-1 and GLP-2. However, the mechanism by which this occurs is not completely understood. The aim of this study was to examine the effects of short-chain fatty acids on butyrate transporters and receptors *in vivo* and *in vitro*. Using NCI-H716 cells, we hypothesized that downregulating GPR43 or T2R38 receptors would inhibit butyrate from increasing proglucagon expression. Cells were transfected with gene-specific siRNA, treated with either 0 or 7.5 mM butyrate, and proglucagon mRNA abundance determined using real-time PCR. Butyrate significantly increased proglucagon expression in non-transfection control wells ($p = 0.0107$); however, when first treated with siRNA, butyrate did not stimulate this same response, with similar proglucagon levels seen with both 0 and 7.5 mM butyrate ($p = 0.5302$ for GPR43-treated cells; $p = 0.7070$ for T2R38-treated cells). We then examined the expression of the butyrate transporters MCT-1, SLC5A8, and SLC5A12 as well as GPR43 in 2 *in vivo* models. In Study 1, neonatal piglets received an 80% jejunoileal intestinal resection and jugular catheterization and were randomized to receive either control TPN or TPN supplemented with 9 mM butyrate for 12 hours, 3 days, or 7 days. In Study 2, neonatal piglets were randomized to receive either sow's milk replacer control formula, formula supplemented with 7.5 g/L soy polysaccharides, or formula supplemented with 7.5 g/L fructooligosaccharides

for 14 days. Transporter abundance was not significantly elevated with butyrate treatment at any time point in Study 1; however, ileal SLC5A8 abundance increased with FOS treatment ($p = 0.05$) and colonic MCT-1 expression was elevated with soy polysaccharide treatment ($p = 0.023$) in Study 2 piglets. Therefore, these results reveal that SCFAs are capable of upregulating their transporters when provided luminally, but not systemically, and that butyrate may be mediating its effects on the proglucagon expression via GPR43 and T2R38 receptors on the L cell.

INTRODUCTION

Short-chain fatty acids (SCFAs) are products of microbial fermentation in the distal intestine and include acetate, propionate, and butyrate. Of interest, butyrate is a 4-carbon SCFA that is particularly important in modulating epithelial structure and mucosal functionality. While acetate and propionate are absorbed and transported to the liver for metabolism, butyrate remains in the intestine, serving as a primary energy source in the colonocytes. In fact, butyrate oxidation accounts for 70% of oxygen consumption within the colon, thus sparing glucose and glutamine for use elsewhere within the intestine (Topping and Clifton, 2001; Wong et al., 2006).

Butyrate may play a particularly important role in patients suffering with short bowel syndrome (SBS), as both an energy source and trophic factor. With less intestinal absorptive surface area in general, more nutrients reach the colon undigested than seen in a normal, intact intestine, resulting in increased fermentation and thus increased SCFA production (Nordgaard et *al.*, 1994). Indeed, butyrate has been found to be trophic to the intestine, as both intravenous and intracecal administration of SCFAs stimulated increased mucosal weight, RNA, DNA, and protein concentrations as well as upregulated nutrient transporters and uptake (Koruda et al., 1990; Tappenden et al., 1997). Oftentimes, supplementation of SCFAs or butyrate specifically

has resulted in increased proglucagon mRNA and plasma GLP-2 concentration (Tappenden et al., 1998; Tappenden and McBurney, 1998; Bartholome et al., 2004); therefore, the adaptive responses associated with SCFA administration may be the result of stimulating this intestinotrophic hormone. However, the mechanism by which butyrate interacts with the L cell remains unknown, as the cells could be transporting butyrate directly and stimulating GLP-2 release or as the L cell is an open type enteroendocrine cell, it may actually be sampling the luminal environment and responding accordingly by releasing GLP-2 in the presence of butyrate.

With the discovery of the importance of butyrate in the prognosis in various models of intestinal injury, it has become clear that a better understanding of the role of butyrate on short-chain fatty acid transporters and receptors is warranted. Therefore, we have utilized 2 *in vivo* models to examine the butyrate-mediated effects on butyrate transporters and receptors within the intestine, including the H+-coupled monocarboxylate transporter MCT-1, the high-affinity Na+-coupled monocarboxylate transporter SLC5A8 (SMCT1), the low-affinity Na+-coupled monocarboxylate transporter SLC5A12 (SMCT2), and the short-chain fatty acid receptor GPR43. In addition, we have also downregulated 2 receptors, the SCFA receptor GPR43 and the bitter taste receptor T2R38, *in vitro* to explore a potential butyrate sensing mechanism by the L cell, ultimately leading to proglucagon activation.

MATERIALS AND METHODS

In Vivo Studies

Study 1 Experimental Design

Forty-eight hour old piglets (n = 36; 6/group) were obtained from the Imported Swine Research Laboratory (University of Illinois, Urbana, IL) and received superior vena cava

cannulation, swivel placement, and an 80% jejunoileal resection as previously described (Bartholome et al., 2004). Following resection, piglets were randomly assigned to one of the following treatment groups: 1) control TPN or 2) an isoenergetic and isonitrogenous TPN supplemented with 9 mM butyrate. Within each group, piglets were further randomized to receive treatment for 3 different time points including 12 hours, 3 days, and 7 days.

Study 2 Experimental Design

Forty-eight hour old piglets (n = 18; 6/group) were obtained from the Imported Swine Research Laboratory (University of Illinois, Urbana, IL) and were randomized to receive one of the following diets for 14 days: 1) sow's milk replacer formula (Advance Baby Pig Liqui-Wean; Milk Specialties, Dundee, IL); 2) formula supplemented with 7.5 g/L of soy polysaccharides (SPS); or 3) formula supplemented with 7.5 g/L fructooligosaccharides (FOS). Daily formula intake was 15 mL/kg/hr, which was provided as equal amounts every 12 hours.

Sample Collection

Upon completion of the treatment period, piglets were administered sodium pentobarbital (Fatal Plus; Veterinary Laboratories, Lenexa, KS); Study 1 piglets received this intravenously while Study 2 piglets received this through intracardiac injection. The intestine was quickly removed and rinsed with 0.9% saline. Briefly in Study 1, intestinal tissue located between the ligament of Treitz and the anastomosis was designated as jejunum; ileum was designated as the intestinal segment located between the anastomosis and the ileocecal valve. In Study 2, the entire small intestine between the ligament of Trietz and ileocecal valve was removed and partitioned into the thirds; the most proximal third was designated as jejunum and the most distal

third as the ileum. In both studies, the colon was removed distal to the ileocecal valve. Once excised, tissue and mucosal scrapings of each segment were taken, snap frozen in liquid nitrogen, and stored at -80° C until analysis.

RNA Isolation and Integrity

Total cellular RNA was isolated from the tissue samples using Trizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol and RNA concentration quantified using a spectrophotometer (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE). In order to check RNA integrity, RNA gel electrophoresis was run to examine 18S and 28S rRNA bands. Briefly, 10 µg of RNA was loaded onto a denaturing agarose gel. The samples were run at 100 V until bromophenol blue dye reached the end of the gel. Integrity of the bands was examined using a UV illumination system (Fotodyne Foto/Analyst Image Capture System; Fotodyne, Hartland, WI).

Determination of Transporter and Receptor mRNA Abundance with Real-Time PCR

Reverse transcription reactions were performed in a Gene Mate Genius thermocycler (ISC Bioexpress, Kaysville, UT) in a total volume of 20 µl containing 5 µg RNA, 250 ng Random Primers, 0.5 mM dNTP mix, 1x First-Strand Buffer, 0.01 M DTT, 40 units RNase Out, and 200 units Superscript II reverse transcriptase (all reverse transcription reagents were obtained from Invitrogen, Carlsbad, CA). RNA, random primers, and dNTPs were heated to 65° C for 5 minutes after which the remaining reagents were added and reactions incubated at 25° C for 10 minutes followed by 42° C for 50 minutes and then finally inactivated at 70° C for 15 minutes.

Resultant cDNA was diluted 1:50 and used for semi-quantitative real-time PCR reactions. Reactions were performed with primers multiplexed in each well, with a total volume of 10 µl containing 2 µl cDNA, 1x TaqMan Universal Master Mix, 1x FAM-labeled Sus scrofa Custom TaqMan Gene Expression Assay primer-probe set, and 1x VIC-labeled eukaryotic 18S rRNA Endogenous Control TaqMan Gene Expression Assay primer-probe set (all real-time PCR reagents were obtained from Applied Biosystems, Foster City, CA). Primers and probes were designed according to Applied Biosystems Custom TaqMan Genomic Assays Service. Briefly, Sus scrofa sequences were obtained for GPR43, MCT-1, SLC5A8, and SLC5A12 using National Center for Biotechnology Information (NCBI) databases (http://www.ncbi.nlm.nih.gov/). Quality of the sequences was assessed using Repeat Masker (http://www.repeatmasker.org), NCBI Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov/BLAST/), NCBI Single Nucleotide Polymorphism (SNP) database (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_blastByOrg.cgi), and the Vertebrate Genome Annotation (VEGA) database (http://vega.sanger.ac.uk/index.html). The primer and probe sequences were as follows: GPR43 (forward) 5'-GCCTGACGCTGGCAGA-3', (reverse) 5'-TCCCGGGCAGGTACCA-3', and (probe) CCGCGTCTAACTTC; MCT-1 (forward) 5'-TGGCAGTCGTCCAGTCATG-3', (reverse) 5'-TCAAGCCACAGCCTGACAAG-3', and (probe) CAGCCGCCAATAAT; SLC5A8 (forward) 5'-TGGGACAAATTGGATGACAACCA-3', (reverse) 5'-AGTGGAGTCCTTTCAATGTT GTGT-3', and (probe) CAGAAGTGCCATTTTC; SLC5A12 (forward) 5'-GTGGGACTCTGAGCACTGTAG-3', (reverse) 5'-CCTCAAAGGTCACTGTTGCT AAGG-3', and (probe) CTGCCAGCATCAATG. Samples were run in a 384-well plate in a TaqMan ABI 7900 real-time PCR machine and analyzed with Sequence Detection Systems 7900HT version 2.2.1 software (Applied Biosystems, Foster City,

CA) Briefly, samples were heated to 50° C for 2 minutes and then 95° C for 10 minutes to allow for DNA polymerase activation followed by 40 cycles of 95° C for 15 seconds and 60° C for 1 minute to allow for denaturing, annealing, and extension. Data were analyzed using the standard curve method with pooled cDNA serving as the standard. Transporter levels were normalized to 18S rRNA.

In vitro studies

Culturing NCI-H716 Cells

NCI-H716 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained as described. Briefly, cells were maintained in 75 cm^2 tissue culture flasks (TPP, St. Louis, MO) in RPMI-1640 media containing 2.0 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10 mM HEPES buffer, 1.0 mM sodium pyruvate, and 4.5 g/L glucose and supplemented with 10% fetal bovine serum (all media reagents obtained from Hyclone, Logan, UT) in a 37° C incubator injected with 5% CO_2.

Transfection of NCI-H716 Cells

In order to examine the role of specific receptors in butyrate-stimulated proglucagon upregulation, NCI-H716 cells were transiently transfected with Silencer Pre-designed siRNA specific for either the short-chain fatty acid receptor GPR43 (siRNA ID # 2192) or the bitter taste receptor T2R38 (siRNA ID # 43301; Ambion, Austin, TX). Briefly, on the day of transfection, cells were pelleted by centrifugation (Centrific Centrifuge; Fisher, Pittsburgh, PA), washed in 1x PBS (Hyclone, Logan, UT), and resuspended in serum-free media. After determining cell concentration, 5 x 10^4 cells in 0.5 mL were seeded into 24-well plates. To prepare siRNA for

transfection, 0.018 nmol of siRNA (a final concentration of 30 nM/well) was diluted in 100 µl of serum-free media. In order to enhance transfection efficiency, siRNA was incubated with 0.5 µl of PLUS reagent (Invitrogen, Carlsbad, CA) for 5 minutes at room temperature. Following incubation, 3 µl of Lipofectamine LTX (Invitrogen, Carlsbad, CA) were added to each reaction and allowed to incubate at room temperature for 30 minutes to allow for complex formation after which the DNA-Lipofectamine LTX complexes were added to the wells. The plate was incubated at 37° C in a CO_2 incubator for 4 hours to allow for transfection after which the media was changed. Cells were then allowed to further incubate for 24 hours to allow for gene silencing. Following incubation, cells were pelleted and resuspended in either 0 or 7.5 mM butyric acid (Sigma, St. Louis, MO), and incubated for 2 hours prior to testing gene expression. This procedure was repeated on 5 separate occasions.

Determination of Proglucagon mRNA Abundance with Real-Time PCR

Following the treatment period, cells were pelleted by centrifugation at speed 7 (Centrific Centrifuge; Fisher, Pittsburgh, PA) and washed in 1x PBS (Hyclone, Logan, UT). Total cellular RNA was isolated from the cell pellets using the Total RNA Purification Kit (Norgen Biotek, Thorold, Ontario, Canada) according to manufacturer's protocol and RNA concentration quantified using a spectrophotometer (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE). Reverse transcription reactions were performed in a Gene Mate Genius thermocycler (ISC Bioexpress, Kaysville, UT) as previously described for the *in vivo* studies using 0.2 µg RNA. Resultant cDNA was used for semi-quantitative real-time PCR reactions as previously described for the *in vivo* studies using the FAM-labeled human proglucagon mRNA TaqMan Gene Expression Assay (Assay ID Hs00174967_m1) and the VIC-labeled human 18S rRNA (Assay

ID Hs99999901_s1) TaqMan Gene Expression Assay primer-probe set (all real-time PCR reagents were obtained from Applied Biosystems, Foster City, CA). Data were analyzed using the standard curve method with pooled cDNA serving as the standard. Proglucagon levels were normalized to 18S rRNA.

Statistical Analysis

For the Study 1 transporter analysis, statistical differences between treatments were determined using a two-way analysis of variance (ANOVA) design in which the effect of the experimental variation between litters was blocked for (block = litter; main effects = treatment and time; interaction = treatment x time). For Study 2, the effect of litter was blocked for (block = litter; main effect = treatment) and a one-way ANOVA was used to determine significance of the treatment groups. In the siRNA assays, the effect of plate was blocked for (block = plate; main effect = treatment) and a one-way ANOVA used to determine significance. Analysis was carried out using a mixed model in SAS (Version 9.1; SAS Institute, Cary, NC). All values are reported as mean ± SEM and statistical significance was defined as $p \leq 0.05$.

RESULTS

In vivo studies

Study 1 Transporter and Receptor mRNA Abundance

Semi-quantitative real-time PCR was performed to determine mRNA abundance of the butyrate transporters MCT-1, SLC5A8 (SMCT1), and SLC5A12 (SMCT2) as well as the short-chain fatty acid receptor GPR43 in jejunum, ileum, and colon tissue samples isolated from piglets receiving 12 hours, 3 days, or 7 days of treatment (**Tables 3.1 and 3.2**). Slight increases

were seen in some of the transporters; however, none of the Study 1 data was significantly different for any treatment or time ($p > 0.05$). MCT-1 abundance remained unchanged despite butyrate supplementation in all 3 segments and at all timepoints. SLC5A8 was unaffected in the jejunum; however, abundance tended to increase with butyrate in the ileum at 3 days (0.3746 ± 0.4860 vs. 0.5891 ± 0.4860 SLC5A8 mRNA/18S rRNA for Control and 9 mM Butyrate, respectively) and in the colon at 7 days (0.000160 ± 0.01236 vs. 0.02534 ± 0.01353 SLC5A8 mRNA/18S rRNA for Control and 9 mM Butyrate, respectively). Little change in SLC5A12 was seen in the more distal ileum and colon, but jejunal SLC5A12 abundance tended to increase with butyrate treatment, particularly at the 7 day timepoint (0.8633 ± 0.3268 vs. 1.8118 ± 0.3580 SLC5A12 mRNA/18S rRNA for Control and 9 mM Butyrate, respectively). GPR43 abundance was unaffected in the proximal intestine by butyrate, but tended to increase in the colon following 7 days of treatment (0.4373 ± 0.2160 vs. 0.8230 ± 0.2206 GPR43 mRNA/18S rRNA for control and 9 mM Butyrate, respectively).

Study 2 Transporter and Receptor mRNA Abundance

Semi-quantitative real-time PCR was performed to determine mRNA abundance of the butyrate transporters MCT-1 and SLC5A8 (SMCT1) as well as the short-chain fatty acid receptor GPR43 in ileum and colon tissue samples isolated from piglets receiving 14 days of treatment (**Table 3.3**). MCT-1 abundance did not change in the ileum. However, in the colon, MCT-1 expression was significantly elevated with supplementation of soy polysaccharides; expression in the FOS treated animals was intermediate, but not significantly different from the control group (1.3543 ± 0.3555 vs. 2.6698 ± 0.3555 vs. 1.8435 ± 0.3277 MCT-1 mRNA/18S rRNA for Control, SPS, and FOS, respectively; $p = 0.023$, **Figure 3.1**). The abundance of

SLC5A8 was significantly elevated in the ileum with FOS fermentation while SPS had no effect (0.3877 ± 0.3028 vs. 0.3133 ± 0.2890 vs. 0.8801 ± 0.2890 SLC5A8 mRNA/18S rRNA for Control, SPS, and FOS, respectively, $p = 0.05$, **Figure 3.2**). SLC5A8 levels did not differ among treatment groups in the colon. GPR43 abundance remained unchanged in both ileum and colon across the treatment groups ($p > 0.05$).

In Vitro Studies

Proglucagon Expression Following siRNA Treatment

Real-time PCR was utilized to verify expression of GPR43 and T2R38 receptors within the NCI-H716 cell line (**Figure 3.3**). Once expression was determined, siRNA-treated and control cells were treated with either 0 or 7.5 mM butyrate and proglucagon expression determined. Control wells responded appropriately to butyrate treatment, with a 2-fold increase in abundance observed (0.4778 ± 0.1284 vs. 0.9528 ± 0.1284 proglucagon mRNA/18S rRNA for 0 and 7.5 mM butyrate; $p = 0.0107$). However, when GPR43 siRNA was transfected into the cells, proglucagon abundance did not increase with butyrate treatment as previously reported (0.5330 ± 0.1284 vs. 0.4185 ± 0.1284 proglucagon mRNA/18S rRNA for 0 and 7.5 mM butyrate, respectively; $p = 0.5302$, **Figure 3.4**). Similar effects were seen upon T2R38 siRNA transfection (0.4377 ± 0.1436 vs. 0.3611 ± 0.1436 proglucagon mRNA/18S rRNA for 0 and 7.5 mM butyrate, respectively, $p = 0.7070$, **Figure 3.5**).

DISCUSSION

It has been estimated that over 10,000 people in the United States alone suffer with SBS (Buchman, 2006). As a result, there is less intestinal absorptive surface area available and an

increasing amount of nutrients reach the distal intestine that may be fermented. Recently, the concept of nutrient sensing was introduced in which cells within the gastrointestinal tract detect the presence of specific nutrients and respond accordingly, regulating proper digestion and absorption, insulin secretion, food intake, and satiety, for example. Indeed, the presence of lipid and protein in the diet will stimulate the secretion of hormones such as CCK and secretin, ultimately leading to release of digestive enzymes. Absorptive mechanisms are similarly affected, as detection of sucralose by sweet taste receptors in rat enterocytes will double glucose absorption by increasing apical GLUT2 protein abundance (Mace et al., 2007). While nutrient sensing is oftentimes thought to occur more proximally where the majority of nutrients are digested and absorbed, distal nutrient sensing is also particularly important. For instance, nutrient stimulation of distal L cells will stimulate the release of hormones such as PYY, which will act as a part of the ileal brake mechanism to slow gastric emptying and intestinal transit rate, and GLP-2, which will stimulate trophic effects on the intestinal epithelium, both of which are particularly important in SBS patients with higher concentrations of nutrients interacting with the ileum and colon than usual.

Multiple mechanisms by which nutrients can interact with endocrine cells have been proposed including: 1) endocrine cells directly monitor the lumen using specific receptors and transporters located on the microvilli; 2) the enteric nervous system is activated by the nutrients, which in turn activates the endocrine cells; and 3) epithelial cells detect the nutrients and release factors that interact and stimulate the neighboring endocrine cells (Buchan, 1999). Recently, 2 types of receptors were localized on the L cells, indicating that they may be directly monitoring luminal contents. Originally localized within leukocytes and neutrophils (Le Poul et al., 2003; Nilsson et al., 2003), the G protein-coupled receptor GPR43 is a SCFA receptor (Brown et al.,

2003), which has since been found in human and rat enteroendocrine L cells (Karaki et al., 2006; Karaki et al., 2008). As it will respond to SCFAs and is located on the L cell, GPR43 is an excellent candidate for monitoring the changes in the luminal environment and stimulating hormone-mediated events. Similarly, while usually thought to be located within specialized neuroepithelial cells of the tongue, the bitter taste receptor T2R38 has also been localized within human enteroendocrine cells along with its G protein gustducin (Rozengurt et al., 2006). Thus, these receptors may be particularly important for detecting and appropriately responding to dietary fibers, resistant starches, and malabsorbed nutrients that escape digestion and are fermented into SCFAs, thereby stimulating the release of hormones, including the intestinotrophic GLP-2.

As GLP-2 is encoded by the proglucagon gene, we examined the effects of downregulation of either GPR43 or T2R38 receptors on proglucagon gene expression following treatment with the SCFA butyrate. Previous *in vivo* studies have established that fermentable substrates and butyrate specifically will drive increased proglucagon abundance (Gee et al., 1996; Reimer and McBurney, 1996; Bartholome et al., 2004). In the human colon cancer cell line NCI-H716, 7.5 mM butyrate treatment stimulated a nearly 2-fold increase in proglucagon mRNA abundance compared to controls. However, when the GPR43 receptor was first downregulated, butyrate treatment was no longer able to stimulate this same response in proglucagon, with virtually identical proglucagon abundance reported in 0 and 7.5 mM treatment groups. Similar effects were seen when the T2R38 receptor was first silenced, with little change in proglucagon mRNA abundance reported. The receptors were down-regulated with gene-specific siRNA. The siRNA is designed to bind to target mRNA only and does not impact RNA abundance globally. This is supported by the fact that proglucagon mRNA abundance was not

reduced by the receptor siRNA, as indicated by similar levels of proglucagon in the controls and siRNA-treated groups as opposed to a further reduction in proglucagon following treatment with siRNA as compared to the controls. Rather, down-regulation of the receptors was having a downstream effect on proglucagon mRNA abundance following butyrate treatment. Thus, these results indicate that GPR43 and T2R38 receptors may be mediating butyrate's effects on the proglucagon gene through cell signaling cascades.

When Le Poul and colleagues examined GPR43 receptor signaling, they found that its activation by SCFAs was linked to formation of inositol 1,4,5-trisphosphate, mobilization of intracellular calcium, and activation of the ERK 1/2 pathway (Le Poul et al., 2003). Similarly, stimulation of the T2R38 receptor will increase the intracellular calcium concentration (Rozengurt et al., 2006). As we have shown that inhibiting receptor abundance decreases proglucagon expression, these intracellular events may be mediating proglucagon activation. While the mechanism is not completely understood, proglucagon transcription may involve these signaling pathways. Indeed, multiple calcium-dependent elements have been identified in the proglucagon gene. Calcium influx will stimulate phosphorylation of cAMP response element binding protein (CREB), which in turn binds the cAMP response element (CRE) and induces transcription (Schwaninger et al., 1993). Additional calcium response elements have also been identified, including the G2 element in which calcium stimulates the binding of NFATp and HNF-3β transcription factors to the G2 element, ultimately resulting in gene transcription (Furstenau et al., 1999). Similarly, the ERK 1/2 pathway has also been implicated as stimulation of the cAMP-dependent Epac pathway activates ERK 1/2 phosphorylation and ultimately proglucagon gene transcription (Lotfi et al., 2006). Moreover, increased calcium is also associated with stimulating exocytosis of hormones; therefore, the signaling induced by these

receptors may be involved in both proglucagon transcription as well as GLP-2 secretion. Further research is needed to determine the exact role of these secondary messengers in the regulation of proglucagon by butyrate.

After determining that butyrate may be mediating its effects through GPR43 and T2R38 receptors *in vitro*, we then wanted to explore the effects of butyrate on receptors and transporters using 2 *in vivo* models. In the first study, 2-day old piglets received an 80% jejunoileal resection and jugular catheterization and were randomized to receive either control TPN or TPN supplemented with 9 mM butyrate for either 12 hours, 3 days, or 7 days. In the second study, 2-day old piglets were randomized to either control formula, formula supplemented with 7.5 g/L SPS, or formula supplemented with 7.5 g/L FOS. Thus, in Study 1, butyrate specifically was provided systemically in a model of intestinal compromise whereas in Study 2, butyrate and the other SCFAs were produced in the intestine through fermentation reactions of the moderately fermentable SPS and highly fermentable FOS fibers in a model of intestinal development. Intravenous butyrate did not significantly change mRNA abundance of any of the genes examined; however, luminal SCFA production significantly increased ileal SLC5A8 expression 2.3-fold with FOS treatment and colonic MCT-1 expression by 2-fold with SPS treatment for 14 days. Therefore, it appears that increased abundance of the transporters is substrate-driven.

Indeed, studies have revealed both MCT-1 and SLC5A8 are increased in the presence of SCFAs. When rats were given the prebiotic germinated barley foodstuff for 2 weeks, cecal butyrate concentrations were significantly elevated by nearly 2-fold, which was associated with increased SLC5A8 expression compared to controls (Kanauchi et al., 2008). Fiber supplementation had similar effects on MCT-1 expression in rats, as 2 weeks on a pectin-containing diet increased MCT-1 protein in the stomach, duodenum, jejunum, ileum, cecum, and

colon, with greatest effects reported in the cecum and colon (Kirat et al., 2009). Furthermore, butyrate specifically increased MCT-1 mRNA abundance 5.7-fold and protein abundance by 5.2-fold in the human colonic epithelial cell line AA/C1 (Cuff et al., 2002). It was previously established that the piglets fed enterally produced significantly more acetate, propionate, and butyrate when on the fermentable diets, FOS and SPS (Correa-Matos, 2006). FOS is a highly fermentable fiber which will be rapidly used by the bacteria whereas SPS is only moderately fermentable. Therefore, it appears that when provided luminally, SCFAs will increase transporter mRNA abundance, particularly in the intestinal segment in which the fiber was fermented, with the rapidly fermented FOS stimulating upregulation in the distal ileum whereas the moderately fermentable SPS produced changes in the colon. As a result, these changes in receptor abundance and, therefore, butyrate uptake, may stimulate GLP-2 necessary for intestinal development and maintenance.

Significant changes in transporter abundance were not seen when butyrate was provided systemically. Yet, intestinal adaptation still occurred in these piglets receiving butyrate and was associated with elevated plasma GLP-2 levels (Bartholome et al., 2004), indicating that an interaction is occurring between butyrate and the enteroendocrine L cells. Both SLC5A8 and MCT-1 have been localized to the apical membrane of epithelial cells in the small and large intestine (Gill et al., 2005; Iwanaga et al., 2006). Changes may not have occurred in these transporters because they were not in use, despite the presence of substrate in the body. Research has shown that the lack of luminal stimulation through the gut will result in intestinal atrophy (Koruda et al., 1990), affecting not only intestinal structure, but functional capacity as well. Thus, another route of SCFA transport was likely utilized, such as passive diffusion or other exchangers and transporters, which would have allowed butyrate to follow the

concentration gradient from the blood into intestinal cells and stimulate the L cells to release GLP-2. Burrin and colleagues found that in a model of TPN-induced mucosal atrophy, 40% of nutrient intake had to be provided enterally in piglets in order to stimulate mucosal improvements associated with elevated GLP-2 levels (Burrin et al., 2000). Therefore, it is possible that luminal nutrients are required to increase abundance of these particular receptors and transporters in models of intestinal compromise.

To conclude, we have shown that butyrate-stimulated increases in proglucagon mRNA abundance may be mediated by GPR43 and T2R38 receptor signaling. Additionally, we also found that the presence of SCFAs in the lumen drove increased expression of SLC5A8 and MCT-1 transporters in healthy piglet intestine; however, these same effects were not seen when butyrate was provided intravenously in a model of intestinal injury despite increased GLP-2 levels. We believe these receptors and transporters may be integral parts of nutrient sensing mechanisms in the distal gut, with increased signaling and transport occurring not only to salvage important energy sources, but also to monitor the nutritional environment within the lumen, triggering hormone release as needed. While GPR43 and T2R38 are known to be expressed in L cells, the presence of SLC5A8 and MCT-1 has only been established in epithelial cells and has yet to be confirmed in enteroendocrine cells. Thus, it is also unknown whether butyrate is capable of interacting directly with the L cells via SLC5A8 and MCT-1 or if these transporters in epithelial cells stimulate paracrine effects on the endocrine cells. Therefore, further research is needed to determine the precise mechanism by which these receptors and transporters act on the L cells, ultimately providing a greater understanding of the mechanism by which butyrate interacts with proglucagon to stimulate intestinal adaptation. We believe this

information may be particularly important for providing insight into further nutritional targets for patients suffering with intestinal dysfunction.

Table 3.1 Effects of systemic butyrate on transporter and receptor mRNA expression in the small intestine following intestinal resection[1]

	12 hours		3 days		7 days		
	CONTROL	**BUTYRATE**	**CONTROL**	**BUTYRATE**	**CONTROL**	**BUTYRATE**	***p-value***
MCT-1 (MCT-1 mRNA/18S rRNA)							
Jejunum	1.9162 ± 0.4267	2.2047 ± 0.4267	1.3179 ± 0.4259	1.1877 ± 0.4000	0.9176 ± 0.2261	0.9629 ± 0.2528	ns
Ileum	0.8973 ± 0.4000	1.1575 ± 0.4000	0.4567 ± 0.4000	0.5766 ± 0.4000	0.6401 ± 0.1270	0.5896 ± 0.1391	ns
SLC5A8 (SLC5A8 mRNA/18S rRNA)							
Jejunum	2.7630 ± 0.5171	2.1285 ± 0.4860	1.4350 ± 0.5171	1.7010 ± 0.4860	2.1133 ± 0.5793	2.4511 ± 0.5903	ns
Ileum	0.8224 ± 0.4860	0.5707 ± 0.4860	0.3746 ± 0.4860	0.5891 ± 0.4860	0.5418 ± 0.1866	0.9070 ± 0.2044	ns
SLC5A12 (SLC5A12 mRNA/18S rRNA)							
Jejunum	1.9280 ± 0.4123	1.5337 ± 0.4123	2.2066 ± 0.4123	1.3471 ± 0.4123	0.8633 ± 0.3268	1.8118 ± 0.3580	ns
Ileum	0.4112 ± 0.4123	0.7419 ± 0.4123	0.6746 ± 0.4123	0.6913 ± 0.4123	1.5724 ± 0.3171	1.7933 ± 0.3423	ns
GPR43 (GPR43 mRNA/18S rRNA)							
Jejunum	2.0976 ± 0.6393	1.6087 ± 0.6393	1.3147 ± 0.6345	1.5239 ± 0.5836	1.3502 ± 0.6753	1.3882 ± 0.6930	ns
Ileum	1.5044 ± 0.6393	0.8067 ± 0.6393	0.7641 ± 0.5836	1.5696 ± 0.5836	0.8309 ± 0.1401	0.7354 ± 0.1513	ns

[1] Data are expressed as mean ± SEM.

Table 3.2 Effects of 7 days of systemic butyrate on transporter and receptor mRNA expression in the colon following intestinal resection[1]

	CONTROL	BUTYRATE	*p-value*
MCT-1 (MCT-1 mRNA/18S rRNA)	1.2820 ± 0.2824	0.7295 ± 0.2938	ns
SLC5A8 (SLC5A8 mRNA/18S rRNA)	0.000160 ± 0.01236	0.02534 ± 0.01353	ns
SLC5A12 (SLC5A12 mRNA/18S rRNA)	0.05437 ± 0.04356	0.08081 ± 0.04349	ns
GPR43 (GPR43 mRNA/18S rRNA)	0.4373 ± 0.160	0.8230 ± 0.2206	ns

[1] Data are expressed as mean ± SEM.

Table 3.3 Effects of fermentable fibers on transporter and receptor mRNA expression in the ileum and colon[1]

	CONTROL	SPS	FOS	*p-value*
MCT-1 (MCT-1 mRNA/18S rRNA)				
Ileum	0.4185 ± 0.1815	0.2294 ± 0.1815	0.1502 ± 0.1815	ns
Colon[2]	1.3543 ± 0.3555[b]	2.6698 ± 0.3555[a]	1.8435 ± 0.3277[b]	0.023
SLC5A8 (SLC5A8 mRNA/18S rRNA)				
Ileum[3]	0.3877 ± .3028[b]	0.3133 ± 0.2890[b]	0.8801 ± 0.2890[a]	0.05
Colon	0.05576 ± 0.03887	0.05484 ± 0.03064	0.05545 ± 0.03233	ns
GPR43 (GPR43 mRNA/18S rRNA)				
Ileum	1.4335 ± 0.4291	1.0026 ± 0.3878	0.8549 ± 0.3567	ns
Colon	0.7242 ± 0.2802	0.8564 ± 0.1772	0.8740 ± 0.1772	ns

[1] Data are expressed as mean ± SEM. Within each dependent variable, means with different letters are statistically different from each other.

[2] Within this dependent variable, a significant treatment effect existed, indicating that SPS had a higher treatment mean than either Control or FOS.

[3] Within this dependent variable, a significant treatment effect existed, indicating that FOS had a higher treatment mean than either Control or SPS.

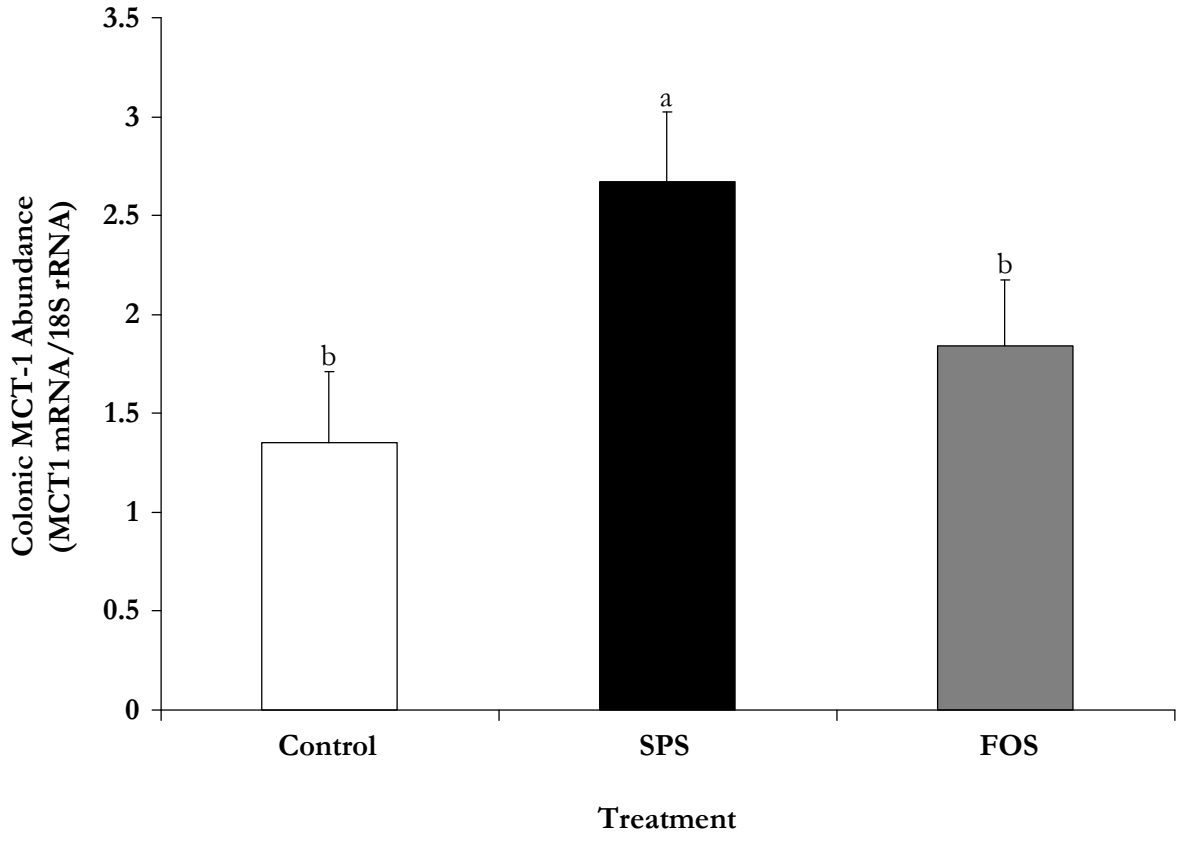


Figure 3.1 The effect of fermentable fibers on colonic MCT-1 mRNA abundance. The addition of the moderately fermentable fiber soy polysaccharides for 14 days significantly increased MCT-1 expression in the colon compared to a sow's milk replacer control formula in healthy piglets ($p = 0.023$). FOS treatment slightly increased MCT-1, but was not significantly different from piglets receiving the Control diet.

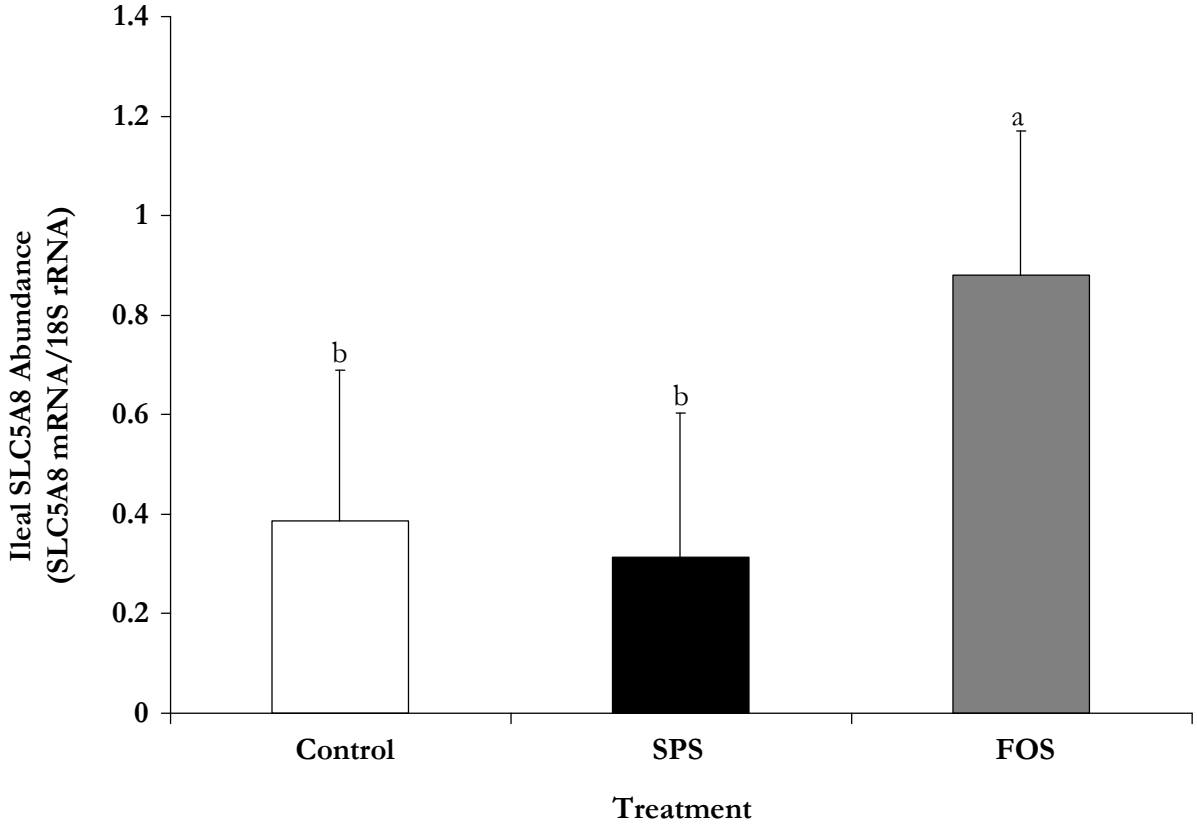


Figure 3.2 The effect of fermentable fibers on ileal SLC5A8 mRNA abundance. The addition of the highly fermentable fiber FOS for 14 days significantly increased SLC5A8 abundance in the ileum compared to a sow's milk replacer control formula in healthy piglets ($p = 0.05$). SPS treatment was not significantly different than the control piglets.

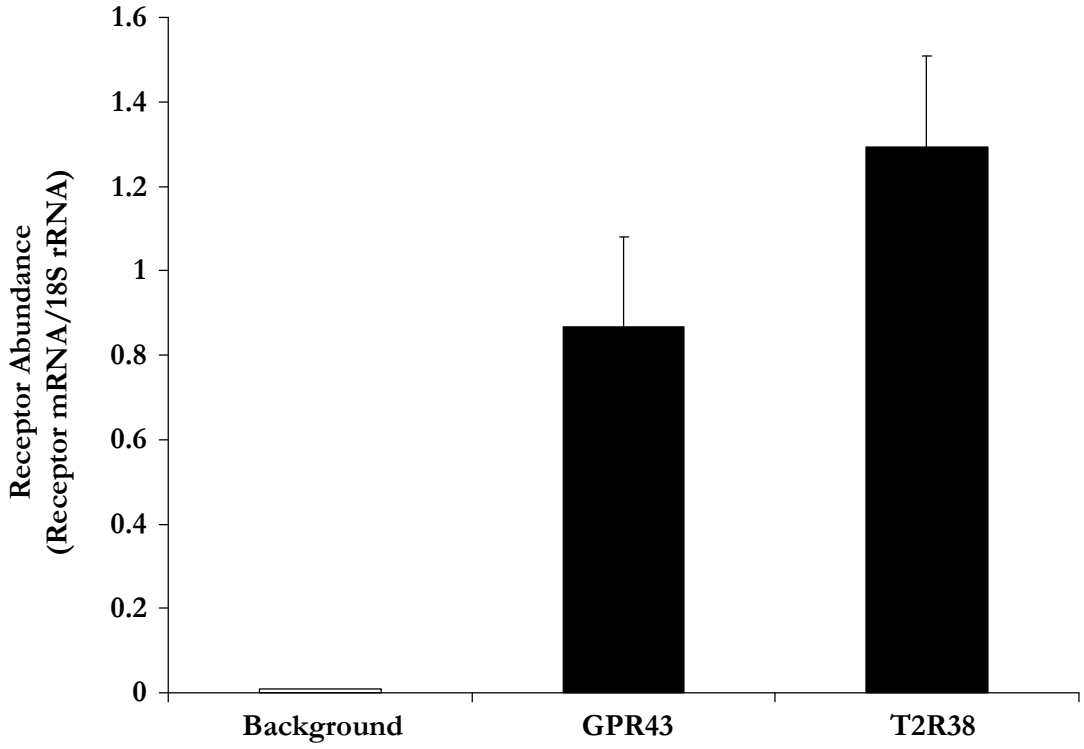


Figure 3.3 The expression of GPR43 and T2R38 receptors in the NCI-H716 cell line. Using real-time PCR, it was verified that the 2 receptors were, in fact, expressed in NCI-H716 cells compared to background levels.

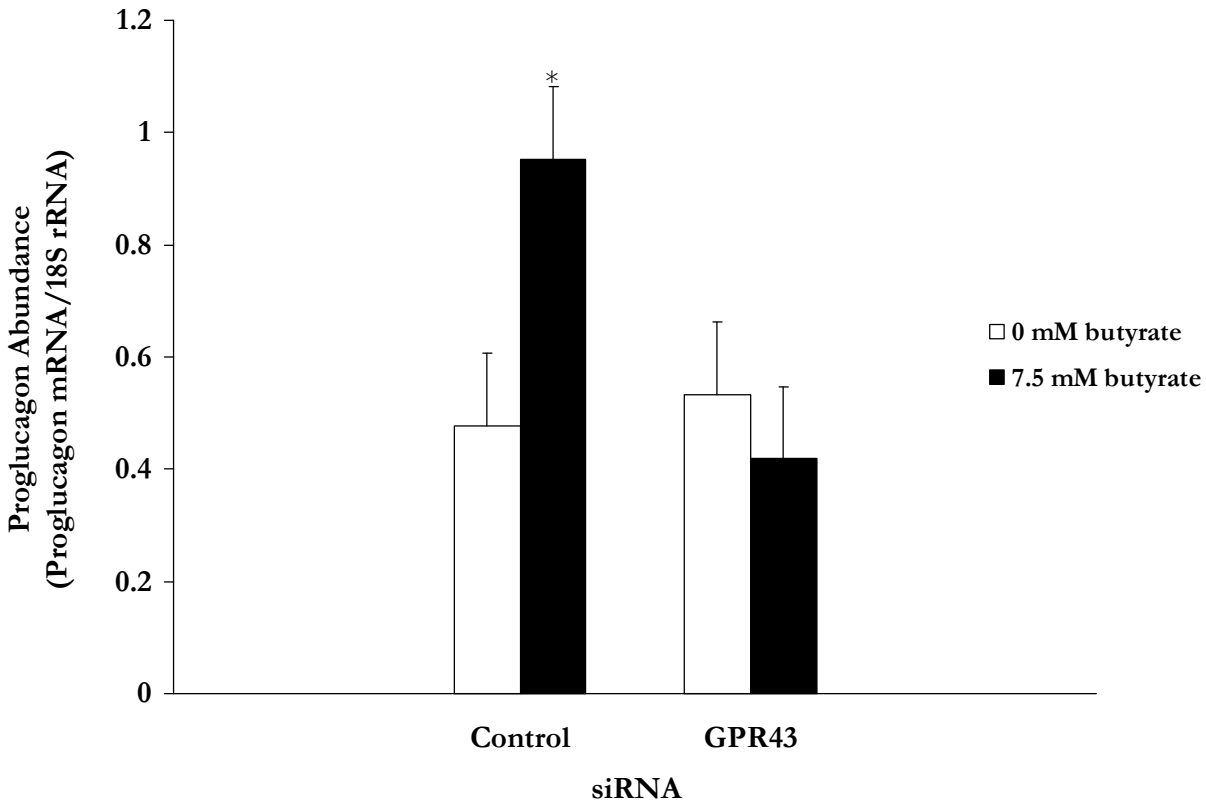


Figure 3.4 The effect of GPR43 siRNA on proglucagon mRNA abundance in NCI-H716 cells. Treatment with 7.5 mM butyrate in non-transfection control wells significantly increased proglucagon mRNA abundance by 2-fold ($p = 0.0107$). However, when cells were first treated with GPR43 to effectively downregulate receptor expression, butyrate was no longer able to stimulate an increase in proglucagon abundance, with no difference compared to control wells ($p = 0.5302$).

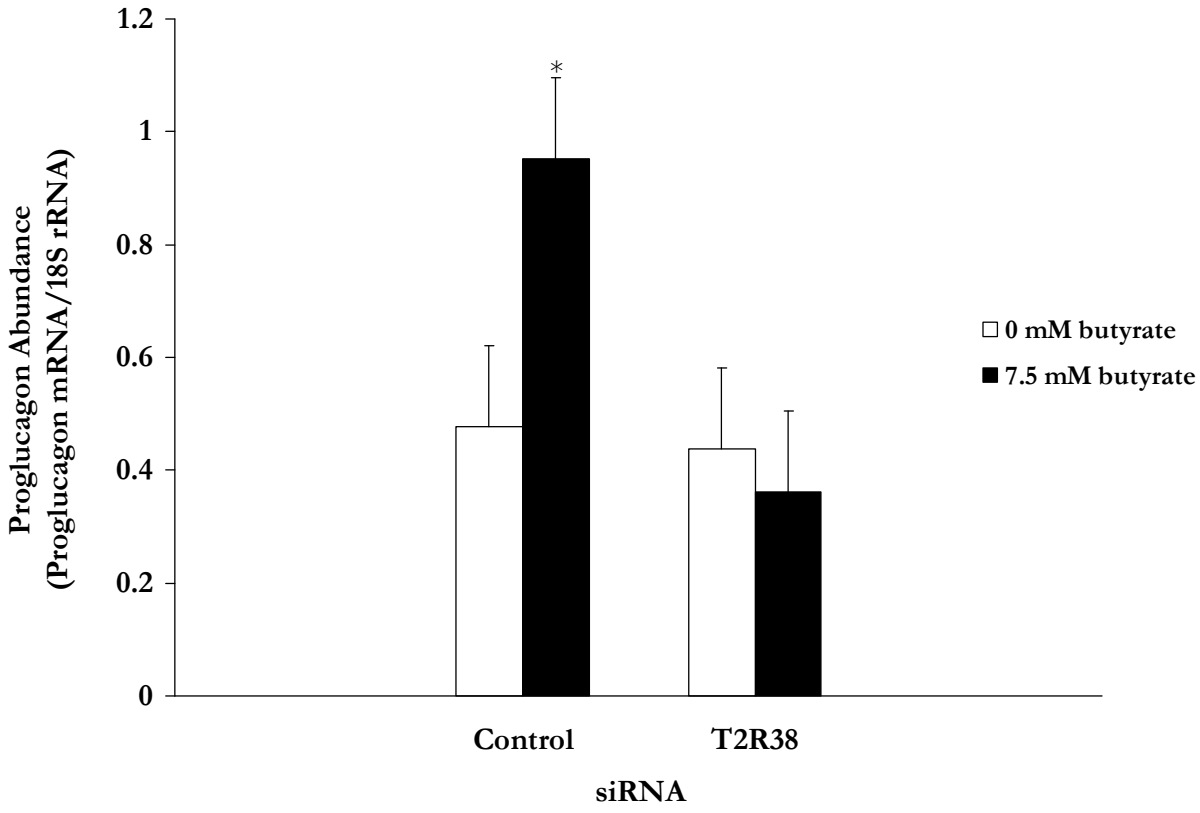


Figure 3.5 The effect of T2R38 siRNA on proglucagon mRNA abundance in NCI-H716 cells. Proglucagon mRNA abundance significantly increased in non-transfection control wells following treatment with 7.5 mM butyrate ($p = 0.0107$). However, decreased expression of T2R38 prevented butyrate from stimulating a similar increase in proglucagon abundance, with no difference compared to control wells ($p = 0.7070$).

CHAPTER 4

BUTYRATE ENHANCES STRUCTURAL MEASURES OF INTESTINAL GROWTH FOLLOWING LUMINAL ADMINISTRATION INTO THE DISTAL INTESTINE OF ADULT RATS

ABSTRACT

Dietary fibers are important for maintaining mucosal homeostasis and are known to be trophic to the intestine during periods of intestinal compromise. Previous research suggests these effects are due to fermentation products, specifically SCFAs. However, the exact effects of SCFAs, and butyrate in particular, in the lumen of the gut are not completely understood. Therefore, the aim of this study was to administer SCFAs into the distal intestine in the absence of other fermentation products and determine their effects on structural parameters and functional capacity of the intestine. Sprague-Dawley rats were randomized to 1 of 2 infusion groups: 1) ileal infusion; or 2) colonic infusion. Following surgery, rats were further randomized to 1 of 3 treatment groups: 1) 0.9% saline; 2) 60 mM butyrate; or 3) a SCFA cocktail consisting of 240 mM acetate, 100 mM propionate, and 60 mM butyrate. Rats received infusions 3 times a day for 7 days. Jejunal, ileal, and colonic weights, but not lengths, were significantly impacted by the infusions and were accompanied by increases in mucosal DNA, RNA, and protein concentrations. These effects are believed to be the result of mucosal expansion, as evidenced by significantly increased jejunal villus height and surface area, ileal villus height, and colonic crypt depth following butyrate infusions. Semi-quantitative real PCR analysis of proglucagon expression revealed butyrate infusions to significantly increase proglucagon mRNA abundance in ileal mucosa, suggesting that proglucagon and its peptide GLP-2 are driving these trophic responses within the intestine. However, functional analysis of butyrate transporters and receptors including MCT-1, SLC5A8, GPR43, and T2R38 revealed

little effect of infusions, suggesting structure is expanding, but the cells are not completely mature and expressing mRNA for these proteins. The results of these experiments revealed that luminal butyrate is capable of driving intestinal adaptation and is likely mediating its effects through proglucagon and GLP-2, thereby furthering our understanding of the intestinotrophic effects of fibers and their fermentation products.

INTRODUCTION

Proper intestinal growth, development, function, and adaptation are driven by enteral nutrition. The presence of nutrients in the lumen not only provides energy sources for the mucosa, such as glucose, amino acids, and SCFAs, but also stimulates release of a variety of gastrointestinal hormones which trigger cell signaling cascades responsible for tissue homeostasis and adaption. Indeed, research has shown starvation, fasting, and even partial dietary restriction to decrease intestinal weights, particularly at the mucosal level (Steiner et al., 1968; Chappell et al., 2003). However, this reaction is not necessarily due to a lack of adequate nutrition. TPN administration also induces mucosal atrophy in both piglet and human models despite meeting nutritional needs (Morgan et al., 1987; Buchman et al., 1995). In fact, in neonatal piglet models, 60% of nutrients needed to be provided enterally in order to maintain normal intestinal growth (Burrin et al., 2000; Stoll et al., 2000).

Of the many classes of nutrients, dietary fibers are particularly important for the intestinal mucosa. Previous research has established that when animals are provided with elemental diets which lack fiber, intestinal atrophy will ensue, particularly in the distal intestine as the majority of nutrients will be absorbed very quickly in the proximal intestine (Janne et al., 1977; Morin et al., 1980; Ecknauer et al., 1981). Moreover, the addition of fiber to the diet has shown to be

important for driving improvements in crypt-villus architecture (Kelberman et al., 1995; Thulesen et al., 1999), nutrient absorption (Buddington et al., 1999; Kanauchi et al., 2008; Kirat et al., 2009), and barrier function (Mariadason et al., 1999; Ito et al., 2009).

One of the characteristic properties of fibers is that they escape digestion in the small intestine and are then fermented by the microbiota of the gut. Comparison of germ-free and conventional animals suggest that the improvements in the intestine are not mediated by the presence of fiber itself, but rather, the products of fermentation in the lumen, such as SCFAs, carobon dioxide, and hydrogen ions (Goodlad et al., 1989). Further evidence suggests that SCFAs, in particular butyrate, may be responsible for stimulating these effects by acting through the intestinotrophic hormone GLP-2 (Koruda et al., 1988; Koruda et al., 1990; Tappenden et al., 1997; Tappenden et al., 1998; Bartholome et al., 2004). In order to explore the effects of luminal SCFAs and butyrate specifically, we have utilized an intestinal intubation model to examine the structure and functional capacity of the intestine in adult Sprague-Dawley rats on a fiber-free diet. We hypothesized that the addition of SCFAs would stimulate increases in structural and functional indices within the intestine. Additionally, we believed that these increases would be the result of butyrate specifically and would be mediated by GLP-2.

MATERIALS AND METHODS

Experimental Design

In order to examine the effects of luminal butyrate specifically, adult male Sprague-Dawley rats (n = 48) were randomized to 1 of 2 surgical groups: 1) ileal intubation; or 2) colonic intubation. Following surgeries, they were further randomized to 1 of 3 treatment infusion groups: 1) 0.9 % saline; 2) 60 mM butyrate; or 3) a SCFA mixture consisting of 240 mM

acetate, 100 mM propionate, and 60 mM butyrate in the molar proportions found in the human colon. All rats were maintained on a fiber-free elemental diet and euthanized after 7 days of treatment. All procedures were performed in accordance to the Institutional Animal Care and Use Committee at the University of Illinois Urbana-Champaign.

Animals

Forty-eight adult male Sprague-Dawley rats (250 – 300 g; Harlan, Indianapolis, IN) were housed individually in wire-bottom cages in a temperature- and humidity-controlled facility with 12-hour light and dark cycles at the Institute of Genomic Biology Vivarium (University of Illinois, Urbana, IL). Animals were allowed to acclimatize for 3 days prior to surgery during which they had free access to a nutritionally complete elemental diet and tap water. The elemental diet was provided during the acclimation period in order to reduce gastrointestinal residue prior to surgery and to allow the animals to adapt to the diet prior to the experimental period.

Experimental Surgery

Twelve hours prior to surgery, rats were withdrawn from food, but not water. Animals were then anesthetized with ketamine/xylazine (87 mg/kg body weight Ketaset, Fort Dodge Animal Health, Fort Dodge, IA; and 13 mg/kg body weight Anased, Lloyd Laboratories, Shenandoah, IA) via intramuscular injection. Upon achieving surgical plane anesthesia as confirmed by absence of toe-pinch reflex, deep, stable respiration, and absence to response to external stimuli, animals were administered enrofloxacin antibiotic (5 mg/kg body weight Baytril; Bayer Animal Health, Shawnee, KS) subcutaneously, the abdomen was clipped and

cleansed with betadine (10% povidone iodine; Purdue Frederick, Norwalk, CT), and petrolatum ophthalmic ointment (Puralube Vet Ointment; Dechra Veterinary Products, Overland Park, KS) was applied. Sterile instruments and aseptic techniques were used at all times. Animals underwent laparotomy and infusion catheters (Argyle 3.5 French polyvinyl chloride catheter with luer lock hub; Covidien, Mansfield, MA) were placed in either the distal ileum (30 cm proximal to the ileocecal valve) or the proximal colon (3 cm distal to the cecocolonic junction). Briefly, the intestine was measured using sterile string placed along the anti-mesenteric border and catheters were inserted at either 33 cm from the ileocecal valve or into the colon and then threaded down 3 cm to their respective locations. Catheters were flushed with approximately 2 ml of sterile saline. Infusion catheters were then secured in the intestine to the bowel wall and again to the abdominal muscle wall using 5-0 silk sutures (Ethicon, Sommerville, NJ) and then tunneled subcutaneously to the subscapular region for externalization. The abdomen was then closed with interrupted 3-0 silk sutures (Ethicon, Sommerville, NJ). Following surgery, animals were fitted with small animal jackets, administered 3 ml of warm Lactated Ringer's solution (Abbott Animal Health, Abbott Park, IL), and placed individually into standard rodent shoebox cages overnight. Animals were monitored to ensure proper recovery following surgery and upon recovery, buprenorphine analgesic (0.01 mg/kg body weight Buprenex, Reckitt & Colman Inc., Richmond, VA) was administered subcutaneously to minimize any pain and discomfort resulting from the surgery.

Experimental Diets

All rats were maintained on a fiber-free elemental diet (70 % carbohydrate, 18 % protein, and 12 % fat; Research Diets, New Brunswick, NJ; **Table 4.1**) during the experimental period to

prevent any fermentation from occurring. Therefore, the only SCFAs present in the intestinal lumen were those directly infused. Three ml of infusate were slowly administered 3 times a day for 7 days. Using a bolus approach, these concentrations administered mmol levels of SCFAs similar to previous continuous infusion studies, which noted markedly improved structural and functional parameters (Kripke et al., 1989; Koruda et al., 1990; Tappenden et al., 1998; Tappenden and McBurney, 1998), while remaining below concentrations required to induce colitis in rats and mice (MacPherson and Pfeiffer, 1978; McCafferty and Zeitlin, 1989; Daneshmand et al., 2008). Short-chain fatty acid solutions (Sigma, St. Louis, MO) were prepared daily in a laminar flow hood and syringe-filtered prior to administration.

Sample Collection

Following 7 days of treatment, rats were euthanized with 0.5 ml sodium pentobarbital (Fatal Plus; Veterinary Laboratories, Lenexa, KS) administered via cardiac puncture and blood and intestinal samples collected. Briefly, blood samples were drawn by cardiac puncture and collected into vacutainers containing EDTA (3.6 mg; Fisher Scientific, Pittsburgh, PA) and diprotin-A (0.1 mM; Sigma, St. Louis, MO). Plasma was separated by centrifugation, aliquoted, and stored at -80° C until further use. The GI tract was quickly removed and weight and length measured. Two-cm segments of duodenum, jejunum, ileum, and colon were be opened longitudinally and placed into 10% formalin for 24 hours and then transferred to 50% ethanol for further histomorphology analyses. Two-cm segments were also opened longitudinally and placed into Tissue-Tek OCT embedding compound (Fisher Scientific, Pittsburgh, PA). The remainder of the segments was opened longitudinally and mucosa scraped and snap frozen in

liquid nitrogen for analysis of DNA, RNA, and protein concentrations as wells as transporter and proglucagon mRNA abundance. Samples were stored at -80° C until analysis.

Gross Morphological Changes

Gross intestinal structure was evaluated through measurements of segmental wet weight and length, as measured by suspending the intestine longitudinally with a 10 g weight attached to the end to provide equal amounts of tension to each tissue from each animal. Changes in mucosal/submucosal proportions were determined by scraping mucosa from 1-cm segments and measuring mucosal and submucosal dry weights.

DNA, RNA, and Protein Concentrations

In order to quantify DNA, RNA, and protein concentrations, mucosa samples were diluted 1:10 w/v in DEPC H_2O and homogenized. DNA content of the samples was determined using the Hoechst microplate method (Latt and Stetten, 1976). Briefly, 2 µl of mucosal homogenate was combined with 200 µl of a buffer solution containing 10 mM Tris, 1mM EDTA, 200 mM NaCl, and 1.6 nmol 33258 bisBenzimide Hoechst dye at pH 7.4. Samples were read at an excitation of 360 nm and emission of 450 nm using the SpectraMax Gemini XS fluorometer (Molecular Devices, Sunnyvale, CA) and concentration calculated using a Herring sperm DNA (Promega, Madison, WI) standard curve.

Mucosal RNA concentration was determined spectrophotometrically. Briefly, 100 mg of each mucosal sample was homogenized in 1 ml of Trizol Reagent (Invitrogen, Carlsbad, CA). RNA was isolated according to the manufacturer's protocol and concentration was quantified using a spectrophotometer (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE).

Mucosal protein concentration were calculated using the Bradford (Bio-Rad) microplate method (Bradford, 1976). Briefly, 10 µl of homogenized mucosal sample will be combined with 200 µl 1x Bio-Rad dye reagent (Bio-Rad, Hercules, CA). The plate was then incubated at room temperature for 5 minutes and absorbance measured at 595 nm in the SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA). Protein concentration were calculated from a bovine serum albumin (Sigma, St. Louis, MO) standard curve.

Histomorphology

Formalin-fixed samples of duodenum, jejunum, ileum, and colon were embedded in paraffin and sectioned to a thickness of approximately 5 µm using a microtome. Sections were stained with hematoxylin and eosin and villus height, mid-villus width, villus surface area and crypt depth were determined by measuring 6 – 8 intact crypt and villi using an Axioskop 40 microscope and AxioVision software (Carl Zeiss Light Microscopy, Goettingen, Germany).

Determination of Transporter, Receptor, and Proglucagon mRNA Abundance with Real-Time PCR

Relative mRNA abundance will be determined with real-time PCR. Briefly, 100 mg of each ileal and colonic mucosal sample were homogenized in 1 ml of Trizol Reagent (Invitrogen, Carlsbad, CA) and RNA isolated according to the manufacturer's protocol. RNA concentration was then quantified using a spectrophotometer (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE) and used for reverse transcription reactions. Reverse transcription was performed in a Gene Mate Genius thermocycler (ISC Bioexpress, Kaysville, UT) in a total volume of 20 µl containing 5 µg RNA, 250 ng Random Primers, 0.5 mM dNTP mix, 1x First-

Strand Buffer, 0.01 M DTT, 40 units RNase Out, and 200 units Superscript II reverse transcriptase (all reverse transcription reagents were obtained from Invitrogen, Carlsbad, CA). RNA, random primers, and dNTPs were heated to 65° C for 5 minutes after which the remaining reagents were added and reactions incubated at 25° C for 10 minutes followed by 42° C for 50 minutes and then finally inactivated at 70° C for 15 minutes.

Resultant cDNA was appropriately diluted and used for semi-quantitative real-time PCR reactions. Reactions were performed with primers multiplexed in each well, with a total volume of 10 µl containing 2 µl cDNA, 1x TaqMan Universal Master Mix, 1x FAM-labeled Rattus norvegicus TaqMan Gene Expression Assay primer-probe set (Assay ID Rn01503812_m1 for SLC5A8, Rn00562332_m1 for MCT-1, Rn02345824_s1 for GPR43, Rn02396417_s1 for T2R38, or Rn00562293_m1 for proglucagon), and 1x VIC-labeled eukaryotic 18S rRNA Endogenous Control TaqMan Gene Expression Assay primer-probe set (Assay ID Hs99999901_s1; all real-time PCR reagents were obtained from Applied Biosystems, Foster City, CA). Samples were run in a 384-well plate in a TaqMan ABI 7900 real-time PCR machine and analyzed with Sequence Detection Systems 7900HT version 2.2.1 software (Applied Biosystems, Foster City, CA). Briefly, samples were heated to 50° C for 2 minutes and then 95° C for 10 minutes to allow for DNA polymerase activation followed by 40 cycles of 95° C for 15 seconds and 60° C for 1 minute to allow for denaturing, annealing, and extension. Data was analyzed using the standard curve method with pooled cDNA serving as the standard. Target gene levels were normalized to 18S rRNA.

Statistical Analysis

Statistical differences between treatments were determined using a completely randomized block two-way analysis of variance (ANOVA). Sources of variation included infusion site (n = 2), treatment (n = 3), and an infusion site x treatment interaction. Analyses were blocked within surgery round (n = 4). Analysis was carried out using a mixed model in SAS (Version 9.1; SAS Institute, Cary, NC). All values were reported as mean ± SEM and statistical significance defined as $p \leq 0.05$.

RESULTS

Nutritional Support

All rats were provided a fiber-free elemental diet *ad libidum* to meet nutritional needs. Following acclimation to the diet and at the time of surgery, body weight did differ among groups ($p < 0.0001$; **Figure 4.1**); however, average daily change in body weight did not differ. Additionally, organ weights did not differ upon euthanization (data not shown).

Gross Morphological Changes

Gross morphology was assessed using measurements of intestinal and mucosal weights and lengths at the time of euthanasia. Small and large intestinal lengths (cm/kg body weight) did not differ among groups. However, the model did have an impact on intestinal weights (mg/cm/kg body weight; **Table 4.2**). A treatment by infusion site intereaction existed for jejunal weight, revealing significantly increased weight following ileal infusions of SCFAs compared to all other groups ($p = 0.020$). Additionally, regardless of treatment, an infusion site main effect

was seen with ileal infusions produced higher jejunal weights (293.7 ± 21.4 vs. 243.4 ± 18.0 mg/cm/kg body weight for ileal and colonic infusions, respectively; $p < 0.00085$).

An interaction between treatment and infusion site was observed in ileal weights. Ileal infusions of SCFAs had localized effects, stimulating increased ileal weight compared to controls; butyrate had an intermediate effect on ileal weight, but was not significantly different from either group (316.6 ± 22.1 vs. 354.2 ± 26.1 vs. 399.3 ± 29.5 mg/cm/kg body weight for ileal infusions of Control, Butyrate, and SCFAs, respectively; $p = 0.03755$). Together, this also resulted in an infusion site main effect, with significantly higher ileal weights following ileal infusions regardless of treatment (355.1 ± 17.7 vs. 255.4 ± 12.5 mg/cm/kg body weight for ileal and colonic infusions, respectively; $p < 0.0001$).

A main effect of infusion site also existed for colonic weight. Colonic infusions had localized effects, producing significantly higher colonic weights than ileal infusions, irrespective of treatment (417.7 ± 37.3 vs. 580.8 ± 42.0 mg/cm/kg body weight for ileal and colonic infusions, respectively; $p = 0.0024$). Taken together, these results show that in general, segmental weights increase at the site of infusion, with treatment-specific changes induced in the jejunum and ileum following ileal infusions of SCFAs.

DNA, RNA, and Protein Concentrations

To assess morphology effects at the cellular level, total DNA, RNA, and protein concentration within each segment were quantified using mucosal samples (**Table 4.3**). Within the duodenum, a treatment by infusion site interaction existed for DNA, RNA, and protein levels. Colonic butyrate infusions significantly increased DNA concentration compared to controls; colonic SCFAs were not different from either group (3.2 ± 0.2 vs. 4.1 ± 0.2 vs. 3.5 ± 0.2 mg

mucosal DNA for Control, Butyrate, and SCFA colonic infusions, respectively; $p = 0.0058$). Duodenal RNA levels were significantly reduced following SCFA infusions into the ileum compared to ileal controls, but were similar to all other groups (101.3 ± 15.3 vs. 56.1 ± 16.1 mg mucosal RNA for Control and SCFA ileal infusions, respectively; $p = 0.0371$). Similarly, duodenal protein levels were also significantly reduced following SCFA infusions into the ileum compared to all other groups ($p = 0.001$). However, regardless of treatment, an infusion site main effect existed, with colonic infusions induced significantly higher duodenal protein conentrations than ileal infusions (53.7 ± 3.2 vs. 72.3 ± 3.1 mg mucosal protein for ileal and colonic infusions, respectively; $p = 0.0001$).

Within the jejunum, a significant treatment by infusion site interaction was observed for DNA concentration. Ileal infusions of SCFAs significantly elevated DNA concentrations compared to controls (7.9 ± 0.9 vs. 10.0 ± 0.9 mg mucosal DNA for Control and SCFA ileal infusions, respectively; $p = 0.00435$). Additionally, an infusion site main effect was seen with RNA, as jejunal RNA levels were elevated following ileal infusions compared to colonic infusions, regardless of treatment (163.5 ± 15.3 vs. 122.8 ± 14.9 mg mucosal RNA for ileal and colonic infusions, respectively; $p = 0.0045$).

Within the ileum, infusion site main effects were observed for DNA and protein concentrations. Ileal infusions produced significantly higher mucosal DNA and protein levels than colonic infusions, regardless of treatment (6.9 ± 1.0 vs. 4.3 ± 0.9 mg mucosal DNA for ileal and colonic infusions, respectively; $p = 0.00045$ and 135.9 ± 19.4 vs. 104.0 ± 19.1 mg mucosal protein for ileal and colonic infusions, respectively; $p = 0.0273$). A treatment main effect on ileal RNA was also observed, with higher levels seen in the butyrate- and SCFA-treated rats

compared to controls (79.8 ± 14.9 vs. 105.3 ± 15.3 vs. 122.3 ± 15.1 mg mucosal RNA for Control, Butyrate, and SCFAs, respectively; p = 0.01345).

Within the colon, a treatment main effect existed for DNA concentrations. DNA levels were significantly elevated following treatment with SCFAs regardless of infusion site; butyrate levels were elevated but not significantly different than controls (1.1 ± 0.1 vs. 1.3 ± 0.1 vs. 1.9 ± 0.1 mg mucosal DNA for Control, Butryate, and SCFAs, respectively; p = 0.004). Treatment main effects were also seen with colonic RNA concentrations. Both butyrate- and SCFA-treated rats had significantly elevated mucosal RNA compared to controls regardless of infusion site (37.5 ± 10.1 vs. 75.6 ± 10.4 vs. 62.3 ± 10.4 mg mucosal RNA for Control, Butyrate, and SCFAs, respectively; p = 0.01915). Additionally, colonic infusion significantly increased local RNA concentrations compared to ileal infusions (42.7 ± 8.6 vs. 74.2 ± 8.2 mg mucosal RNA for ileal and colonic infusions, respectively; p = 0.0059). There were no differences observed in colonic protein concentrations. Therefore, to summarize, SCFA and butyrate infusions significantly impacted DNA and RNA concentrations in all intestinal segments. Again, infusion site main effects were also observed, with ileal infusions impacting both jejunal and ileal measures of mucosal cellular content whereas colonic infusions had greater effects within the colon.

Histomorphology

Effects of distal SCFAs on epithelial expansion were determined through measurements of the crypt-villus architecture in all segments of intestine (**Table 4.4**). Butyrate infusion into the ileum tended to increase duodenal villus height (p = 0.0756) and crypt depth (p = 0.0887), although this did not achieve significance. However, infusion site main effects did exist, with ileal infusions produced significantly higher duodenal villus surface area compared to colonic

infusions regardless of treatment (41143 ± 2339.2 vs. 34920 ± 2230.4 μm^2 for ileal and colonic infusions, respectively; $p = 0.03105$).

Within the jejunum, there were no significant changes in either villus width or crypt depth; however, treatment main effects were seen in villus height and surface area. Regardless of infusion site, butyrate significantly increased jejunal villus height compared to controls; SCFAs increased villus height as well and were not significantly different than butyrate infusions (336.6 ± 14.9 vs. 394.7 ± 16.0 vs. 381.5 ± 16.1 μm for Control, Butyrate, and SCFAs, respectively; $p = 0.01405$). Similarly, this drove changes in jejunal villus surface area, with both butyrate and SCFAs being significantly elevated compared to controls, although not different from one another (40196 ± 3420.1 vs. 51663 ± 3656.2 vs. 50676 ± 3694.1 μm^2 for Control, Butyrate, and SCFAs, respectively; $p = 0.02415$).

SCFA infusions also impacted ileal histomorphology, with localized effects observed. For villus height, a significant treatment by infusion site interaction existed, with butyrate infusion into the ileum significantly increasing villus height above both control and SCFA infusions (238.2 ± 11.9 vs. 272.1 ± 13.6 vs. 220.8 ± 12.8 μm for Control, Butyrate, and SCFA infusions into the ileum, respectively; $p = 0.033$). Infusions into the colon did not impact ileal villus height, as all colonic treatment groups were similar to the ileal control group. Infusion site main effects were also observed, with ileal infusions having localized effects on villus width, villus surface area, and crypt depth. Villus width was significantly higher following infusion into the ileum, irrespective of treatment group (121.9 ± 5.1 vs. 109.2 ± 5.0 μm for ileal and colonic infusions, respectively; $p = 0.032$). As a result of elevated villus heights and widths, ileal villus surface area was also significantly higher with ileal infusions than colonic infusions (29563 ± 1721.7 vs. 22348 ± 1677.0 μm^2 for ileal and colonic infusions, respectively; p =

0.0016). Additionally, crypt depth was also greater after infusing into the ileum (98.6 ± 3.3 vs. 88.7 ± 3.2 μm for ileal and colonic infusions, respectively; p = 0.019).

Localized effects of SCFA infusions also significantly impacted colonic crypts. For colonic crypt depth, a significant treatment by infusion site interaction existed, with butyrate infusion into the colon significantly increasing crypt depth compared to controls; SCFAs induced a similar effect and were not significantly different from butyrate (144.1 ± 16.5 vs. 232.8 ± 16.5 vs. 204.9 ± 17.6 μm for Control, Butyrate, and SCFA infusions into the colon, respectively; p = 0.0336). Infusions into the ileum did not impact colonic crypt depth, as all ileal treatment groups were similar to the colonic control group. In summary, butyrate significantly improved villus height and crypt depth to the same degree as SCFAs. Infusion site also impacted morphology, with ileal infusions affecting the small intestine and colonic infusions affecting the large intestine.

Transporter and Receptor mRNA Abundance

Relative abundance of the transporters MCT-1 and SLC5A8 as well as the receptors GPR43 and T2R38 was determined using semi-quantitative real-time PCR (**Tables 4.5 and 4.6**). There were no significant changes in either ileal or colonic MCT-1 expression, regardless of treatment or infusion site (p > 0.05).

With colonic infusions, ileal SLC5A8 was significantly higher with butyrate than SCFAs; however, neither group was different than colonic control infusions (0.0498 ± 0.039 vs. 0.0631 ± 0.050 vs. 0.0198 ± 0.015 SLC5A8 mRNA/18S rRNA for Control, Butyrate and SCFA infusions into the colon, respectively; p = 0.04675). Colonic SLC5A8 levels did not change, regardless of treatment or infusion site.

Ileal GPR43 expression was affected by ileal infusions, with a downregulation of GPR43 mRNA following ileal infusions of SCFAs (0.2879 ± 0.12 vs. 0.2771 ± 0.12 vs. 0.1003 ± 0.044 GPR43 mRNA/18S rRNA for Control, Butyrate, and SCFAs, respectively; $p = 0.0191$). Colonic infusions did not have any effect on ileal GPR43 mRNA. With ileal infusions, colonic GPR43 levels were significantly higher with SCFAs than butyrate; however, neither group was different than ileal control infusions (1.0032 ± 0.28 vs. 0.9057 ±0.27 vs. 1.7187 ± 0.52 GPR43 mRNA/18S rRNA for Control, Butyrate, and SCFA infusions into the ileum, respectively; $p = 0.05$).

Changes were observed in ileal, but not colonic, T2R38 expression. With ileal infusions of both butyrate and SCFAs, T2R38 levels were significantly decreased (1.4431 ± 0.38 vs. 0.4319 ± 0.22 vs. 0.1488 ± 0.13 T2R38 mRNA/18S rRNA for Control, Butyrate, and SCFA ileal infusions, respectively; $p = 0.0103$); however, colonic infusions had no effect on T2R38 expression. Thus, when transporter and receptor abundance was significantly different than controls, there was actually a downregulation of expression following treatment with butyrate or SCFAs.

Proglucagon mRNA Abundance

Proglucagon mRNA abundance was determined using semi-quantitative real-time PCR (**Figure 4.2**). Ileal infusions of butyrate significantly increased ileal proglucagon mRNA compared to controls (0.3126 ± 0.1603 vs. 0.6782 ± 0.1515 proglucagon mRNA/18S rRNA for Control and Butryate, respectively; $p = 0.0452$). However, there were no significant differences observed in ileal expression of proglucagon following colonic infusions.

Colonic proglucagon mRNA abundance was elevated following treatment with SCFAs into the ileum (0.7382 ± 0.2526 vs. 1.2783 ± 0.2452 proglucagon mRNA/18S rRNA for Control and SCFAs, respectively; $p = 0.0579$). Again, treatment induced no change in proglucagon expression following colonic infusion. To summarize, ileal infusions significantly increased both ileal and colonic proglucagon abundance while colonic infusions had no effect.

DISCUSSION

It has been established that fermentable substrates maintain and stimulate adaptation when provided in the diet. Previous research has revealed that the fermentation end-products SCFAs are likely responsible for these effects. The addition of SCFAs to TPN has proven to positively impact the gut, stimulating increases in villus height and crypt depth, RNA, DNA, and protein concentrations as well as GLUT2 and SGLT-1 expression in both adult rat and neonatal piglet models (Koruda et al., 1988; Koruda et al., 1990; Tappenden et al., 1997; Tappenden et al., 1998; Bartholome et al., 2004). These effects are likely mediated by the intestinotrophic hormone GLP-2, as indicated by significant increases in proglucagon mRNA following addition of fermentable fibers to the diet or supplementation of SCFAs, including butyrate specifically, to TPN (Gee et al., 1996; Reimer and McBurney, 1996; Tappenden et al., 1996; Bartholome et al., 2004). Indeed, previous research from our lab suggests that the trophic effects of SCFAs may be attributable to butyrate specifically and its ability to increase proglucagon mRNA abundance by activating its gene promoter following an interaction with various receptors and transporters such as GPR43, T2R38, SLC5A8, or MCT-1, ultimately leading to elevated plasma GLP-2 levels.

In order to explore this relationship further, we utilized an *in vivo* model which provided SCFAs and butyrate specifically into the distal intestine. We infused either 0.9% saline, 60 mM

butyrate, or a combination of 240 mM acetate, 100 mM propionate, and 60 mM butyrate 3 times a day for 7 days. While these concentrations appear higher than typically reported, they are mimicking physiological levels of SCFAs into the intestinal lumen. The infusions delivered very similar mmol levels to previous studies involving SCFA infusions into rats, including both systemic and cecal administration (Kripke et al., 1989; Koruda et al., 1990; Tappenden et al., 1998; Tappenden and McBurney, 1998). These mmol levels have shown to have positive effects on structural and functional indices within the intestine and approximate SCFA levels in the normal rat colon (Sakata and von Engelhardt, 1983; Koruda et al., 1990).

Our results indicate that the presence of luminal SCFAs can increase structural components not only locally at the site of infusion, but also at more distant locations in the intestine. The addition of SCFAs, either as a mixture or as butyrate alone, positively impacted a number of structural indices including: duodenal DNA; jejunal weight, villus height, and surface area; ileal weight, RNA, and villus height; and colonic DNA, RNA, and crypt depth. Research has established the ability of SCFAs to impact the mucosa in the surgically-altered large intestine. Using a model of colonic atrophy in which the colon was defunctioned via a diverting colostomy, Kissmeyer-Nielsen and colleagues found that administering SCFA enemas (225 mM acetate, 105 mM propionate, and 60 mM butyrate) for 14 days increased mucosal weight by 22% and mucosal surface by 31% compared to placebo controls (Kissmeyer-Nielsen et al., 1995). Additionally, Sakata and von Engelhardt revealed that a combination of intraluminal SCFAs (75 mM acetate, 35 mM propionate, and 20 mM butyrate) increased the colonic mitotic index following 60 minutes of exposure in surgerically created pouches (Sakata and von Engelhardt, 1983). Similarly, we found SCFAs to induce an expansion of mucosal epithelium, as evidenced

by increases in mucosal DNA and RNA concentrations resulting in growth of the crypt-villus architecture.

Moreover, further examination of the results indicates that oftentimes when SCFA treatment increased these structural measures, the effects of butyrate alone were not significantly different from or were better than the SCFA mixture, suggesting that the butyrate present in the mixture is likely responsible for stimulating the trophic effects. Of the SCFAs, butyrate specifically remains in the intestine as one of the primary energy sources (Wong et al., 2006); therefore, we expected it to be responsible for inducing intestinal changes in this model. Indeed, this is supported by the fact that jejunal mucosal DNA, ileal mucosal protein, and colonic mucosal DNA, RNA, and protein concentrations did not differ from each other when cecectomized rats were continuously administered either butyrate (20, 40, or 150 mM) or a combination of SCFAs (70 mM acetate, 35 mM propionate, and 20 mM butyrate) into the colon for 7 days (Kripke et al., 1989). Additionally, studies have also shown butyrate alone to impact proliferation rates. Intracolonic administration of butyrate (60 mM), but not acetate (100 mM) or propionate (20 mM), increased jejunal crypt cell proliferation rates following 2 days of treatment in rats (Sakata, 1987). Likewise, 80 mM luminal butyrate increased colonic crypt depth (37.9 vs. 44.7 cells for 0 and 80 mM, respectively) and mitotic index from 4.4 to 8.4% after 4 days administration to rats (Sengupta et al., 2002). Kien and collegues found similar effects in a piglet model, revealing butyrate's ability to increase proliferation in the jejunum (111%), ileum (78%), and colon (89%) compared to controls (Kien et al., 2007). Thus, although proliferation rates were not measured directly in this study, these results suggest that butyrate and the SCFA mixture may able to stimulate hyperplasia, resulting in the increased mucosal cellular content, mucosal architecture, and gross intestinal morphology observed.

Dietary fibers and SCFAs have proven to increase proglucagon mRNA and plasma GLP-2 levels in previous models of intestinal adaptation. Therefore, we hypothesized that butyrate's effects would be associated with elevated proglucagon and thus GLP-2 was likely mediating both the local and distant intestinotrophic effects. Indeed, we found that within the ileum, ileal infusions of butyrate alone were able to stimulate a nearly 2-fold increase in proglucagon mRNA abundance compared to saline control infusions into the ileum. Colonic proglucagon mRNA abundance also responded to ileal infusions of SCFAs, increasing again by nearly 2-fold. As proglucagon levels increase, it will be processed into the PGDPs, which includes GLP-2. Studies have shown luminal nutrients stimulate the release of GLP-2 (Xiao et al., 1999). Thus, the presence of SCFAs and butyrate likely caused the release of GLP-2 into circulation.

Once in circulation, GLP-2 is known to stimulate a number of intestinotrophic effects, both structurally and functionally. GLP-2 is known to dose-dependently increase small intestinal weight, DNA and protein content, and villus height (Burrin et al., 2005). The role of GLP-2 in stimulating these effects was further demonstrated when weanling rats were administered anti-GLP-2 serum for 2 weeks, ultimately resulting in inhibition of the proliferation and elongation of the intestine that was reported in control animals (Ishizuka et al., 2009). GLP-2 exerts its effects by upregulating the GLP-2 receptor, leading to greater cell signaling cascades (Koopmann et al., 2008). In particular, the modulated cell signaling cascades are related to both proliferation and apoptosis, with increases in ErbB signaling molecules and phosphorylation of PKA, PKB, ERK1/2, CREBP, and c-Fos ultimately stimulating more proliferation and decreases in caspase-3 and -6 and increases in Bcl-2 decreasing apoptosis rates (Burrin et al., 2005; Burrin et al., 2007; Yusta et al., 2009). Along with structural improvements, GLP-2 also increases functional capacity, including increased sucrase activity (Liu et al., 2006), lactose digestion and glucose

absorption via SGLT-1 (Cottrell et al., 2006), lipid absorption and chylomicron production (Hsieh et al., 2009), and improved intestinal permeability and tight junction protein expression (Cani et al., 2009). Based on these reports, our findings of increased structural parameters following infusions of SCFAs and butyrate are likely being induced by GLP-2, which is supported by the increased proglucagon expression.

Surprisingly, ileal infusions, but not colonic infusions, were able to stimulate increases in proglucagon mRNA abundance despite the fact that a gradient in L cell density exists, with a higher concentration of L cells located in the colon (Arantes and Nogueira, 1997). However, data from SBS studies reveals that the L cells in the ileum may be particularly important for stimulating intestinal adaptation. When given a standard breakfast containing dietary fiber, there was no change in plasma GLP-2 levels in patients with an ileal resection (baseline of 5 pmol/L, peak of 10 pmol/L) whereas patients with an intact ileum saw a significant increase in GLP-2 concentrations (baseline of 9 pmol/L, peak of 59 pmol/L) when measured over 180 minutes following the meal (Jeppesen et al., 1999). Additionally, in *in vivo* models comparing proximal and distal small bowel resections, mucosal cross-sectional area and plasma GLP-2 levels were decreased in the distally-resected rats as compared to the proximally-resected rats (Thulesen et al., 2001). Similarly, when the ileum and cecum were resected, the remaining colonic L cells were not able to produce GLP-2 at levels sufficient to stimulate intestinal adaptation in the jejunum in rats (Koopmann et al., 2009). Together, these results suggest that despite a lower L cell density than the colon, the ileum has a much stronger ability to produce GLP-2 and stimulate adaptation, providing rationale for why our ileal, but not colonic, infusions activated proglucagon and were associated with greater adaptive responses.

We had hypothesized that butyrate was acting through transporters such as MCT-1 and SLC5A8 and receptors including GPR43 and T2R38 to activate proglucagon mRNA production. However, we did not see butyrate or SCFA infusions increasing mRNA abundance of these transporters and receptors. In fact, we report a downregulation of ileal SLC5A8, GPR43, and T2R38 and no changes in expression patterns in the colon. Research has shown that the presence of fiber in the diet will stimulate increased SCFA transporter expression in the distal intestine. Indeed, when the fermentable substrate germinated barley foodstuff was supplemented into a rat diet for 14 days, colonic SLC5A8 significantly increased (Kanauchi et al., 2008). Moreover, supplementation with 2.5% pectin for 14 days increased MCT-1 expression, particularly in the cecum and colon (Kirat et al., 2009). These increases in MCT-1 and SLC5A8 were associated with elevated butyrate concentrations in the intestine, even with the presence of acetate and propionate. Additionally, *in vitro* work has also shown that MCT-1 is upregulated specifically by butyrate (Cuff et al., 2002). Research has also demonstrated that the receptors GPR43 and T2R38 are present on the L cell and respond to SCFAs (Karaki et al., 2006; Rozengurt et al., 2006; Karaki et al., 2008). Therefore, we had anticipated that the substrate could regulate abundance, as more transporters and receptors would be needed to keep up with substrate availability.

It is possible that butyrate and SCFAs are not involved in regulating transporter and receptor abundance. However, it is more likely that the lack of response in mRNA abundance was a result of the time point examined in the study, with a possibility of being either too late or too early to observe the desired effects. We had hypothesized that these receptors and transporters may be mediating butyrate's effects on the proglucagon gene. Therefore, it is conceivable that the mRNA abundance was upregulated early on in the study period in order to

activate proglucagon and induce structural adaptations. By 7 days, we were observing significant changes in the crypt-villus architecture. Thus, the receptors and transporters may have been downregulated by the end of the study because structural adaptations had already been stimulated. Had we examined the abundance at a more acute time point, we may have observed increases in the transporters and receptors necessary for proglucagon activation due to increased butyrate availability.

Conversely, it is also possible that SCFAs had few effects on mRNA abundance due to a discrepancy between the timing of structural and functional adaptations. In resection models, structural adaptations occur fairly rapidly within a few weeks whereas functional adaptation occurs more slowly over a period of months (Hanson et al., 1977; Gouttebel et al., 1989). Thus, by rapidly increasing the cellular content, we may have effectively diluted out some of the effect in function because the cells may not be fully mature and expressing the transporters and receptors despite the increased presence of substrates. Given more time, we may have seen increases in the transporter and receptor abundance associated with butyrate administration and proglucagon elevation.

Along with butyrate- and SCFA-induced effects, we also reported a number of infusion effects where, regardless of treatment, ileal infusions impacted the duodenum, jejunum, and ileum whereas colonic infusions seemed to be limited to the colon itself. Bowel dilation is a part of the natural adaptive process in response to intestinal resection and may further the intestinotrophic response. Therefore, it is conceivable that luminal distension caused by the infusions themselves may have been stimulating some adaptation. Indeed, using Thiry-Vella loops in rats, mechanical stimulation was shown to reduce atrophy in intestinal mass (Fenyo, 1976). Saline infusions for 1 week into rat jejunum taken out of continuity increased length by

32%, weight by 375%, and protein by 510% as compared to controls receiving no infusions (Puapong et al., 2006). Moreover, saline enemas given twice daily to humans were shown to result in a lengthening of colonic crypts (Gibson et al., 1998). Thus, it is conceivable that ileal infusions were likely able to stimulate adaptation not only through mechanical distension, but also through activation of proglucagon and GLP-2 resulting in adaptation throughout the intestine. Through luminal distension, colonic infusions also affected adaptation, but because colonic proglucagon is less responsive, effects tended to be localized in the colon only and not throughout the intestine.

To summarize, administration of SCFAs or butyrate alone into the lumen of the distal intestine for 7 days enhances structural, but not functional, indices of intestinal adaptation. We have shown that of the SCFAs, butyrate is likely responsible for inducing these trophic effects which are mediated by proglucagon and GLP-2. Additionally, we have shown that ileal L cells tend to have a greater impact on the intestine than colonic L cells. Together, these results have important implications for stimulating intestinal adaptation, including the types of substrates and target locations best suited for producing an adaptive response.

Table 4.1 The composition of the rodent fiber-free elemental diet.

Ingredient	g/950 g diet
Dextrose	675.5
Soybean Oil	50
L-Arginine	10
L-Histidine-HCl-H_2O	6
L-Isoleucine	8
L-Leucine	12
L-Lysine-HCl	14
L-Methionine	6
L-Phenylalanine	8
L-Threonine	8
L-Tryptophan	2
L-Valine	8
L-Alanine	10
L-Asparagine-H_2O	5
L-Aspartate	10
L-Cysteine	4
L-Glutamic Acid	30
L-Glutamine	5
Glycine	10
L-Proline	5
L-Serine	5
L-Tyrosine	4
Mineral Mix[1]	35
Sodium Bicarbonate	7.5
Vitamin Mix[2]	10
Choline Bitartrate	2

Composition is based on a standard rat chow, but provides nutrients in highly digestible forms to prevent fermentation from occurring. The diet consists of 70% carbohydrate, 18% protein, and 12% fat and provides 4.1 kcal/g. [1]Mineral mix is the standard AIN-76A rodent diet mix and is added in at 35g/kg diet. Mineral mix consists of: calcium phosphate dibasic (500 g), magnesium oxide (24 g), potassium citrate-H_2O (220 g), potassium sulfate (52 g), sodium chloride (74 g), chromium postassium sulfate-$12H_2O$ (0.55 g), cupric carbonate (0.3 g), potassium iodate (0.01 g), ferric citrate (6.0 g), manganous carbonate (3.5 g), sodium selenite (0.01 g), zinc carbonate (1.6 g), sucrose (118.03 g). [2]Vitamin mix is the standard AIN-76A rodent diet mix and is added in at 10g/kg diet. Vitamin mix consists of: Vitamin A palmitate (0.8 g), Vitamin D3 (1.0 g), Vitamin E acetate (10.0 g), menadione sodium bisulfite (0.08 g), biotin (2.0 g), cyancocobalamin (1.0 g), folic acid (0.2 g), nicotinic acid (3.0 g), calcium pantothenate (1.6 g), pyridoxine-HCl (0.7 g), riboflavin (0.6 g), thiamin HCl (0.6 g), sucrose (978.42 g). The diet was obtained from Research Diets, New Brunswick, NJ.

Table 4.2 Effects of infusions on gross intestinal structural changes[1]

	Ileal Infusion			Colonic Infusion			
	CONTROL	**BUTYRATE**	**SCFA**	**CONTROL**	**BUTYRATE**	**SCFA**	***p-value***
Duodenum							
Length (cm/kg BW)	38.9 ± 3.8	37.3 ± 3.9	40.8 ± 3.9	35.3 ± 3.8	44.4 ± 3.8	42.1 ± 3.8	0.099
Weight (mg/cm/kg BW)	431.0 ± 23.8	448.5 ± 26.5	493.6 ± 29.2	475.5 ± 26.3	422.2 ± 23.3	456.5 ± 25.2	ns
Jejunum							
Length (cm/kg BW)	156.5 ± 5.6	152.9 ± 5.9	164.9 ± 5.9	160.6 ± 5.6	162.1 ± 5.7	158.4 ± 5.6	ns
Weight (mg/cm/kg BW)[2]	277.9 ± 22.9^{b}	250.3 ± 21.4^{b}	364.2 ± 31.1^{a}	240.5 ± 19.5^{b}	242.6 ± 20.2^{b}	247.1 ± 20.0^{b}	0.020
Ileum							
Length (cm/kg BW)	156.5 ± 5.6	152.9 ± 5.9	164.9 ± 5.9	160.6 ± 5.6	162.1 ± 5.7	158.4 ± 5.6	ns
Weight (mg/cm/kg BW)[3]	316.6 ± 22.1^{bc}	354.2 ± 26.1^{ab}	399.39 ± 29.5^{a}	272.0 ± 18.6^{cd}	236.1 ± 16.9^{d}	259.5 ± 17.7^{d}	.03755
Colon							
Length (cm/kg BW)	51.5 ± 2.5	45.2 ± 2.6	48.4 ± 2.6	45.6 ± 2.5	47.4 ± 2.5	48.4 ± 2.5	0.081
Weight (mg/cm/kg BW)[4]	363.5 ± 57.6	367.9 ± 62.0	532.2 ± 74.5	564.3 ± 71.8	646.2 ± 76.8	534.5 ± 69.9	ns

[1]Data are expressed as mean ± SEM. Within each dependent variable, means with different letters are statistically different from each other.

[2] Within this dependent variable, an interaction between treatment and infusion site existed, indicating that ileal infusions of SCFAs had a higher treatment mean than all other groups.

[3] Within this dependent variable, an interaction between treatment and infusion site existed, indicating that ileal infusions of SCFAs had a higher treatment mean than Control infusions.

[4] Within this dependent variable, regardless of treatment, the main effect of infusion site was significant, indicating that the pooled treatment means within colonic infusions were higher than the pooled treatment means with ileal infusions.

Table 4.3 Effects of infusions on mucosal cellular content[1]

	Ileal Infusion			Colonic Infusion			
	CONTROL	**BUTYRATE**	**SCFA**	**CONTROL**	**BUTYRATE**	**SCFA**	***p-value***
Duodenum							
DNA (total mg)[2]	3.2 ± 0.2^{bc}	2.8 ± 0.2^{c}	2.0 ± 0.2^{d}	3.2 ± 0.2^{bc}	4.1 ± 0.2^{a}	3.5 ± 0.2^{ab}	0.0058
RNA (total mg)[3]	101.3 ± 15.3^{a}	85.3 ± 16.1^{ab}	56.1 ± 16.1^{b}	85.7 ± 15.1^{ab}	89.0 ± 15.4^{ab}	107.6 ± 15.1^{a}	0.0371
Protein (total mg)[4]	67.3 ± 5.3^{ab}	54.7 ± 5.7^{b}	39.2 ± 5.7^{c}	64.0 ± 5.3^{ab}	75.4 ± 5.3^{a}	77.5 ± 5.3^{a}	0.001
Jejunum							
DNA (total mg)[5]	7.9 ± 0.9^{b}	5.0 ± 0.9^{c}	10.0 ± 0. 9^{a}	5.8 ± 0.8^{c}	5.7 ± 0.9^{c}	5.6 ± 0.8^{c}	0.00435
RNA (total mg)[6]	153.8 ± 20.9	138.7 ± 21.9	197.9 ± 21.8	127.0 ± 20.7	126.3 ± 21.1	115.2 ± 20.7	0.0694
Protein (total mg)[7]	164.9 ± 16.9^{a}	108.4 ± 17.9^{b}	182.0 ± 17.9^{a}	127.8 ± 16.7^{ab}	145.9 ± 17.1^{ab}	121.4 ± 16.7^{b}	0.0055
Ileum							
DNA (total mg)[6]	7.1 ± 1.2	7.5 ± 1.2	6.2 ± 1.2	5.5 ± 1.2	4.1 ± 1.2	3.4 ± 1.2	ns
RNA (total mg)[6, 8]	81.1 ± 18.3	118.4 ± 19.1	138.1 ± 19.1	78.5 ± 18.1	92.2 ± 18.5	106.5 ± 18.1	ns
Protein (total mg)[6]	151.0 ± 24.9	120.7 ± 25.8	136.0 ± 25.8	118.8 ± 24.7	108.4 ± 25.0	84.8 ± 24.7	ns
Colon							
DNA (total mg)[9]	1.2 ± 0.2	1.0 ± 0.2	1.8 ± 0.2	1.1 ± 0.2	1.6 ± 0.2	2.1 ± 0.2	ns
RNA (total mg)[8, 10]	29.5 ± 14.3	49.4 ± 15.2	49.2 ± 15.2	45.5 ± 14.3	101.8 ± 14.3	75.4 ± 14.3	ns
Protein (total mg)	31.2 ± 6.6	36.2 ± 7.0	50.2 ± 7.0	41.4 ± 6.6	47.4 ± 7.0	45.2 ± 6.6	ns

1 Data are expressed as mean ± SEM. Within each dependent variable, means with different letters are statistically different from each other.

2 Within this dependent variable, an interaction between treatment and infusion site existed, indicating that colonic infusions of Butyrate had a higher treatment mean than Control infusions.

3 Within this dependent variable, an interaction between treatment and infusion site existed, indicating that ileal infusions of SCFAs had a lower treatment mean than Control infusions.

4 Within this dependent variable, an interaction between treatment and infusion site existed, indicating that ileal infusions of SCFAs had a lower treatment mean than all other groups.

Table 4.3, continued

[5] Within this dependent variable, an interaction between treatment and infusion site existed, indicating that ileal infusions of SCFAs had a higher treatment mean than all other groups.

[6] Within this dependent variable, regardless of treatment, the main effect of infusion site was significant, indicating that the pooled treatment means within ileal infusions were higher than the colonic infusion means.

[7] Within this dependent variable, an interaction between treatment and infusion site existed, indicating that ileal infusions of Butyrate had a lower treatment mean than Control infusions.

[8] Within this dependent variable, regardless of infusion site, the main effect of treatment was significant, indicating that the pooled infusion site means of Butyrate and SCFAs were higher than the Control mean.

[9] Within this dependent variable, regardless of infusion site, the main effect of treatment was significant, indicating that the pooled infusion site mean of SCFAs was higher than either Control or Butyrate means.

[10] Within this dependent variable, regardless of treatment, the main effect of infusion site was significant, indicating that the pooled treatment means within colonic infusions were higher than the ileal infusion means.

Table 4.4 Effects of infusions on mucosal architecture[1]

	Ileal Infusion			Colonic Infusion			
	CONTROL	**BUTYRATE**	**SCFA**	**CONTROL**	**BUTYRATE**	**SCFA**	***p-value***
Duodenum							
Villus Height (μm)	319.2 ± 24.7	406.1 ± 28.5	354.0 ± 28.5	339.5 ± 28.5	320.6 ± 24.7	332.8 ± 24.7	0.0756
Villus Width (μm)	110.0 ± 7.3	118.6 ± 8.4	115.5 ± 8.4	99.3 ± 8.4	108.3 ± 7.3	106.8 ± 7.3	ns
Villus Surface Area (μm^2)[2]	35484 ± 3664.9	47862 ± 4231.8	40082 ± 4231.8	33528 ± 4231.8	35222 ± 3664.9	36009 ± 3664.9	ns
Crypt Depth (μm)	209.5 ± 21.4	276.5 ± 24.8	216.4 ± 24.2	229.4 ± 24.7	221.3 ± 21.6	223.2 ± 21.1	0.0887
Jejunum							
Villus Height (μm)[3]	329.4 ± 21.2	389.1 ± 22.6	373.6 ± 24.5	343.8 ± 21.2	400.3 ± 22.6	389.5 ± 21.2	ns
Villus Width (μm)	129.6 ± 9.9	129.6 ± 10.4	124.7 ± 11.0	111.8 ± 9.7	130.2 ± 11.1	137.3 ± 9.7	ns
Villus Surface Area (μm^2)[3]	41887 ± 4836.8	50904 ± 5170.7	46650 ± 5585.0	38504 ± 4836.8	52422 ± 5170.7	54701 ± 4836.8	ns
Crypt Depth (μm)	106.9 ± 10.9	119.1 ± 11.4	115.8 ± 12.0	109.7 ± 10.7	103.0 ± 11.8	112.8 ± 10.7	ns
Ileum							
Villus Height (μm)[4]	238.2 ± 11.9^{b}	272.1 ± 13.6^{a}	220.8 ± 12.8^{bc}	215.3 ± 11.9^{bc}	196.3 ± 12.8^{c}	198.5 ± 11.9^{c}	0.033
Villus Width (μm)[2]	117.7 ± 8.2	120.3 ± 8.7	127.7 ± 8.7	104.1 ± 8.1	109.2 ± 8.7	114.3 ± 8.1	ns
Villus Surface Area (μm^2)[2]	28403 ± 2779.0	31751 ± 2955.4	28535 ± 2961.7	22771 ± 2750.4	21179 ± 2955.4	23095 ± 2750.4	ns
Crypt Depth (μm)[2]	100.1 ± 5.5	104.5 ± 5.9	91.4 ± 5.9	88.7 ± 5.5	89.2 ± 5.9	88.3 ± 5.5	ns
Colon							
Crypt Depth (μm)[5]	154.9 ± 16.5^{c}	175.2 ± 17.6^{bc}	144.2 ± 17.6^{c}	144.1 ± 16.5^{c}	232.8 ± 16.5^{a}	204.9 ± 17.6^{ab}	0.0336

[1] Data are expressed as mean ± SEM. Within each dependent variable, means with different letters are statistically different from each other.

[2] Within this dependent variable, regardless of treatment, the main effect of infusion site was significant, indicating that the pooled treatment means of ileal infusions were higher than the colonic infusion means.

[3] Within this dependent variable, regardless of infusion site, the main effect of treatment was significant, indicating that the pooled infusion site means of Butyrate and SCFAs were higher than the Control mean.

Table 4.4, continued

[4] Within this dependent variable, an interaction between treatment and infusion site existed, indicating that ileal infusions of Butyrate had a higher treatment mean than Control or SCFAs.

[5] Within this dependent variable, an interaction between treatment and infusion site existed, indicating that colonic infusions of Butyrate and SCFAs had a higher treatment mean than Control.

Table 4.5 Effects of ileal infusions on transporter and receptor mRNA expression in the ileum and colon[1]

	Treatment Group			
	CONTROL	**BUTYRATE**	**SCFA**	***p-value***
MCT-1 (MCT-1 mRNA/ 18S rRNA)				
Ileum	0.2169 ± 0.14	0.2663 ± 0.17	0.1896 ± 0.12	ns
Colon	1.8154 ± 0.43	2.316 ± 0.45	2.0788 ± 0.45	ns
SLC5A8 (SLC5A8 mRNA/ 18S rRNA)				
Ileum	0.0163 ± 0.013	0.0205 ± 0.017	0.0123 ± 0.010	ns
Colon	1.3417 ± 0.36	1.9148 ± 0.38	1.959 ± 0.38	ns
GPR43 (GPR43 mRNA/ 18S rRNA)				
Ileum[2]	0.2879 ± 0.12[a]	0.2771 ± 0.12[a]	0.1003 ± 0.044[b]	0.0191
Colon[3]	1.0032 ± 0.28[ab]	0.9057 ± 0.27[b]	1.7187 ± 0.52[a]	0.05
T2R38 (T2R38 mRNA/ 18S rRNA)				
Ileum[4]	1.4431 ± 0.38[a]	0.4319 ± 0.22[b]	0.1488 ± 0.13[b]	0.0103
Colon	0.2190 ± 0.13	0.2515 ± 0.13	0.1782 ± 0.10	ns

1 Data are expressed as mean ± SEM. Within each dependent variable, means with different letters are statistically different from each other.

[2] Within this dependent variable, an interaction between treatment and infusion site existed, indicating that SCFAs had a lower treatment mean than either Control or Butyrate following ileal infusion.

[3] Within this dependent variable, an interaction between treatment and infusion site existed, indicating that SCFAs had a higher treatment mean than Butyrate following ileal infusion.

[4] Within this dependent variable, an interaction between treatment and infusion site existed, indicating that SCFAs and Butyrate had a lower treatment mean than Control following ileal infusion.

Table 4.6 Effects of colonic infusions on transporter and receptor mRNA expression in the ileum and colon[1]

	Treatment Group			
	CONTROL	**BUTYRATE**	**SCFA**	***p-value***
MCT-1 (MCT-1 mRNA/ 18S rRNA)				
Ileum	0.2205 ± 0.15	0.3159 ± 0.22	0.1824 ± 0.11	ns
Colon	1.7318 ± 0.42	2.009 ± 0.43	2.2556 ± 0.42	ns
SLC5A8 (SLC5A8 mRNA/ 18S rRNA)				
Ileum[2]	0.0498 ± 0.039[ab]	0.0631 ± 0.050[a]	0.0198 ± 0.015[b]	0.04675
Colon	1.3693 ± 0.35	1.9117 ± 0.36	1.6315 ± 0.35	ns
GPR43 (GPR43 mRNA/ 18S rRNA)				
Ileum	0.2504 ± 0.11	0.2945 ± 0.14	0.3003 ± 0.12	ns
Colon	1.0817 ± 0.30	0.9594 ± 0.27	1.3909 ± 0.39	ns
T2R38 (T2R38 mRNA/ 18S rRNA)				
Ileum	0.5196 ± 0.26	1.0314 ± 0.41	0.7247 ± 0.34	ns
Colon	0.2109 ± 0.10	0.2485 ± 0.12	0.1672 ± 0.09	ns

1 Data are expressed as mean ± SEM. Within each dependent variable, means with different letters are statistically different from each other.

2 Within this dependent variable, an interaction between treatment and infusion site existed, indicating that SCFAs had a lower treatment mean than Butyrate following colonic infusion.

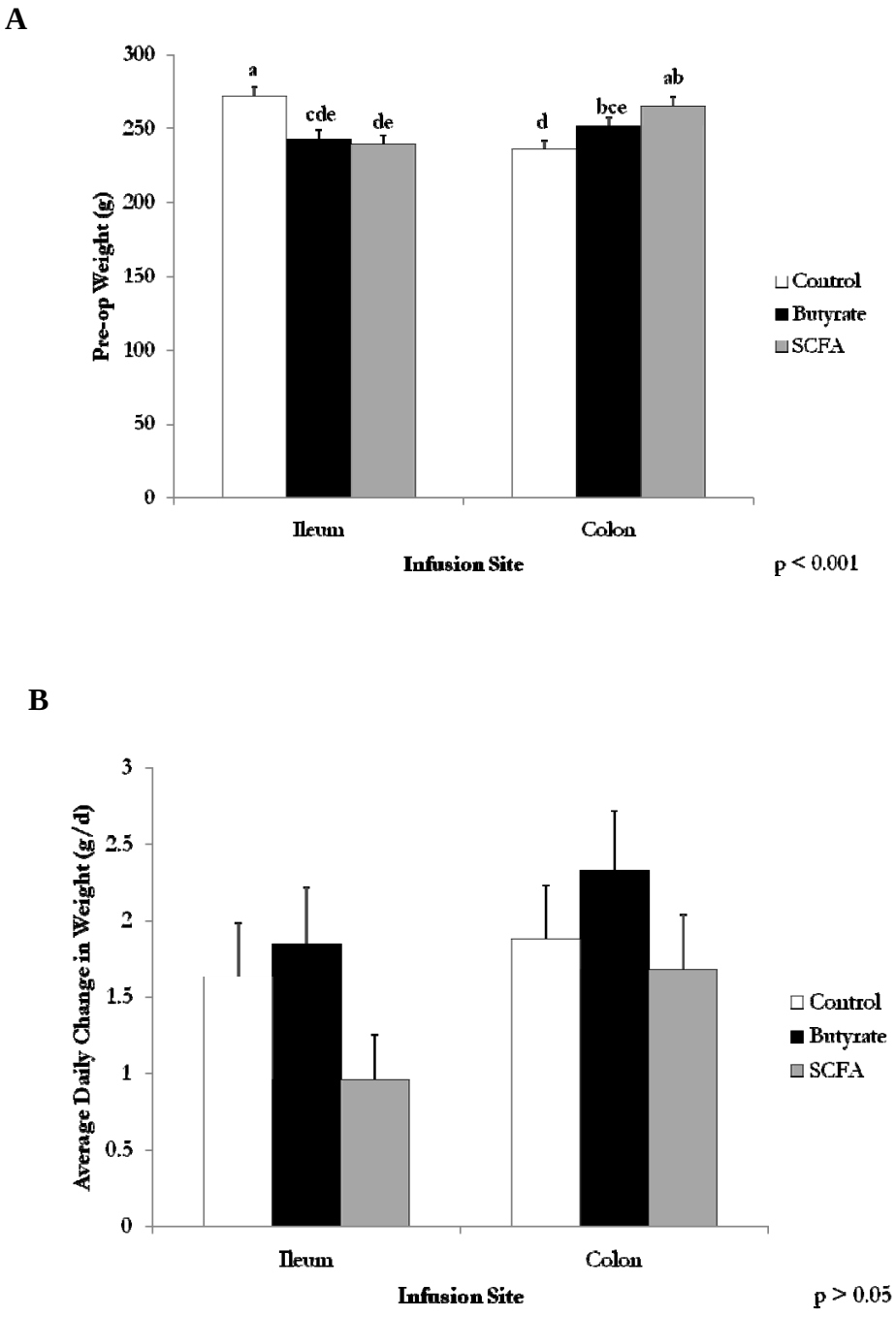


Figure 4.1 The effects of infusions on body weights. **A** Following acclimation to the diets, body weights at the time of surgery were significantly different ($p < 0.001$). **B** However, the average daily change in body weight was not significantly different between groups ($p > 0.05$).

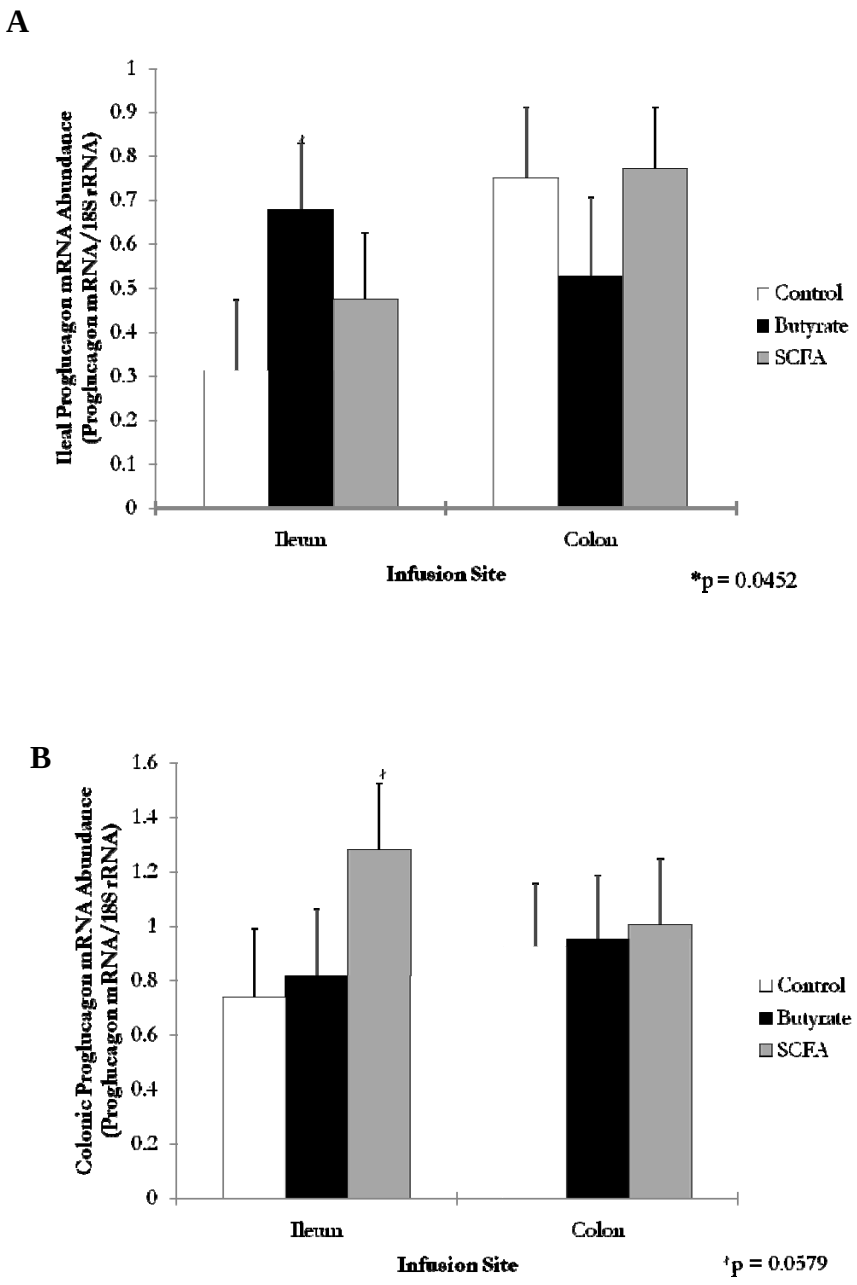


Figure 4.2 The effects of infusions on proglucagon mRNA abundance in the distal intestine. **A** Ileal proglucagon mRNA abundance was significantly increased following butyrate infusion into the ileum as compared to control and SCFAs ($p = 0.0452$). Colonic infusions had no effect on ileal proglucagon expression. **B** Colonic proglucagon mRNA abundance was elevated following infusion of SCFAs into the ileum ($p = 0.0579$); however, colonic infusions did not increase colonic proglucagon abundance.

CHAPTER 5

GENERAL DISCUSSION, CONCLUSIONS, AND FUTURE DIRECTIONS

Research has established the importance of enteral nutrition for intestinal maintenance, growth, and adaptation. Of particular interest for this research is the role of dietary fibers and their fermentation end-products SCFAs, including acetate, propionate, and butyrate. Over the past several years, studies have established fibers and systemic SCFAs to mediate trophic responses in the intestine through an interaction with humoral factors, namely GLP-2. However, the relationship between these nutrients and the hormone is less understood. Therefore, the **specific aim** of this thesis research was to examine the effects of SCFAs, specifically butyrate, on proglucagon gene expression in the intestine in both *in vitro* and *in vivo* models. This was explored by investigating the underlying molecular mechanisms of the interaction in cell culture and through the provision of luminal SCFAs in an adult rodent model.

HYPOTHESIS 1

Butyrate upregulates proglucagon mRNA abundance in enteroendocrine L cells.

Treatment of NCI-H716 cells, a human cell culture model of the enteroendocrine L cell, with physiological concentrations of butyrate increased proglucagon mRNA abundance at acute time points, starting to increase expression as early as 1 hour, reaching significance following 2 hours of treatment. Additionally, butyrate was proven to increase mRNA abundance through transcriptional regulation, with elevated promoter activation observed.

While we have shown that butyrate increases proglucagon promoter activation, it would be interesting to examine the promoter for specific transcription factors and cell signaling molecules involved in the upregulation. Previous studies have examined transcriptional regulation of the gene in rodent models. However, research has also uncovered the fact that there is a divergent regulation between the rodent and human genes (Nian et al., 1999; Nian et al., 2002). Therefore, it is necessary to perform the analysis again with the human promoter specifically to gain a better understanding of the mechanisms involved and provide more data on the molecular targets that may be regulated nutritionally.

Additionally, while promoter activation is the most likely way a gene is transcriptionally regulated, it is not the only mechanism. Synthesis of mRNA is only part of the equation; the rate of mRNA degradation is also involved in determining the overall half-life of the mRNA and thus how much protein can be made in a cell at a given point. Therefore, it would be beneficial to examine butyrate's ability to stabilize proglucagon mRNA. It is possible that butyrate also inhibits or lessens degradation of the molecule, ultimately leading to a greater abundance and thus greater GLP-2.

It would also be beneficial to look at the effects of GLP-2 treatment on the cell line. Again, we have shown butyrate to increase proglucagon which would in turn drive increases in GLP-2 production and secretion. GLP-2 receptors are known to be expressed on enteroendocrine cells (Yusta et al., 2000b). Thus, there is potential for the GLP-2 produced to act through a feedforward mechanism via the GLP-2 receptor on the L cell to further increase its own production. It would be interesting to determine how much of the proglucagon mRNA produced is driven by butyrate and how much is due to GLP-2 itself, providing a greater understanding of the mechanisms by which butyrate induces trophic effects in the intestine.

HYPOTHESIS 2

Butyrate activates the proglucagon promoter by interacting with receptors and transporters on the enteroendocrine L cell and these receptors and transporters are upregulated in the presence of SCFAs.

In order for butyrate to stimulate proglucagon promoter activation, it must either act through receptors and their subsequent cell signaling cascades or be directly transported into the L cell. Therefore, we designed both *in vitro* and *in vivo* studies to examine the role of the receptors GPR43 and T2R38 and the transporters SLC5A8 and MCT-1. Using siRNA, *in vitro* downregulation of either GPR43 or T2R38 decreased proglucagon mRNA abundance despite the presence of butyrate, indicating that these 2 receptors may be mediating some of butyrate's effects on the proglucagon gene. We then examined the expression patterns *in vivo* using 2 neonatal piglet models. Receptor and transporter abundance was not influenced by systemic SCFAs or butyrate specifically. However, transporter abundance increased in the presence of fermentable substrates, particularly at the site of fermentation with FOS inducing changes in SLC5A8 in the ileum and SPS increasing abundance of MCT-1 in the colon.

While the present study established *in vitro* that butyrate may be acting through SCFA receptors including GPR43 and T2R38, we have not established what may be occurring intracellularly once butyrate binds to the receptor. Therefore, further studies are needed to examine the signaling involved in this particular interaction. Previous research has noted different G proteins and intermediate molecules that may be associated with these 2 receptors. Examining the effect of butyrate on proglucagon in the presence of inhibitors of specific proteins

and pathways may further elucidate the mechanism by which butyrate increases GLP-2 and provide additional nutritional targets.

Additionally, while we did show that butyrate may be acting through GPR43 and T2R38 *in vitro*, we did not see an upregulation of the receptors in the presence of either systemic SCFAs or provision of fermentable fibers. Thus, it would be interesting to see if a similar effect on proglucagon abundance could be achieved *in vivo* if the receptors were effectively silenced or if these effects can only be achieved in a cell culture system. RNA interference and siRNA technologies are beginning to be explored and applied in animal models to examine gene function within the whole organism. Hence, there is potential to knock down the receptors *in vivo* to determine their true physiological role in this nutrient-gene interaction.

Moreover, there are a number of other taste receptors present in the distal intestine that have the potential to mediate the interaction between butyrate and proglucgon. We chose to explore the SCFA receptor GPR43 and the bitter taste receptor T2R38 based on previous research that had localized them on enteroendocrine L cells (Karaki et al., 2006; Rozengurt et al., 2006; Karaki et al., 2008). However, there are other taste receptors, including both bitter and sweet, which may also warrant further exploration. It is possible that different receptors may act through similar cell signaling cascades ultimately having the same end result and thus be additional targets for stimulating proglucagon activation. Similar experiments should be conducted to examine the role of multiple receptors as butyrate is likely able to stimulate its effects via multiple mechanisms.

Lastly, we have shown that the abundance of the transporters SLC5A8 and MCT-1 was upregulated in the presence of fermentable fibers. However, the current study did not elucidate the potential role of these receptors in mediating butyrate's effects on proglucagon. Along with

interacting with receptors on L cells, they may also directly transport butyrate in. Future studies are needed to examine the mRNA and protein abundance on the L cells specifically to determine if this is a potential mechanism by which butyrate produces its trophic effects.

HYPOTHESIS 3

Luminal butyrate provided into the distal intestine increases structural and functional indices of adaptation by increasing proglucagon and GLP-2 levels in the adult rat.

Previous studies had established the importance of luminal nutrition, particularly fermentable fibers, in intestinal growth and homeostasis. It is believed that the production of SCFAs, especially butyrate, is important in this capacity. We found luminal SCFAs, in particular butyrate alone, to stimulate increases in a number of structural indices including duodenal DNA, jejunal DNA, villus height and surface area, ileal weight, RNA, and villus height, and colonic DNA, RNA, and crypt depth. These changes are believed to be mediated by GLP-2 as evidenced by elevated proglucagon levels.

We have established that luminal SCFAs and butyrate will stimulate increases in intestinal structure, but we did not see positive changes in intestinal functional capacity in this study. We had anticipated that with greater substrate availability, we would see increases in SCFA receptor and transporter abundance. We believe that the reason we did not see this adaptation was due to the discrepancy in the timing of structural and functional maturation of the gut. Therefore, it would be interesting to conduct the study using a time course model to determine at what point the receptors and transporters are impacted by luminal SCFAs.

Additionally, previous studies have established that SCFAs have intestinotrophic effects when provided systemically in models of resection and are particularly important for stimulating adaptation (Tappenden et al., 1996; Tappenden et al., 1997; Bartholome et al., 2004). Thus, it would be interesting to determine the effects of luminal SCFAs in a model of intestinal resection. Luminal SCFAs could be provided with enteral nutrition as in the current study or could be used as minimal enteral feeds, which may have greater clinical applicability.

The current study suggests that butyrate is mediating its intestinotrophic effects through the hormone GLP-2. To explore this further, additional *in vivo* studies are warranted. GLP-2 must interact with the GLP-2 receptor in order to have any effects. It would be beneficial to examine SCFA and butyrate administration in GLP-2 receptor knock out models. This data would not only further substantiate the interaction between butyrate and GLP-2, but could also be informative about any SCFA- and butyrate-stimulated effects that are not mediated by GLP-2.

Lastly, the mechanism by which GLP-2 acts on the intestine is not completely understood. Previous research has suggested that GLP-2 may be stimulating release of other growth factors and hormones which are acting on the intestine, including KGF, IGF-I, and EGF (Orskov et al., 2005; Dube et al., 2006; Yusta et al., 2009). These studies examined exogenous GLP-2 administration in knock out models of the various growth factors. However, butyrate administration in these animal models has not been conducted and thus is warranted. Elucidation of the role of these growth factors would further expand our knowledge of the mechanisms by which butyrate stimulates intestinotrophic effects and may provide additional therapeutic strategies.

SUMMARY

In summary, this thesis research has provided insight into the endocrine function of the gut, specificially the nutrient-gene interaction between butyrate and proglucagon. It further substantiated the trophic effects of butyrate and explored the molecular mechanisms by which this is induced. Thus, the information gained from this research strengthens our understanding of the relationship between PGDPs and SCFAs, providing new nutritional targets for treating disorders impacted by proglucagon, such as intestinal dysfunction, Type 2 diabetes, and obesity.

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CURRICULUM VITAE

JENNIFER N. WOODARD

EDUCATION

2005 – present	**Ph.D. Candidate in Nutritional Sciences** University of Illinois at Urbana-Champaign, Urbana, IL Expected Graduation: May 2010 GPA: 4.0/4.0
2009	**Certificate in Business Administration** University of Illinois at Urbana-Champaign, Urbana, IL
2001 – 2005	**B.S. in Chemistry, Minor in Anthropology** University of Illinois at Urbana-Champaign, Urbana, IL GPA: 3.94/4.0

RESEARCH FOCUS

Advisor: Kelly Tappenden, Ph.D., R.D.
Thesis Research: Butyrate-Induced Expression of Proglucagon: Implications for Enteroendocrine Signaling and Intestinal Growth

- Analysis of the human proglucagon gene promoter in human enteroendocrine cells
    - Applied cell culture techniques, subcloning, transfection, and luciferase assays to examine *in vitro* samples
- Evaluation of signaling mechanisms involved in proglucagon activation
    - Employed semi-quantitative real-time PCR and siRNA to study relationship between butyrate transporter and receptor expression and proglucagon *in vivo* and *in vitro*
- Assessment of luminal short-chain fatty acids on structure and function in the adult rodent intestine
    - Utilized surgical techniques, RNA, DNA, and protein quantification assays, real-time PCR, and histomorphology to quantify adaptive response *in vivo*

PROFESSIONAL EXPERIENCE

Research Fellow *2005-present*
Division of Nutritional Sciences
University of Illinois at Urbana-Champaign, Urbana, IL

- Utilized molecular techniques to analyze and quantify proglucagon regulation and intestinal adaptation *in vivo* and *in vitro*
- Organized and directed research projects including an animal study involving management of personnel, surgeries, animal care, and sample collection

- Assisted fellow lab members with animal husbandry, preparation of parenteral nutrition, lab equipment, troubleshooting, and assays including immunohistochemistry, FISH staining, and Ussing chambers
- Developed research study designs and experimental protocols

Industry Internship *2009-present*
Abbott Nutrition Research and Development – Pediatric Nutrition Strategic Research
Research Park, Champaign, IL
- Evaluated the scientific literature on efficacy, tolerability, processing, and sourcing of factors to be included in potential products
- Wrote white paper and presented findings to colleagues

Teaching Assistant *2007-2008*
Department of Food Sciences and Human Nutrition
University of Illinois at Urbana-Champaign, Urbana, IL
- **FSHN 420 Nutritional Aspects of Disease, Fall 2007, 2008**
  - Prepared and presented lectures on Celiac Disease during Gastrointestinal Physiology and Disorders portion of the course
- **FSHN 220 Principles of Nutrition (Head TA), Spring 2008**
  - Supervised 5 discussion leaders, wrote and graded exams, created discussion section materials, ran review sessions, assisted students with questions regarding class materials

Undergraduate Assistant *2004-2005*
- Assisted professor with office tasks including transcribing lectures, proofreading and editing materials and reference citations, searching out and reading current research, organization of files

SCHOLARSHIPS, HONORS, AND AWARDS

Abbott Nutrition Certificate in Business Administration Scholarship
University of Illinois, Division of Nutritional Sciences, 2009

National Institutes of Health Ruth L. Kirschstein National Service Research Fellowship
University of Illinois, Division of Nutritional Sciences, 2008 - 2010

Margin of Excellence Research Award
University of Illinois, Division of Nutritional Sciences, 2008

Margin of Excellence Travel Award
University of Illinois, Division of Nutritional Sciences, 2008, 2009, 2010

Gamma Sigma Delta Honor Society
University of Illinois, College of Agricultural, Consumer, and Environmental Sciences, 2006

University Fellowship
University of Illinois, Graduate College, 2005

Bronze Tablet (University Honors)
University of Illinois, College of Liberal Arts and Sciences, 2005

Reynold Clayton Fuson Award
University of Illinois, Department of Chemistry, 2005

Clarence E. Brehm Award
University of Illinois, College of Liberal Arts and Sciences, 2004, 2003

John E. Scott Award
University of Illinois, Department of Chemistry, 2003

Harold and Edith Hoots Award
University of Illinois, Department of Chemistry, 2003

Dean's List
University of Illinois, College of Liberal Arts and Sciences, 2001-2005

Michael K. Magill Award
Crawford County, Illinois, 2001

American Legion Post 69 Scholarship Award
Crawford County, Illinois, 2001

Jackson Wapner Memorial Scholarship Award
Robinson High School, Robinson, IL, 2001

SOCIETY MEMBERSHIPS

American Gastroenterological Association, Student Trainee Member, 2007-present
American Society for Nutrition, Student Member, 2009-present

PUBLICATIONS

Woodard JN, Tappenden KA. The Role of Humoral Factors in Intestinal Adaptation. In: *Intestinal Failure: Diagnosis, Management and Transplantation.* Blackwell Publishing, 2008: 223-228.

ABSTRACTS

Woodard JN, Tappenden KA. Butyrate upregulates proglucagon mRNA abundance through activation of the proglucagon gene promoter in NCI H716 cells. The FASEB Journal 2008; 22: lb135.

Woodard JN, Tappenden KA. Downregulation of GPR43 and T2R38 receptors prevents butyrate-stimulated increases in proglucagon mRNA abundance in NCI H716 cells. Clinical Nutrition Week, New Orleans, LA, February 2009.

Woodard JN, Correa-Matos NJ, & Tappenden KA. The provision of the fermentable fibers soy polysaccharides and fructooligosaccharides increases short-chain fatty acid transporter abundance in healthy piglets. Experimental Biology, Anaheim, CA, April 2010.