BUTYRATE-INDUCED UPREGULATION OF INTESTINAL GLUCOSE TRANSPORT AND SIGNALING PATHWAYS REPRESENT A POSSIBLE NUTRIENT THERAPY FOR INDIVIDUALS WITH MALABSORPTIVE DISORDERS

BY

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DISSERTATION

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ABSTRACT

Individuals with significant intestinal malabsorption face challenges digesting and absorbing sufficient nutrients. A number of intestinal diseases, including celiac disease, short bowel syndrome (SBS), necrotizing enterocolitis (NEC), and the inflammatory bowel diseases, Crohn's disease and ulcerative colitis, contribute to malabsorption. The treatment of severe malabsorption often requires medical nutrition support strategies, including parenteral nutrition (PN), that are costly and associated with complications when administered as a long-term solution. Beyond the financial burden of intestinal diseases, there is an added price of psychological stress, medical complications, and limitations in lifestyle. Interventions aimed at increasing intestinal transport capacity may mitigate the negative consequences of a damaged intestine, and reduce the need or intensity of costly supportive therapies.

Attaining nutrients is a careful orchestration of diet, digestion, and absorption. In a typical "Western" diet, approximately half the calories are derived from carbohydrates that are digested to monosaccharides (glucose, fructose, and galactose) in preparation for absorption. D-glucose is the major transport form of fuel for most tissues and, therefore, the focus of these studies. It is transported by the hexose transporters sodium-dependent glucose cotransporter-1 (SGLT1) and glucose transporter 2 (GLUT2). GLUT2 is a high-capacity, facilitative intestinal monosaccharide transporter that is upregulated by the short-chain fatty acids (SCFA) derived from the intestinal microbiota during fermentation.

The objective was to identify the impact of the SCFA, butyrate, upon intestinal glucose transport capacity. The hypothesis that ileal butyrate would increase glucose transport capacity and that this would occur through increased GLUT2 abundance, or signaling, was tested.

Further, possible mechanisms through stimulation of enteroendocrine L-cells by butyrate were examined.

The initial studies used Caco2-BBE monolayers as an *in vitro* model for the human intestine to test for the upregulation of GLUT2 mRNA abundance by butyrate and other SCFA. Next, the impact of butyrate on glucose transport by the intestine was examined using the Sprague Dawley rat as an *in vivo* model. Finally, proglucagon mRNA, and the G-protein coupled receptors (GPCR), Free Fatty Acid Receptor FFAR2) and Free Fatty Acid Receptor 3 (FFAR3), were measured.

To establish that butyrate increased the glucose transport capacity of the intestine through upregulation of GLUT2, mRNA abundance and promoter activation were measured in differentiated Caco2-BBe monolayers. Caco2-BBe monolayers were treated for 0.5-24 h with 0-20 mM butyrate and GLUT2 mRNA was measured using quantitative reverse transcription–polymerase chain reaction (RT-PCR). GLUT2 mRNA abundance was higher with 1-4 h of exposure to 2.5, 7.5, and 10 mM butyrate ($P < .0001$). Following transient transfection of the Caco2-BBe monolayers with the pGL3 luciferase reporter vector, butyrate treatment induced promoter activity in a dose-dependent fashion ($P < 0.0001$). Analysis of the GLUT2 promoter indicated that regions –282/+522, –216/+522, and –145/+522 had a heightened ($P < 0.05$) response to butyrate compared with 1135/+522 and 564/+522. These results demonstrated that butyrate upregulates GLUT2 mRNA abundance in Caco2-BBe monolayers by activating specific regions within the human GLUT2 promoter.

Next, butyrate induced increases in GLUT2 mRNA were tested *in vivo*. Sprague Dawley rats were surgically modified with an ileal cannula for infusion of either 10 mM butyrate or saline solution (for 96 or 156 min) and a portal cannula for blood sampling. All rats received the

tracer (3-O-[methyl-^{14}C] –D-glucose (8 uCi) within a liquid meal, but were randomized to be FED (3 mg/kg BW D-glucose) or FASTED (0 mg/kg BW D-glucose). Glucose absorption by the proximal intestine decreased after extended ileal saline infusion compared to a shorter saline infusion ($P< 0.05$), but butyrate infusion attenuated this decrease. The mRNA abundance of hexose transporters responsible for glucose absorption also were measured. Butyrate increased jejunal GLUT2 (by 23%; $P<0.05$) but not SGLT1 mRNA. Within the L-cell, proglucagon mRNA abundance (the transcript coding for GLP-1 and GLP-2) was 2-fold higher in the cecum of butyrate-treated rats ($P<0.0014$). Butyrate also increased FFAR2 and FFAR3 mRNA abundance in the cecum. Taken together, butyrate increased glucose transport by the proximal intestine supported by an increase of jejunal GLUT2 and stimulation of FFAR2, FFAR3, and proglucagon within the L-cells in the cecum.

Unexpectedly, the prolonged infusion of saline into the ileum decreased glucose transport by the proximal intestine. It is likely that saline prevented contact of intestinal contents with the mucosa and dramatically reduced stimulation of the mucosal cells. Treatment of the distal intestine with butyrate attenuated the loss of glucose transport capacity attributed to extended ileal saline. Thus, 10 mM butyrate, a physiologically relevant concentration, effectively maintained glucose absorption. The contact of butyrate with the mucosa of the distal ileum, cecum, and colon stimulated the intestinal L-cell to increase the abundance of proglucagon and the GPCR FFAR2 and FFAR3. These finding focus attention on butyrate, proglucagon, and its derived peptides (GLP-1, GLP-2) along with the taste receptors FFAR2 and FFAR3 for their important roles in intestinal absorption. The upregulation of glucose transport by butyrate is a nutrient therapy for individuals with malabsorptive disorders.

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ABBREVIATIONS

µg - micrograms
µl – microliters
µm - microns
ANOVA – analysis of variance
AUC- area under the curve
buk – butyrate kinase gene
BW – body weight
cAMP – cyclic adenosine monophosphate
CAT - choloramphenicol acetyltransferase
cDNA – complementary deoxyribonucleic acid
cm – centimeters
Ct – cycle threshold
d – day
DEPC – diethyl pyrocarbonate
DNA – deoxyribonucleic acid
DPP-IV – dipeptidyl peptidase-IV
EN – enteral nutrition
FFAR – free fatty acid receptor
g - grams
GIP – glucose-dependent insulinotrophic peptide
GLP-1 – glucagon-like peptide-1
GLP-2 – glucagon-like peptide-2
GLP-2R – glucagon-like peptide receptor
GLUT2 – glucose transporter 2
GPCR – G-protein coupled receptor
h – hours
HEPA – high efficiency particulate air
IACUC – Institutional Animal Care and Use Committee
IBD – inflammatory bowel disease
IgT - Immunoglobin T cell
IF – intestinal failure
kcal - kilocalories
kg - kilograms
Km – Michaelis Menton constant
lsmeans – least square means
mg - milligrams
min - minutes
ml - milliliters
mM - millimolar
mRNA – messenger ribonucleic acid
NaCl – sodium chloride
NEC – necrotizing enterocolitis
nm - nanometers
nM – nanomolar

PEN – partial enteral nutrition
PN – PN
Pcsk – prohormone convertase
PYY – peptide YY
R – Resistance
RLU – relative light units
RNA – ribonucleic acid
RT-PCR – real time polymerase chain reaction
SBS – short bowel syndrome
SCFA – short chain fatty acid
SEM – standard error of the mean
SGLT1 – sodium dependent glucose transporter-1
SNP – single nucleotide polymorphism
T2D – type 2 Diabetes
UC – ulcerative colitis
US – United States
wk - week

Chapter 1 LITERATURE REVIEW

INTESTINAL MALABSORPTIVE DISORDERS

When the intestine is damaged through disease, inflammation, traumatic injury, or shortened as the result of surgical interventions, the natural capacity for digestion and absorption is decreased. The location and extent of the insult dictates the risk of nutrient malabsorption. For example, vitamin B_{12} and bile acids are absorbed by the ileum, so when the ileum is resected, this capacity is removed. If less than 200 cm of small intestine remains, there is a high risk of short bowel syndrome (SBS) (Van Gossum et al., 2009). In response to damage or shortening, the intestine begins the process of adaptation wherein physical and functional changes occur to reduce malabsorption and increase nutrient transport. The adaptation process can span years (Gouttebel et al., 1989; Pironi et al., 2008; Amiot et al., 2013). Resections leaving portions of the ileum and colon allow for high levels of adaptation (Sigalet and Martin, 1998). The ileum has a greater capacity for adaptation than the jejunum (Booth et al., 1959; Dowling and Booth, 1966, 1967; Thompson et al., 1999). The Small Bowel and Nutrition Committee of the British Society of Gastroenterology state in their published guidelines for patient care that patients without an ileum or colon do not adapt well (Nightingale and Woodward, 2006). Thus, the distal intestine is indicated as a region that is associated with positive change during intestinal adaptation.

Early experiments exploring the adaptation of the intestine following resection demonstrated that the presence of the distal intestine rather than the proximal intestine was the most important to recovery of structure and function. As early as 1959, Booth and colleagues established that there was hypertrophy of the remaining small bowel when the proximal small intestine of a rat was removed, but only modest changes occurred when the distal small intestine

was excised (Booth et al., 1959). Follow-up studies investigating the impact of gastrointestinal resection and refeeding regimens showed that luminal nutrients influenced the absorptive capacity of the intestine and the health of the animal. In rats bearing a resected small intestine (60% distal region) that were either pair-fed to control (sham-operated) rats, or fed ad libitum, the free-fed resected rats exhibited significantly higher and wider villi, with deeper crypts, and absorbed more glucose (Menge et al., 1975). Although the pair-fed resected rats showed a trend towards increased villi height, they did not significantly differ from the sham controls. This early report is one of the first that relates the glucose absorption capacity of the intestine to luminal nutrients in a malabsorptive model.

During nutrient malabsorption, carbohydrates and other nutrients that are not absorbed by the proximal intestine continue to the terminal ileum and colon where they are fermented by the microbiota to short chain fatty acids (SCFA). SCFA are absorbed by the intestine and provide a means of rescuing energy lost to the proximal intestine, but also participate in signaling pathways. One signaling pathway is the "ileal brake", which slows the transit of nutrients through the proximal intestine through communications from the ileum (van Citters et al., 2002; Maljaars et al., 2008). This process slows the release of digesta from the stomach, the motility of the proximal intestine and reduces gastric, pancreatic and biliary secretions. The net effect is to increase nutrient absorption and feelings of satiety. The ileal brake was first demonstrated in humans through infusion of liquid lipid into the ileum (Read et al 1984; Spiller et al. 1984,) and confirmed by several later studies. GLP-1 activates the ileal brake (Cuche et al., 2000; Holst, 2007) .

Recently, SCFA, and specifically, butyrate, stimulation of intestinal taste receptors were investigated as a means for the distal intestine to sense luminal nutrients. Taste receptors that

have SCFA as a ligand, such as FFAR2 and FFAR3 may serve as the mechanism connecting sensing of luminal contents to the implementation of intestinal responses.

THE IMPORTANCE OF GLUCOSE IN METABOLISM

Carbohydrate as a major macronutrient fuel

Carbohydrates within the intestine originate in foods that are consumed and digested yielding the monosaccharides glucose, fructose, and galactose. A typical "Western Diet" is composed of approximately 50% carbohydrate (National Center for Health Statistics 2014). Thus, carbohydrates are a major source of metabolic energy for people.

Digestion of carbohydrates begins with mastication, lubrication with saliva containing salivary amylase, followed by acidic attack, and physical disruption within the stomach. The exit of chyme from the stomach is carefully titrated by hormones released by the intestine as it senses nutrients present within the bowel. Once in the duodenum, pancreatic enzymes are released as zymogens that are then activated and bile is also released to ensure the breakdown of the digesta. Pancreatic insufficiency contributes to malabsorption of nutrients since lack of peptidase, protease, and lipase can disrupt the digestive process. The hydrolytic enzymes, sucrase, maltase, lactase, α-dextrinase, and the peptidases reside within the brush border located on the luminal surface of the intestine and catalyze the release of monosaccharides and amino acids that are absorbed by the enterocyte and transported to the portal vein for delivery to the liver. There, glucose is either stored as glycogen or transported in systemic circulation to extrahepatic tissues. In response to a meal, insulin is secreted by the pancreas and stimulates the uptake of glucose by non-hepatic tissues.

Of the monosaccharides released by digestion, D-glucose is used as the major energy source by most tissues except for the heart that uses lactate and skeletal muscle that prefers free fatty acids while at rest or in moderate exercise, enterocyte that uses glutamine, and colonocytes that preferentially use butyrate (Bing, 1954; Andres et al., 1956; Cummings et al., 1987) Glucose is the major source of energy used by the brain (Scheinberg and Stead, 1949). The synthesis of glucose is restricted to liver, kidney, and intestine, tissues that express the enzyme glucose-6-phosphatase and are able to conduct gluconeogenesis to maintain blood glucose during fasting (Mithieux, 2005). Since the major acquisition of glucose stems from the digestion of dietary carbohydrate and absorption of glucose from the intestine, it is this pathway we examine.

Glucose Transporters

Within the intestine, GLUT2 and SGLT1 are the nutrient transporters responsible for glucose absorption by the enterocyte. The classical theory of glucose absorption from the lumen of the intestine includes SGLT1 embedded within the luminal facing brush border transporting glucose into the enterocyte against a concentration gradient via coupling with a sodium pump (Hediger et al., 1987; Cheeseman, 1997; Hirsh and Cheeseman, 1998). GLUT2, a high capacity, low affinity transporter, passively transports glucose across the basolateral membrane down a concentration gradient out into the blood in the capillary bed within the villus (Bell et al., 1990). The observation of GLUT2 within the brush border membrane of the enterocyte fueled the alternative theory of GLUT2-assisted transport under high sugar concentrations (Corpe et al., 1996; Kellett et al., 2008). While several rigorous studies disagree with the presence of GLUT2 in the brush border membrane, its presence has been confirmed in conditions of high luminal glucose using membrane vesicles, luminal biotintylation of proteins, and immunohistology (Au

et al., 2002; Gouyon et al., 2003; Shepherd et al., 2004; Kellett and Brot-Laroche, 2005; Grefner et al., 2006; Gromova et al., 2006; Morgan et al., 2007; Tobin et al., 2008; Grefner et al., 2010; Wei et al., 2011; Chaudhry et al., 2012; Zheng et al., 2012). Currently, there is an active debate among different laboratories regarding the role and even the existence of GLUT2 within the brush border of the enterocyte.

DIETARY FIBER AND SCFA

SCFA are released from the fermentation of dietary fiber, resistant starch, intestinal secretions, and other undigested material fermented by microbial populations mainly within the distal intestine. The consumption of a diet high in fermentable fiber is associated with gastrointestinal health and glycemic control (Burkitt et al., 1974; Marlett et al., 2002; Slavin, 2008; Wolfram and Ismail-Beigi, 2011). It is thought that these benefits are conferred, in part, through SCFA.

The primary SCFA produced in the intestine are acetate, propionate, and butyrate that are present in a fairly consistent molar ratio of 60:22:21, respectively (Cummings et al., 1987). SCFA are trophic to the intestinal mucosa, increase mucosa weight, DNA, RNA protein concentration, and are associated with upregulated glucose uptake (Kripke et al., 1989; Koruda et al., 1990; Tappenden et al., 1997).

Exact concentrations of microbial populations and the SCFA they produce depend on the site within the colon as well as the substrates available to them. Since microbe density is highest within the colon where there may be up to 10^{12} per gram digesta, the colon is the primary site of SCFA production, followed by the distal ileum. This varies according to the type of fiber that can dictate the resistance to and rate of fermentation. In humans, colonic SCFA concentrations

are estimated at 123 mM/kg of intestinal contents in the proximal colon and fall to 100 mM in the distal colon. Ileal SCFA concentrations fall far below the colon, but are measurable (Cummings et al., 1987).

Impact of dietary fiber on glucose metabolism

Fiber has been investigated as a means to reduce fasting blood glucose, properties of considerable value to individuals suffering from Type 2 Diabetes (T2D). Inclusion of soluble fiber at 50 g/d through the consumption of foods, increased glycemic control and decreased the area under the curve (AUC) of peripheral blood glucose and blood insulin in human subjects after a meal (Chandalia et al., 2000). Lower peripheral blood glucose concentrations have been measured in humans consuming diets supplemented by fiber (Jenkins et al., 2008; De Natale et al., 2009). In a randomized trial, HbA_{1c}, a measure of long-term glycemic control, was significantly reduced in individuals with T2D after 6 mo of consuming either a low-glycemic index diet (-0.50 % absolute HbA_{1c}) or high-cereal diet (-0.18 % absolute HbA_{1c}), (Jenkins 2008). Attempts to identify mechanisms improving glycemic control by dietary fiber center on the physical properties of fiber and upon SCFA (Murray et al., 1999; Chutkan et al., 2012; de Godoy et al., 2013). Dietary fiber that is considered soluble or viscous decreased and delayed glucose absorption (Jenkins et al., 1978; Serena et al., 2009).

Reports from *in vivo* studies measuring the impact of fiber on glucose transport contain variable results that may stem from differences in the routes of administration, techniques used to measure glucose transport, and the physical characteristics of various dietary fiber sources. Further, glucose measurements include the response to insulin and site of sampling. Therefore,

these studies measure the overall response to fiber and SCFA, but do not provide the specific information about glucose transport capacity of the intestine in response to butyrate alone.

SCFA and Butyrate

Butyrate is used preferentially by colonocytes for energy (Ritzhaupt 1998, 1998a), and has been examined for other metabolic activities. It reduces inflammation, primarily through inhibiting NFKβ (Andoh 1999, Vanhouloin 2009) and spurs cell differentiation *in vivo* and proliferation *in vitro* (Mariadason 2000). Butyrate impacts transcriptional activities within the cell through butyrate response elements and the inhibition of histone deacetylase activity (Gibson et al., 1999; Davie, 2003).

Investigations of the impact of butyrate treatment of the intestine on its capacity to absorb glucose are limited. The few published reports show increased glucose absorption in response to SCFA or butyrate treatment. When butyrate, or a combination of SCFA, was administered intravenously via PN to rats recovering from an 80% jejunoileal resection, there was increased glucose absorption within the ileum (Tappenden et al., 1997). The observation of higher D-glucose (4-64 mM) transport, measured by uptake in brush border membrane vesicles, was accompanied by significantly higher GLUT2 mRNA abundance compared to controls on the third day post-surgery (10.1 vs. 3.7 relative densitometer units, SCFA and control respectively). SGLT1 abundance showed a non-significant trend towards an increase in association with SCFA treatment ($P<0.09$). This was confirmed when rats with intact intestines received PN infusions containing SCFA showed higher jejunal GLUT2 mRNA abundance ($P<0.03$) and ileal protein levels ($P<04$) after 24 or 72 h of treatment (Tappenden et al., 1998). In these rats, ileal proglucagon mRNA also increased with SCFA treatment ($P<0.05$). A similar experiment, using

rats with intact intestines receiving PN containing a combination of SCFA or butyrate alone were compared to an oral nutrition or PN (no SCFA) control group, showed that ileal GLUT2 mRNA abundance was increased 3-fold after SCFA treatment (Drozdowski et al., 2002). Rats receiving the butyrate treatment showed a non-significant rise in ileal GLUT2 mRNA abundance compared to the orally fed rats (0.5 vs 1.2 arbitrary densitometer units for oral verses 9 mM butyrate treatment). Neither the SCFA nor butyrate treatments were significantly different than the PN control group. The addition of a combination of SCFA or butyrate within PN also was examined in a neonatal short bowel model. In this study, piglets were supported with PN containing SCFA or butyrate following an 80% jejunoileal resection. Butyrate treatment increased villus height and cellular proliferation in the residual intestine (Bartholome et al., 2004). SCFA- and butyrate-treated piglets had greater amounts of glucose transported by the small intestine than controls (Albin et al., 2003).

In vitro studies using Caco2-BBe mature monolayers showed that butyrate increased the abundance of GLUT2 mRNA beginning at concentrations of 2.5 through 10 mM. The GLUT2 promoter activation was increased 7.5 fold compared to controls in response to a 10 mM butyrate treatment (Mangian and Tappenden, 2009). Upregulation of GLUT2 mRNA abundance occurred within 1 h of treatment (Mangian and Tappenden, 2009). The upregulation of GLUT2 in response to luminal and systemic factors including glucose and fructose was tested in neonatal rat pups (Cui et al., 2003). Cui found that GLUT2 mRNA abundance was rapidly induced, within on hour of treatment by glucose or fructose (molecules transported by GLUT2). Further, GLUT2 abundance was found to be transcriptionally, not translationally controlled.

Typical measures of glucose transport capacity of the intestine are 1) the uptake of labeled glucose by brush border or basolateral vesicles formed from isolated portions of the

intestinal mucosa or everted sleeves formed from intestinal tissue, 2) electrogenic measures of transport using tissue sections mounted in Ussing chambers, and 3) glucose concentrations found in plasma taken from peripheral or portal blood. The experiments described herein focus on glucose absorption from the lumen of the intestine into the portal blood stream.

PROGLUCAGON AND GLUCAGON-LIKE PEPTIDES

The enteroendocrine L-cell of the intestine produces peptides that effect changes to the structure and function of the intestine. Located principally in the distal intestine and colon, they have a pyramid shape that allows them contact with the lumen of the intestine at their narrow brush border and secretion of peptides from their wide basolateral base. Proglucagon mRNA is produced in L-cells and translated into the enteroglucagon peptide. Post translational processing of enteroglucagon by prohormone convertase 1/3 yields several important peptides including GLP-1 and GLP-2. GLP-1 is an incretin and also slows gastric emptying, while GLP-2 is trophic to the intestine. Cleavage of enteroglucagon occurs through the secretory pathway where GLP-1 and GLP-2 are packaged into vesicles in preparation for secretion (Brubaker and Anini, 2003). GLP-1 is produced and secreted in a 1:1 ratio with GLP-2 (Orskov et al., 1986; Hartmann et al., 2000). Secretion of GLP-1 and GLP-2 occurs through separate pathways (Orskov et al., 1986). Only a small percentage of GLP-1 and GLP-2 leave the intestinal mucosa (<25%) (Drucker, 1998). Both are rapidly degraded by dipeptidase IV (DPPIV), an important control point for their activity. The half life for GLP-1 in plasma is less than 2 min, while the half life for GLP-2 is less than 7 min (Deacon et al., 1995; Kieffer et al., 1995). Butyrate treatment (1, 2, 5, 10 mM) increases the *in vitro* secretion of GLP-1 from NCI-716 enteroendocrine L-cells as

well as the abundance of proglucagon, and prohormone convertase 1/3 (Woodard, 2010; Yadav et al., 2013).

GLP-2 is trophic to the intestine (Drucker et al., 1997; Kato et al., 1999; Jasleen et al., 2002). Its principal actions are to promote adaptation after intestinal resection or damage (Brubaker et al., 1997; Drucker et al., 1997; Tsai et al., 1997). Treatment with GLP-2 analogs increases the structure of the intestine that supports its function in both human and animal models of SBS (Brubaker et al., 1997; Tappenden et al., 2003; Jeppesen et al., 2005; Jeppesen et al., 2011; Benight et al., 2013). Administration of GLP-2 mainly impacts the growth of the small intestine, but the colon also benefits. Although the primary function of GLP-2 is intestinotrophic, there is some evidence that it also increases glucose absorption. In humans, evidence shows that GLP-2 administration improves or, to some degree, ameliorates the malabsorption seen in SBS (Jeppesen et al., 2005). In PN-fed piglets, GLP-2 treatment provided potent protection against malabsorption of hexoses (Cottrell et al., 2006).

Nutrient transport studies, albeit few, show increased glucose transporter activity following GLP-2 treatment. GLP-2 increased SGLT-1 activity within the small intestine when administered to rats (Cheeseman, 1997). In this study, brush border membrane vesicles that were treated with GLP-2 more than doubled glucose uptake. A significant change was observed after 30 min and reached a maximum increase at 60 min. Rapid trafficking and insertion of SGLT1 into the membrane in response to GLP-2 was thought to be responsible for the rise. GLP-2 administration promoted the insertion of GLUT2 into the small intestine brush border membrane of rats as demonstrated in vivo and using membrane vesicles (Au et al., 2002). Thus, GLP-2 demonstrates potential to exert a positive effect on the glucose transport capacity of the intestine through increasing structure as well as function (Estall and Drucker, 2006).

G-PROTEIN COUPLED RECEPTORS/SENSORS

Taste Receptors

G-protein coupled receptors (GPCR), located in cellular membranes, are activated by chemical ligands binding their active sites. They translate chemical signals into metabolic regulation through interaction with G-proteins and their effectors. The effectors include secondary messenger systems or ion channels used for internal cell signaling or cell-to-cell communication through the release of hormones, and stimulation of neurons. Studies linking particular GPCR within the digestive tract to chemical ligands that are perceived as "sweet" or "bitter", combined with the location of some GPCR within taste buds, earn their name as "taste receptors". The sensing of luminal nutrients as chemical ligands binding the GPCR, and the subsequent stimulation of signaling cascade, is a process likely to orchestrate the complexities of nutrient absorption. These pathways offer the potential to control specific aspects of metabolism and make GPCR, their ligands, and allosteric modulators attractive targets for therapeutic drug development (Rask-Andersen et al., 2011; Urwyler, 2011).

FFAR2 and FFAR3

FFAR2 and FFAR3 were discovered during a search for mechanisms controlling galanin abundance. The sequences for four novel GPCR were discovered on chromosome 19, located in continuous order. Originally, the orphan receptors were named GPR40, GPR41, GPR42, and GPR43 (Sawzdargo et al., 1997); however, the preferred nomenclature was changed to FFAR1 (GPR40), FFAR2 (GPR43), and FFAR3 (GPR41), respectively, to more accurately tie their names to their physiological function (Kaemmerer et al., 2010). GPR42 is assumed to be a pseudo gene that has no associated function. The agonists for two of the orphaned protein

receptors, GPR41 and GPR43, were reported as SCFA by three independent laboratories that brought them under scrutiny for their potential link to intestinal nutrient sensing and signaling pathways (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). Recognition of the powerful potential of a drug(s) harnessing the control of metabolism, FFAR2 and FFAR3 led to the development of agonists and antagonists (Lee et al., 2008; Brantis, 2011; Schmidt et al., 2011) for use in appetite control and diabetes.

FFAR2 and FFAR3 are seven transmembrane receptors found in several tissues including enteroendocrine, enterocytes, enteric neurons, leukocytes, mast and adipose cells (Raybould, 1998; Conigrave and Brown, 2006; Covington et al., 2006; Egan and Margolskee, 2008; Engelstoft et al., 2008; Egerod et al., 2012; Nohr et al., 2013). FFAR2 is also found in neutrophils and eosinophils (Maslowski et al., 2009). FFAR2 has greater affinity for propionate than butyrate or acetate; FFAR3 has equal affinity for propionate, butyrate, and pentanoate.

Within the intestine, FFAR2 and FFAR3 are located on the luminal-facing membrane of L-cells where SCFA, their ligands, bind them (Karaki et al., 2008; Tazoe et al., 2009; Nohr et al., 2013). Since their ligand spectrum overlap and they have a similar expression pattern, their metabolic purposes may overlap (Blad et al., 2012). It is hypothesized that they provide the intestine with a means of sensing metabolic products from nutrient consumption, microbiota, or fermentation products.

Both FFAR2 and FFAR3 are linked to Gi type G-proteins, and FFAR2 is also linked to Gq/G11 type G-proteins (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). They both activate second messenger systems using cAMP, while FFAR2 also activates protein C kinase and serotonin signaling pathways. Since FFAR2 is also found in pancreatic beta cells, it is

of particular interest for therapies for glucose homeostasis in obesity and diabetes (Engelstoft et al., 2008; Ahren, 2009; Reimann et al., 2012)

Studies focusing on FFAR2 and FFAR3 were limited to *in vitro* systems until new mouse knockouts models were created. Two recent studies used mice with $FFAR2^{-/-}$ or $FFAR3^{-/-}$ to examine their roles in SCFA stimulated GLP-1 secretion. Tolhurst et al. (2012) found that FFAR2 and FFAR3 were colocalized with GLP-1 in L-cells. In these cells, SCFA-triggered secretion of GLP-1 by a Gq pathway, not a Gi-mediated pathway (Tolhurst et al., 2012). Therefore, FFAR2 is the GPCR implicated in SCFA upregulation of GLP-1 secretion by L-cells (Reimann et al., 2012). Mice deficient in FFAR2 exhibited less SCFA-triggered GLP-1 secretion along with less insulin and glucose intolerance (Tolhurst et al., 2012). In contrast, Bjursell et al. (2011) found that $FFAR2^{-/-}$ mice had improved glucose tolerance and insulin sensitivity compared to wild type mice.These FFAR2-deficient mice had decreased body fat mass and increased lean body mass. The results of these two studies with FFAR2-deficient mice are not in agreement with regard to the impact of lack of FFAR2 on glucose tolerance. This may be due to the different metabolic profiles of the foundation stock of C57/B6 compared to C57/B6/129 used when each laboratory derived their strain of $FFAR2^{-/-}$ mice (Bjursell et al., 2011; Lin et al., 2012), or the composition of the diets fed. FFAR3 does not play a role in butyrate or propionate induced GLP-1 secretion (Lin et al., 2012). The delivery of butyrate to the intestine via probiotic therapy (probiotic VSL#3) was successful in increasing GLP-1 mRNA abundance by intestinal L-cells (Yadav et al., 2013). Changes in nutrient transport due to SCFA induced intestinal motility also have been examined for potential signaling through FFAR2 and FFAR3. At present, there are contradictory reports regarding motility linked through GPR41 activated signaling (Dass et al., 2003; Dass et al., 2007).

Taken together, this information focuses attention on FFAR2 and FFAR3 for their potential in nutrient sensing the SCFA within the intestine and stimulating a response with the L-cell. The L-cell is uniquely equipped to produce and secrete peptides that can delay gastric emptying, produce incretin effects, and are trophic to the intestine. All of these actions contribute to the absorptive capacity of the intestine. Since SCFA are produced from the fermentation of undigested nutrients, providing a response based upon SCFA give fundamental feedback control of the absorptive capacity of the proximal intestine to the distal intestine.

Chapter 2 RATIONALE AND OBJECTIVES OF RESEARCH

SIGNIFICANCE

Medical interventions for malabsorption are costly and associated with complications when administered as a long-term solution. Approximately 225,000 individuals in the US require some nutritional support to maintain their health. Since there are limitations in diagnosis and reporting, this number may be grossly underestimated (Evans, 2009). Malabsorption may stem from various intestinal diseases, including celiac disease, SBS, necrotizing enterocolitis (NEC), the inflammatory bowel diseases (IBD), Crohn's disease, and ulcerative colitis (UC). The worldwide incidence ranges from 0.5 to 24.5/100,000 for UC, 0.1 to 16/100,000 for Crohn's disease, and 396/100,000 for IBD (Loftus, 2004; Lakatos, 2006). Traumatic injuries and infectious disease also contribute to the incidence of decreased intestinal function and malabsorption of nutrients.

The incidence of malabsorption and associated medical expenses is expected to rise. The cost of nutritional support is not limited to nutrients alone, but also includes compounding, supporting devices, and consumable supplies. Annual medical charges for patients supported by PN range across time, geographic location, and health needs from $75,000 to $300,000 annually (Evans, 2009). Medical care to treat inflammatory bowel disease was 1.7 billion dollars in the 1980's (Sonnenberg, 1989). By 2008, the sale of clinical nutrition products increased to 13.6 billion dollars where 2.0 billion dollars purchased PN products within the US (GBI Research, 2012). Market projections estimate the 2.9 billion dollar global market for PN will expand to 4.86 billion dollars in 2018 (GBI Research, 2012) . This will represent a rise from 1.47 billion dollars in 2011 to 2.49 billion in 2018 within the US health care market. These predictions are

based on the increase in gastrointestinal disease, cancer, and AIDs (Lakatos, 2006; Prevention, 2010; GBI Research, 2012; Phillips et al., 2013) .

The human costs of malabsorption severely curtail the quality of life. Individuals may suffer from lack of growth (children), neurological disorders, bone demineralization, vitamin and mineral deficiencies, renal stones, diarrhea, and inflammation of joints, skin, mouth, and eye (Greenstein et al., 1976; Nightingale and Woodward, 2006). Long-term PN may cause the destruction of liver function and lead to the need for organ transplantation (Schalamon et al., 2003; Bishay et al., 2012).

Liberation from intensive therapies can improve the quality of life and increase independence for individuals with significant long-term nutritive treatments. Interventions aimed at increasing intestinal transport capacity may mitigate the negative consequences of a damaged intestine, and reduce the need or intensity of costly supportive therapies. This study, described herein, assesses the impact of ileal butyrate on glucose transport capacity by the proximal intestine. The focus is to identify the mechanisms that support increased transport with a goal of identifying new targets that are responsive to butyrate. Establishing the impact of butyrate on glucose transport capacity and illuminating the mechanistic pathways supplies vital information for effective new therapies.

RATIONALE OF RESEARCH

Nutrient absorption involves the interplay of diet, digestion, and absorption. Within our "Western Diet", carbohydrates compose about 50% of the calories ingested (National Center for Health Statistics 2014). These macromolecules are digested into monosaccharide molecules in preparation for absorption. Of the three monosaccharides (glucose, fructose, and galactose), D-

glucose is transported as a major metabolic fuel. Increasing the capacity of the intestine to absorb D-glucose provides a significant increase in energy available to living tissues.

Glucose is transported from the lumen of the intestine into the absorptive enterocyte via SGLT1 and across the basolateral membrane into the mesenteric blood via GLUT2. When glucose concentrations within the lumen of the intestine are high, GLUT2 contained in vesicles within the enterocyte are translocated to the brush border where they are inserted and assist in transporting glucose into the enterocyte, down a concentration gradient. At these times GLUT2 assists in transporting glucose into the cell as well as out of the cell. Prior research established that GLUT2, and not SGLT1, is upregulated by SCFA and, specifically, butyrate. Thus, butyrate- induced GLUT2 up-regulation represents a possible means of increasing the glucose transport capacity of the intestine.

The pathways involved in butyrate-induced GLUT2 upregulation may involve direct stimulation, or other pathways. Butyrate is used as a preferred fuel by colonocytes but is currently investigated in other roles. It impacts the production and release of hormones within intestinal endocrine L-cells that may also influence glucose absorption. Proglucagon mRNA, produced by the L-cells, codes for several biologically active peptides including GLP-1, and GLP-2. These peptides could mediate the effects of butyrate on absorption by the intestine. The GPCR, FFAR2 and FFAR3, are also found within the membranes of the intestinal L-cell and have SCFA as ligands. These may respond to contact with butyrate by initiating a cascade of secondary messengers.

AIMS AND SCOPE OF RESEARCH

The overall aim of this research was to demonstrate increased glucose transport capacity within the proximal intestine in response to butyrate within the distal ileum and large intestine. Further, potential mechanisms supporting butyrate stimulated glucose transport were examined within intestinal L-cells and taste receptors. The ultimate goal was to provide the basis for new or refined therapies for individuals with intestinal failure for improved survival and quality of autonomous life.

Our central hypothesis was that glucose absorption from the intestine is acutely up-regulated by intestinal butyrate through increased glucose transporter capacity. The signals for the coordination of this response may be mediated through pathways of the GPCR FFAR2 or FFAR3 (taste receptors) and implemented through the increased abundance of proglucagon mRNA and intestinal hormones GLP-1 and GLP-2.

Hypothesis 1: Butyrate in the lumen of the ileum and distal large intestine acutely increases glucose absorption by the proximal small intestine through increased glucose transporter capacity. These experiments establish and quantify butyrate induced *in vivo* up-regulation of intestinal glucose transport through the appearance of labeled glucose in the portal blood.

a. *In vitro:* Butyrate alone up-regulates GLUT2 mRNA abundance through the activation of specific elements encoding transcription factor binding sites within the GLUT2 promoter.

b. *In vivo:* Glucose absorption is acutely increased by intestinal butyrate *in vivo*.

Chapter 3 provides *in vitro* evidence that butyrate increases GLUT2 mRNA abundance through activation of specific regions of its promoter containing transcription factor binding sites.

Chapter 4 provides evidence that within the *in vivo* model of the rat, butyrate, supplied to the distal ileum and large intestine stimulated glucose uptake by the proximal small intestine.

Hypothesis 2: Exposure of the distal ileum and large intestine to butyrate upregulates GLUT2 mRNA abundance, while SGLT1 mRNA abundance remains unchanged. Thus, the glucose transporter mRNA abundance (GLUT2, SGLT1), as measured through real-time reverse transcription polymerase chain reaction (RT-PCR), will reveal a positive impact of butyrate on the GLUT2 mRNA in mucosal cells from the site of administration in the distal intestine (ileum, cecum, and colon) and on the site of glucose absorption in the proximal intestine (duodenum, jejunum).

Chapter 5 reports that butyrate, supplied to the distal ileum and large intestine, stimulated glucose uptake and increased GLUT2 mRNA abundance in the proximal small intestine.

Hypothesis 3: Butyrate up-regulates proglucagon mRNA abundance. This may be mediated through increased abundance of the GPCR FFAR2 or FFAR3 (also known as GPR43 and GPR41, respectively). There will be increased abundance of FFAR2 and FFAR3 mRNA in the ileum of rats treated with butyrate when compared to those treated with a saline control**.**

Chapter 5 provides evidence that proglucagon abundance is increased by butyrate and also shows increases in the mRNA abundance of GPCR FFAR2 and FFAR3.

Chapter 6 summarizes the overall findings of this thesis research and the future directions for this line of research.

INNOVATION

A novel experimental design allowed for the study of luminal butyrate within the distal ileum and large intestine on the glucose transport capacity of the proximal intestine. Glucose transport was measured using the appearance of labeled 3-O-methyl glucose in the portal vein after absorption from the proximal intestine. This model eliminated confounding variables introduced by the metabolism of D-glucose by the enterocyte, because 3-O-methyl glucose is handled by glucose transporters in the same way as D-glucose, but does not enter metabolism. The intact tissues and neuronal pathways of a living rat embraced the intricacies of their communication and signaling. Despite a growing body of evidence demonstrating associations between fiber-containing diets, intestinal SCFA, and increased intestinal absorptive capacity, the direct *in vivo* evidence measuring glucose transport stimulated by known quantities of butyrate is lacking.

Blood collection through portal cannulation and glucose transport measured by 3-O-methyl glucose are well-established models. Combining ileal cannulation to deliver butyrate to the distal ileum and large intestine with portal vein sampling of 3-O-methyl glucose was a new application. In addition, the response of the intestine to conditions that mimic postprandial and fasted states were examined using glucose containing-meals. Since the presence of glucose in

the intestine exerts a positive relationship to its own absorption, the additive effect of luminal butyrate was examined. Butyrate was infused for 1h prior to a meal, or directly with the meal. This helped to distinguish between immediate responses that may be attributed to signaling pathways, or responses that occurred after prolonged exposure representing possible upregulation in transcription or translation. Furthermore, the correlation of glucose transport capacity to changes in mechanistic pathways of the L-cell was possible. This facilitated the identification of receptors and peptides to increase the adaptive response of the intestine and glucose transport.

Chapter 3 BUTYRATE INCREASES GLUT2 MRNA ABUNDANCE BY INITIATING TRANSCRIPTION IN CACO2-BBE CELLS[1]

INTRODUCTION

Nutrient transport is a key component of gastrointestinal function. Located within the membranes of intestinal epithelial cells, transporters serve to move nutrient molecules across those membranes. The absorption of nutrients from within the lumen of the intestine and their subsequent availability for use by the intestine and other tissues is facilitated by these transporters. Among nutrient transporters, glucose transporter 2 (GLUT2) has been identified as a high capacity facilitative transporter responsible for glucose transport (Thorens et al., 1988; Bell et al., 1990) and has been identified in the liver, pancreas, kidney, brain and intestine. Ultimately, GLUT2 ensures the movement of glucose across the enterocyte by spanning the basolateral membrane and translocating to the brush border upon stimulation of the cell by glucose (Bell et al., 1990; Kellett and Helliwell, 2000; Affleck et al., 2003; Gouyon et al., 2003; Kellett and Brot-Laroche, 2005). Further, GLUT2 plays a role in glucose homeostasis by contributing to glucose sensing and signaling pathways in the enterocyte and pancreas (Burcelin et al., 2000; Burcelin and Thorens, 2001; Le Gall et al., 2007). Understanding the regulatory mechanisms stimulating absorptive function will allow for optimization of the therapies provided to individuals with intestinal failure. This in turn, may reduce their long-term dependence on PN (PN) by enhancing their tolerance to and absorption of enteral nutrients.

[1] The final, definitive version of this paper has been published in the Journal of Parenteral and Enteral Nutrition, November 2009 by SAGE Publications, Inc., All rights reserved. © 2009 A.S.P.E.N. Mangian HF, Tappenden KA. Butyrate increases GLUT2 mRNA abundance by initiating transcription in Caco2-BBe cells, JPEN J Parenter Enteral Nutr. 2009; Nov-Dec;33(6):607-17; discussion 617. doi: 10.1177/0148607109336599.

Short chain fatty acids (SCFA), such as acetate, propionate, and butyrate, are products of bacterial fermentation in the intestine and have been shown to enhance structural and functional adaptation of the residual intestine following partial enterectomy (Tappenden et al., 1997), including increased abundance of ileal GLUT2 mRNA and protein (Tappenden et al., 1997; Tappenden et al., 1998; Tappenden and McBurney, 1998; Drozdowski et al., 2002). Recently, we have reported that butyrate is the SCFA able to stimulate structural adaptations that are induced in a neonatal piglet short bowel syndrome model (Bartholome et al., 2004), however the effects of butyrate alone on GLUT2 abundance are not known.

The objective of our study was to examine the mechanism regulating the cellular and molecular alterations underlying the acute responses of GLUT2 to butyrate. We used a Caco2-BBe model for the human intestine and measured the GLUT2 mRNA abundance following butyrate treatment. Next, we quantified the activation of the GLUT2 promoter in a transient transfection Caco2-BBe model and designed experiments to answer the following questions:

1. **Does butyrate increase GLUT2 mRNA abundance in Caco2-BBe monolayers?**
2. **Does butyrate alone activate the GLUT2 promoter or are all three SCFA (acetate, propionate, and butyrate) required?**
3. **Does butyrate exert its effect on the GLUT2 promoter through a sequence specific portion of the promoter, thus inferring interaction with a specific transcription factor?**

We hypothesized that butyrate alone would up-regulate GLUT2 mRNA abundance through the activation of specific elements encoding transcription factor binding sites within the GLUT2 promoter and that butyrate would exert a larger effect than either propionate or acetate.

ABSTRACT

Background: GLUT2 is a high capacity, facilitative intestinal monosaccharide transporter, known to be upregulated by short-chain fatty acids (SCFA) derived from the intestinal microbiota during fermentation. Understanding the mechanisms regulating intestinal function is important to allow for optimization of therapies provided to individuals with intestinal failure and ultimately reduce their dependence on PN.

Objective: Our objective was to examine the mechanism regulating the underlying the response of GLUT2 to the SCFA, butyrate.

Methods: GLUT2 mRNA abundance was measured in differentiated Caco2-BBe monolayers treated for 0.5-24 hours with 0-20 mM butyrate using quantitative RT-PCR. Activation of the human GLUT2 promoter was measured using luciferase reporting in transiently transfected Caco2-BBe monolayers.

Results: GLUT2 mRNA abundance was higher ($P< 0.0001$) with 1-4h exposure to 2.5, 7.5, and 10 mM butyrate. Butyrate induced ($P< 0.0001$) promoter activity in a dose dependent fashion. Analysis of the GLUT2 promoter indicated that regions -282/+522, -216/+522 and -145/+522 had a heightened ($P<0.05$) response to butyrate compared to -1135/+522 and -564/+522.

Conclusions: Butyrate upregulates GLUT2 mRNA abundance in Caco2-BBe monolayers by activation of specific regions within the human GLUT2 promoter. These results identify a cellular mechanism wherein butyrate upregulates intestinal absorption that may be relevant to patients with reduced function. Additional work is necessary to understand cellular targets of

butyrate therapy and define clinically appropriate means of providing such strategies, such as consuming pre- and probiotics.

MATERIALS AND METHODS

Experimental Design. The experiments outlined below were conducted to answer our stated questions.

Question 1. **Does butyrate increase GLUT2 mRNA abundance in Caco2-BBe monolayers?**

GLUT2 mRNA abundance was measured in differentiated Caco2-BBe monolayers after treatment with butyrate (0, 2.5, 5, 7.5, 10, 20 mM) for acute periods of time (0.5, 1, 2, 4, 6, 24 hours), as described below.

Question 2. **Does butyrate alone activate the GLUT2 promoter or are all three SCFA (acetate, propionate, and butyrate) required?**

The full length promoter construct (pGL3 -1296/+522 GLUT2 described below) was transfected into differentiated Caco2-BBe monolayers, and randomized to one of the following five treatments (n=12/treatment) for 48 hours:

1) **5 mM acetate** (A);

2) **5 mM butyrate** (B);

3) **5 mM propionate** (P);

4) **5 mM SCFA** (composed of 3 mM A, 1.25 mM P, and 0.75 mM B , to mimic the physiological ratio produced in the colon), or;

5) **15 mM SCFA** (5 mM each A, B, P, to test for cumulative effects observed with individual SCFA).

Upon harvesting the cells, activation of the GLUT2 promoter was measured as described below. These experiments were also conducted in STC-1 cells, a mouse enteroendocrine cell line, which served as a negative control in that it is a non-absorptive intestinal epithelium cell line.

In addition, to identify the optimal dose of butyrate to use in subsequent experiments analyzing the GLUT2 promoter (Question 3), differentiated Caco2-BBe monolayers were transfected with the full length GLUT2 promoter reporter vector and treated with 0, 2.5, 5, 7.5, 10 or 20 mM sodium butyrate for 48 hours.

Question 3. **Does butyrate exert its effect on the GLUT2 promoter through a sequence specific portion of the promoter, thus inferring interaction with a specific transcription factor?**

To investigate the region of the GLUT2 promoter that is activated by butyrate, 21 day Caco2-BBe monolayers were transiently transfected with either the full length promoter, or sequential portions of the promoter exhibiting 5' deletions **(Figure 1 and 2).** The transfected monolayers were treated with either 0 or 10 mM sodium butyrate (the optimal does identified in the dose experiments outlined above) for 48 hours.

Cell Lines and Culture. Well-differentiated, mature monolayers of Caco2-BBe cells provide a model for the absorptive enterocyte (Peterson and Mooseker, 1992; Peterson et al.,

1993; Basson et al., 1994; Sambuy et al., 2005) and were used to study the regulation of GLUT2 mRNA abundance and promoter activation by butyrate. Cells from the Caco2-BBe (CRL-2102, American Type Culture Collection, ATCC, Manassas, Virginia) clone of Caco-2 (ATCC: ATB-37) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, supplemented with 0.01 mg/ml human apotransferrin (Sigma, St. Louis, MO, USA), and 10 % fetal bovine serum (FBS, Life Technologies, USA). All cells were and grown at 37 ^{o}C, with 5 % CO_2. The cells were seeded into 24 well plates at a density of 5 x 10^5 and grown to mature, differentiated monolayers for 21 days prior to treatment. Growth medium was replaced as needed, but no less than twice per week (Peterson and Mooseker, 1992). In experiments, SCFA treated monolayers had growth medium removed and replaced with 4 mL of growth medium supplemented with SCFA, as described above.

Mouse enteroendocrine cells, STC-1, were a gift from P. Brubaker at the University of Toronto and used as a negative control in that these cells derived from mice, rather than humans, and do not constitutively express abundant amount of GLUT2 (Dyer et al., 1997b; Han et al., 2007). STC-1 cells were cultured in DMEM with 10 % horse serum and 2.5 % FBS. Growth medium was replaced as needed, but no less than twice per week, and cells were subcultured weekly. In transfection experiments, 24 well plates were seeded at 5 x 10^5 STC-1 cell density and grown until 80 % confluent prior to treatment. After transfection, the growth medium was removed and replaced with 4 ml of the treatment medium for 48h.

Quantification of GLUT2 mRNA Abundance. Real-time RT-PCR (reverse transcription-polymerase chain reaction) was employed to assess the impact of butyrate on GLUT2 mRNA abundance. After treatment, Caco2-BBe monolayers were harvested and their RNA was isolated using the TRIzol method (Sigma, St. Louis, MO) according to manufacturer's protocol which is based on the guanidium isothiocynate phenol-chloroform method (Chomczynski, 1993). RNA concentration and quality was ascertained through measurement of RNA 260/280 ratio and 230 absorbance spectra (data not shown). Then, total RNA was reverse transcribed using heximer primers and Superscript™II Reverse Transcriptase (Invitrogen, Carlsbad, CA), per manufacturer recommendations.

Real Time PCR amplification reagents, primers and probes were purchased from Applied Biosystems (ABI, Applied Biosystems, Foster City CA). Commercially available, Taqman® Gene Expression assays supplied minor groove binding probes (MGB) and primers for amplification and analysis of human GLUT2 (Hs00165775), Mouse GLUT2 (Mm00446224), and 18S endogenous control (4308329). GLUT2 and 18S endogenous probes were labeled with the fluorescent dyes, FAM and VIC, respectively, to allow for simultaneous quantification within each sample and use of endogenous 18S abundance as an internal control. GLUT2 and 18S mRNA abundance was measured with a Taqman ABI 7900 (Applied Biosystems).

Data were analyzed with ABI Sequence Detection System software (Applied Biosystems, Foster City, CA, Version 2.2.1) using standard curve methodology. In this method, the threshold cycles (Ct) from the GLUT2 target gene were compared to a standard curve generated from a series of dilutions of GLUT2 cDNA spanning six concentrations. The calculated concentration

of the GLUT2 mRNA was then normalized to the 18S RNA concentration for each sample. Each sample was assayed in quadruplicate.

Activation of the GLUT2 Promoter. Cloning techniques were used to produce a luciferase expression vector as a plasmid constructs containing either full-length, or reduced sequences, of the GLUT2 promoter. Transfection of these constructs into eukaryotic cells demonstrates the activation of promoter sequences through the measurement of luciferase synthesized within transfected cells under various conditions.

GLUT2 promoter luciferase reporter vector constructs: The GLUT2 promoter region was amplified from human genomic DNA (Promega, Madison, WI) using the Platinum® P*fx* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and the polymerase chain reaction (PCR) and primers sequences ctgcagaatgtcatgtcactggcaaatgcc (forward) and gtcagttaacagagcaaaaatccttaaaacc (reverse). The PCR reactions yielded a DNA amplicon containing 1296 base pairs (bp) of the 5' flanking region and 522 bp of exon 1 of the GLUT2 gene representing the promoter region (**Figure 1**).

The sequence of the cloned GLUT2 promoter was confirmed through alignment with the reported GLUT2 promoter region for human glucose transporter 2 (GLUT2) gene exon 1 (gi183282), deposited by Takeda et al.(Takeda et al., 1993) , with only 3 mismatched base pairs, and also to Homo Sapiens Bac RPII-115J24 with 100% match using NIH BLAST. The 3 mismatched bases within the GLUT2 exon 1 sequence gi183282 represented single nucleotide polymorphism variants (SNP) that were previously reported in the NCBI SNP Database. This

cloned sequence contained A verses C at 1134 bp, a C verses T at 1225 bp, and A verses G at 1267 bp corresponding to rs5393, rs5394 , and rs5396 respectively (Figure 1) . The sequence of this GLUT2 promoter reporter vector was determined by the SNP variant from the human donor DNA used for the amplification of the promoter sequence.

This GLUT2 promoter DNA was cloned into the pGL3-Basic Luciferase Reporter Vector (Promega, Madison, WI, USA) at the KPN1; XHO1 restriction site to construct the full length GLUT2 promoter reporter vector (pGL3 -1296/+522). Position 0 refers to the major transcription start site for the GLUT2 gene (Takeda et al., 1993) . GLUT2 promoter reporter vector subclones containing portions of the GLUT2 promoter were constructed using restriction digests followed by re-ligation of the plasmid to form nested 5' deletion constructs (Figure 2).

The GLUT2 reporter vector constructs were then transformed into chemically competent Top 10 cells (Promega, Madison, WI) and grown overnight at 35 ^{o}C. DNA for transient transfection experiments was prepared using the Qiagen Maxi Prep (Qiagen Inc., Valencia, CA, USA) according to manufacturer recommendations. The sequence of reporter constructs was confirmed in both directions with a ABI3730*xl* capillary sequencer (Applied Biosystems, Foster City CA) after each DNA preparation and prior to use for transient transfections to ensure no insertions or deletions to the promoter sequence were induced by the bacterial host.

Transient transfection of Caco-2BBe and STC-1 cells: For experiments using Caco2-BBe cells, the full length promoter was co-transfected with pRL-TKRenilla (transfection efficiency control) into differentiated Caco2-BBe monolayers using a cationic liposome, Transfast^{tm} Transfection Reagent (Promega, Madison, WI, USA), according to manufacturer

recommendations. All transfection conditions were selected from optimization experiments included in manufacturer recommendations (data not shown). Tandem transfections using the promoterless pGL3-Basic reporter vector served as treatment controls. Following a two-hour transfection period wherein the reporter construct entered the Caco2-BBe cells, the monolayers were overlayed with growth medium containing the experimental treatments for 48 hours. Then, the cells were harvested, lysed and the luciferase produced from promoter activation was measured with Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Luciferas*e* was expressed as the fold increase in luciferase relative light units (RLU) over the control (no treatment) group, normalized to the DNA concentration (DNA) of the cell lysate (RLU/µg DNA). Each transfection was performed in quadruplicate wells per plate and repeated at least three times on different days ($n \geq 12$/treatment). To enable minor differences between response of monolayers and allow for RLU normalization, DNA (Latt and Stetten, 1976) and protein (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA, USA) concentration of the cell lysates were determined.

For the transfection experiments using STC-1 cells, cells at 80 % confluence were grown in low serum medium (1.2 %) for 24 hours prior to transfection. Cells were then rinsed with serum free media and transfected with pGL3 -1296/+522 at 0.75 ug DNA per well in a 1:1 ratio with Transfast^{tm}. Following a 1.5 hour transfection incubation, treatment medium was applied. After 24 hours of treatment, transfected STC-1 cells were lysed and luciferase RLU, DNA, and protein was measured as before.

Statistical Analyses. The effects of SCFA on the GLUT2 promoter were determined using the Mixed Model as calculated by the SAS program (Version 9; SAS Institute Inc., Cary, NC) followed by orthogonal comparison. All analyses used $P \leq 0.05$ as the minimal criterion of statistical significance and values were reported as means ± SEM. Regression analysis of the butyrate dose effect used the best fit straight line, trendline function of Excel (Microsoft Office 2007, Microsoft Corp.) to determine the R^2 of activation of the GLUT2 promoter (RLU/DNA fold increase over control) for 0-10mM butyrate.

RESULTS

Effect of butyrate on GLUT2 mRNA abundance. GLUT2 mRNA abundance was higher in Caco2-BBe monolayers treated with 2.5 ($P<0.0363$), 7.5 ($P<0.0357$), and 10 mM ($P<0.0001$) butyrate compared to that of control (0.5482 GLUT2/18S mRNA), regardless of incubation time (**Figure 3.3**). The highest ($P<0.0001$) abundance of GLUT2 mRNA was achieved with the 10 mM butyrate treatment at the 1, 2, and 4 hour time points (**Table 3.1**).

Effect of butyrate, and other SCFA, on the GLUT2 promoter. The activation of the GLUT2 promoter was 7.5 -fold higher ($P< 0.0001$) following butyrate treatment (**Figure 3.4**). Neither acetate, nor propionate treatment impacted the GLUT2 promoter. The highest ($P< 0.0001$) level of promoter activation (9-fold) was observed following the 15 mM SCFA and may have resulted from cumulative effects of butyrate and a numerical increase observed in the individual treatments.

Butyrate activated the GLUT2 promoter in a dose-dependent fashion with higher activation achieved in the 5 ($P<0.0006$), 7.5 ($P<0.0001$), 10 ($P<0.0001$), and 20 mM ($P<0.0001$)

groups, compared to control (**Figure 3.5**). The GLUT2 promoter response showed a plateau between 10 mM with no difference observed between the 10 mM and 20 mM groups **(Figure 3.6**). Linear regression of the promoter response for 0 through 10 mM butyrate treatment showed a positive relationship (R^2=0.978).

The protein per DNA ratio was unchanged by any treatment (0.85 mg pro/ug DNA). Although the DNA content was decreased ($P< 0.05$) by 2.5, 5, 7.5, 10 mM butyrate compared to controls (0.2902, 0.2902, 0.2908, 0.2935, vs. 0.3087 ug/ml Caco2-BBe lysate), the protein content was impacted comparably (20.2, 19.6, 19.4, 19.6, 19.9 vs. 21.4 mg/ml Cac02-BBe lystate, $P< 0.05$) in 2.5, 5, 7.5, 10, 20 mM butyrate treatments.

The transfection control, TK Renilla, was activated minimally and equally in like treatments demonstrating the equal transfection efficiency between monolayers (data not shown). The empty, promoter-less pGL3 reporter vector, used as a control for any unforeseen, nonspecific effects of SCFA on the pGL3 reporter vector, did not respond to either the SCFA or butyrate treatments indicating the specificity of the butyrate response to the GLUT2 promoter. Finally, the STC-1 cells, which served as the negative control cell system, did not reveal altered activation of the GLUT2 promoter following incubation with butyrate **(Figure 3.6).** A modest, albeit significant activation of the GLUT2 promoter was observed with the 15 mM SCFA treatment and may reveal enhanced sensitivity of these enteroendocrine cells to the increased presence of nutrients and the osmotic tension produced in this group.

*Effect of butyrate on specific regions of the GLUT2 promoter***.** Various regions of the GLUT2 promoter were activated by butyrate, however varying levels of activation were achieved among regions studies. The highest level of activation was observed in the -282/+522 promoter. The -282/+522, -216/+522 and +145/+522 were activated by a greater extent than -1135/+522 and -564/522 subclones (**Figure 3.7**).

DISCUSSION

The experiments herein were conducted as part of a broader initiative to enhance our understanding of the mechanisms regulating intestinal function so that future therapies for individuals with intestinal failure may be optimized and thereby reduce their dependence on PN. The objective of our current study was to examine the mechanism regulating the cellular and molecular alterations underlying the acute responses of GLUT2 to butyrate. We used a Caco2-BBe model for the human intestine and measured GLUT2 mRNA abundance and promoter activation of the GLUT2 promoter in a transient transfection Caco2-BBe model to obtain answers to the three questions posed earlier.

Question 1. **Does butyrate increase GLUT2 mRNA abundance in Caco2-BBe monolayers?**

Previous studies have demonstrated higher GLUT2 mRNA abundance in the ileum of rats receiving butyrate supplemented PN (Tappenden et al., 1997; Tappenden et al., 1998; Tappenden and McBurney, 1998), following 70% jejunoileal resection (Iqbal et al., 2008) , and *in vitro* in RIN-m5F cells exposed to butyrate supplemented culture media (Fernandez-Mejia and Davidson, 1993). Our results support these reports by confirming that butyrate treatment increased mRNA abundance and provide a validated model system in which to study the cellular

and molecular mechanisms regulating butyrate's impact. While the clinical relevance and systemic physiologic influence inherent to preclinical animal models cannot be overlooked, the development of a well-controlled reproducible *in vitro* model system was lacking for these focused investigations. The validation of this model system eliminates the complications inherent to *in vivo* models such as variations in amount and location of butyrate produced by the intestinal microbiota present, the composition of the diet consumed by the experimental animals, the impact of motility on the presence of nutrients within the intestine, and the diurnal variation well known to impact the expression of intestinal nutrient transporters (Houghton et al., 2008). This model system is irreplaceable for conducted the mechanistic work necessary to understand the regulation of GLUT2 by butyrate, however additional work will be necessary to translate these observations into effective delivery methods and dosing schemes relevant to potential clinical therapies.

Question 2. **Does butyrate alone activate the GLUT2 promoter or are all three SCFA (acetate, propionate, and butyrate) required?** Using the conditions outlined in the current experiments, butyrate treatment alone was effective in activating the full-length GLUT2 promoter. Both acetate and propionate alone were ineffective, as was the physiologically relevant cocktail of acetate, propionate and butyrate. The inability of the 5 mM SCFA cocktail to alter GLUT2 promoter activation may have been because the dose of butyrate provided in this cocktail was 0.75 mM and based on data from the dose experiments likely fell below the effective dose. Certainly within the 15 mM SCFA treatment, wherein 5 mM of butyrate was present, the GLUT2 promoter was markedly impacted. Notably, activation of the GLUT2 promoter by the 15 mM SCFA treatment, exceeded that of the 5 mM butyrate dose, indicative of

additive effects between butyrate and the other SCFA, or the additional osmotic tension produced by the higher nutrients present within this treatment. Regardless, these data indicate that the molecular mechanism(s) whereby butyrate increases GLUT2 mRNA includes transcriptional initiation of the GLUT2 promoter.

The lack of butyrate effect on the GLUT2 promoter in the enteroendocrine cell, STC-1, demonstrated that cell type may be an important factor mediating this response. Derived from a murine intestinal endocrine tumor, the STC-1 cell line displays several functions of intestinal endocrine cells (Rindi et al., 1990), but are functionally distinct from enterocytes. The absence of the butyrate effect on STC-1 cells confirm the specificity of butyrate on up-regulating GLUT2 in the absorptive enterocyte and refute suspicion that butyrate is inducing an indiscriminate response of gene transcription related to inhibition of histone deacetylase. Though activation of the GLUT2 promoter in STC-1 cells was observed with the 15 mM SCFA cocktail, these effects were not attributed to butyrate and demonstrate the specificity of butyrate induction of the GLUT2 promoter in the absorptive epithelium.

Question 3. **Does butyrate exert its effect on the GLUT2 promoter through a sequence specific portion of the promoter, thus inferring interaction with a specific transcription factor?** Our studies demonstrate that discrete regions of the GLUT2 promoter responded to butyrate to blunt or participate transcription of GLUT2. The activation of the human GLUT2 promoter was studied in ßTC-3 insulin producing, and 3T3 non-insulin producing cells by Leibiger (Leibiger and Leibiger, 1995) who showed region specific activation of the promoter and protected areas. These investigators found the highest CAT activation with

the -220/+309, followed by -531/+309 portions of the promoter in ßTC-3 cells. Lower activation was observed in the -220/+309 region. Regions -93/+308 showed high activation levels in both ßTC-3 and 3T3 cells while portions containing 3' deletions of the promoter regions (+19/+138 and +261/+292) showed a 3-fold drop in activation.

The results reported herein identify specific regions of the promoter responsive to butyrate treatment. The regions of the promoter most highly impacted by butyrate were -282/+523, -216/+522, and +145/+523, while those not impacted were -1135/+522 and -564/+523. Based on this knowledge, we examined the GLUT2 promoter sequence for potential *cis*-acting DNA sequence elements using Transfac®7 and found putative sequences including many AP1 potential binding sites in regions -1135/+522, -564/+523. This is consistent with the observation that c-fos and c-jun, subunits of AP1, were up-regulated in response to butyrate (Tappenden and McBurney, 1998) . Butyrate also stimulates the intestinal specific transcription factor Cdx2 homeobox gene (Domon-Dell et al., 2002). There have been several proposed mechanisms for the actions of butyrate within the cell including inhibition of histone deacetylases (Doenecke and Gallwitz, 1982; Wilson et al., 2006), butyrate response elements (Lallemand et al., 1996), the TGF-1 pathway (Barnard and Warwick, 1993; Schroder et al., 1999) , and regulation by SP1/SP3 (Nakano et al., 1997; Sowa et al., 1999; Sowa and Sakai, 2000). *De novo* DNA methylation also down-regulates GLUT2 expression while demethylation rescues GLUT2 expression (Jin et al., 2005). Additional studies will help to further identify the functionally relevant sequences and the transcription factors or binding elements facilitating the response of the GLUT2 promoter to butyrate.

The SNP profile of our promoter sequence has been studied in human subjects for conversion from impaired glucose tolerance (IGT) to type 2 diabetes (Laukkanen et al., 2005). The strongest positive relationship is with SNP rs5393 which is located within the promoter region of the GLUT2 gene and also within our cloned promoter. In individuals impacted by this SNP, dietary intervention eliminated the correlation between IGT and type 2 diabetes. Future studies may reveal that dietary modification, with a focus on consumption of dietary fiber, prebiotics and/or probiotics may impact the responsiveness of this GLUT2 promoter SNP and present an opportunity to modulate effectiveness due to sustained and augmented production of butyrate within the intestinal lumen.

CONCLUSION

Butyrate up-regulated mRNA abundance of the GLUT2 transporter and activated the GLUT2 promoter in Caco2-BBe monolayers, in a manner impacted by both butyrate dose and incubation time. These results identify a cellular mechanism wherein butyrate may up-regulate intestinal absorption that may be relevant to patients with reduced function. Additional work is necessary to understand cellular targets of butyrate therapy and define clinically appropriate means of providing such strategies, such as consuming pre- and probiotics.

TABLES AND FIGURES

Table 3.1 Effect of 0-20 mM butyrate for 0.5-24 h on GLUT2 mRNA/18S abundance.[1]

	Butyrate, mM					
Hours	**0**	**2.5**	**5**	**7.5**	**10**	**20**
0.5	0.564 ± 0.06	0.606 ± 0.07	0.548 ± 0.06	0.677 ± 0.06	0.654 ± 0.06	0.619 ± 0.06
1	0.366 ± 0.06 ^{ab}	0.441 ± 0.06 ^{ab}	0.362± 0.06 ^{a}	0.506 ± 0.06 ^{bc}	0.594 ± 0.06^{c}	0.485 ± 0.06 ^{abc}
2	0.593 ± 0.06 ^{a}	0.690 ± 0.07^{a}	0.616 ± 0.06 ^{a}	0.710 ± 0.07 ^{ab}	0.810 ± 0.07 ^{b}	0.597 ± 0.06^{a}
4	0.865 ± 0.06 ^{a}	0.843 ± 0.06 ^{a}	0.966 ± 0.07 ^{a}	0.866 ± 0.07 ^{a}	1.19 ± 0.06 ^{b}	0.863 ± 0.06 ^{a}
6	0.547± 0.06	0.622 ± 0.06	0.567 ± 0.06	0.548 ± 0.06	0.652 ± 0.06	0.498 ± 0.06
24	0.254 ± 0.06 ^{ab}	0.554 ± 0.06 ^{b}	0.430 ± 0.06 ^{b}	0.488 ± 0.07 ^{b}	0.418 ± 0.07 ^{a}	0.227 ±0.07 ^{a}

[1]GLUT2 mRNA is reported as mean ± SEM . Means within a row with different letters are significantly different ($P<0.05$).

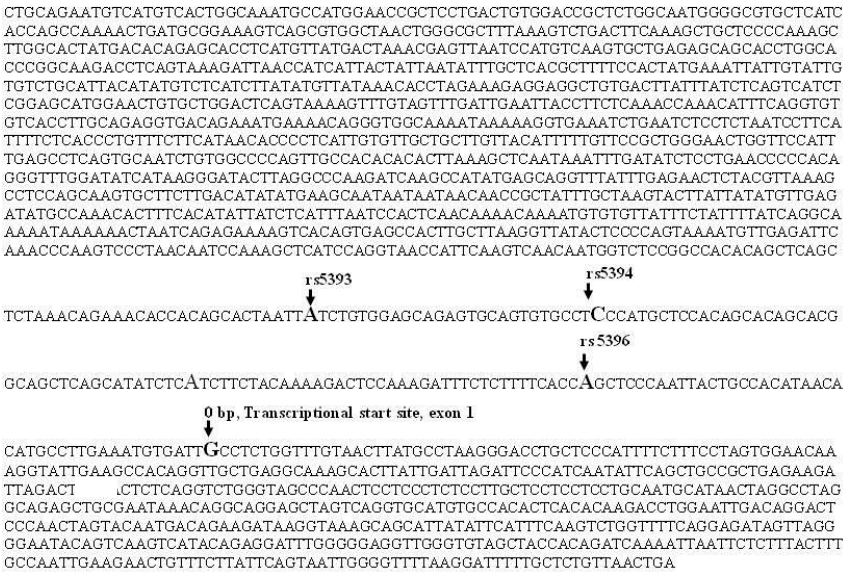

Figure 3.1 The sequence of the cloned GLUT2 promoter.

The promoter sequence was confirmed through alignment with the reported GLUT2 promoter region (gi183282) showing 3 mismatched base pairs, and also to Homo Sapiens Bac RPII-115J24 with 100% match using NIH BLAST. The 3 mismatched bp represent single nucleotide polymorphism variants (SNP) previously reported in the NCBI SNP Database. They are identified above by arrows as A verses C at 1134 bp (rs5393), C verses T at 1225 bp (rs5394) and A verses G at 1267 bp (rs5396) numbered relative to the deposited sequence gi18382 for the human GLUT2.

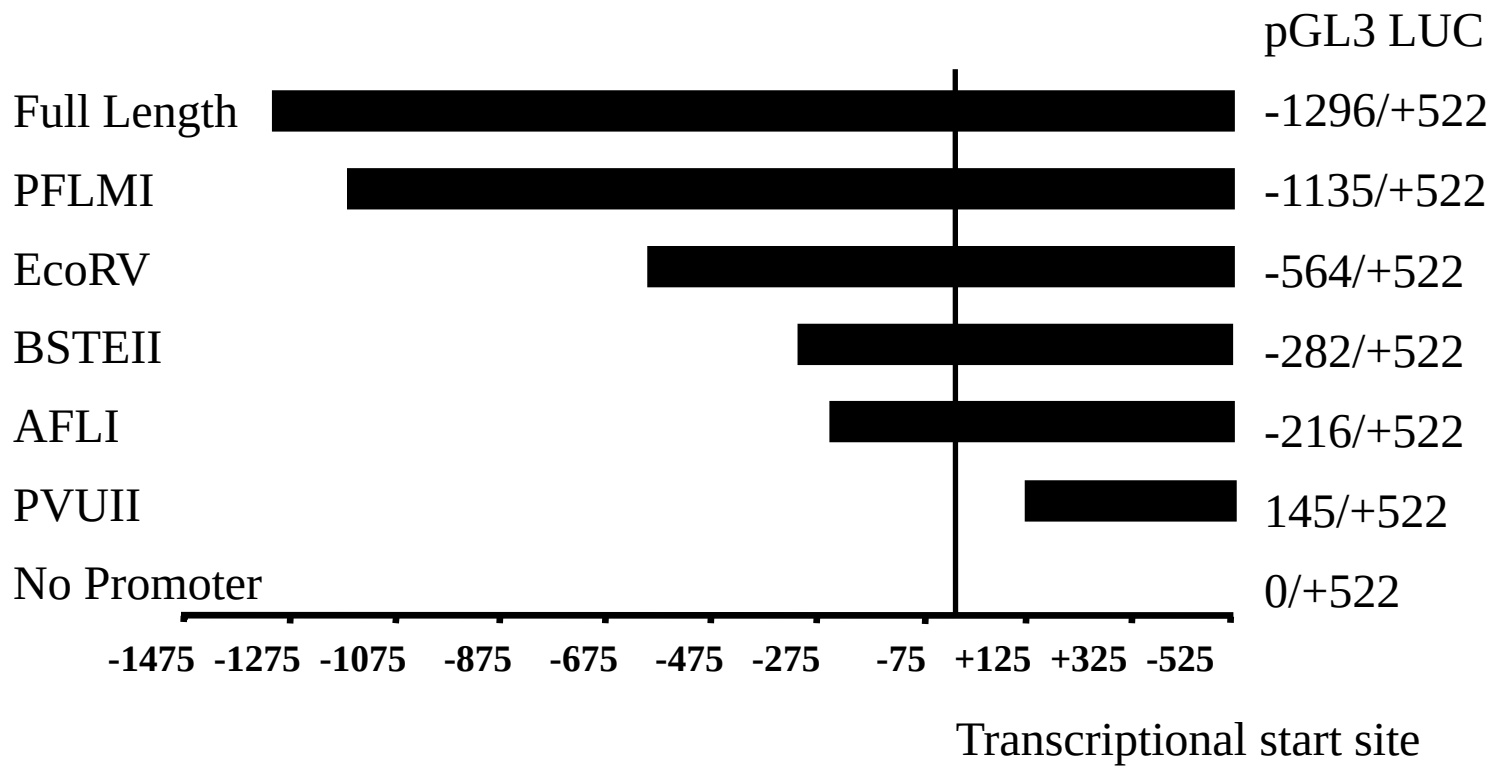

Figure 3.2 Subclones containing portions of the GLUT2 promoter.

The GLUT2 promoter was digested with restriction endonuclease enzymes followed by re-ligation of the plasmid to form nested 5' deletion constructs.

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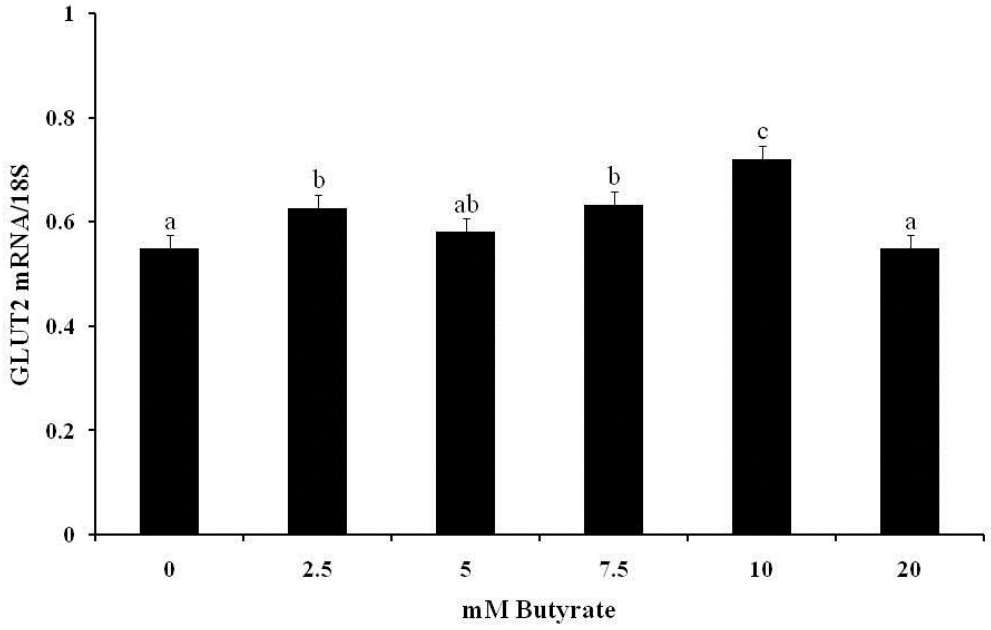

Figure 3.3 GLUT2 mRNA/18S abundance in Caco2-BBE monolayers treated with 0-20 mM butyrate. Data is pooled by time time (0.5-24 h).

GLUT2 mRNA abundance was higher in Caco2-BBe monolayers treated with 2.5, 7.5 and 10 mM butyrate compared to that of control (0.5482 GLUT2/18S mRNA). Means with different letters are significantly different, $P<0.05$, mean ±SEM; n=35.

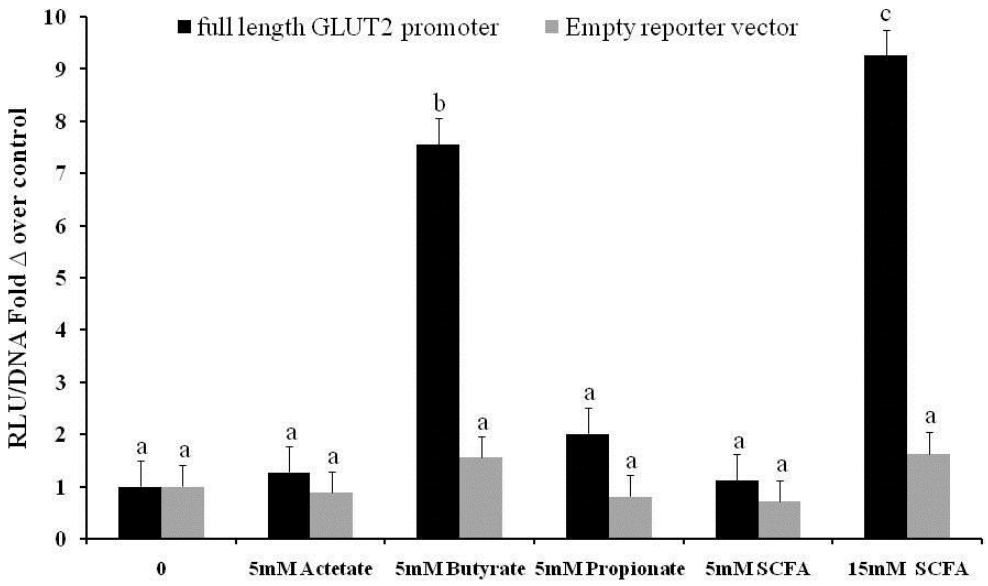

Figure 3.4 GLUT2 promoter activation in Caco2-BBE monolayers treated with SCFA for 48 h.

Butyrate increased the activation of the GLUT2 promoter.

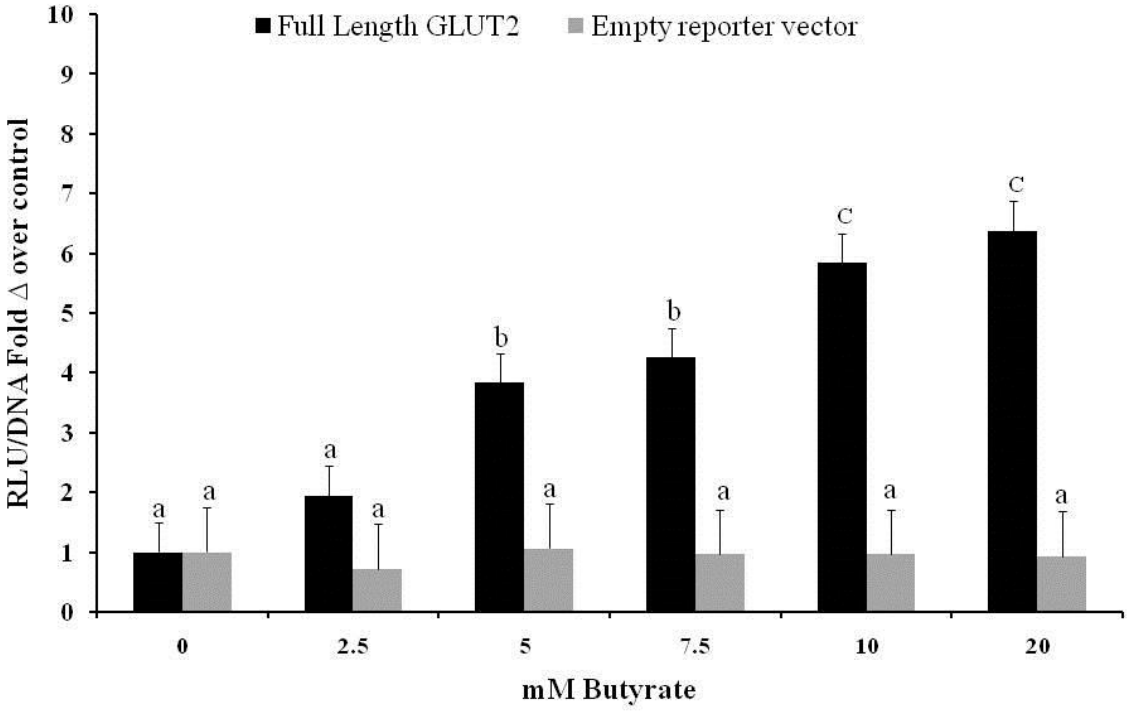

Figure 3.5 The activation of the GLUT2 promoter by butyrate ranging from 0 to 20 mM**.**

Butyrate activated the GLUT2 promoter in a dose-dependent fashion with higher activation achieved in the 5, 7.5, 10, and 20 mM groups $P<0.0001$, compared to the 0 butyrate control. The expression by the empty reporter vector was unchanged by any treatment. Data is expressed as means ± SEM; n= 12.

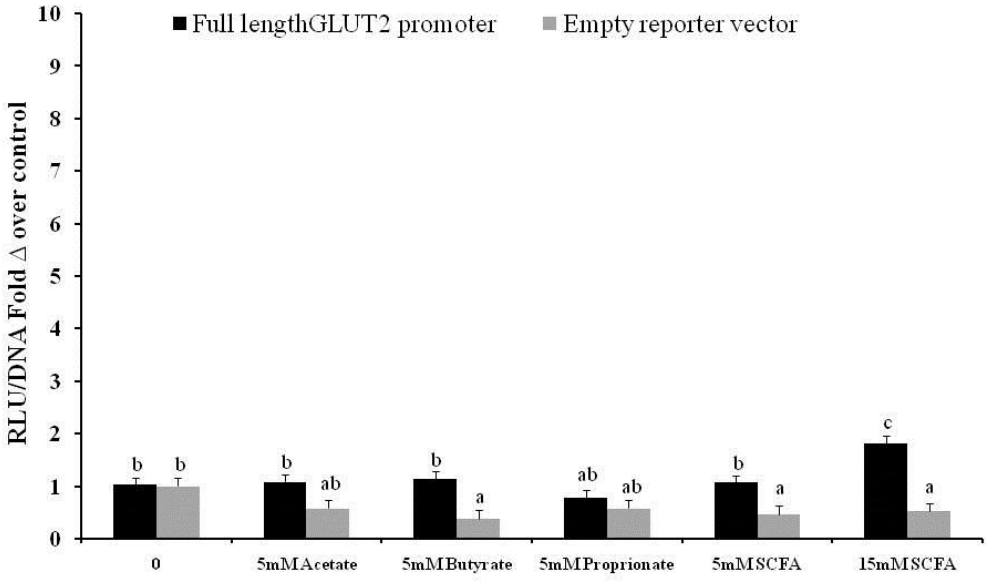

Figure 3.6 GLUT2 promoter activation in STC1 cells following SCFA treatment for 48 h.

Luciferase expression remained at control levels, except for a significant a modest, albeit significant activation of the GLUT2 promoter was observed with the 15 mM SCFA treatment. Data is expressed as means ± SEM; n= 12.

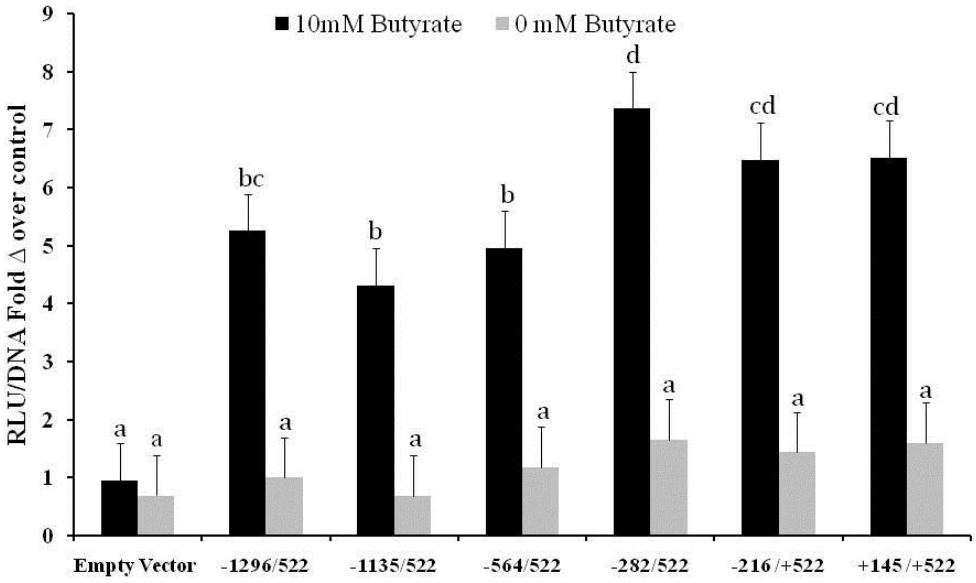

Figure 3.7 Activation of the GLUT2 promoter and portions of the GLUT2 promoter.

Various regions of the GLUT2 promoter were all activated by butyrate, however varying levels of activation were achieved among regions studies. The highest level of activation was observed in the -282/+522 promoter. The -282/+522, -216/+522 and +145/+522 were activated by a greater extent than -1135/+522 and -564/522 subclones. Data is expressed as means ± SEM; n= 12.

Chapter 4 BUTYRATE-INDUCED UPREGULATION OF GLUCOSE TRANSPORT

ABSTRACT

Individuals with intestinal malabsorption face challenges digesting and absorbing sufficient nutrients. Therefore, increasing the capacity for glucose absorption by the intestine would benefit their health. Glucose is a major form of energy transported to, and used by most tissues. While glucose is transported mainly by the proximal intestine, SCFA are predominantly produced within the distal intestine (Cummings et al., 1987). SCFA, the products of microbial fermentation, provide a means of extracting energy from undigested nutrients and also may supply cues to the proximal intestine to increase absorptive capacity when the available substrate and thus SCFA produced are high. Other examples of signals from the distal intestine to the proximal intestine exist. One example is named the ileal brake (Spiller et al., 1984). Nutrients, particularly lipids, within the distal ileum activate the ileal brake slowing gastric emptying and motility within the proximal intestine. The coordination of nutrient absorption through feedback from the distal to the proximal intestine represents a means of changing absorptive capacity and maximizing the efficiency of the intestine.

It has been demonstrated that when SCFA and butyrate were administered intravenously within PN formulas, there was increased absorption of glucose from the small intestine of rats (Tappenden et al., 1997; Tappenden and McBurney, 1998) and piglets (Albin, 2004) compared to controls. *In vitro* studies demonstrated that butyrate upregulated the mRNA abundance of GLUT2, a major glucose transport protein, through promoter activation (Mangian and Tappenden, 2009). Thus, the impact of SCFA, produced within the distal intestine increasing the glucose transport capacity of the proximal intestine was tested.

Objective: The hypothesis that ileal butyrate would increase glucose transport by the proximal intestine and that this response would be acutely regulated and higher in postprandial rats was examined. ***Method:*** Sprague Dawley rats were surgically modified with an ileal

cannula for the infusion of either saline or 10 mM butyrate (for 96 or 156 min) and a portal cannula for blood sampling. All rats received the tracer, 3-O-[methyl-14C] –D-glucose (8 uCi), that was included in an intra-gastric liquid meal, but were randomized to be FED (3 mg/kg BW D-glucose meal) or FASTED (0 mg/kg BW D-glucose meal). FED rats represented the post-prandial state when there is the highest demand for glucose transport. Alternatively, the FASTED rats represented the fasting state with lower demands for glucose transport. ***Results:*** Glucose absorption decreased after an extended ileal saline infusion compared to a shorter saline infusion (AUC 4,640,245 vs. 2,567,799 DPM/ml, $P < 0.05$). Butyrate abolished this decrease. ***Conclusions:*** Treatment of the distal intestine with butyrate increased the glucose absorptive capacity of the proximal intestine. The absence of signals from luminal contents in the distal ileum and large intestine decreased glucose absorption by the proximal intestine.

INTRODUCTION

Interventions aimed at increasing intestinal transport capacity may mitigate the negative consequences of a damaged intestine, and reduce the need and intensity of costly supportive therapies. The contribution of this study is to assess the impact of ileal butyrate on the upregulation of glucose transport capacity and focus on the mechanisms upregulated within the distal ileum and large intestine supporting these changes.

The microbial fermentation of nutrients entering the distal ileum and large intestine produces SCFA that rescue energy, and provide nutrients for the intestine, as well as other tissues (Cummings et al., 1987). Signals from the distal intestine that are instigated by the present of lipids or other nutrients change the motility and absorptive capacity of the proximal intestine via the ileal brake (Spiller et al., 1984). This mechanism slows gastric emptying, and small intestine motility; processes aimed at increasing nutrient absorption by the proximal small intestine. A second example of signaling from the distal intestine is GLP-1 secretion from L-cells. GLP-1 is incretin stimulating insulin release and it increases in response to SCFA (Massimino et al., 1998; Zhou et al., 2008). The ileal brake and GLP-1 secretion represent two pathways stimulated by feedback signals from the distal intestine and result in decreased intestinal motility and increase glucose uptake by peripheral tissues respectively. The SCFA, butyrate, may also act as a signal from the distal intestine to the proximal intestine to increase nutrient absorptive capacity through increased glucose absorption.

The orchestration of nutrient absorption involves diet, digestion, and absorption. Carbohydrates supply about 50% of the typical "Western Diet" and undergo digestion to release the monosaccharides glucose, galactose, and fructose in preparation for absorption. D-glucose is the major transport form of metabolic fuel which is used by most tissues and is the focus of our

studies. Glucose is co-transported across the brush border surface of the enterocyte via the SGLT1 transporter against a concentration gradient in a process that requires ATP and Na^+, and then out of the cell via glucose transporter 2 (GLUT2) in the basolateral membrane (**Figure 4.1**). GLUT2 is a high capacity, facilitative monosaccharide transporter, found within the enterocyte cells lining the intestinal villus. Transcriptional regulation of GLUT2 mRNA abundance occurs after as little as 1 h in response to nutrient treatments (Cui et al., 2003; Mangian and Tappenden, 2009). We found that butyrate increased the abundance of GLUT2 mRNA through activation of its promoter (Mangian and Tappenden, 2009). Butyrate-induced changes are both time-and concentration-dependent (Mangian and Tappenden, 2009). *In vivo*, ileal GLUT2 mRNA abundance is increased by SCFA and butyrate in animals (rat and neonatal pigs) when administered intravenously within PN formulas (Tappenden et al., 1998; Albin et al., 2003).

SCFA are products of dietary fiber fermentation primarily within the distal ileum and proximal colon. Acetate, propionate, and butyrate account for approximately 85% of SCFA and are produced within the intestine lumen in a fairly constant molar ratio of 60 acetate :25 propionate :15 butyrate (Cummings et al., 1987). Butyrate is preferential fuel of colonocytes and also more recently investigated as a signaling molecule (Cummings, 1984). It enhances structural and functional adaptation of residual intestine following partial enterectomy. SCFA provide energy and are associated with gastrointestinal health (Aghdassi et al., 1995).

The microbiota responsible for fermentation are present in lower numbers in the proximal intestine and exponentially greater numbers in the distal intestine. In studies with both rats and piglets, SCFA and butyrate increased GLUT2, but did not change SGLT1 mRNA abundance (Tappenden et al., 1998; Albin, 2004). Tappenden et al. delivered SCFA within PN to rats after an 80% intestinal resection and found that GLUT2 but not SGLT1 expression was significantly

higher in SCFA treated rats at 3 days post-surgery (Tappenden et al., 1997). A strong trend toward higher GLUT2 expression was also observed in neonatal piglets at 4 h after intestinal resection when receiving 9 mM butyrate in TPN (Albin et al., 2003) . Once again, SGLT1 mRNA abundance was not changed by treatment. Thus, GLUT2 is the hexose transporter that is transcriptionally increased by SCFA, and specifically butyrate.

Nutrients that resist digestion pass to the distal ileum and large intestine where they undergo microbial fermentation yielding SCFA, providing energy for the intestine. Butyrate is preferentially used by colonocytes for energy, a small percentage appears in the portal vein, and even less enters peripheral circulation. Butyrate is also examined as an intestinal signaling molecule. It is probable that SCFA, and butyrate in particular, may provide signals to modulate absorption from the proximal intestine. This may be accomplished through the simulation of a hormonal response from the distal intestine to modulate nutrient transport and increase the glucose absorptive capacity of the proximal intestine. This study is designed to infuse butyrate into the distal ileum and large intestine and measure changes in the glucose absorptive capacity of the proximal small intestine.

METHODS

Experimental Model

Sprague Dawley rats were used to measure the absorption of glucose from the proximal intestine into the portal blood supply. The impact of butyrate in the distal ileum and large intestine on glucose absorption was measured under conditions that mimicked the fasted and fed state.

The adult rat was selected as an *in vivo* model for intestinal absorption due to its relatively small size, allowing for the use of minimal quantities of methyl-D-glucose, 3-O-[methyl-^{14}C] as a tracer to measure glucose absorption while also providing sufficient blood volume for the collection of multiple samples (**Figure 4.2**). Since 3-O-methyl-glucose is transported by hexose transporters as is glucose sans metabolism, its use precludes the complicating factors of glucose metabolism (Campbell, 1949, 1952; Campbell and Young, 1952; Uhing and Kimura, 1995).

The rats were surgically modified with an ileal cannula for infusion of either a saline or 10 mM butyrate solution and a portal cannula for blood sampling (**Figure 4.3**). Following a glucose meal, delivered directly into the stomach, the absorption of glucose by the intestine was measured by its appearance in the portal blood over time (0, 1, 2, 4, 8, 16, 32, 62, 96 min, **Figure 4.2**). Within this model, the rats were assigned to receive an ileal infusion of either saline or 10 mM butyrate and further randomized to begin the ileal infusions one hour prior to (Pretreatment) or commence directly upon the glucose meal (Treatment).

Animals

All animal studies were conducted in accordance with University of Illinois IACUC protocol 110334. Adult male Sprague Dawley rats (300 – 350 g), were surgically modified with a portal cannula by Harlan Surgical Services, Harland Laboratories, Indianapolis, IN, and allowed to recover for 72 h, prior to shipment to the University of Illinois. Upon receipt, the rats acclimated to a fiber-free elemental diet and were housed in wire-bottom cages for 7d. These measures were taken to allow for the full recovery from the portal cannulation surgery and to control the SCFA contributed from sources other than the treatments.

The diet was composed of nutrients from highly digestible ingredients including dextrose, all 20 amino acids, and soybean oil (Diet A09061001, Research Diets, NJ, **Table 4.1**). The rodent diet contained 70% of calories from carbohydrate, 18% from protein, and 12% from fat and met the nutritional requirements of the rat. Consumption of a chemically defined, elemental formulation eliminated the potential confounding effects of natural ingredient-based diet such as rat chow. The absence of dietary fiber reduced gastrointestinal residues to ensure that the rats had the lowest possible fermentation rate within the lumen of the intestine. To limit coprophagy, the rats were housed in individual wire-bottom stainless steel cages. Coprophagy would compound the expected treatment effects by providing SCFA contributed from the fermentation of the consumed fecal matter (materials such as bacterial components, proteins secreted into the digestive tract but not reabsorbed during digestion, sloughed epithelial cells, etc.) and the SCFA already present in the feces. Thus, the potential confounding effects stemming from SCFA produced from the microbial fermentation of dietary components and coprophagy of feces were controlled by utilizing a chemically defined diet and specialized caging.

The patency of the portal vein catheter was tested 4 d after arrival in the animal facility as per Harlan protocol. All solutions, syringes, and needles were sterile. A towel was used to allow exploratory behavior and gentle restrain of the rat without inducing stress while evaluating the catheter. The catheter extruded from the skin just behind the shoulder blades and was gripped with non-traumatizing hemostats proximal to the stainless steel plug while the stainless steel plug were gripped by a second of hemostats and removed with gentle force. A 23 gauge blunt tip catheter needle (SAI, Lake Villa, IL) was inserted into the cannula and the cannula lock solution withdrawn. Next a second empty syringe was attached and gentle back pressure was exerted until a blood flash was observed from in the catheter. The cannula was clamped with

non-traumatic hemostats and the syringe was removed. A syringe containing physiological saline was attached to the needle and the cannula was flushed with 2x the cannula volume. The cannula was clamped and the syringe replaced with a syringe containing heparinized glycerol solution (500u/ml) with 1.5 times the cannula volume as a cannula lock solution. The stainless steel plug was returned to the cannula. This process was repeated every 3-4 days to maintain the portal cannula with very minimal stress to the rat.

Treatment assignment

Within the experiment, all rats received the tracer (3-O-[methyl-^{14}C] –D-glucose (8 uCi) in their meal (2 ml volume), and were randomized to be FED (3 mg/kg BW D-glucose) or FASTED (0 mg/kg BW D-glucose). Rats were further randomized to receive an ileal infusion of either saline or 10 mM butyrate beginning either one hour prior to the meal (Pretreatment) or commencing directly upon the glucose meal (Treatment).

Ileal Surgery

Following a 7 d adaptation period (described above), the rats underwent a surgery to place an ileal cannula for the infusion of the treatment solutions (saline or saline plus 10 mM butyrate) into the distal ileum (**Figure 4.3**). Food but not water was withdrawn from the animals on the evening prior to surgery. Each rat was weighed before surgery and rectal body temperature was recorded and monitored throughout surgery. Sterile instruments and aseptic technique were used at all times. All surgical procedures took place in an externally vented exhaust hood on warming pads covered with disposable drapes. Telazol (tiletamine HCL and zolazepam HCL; 6.6 to 9.9 mg/kg; Fort Dodge Animal Health, Iowa, USA), a rapid acting

anesthetic, was used to produce a dissociative pre-anesthesia sedation prior to surgery. During surgery, anesthesia was induced by masked inhalation anesthesia (98% oxygen, 2% isofluorane). Surgical plane anesthesia was confirmed by the absence of toe-pinch reflex, deep stable respiration throughout the surgical procedure and absence of response to external stimuli. After the surgical plane anesthesia was met, preoperative preparations included shaving the abdomen fur, cleansing and covering the intended surgical site with betadine. Lidocaine (15 mg) was administered by subcutaneous injection around the laparotomy site and the rats were draped with sterile gauze. Following laparotomy, the cecum was partially externalized so that the ileocecal valve was visualized. The measurement of tissue from intestinal landmarks was completed by using a sterile string placed along the anti-mesenteric border of the gently stretched intestine to ensure that the cannula placement at 30 cm proximal to the ileocecal valve was consistent in all rats. The ileum was nicked using iris scissors, and a silastic infusion catheter was inserted in the distal ileum at 33 cm proximal to the ileocecal valve and then threaded down 3 cm to the final location. This infusion catheters was secured to the bowel using 5-0 silk sutures. The laparatomy was closed in three layers (peritoneum, muscle, and skin) using sutures but allowing for the extrusion of the cannula. The rats were covered in sterile gauze and maintained on warming pad where rectal body temperature was monitored continually and used to maintain their body temperature.

Experiment

The glucose meal was delivered directly into the stomach of the anesthetized rat. A two ml meal, pre-warmed to body temperature, was injected into the fundus of the stomach using a syringe and a 1.5 inch 20 gauge needle using slow pressure.

The ileal perfusions were delivered pre-warmed to body temperature at a constant rate via syringe perfusion pump apparatus at a rate of 0.25 ml/m at body temperature. The fluid replacement for blood lost through periodic blood sampling occurred through the absorptive surface of the intestine which was continuously perfused with fluid. Following the final blood collection, the rats were euthanized immediately with Fatal-plus solution at 0.23 ml/kg or 1 ml per 10 lbs (Fort Dodge Animal Health, Iowa, USA) prior to regaining consciousness.

Sample Collection

Portal blood samples (0.2 ul) were drawn at 0, 0.5, 1, 2, 4, 8, 16, 32, 64, 96 min post-intra-gastric glucose dose. The blood samples were collected into heparinized tuberculin syringes, transferred to 1.5 microfuge tubes, and the plasma was immediately separated via centrifugation. After each blood withdrawal, the portal cannula was flushed with the volume of saline equal to the cannula volume so as to clear the cannula but not dilute the animal's blood volume. The blood samples were immediately cooled in an ice bath for 2 min, then the plasma was separated via centrifugation at 4 g for 5 min. The plasma was snap frozen in a dry ice bath and stored at -80º until assayed. Syringes were disposed of into a special isotope sharps waste container. Samples of the intestinal tissues collected for histological examination were placed in padded tissue cassettes and 10% buffered formalin for 48 h then 50% ethanol until embedding in paraffin. Intestine samples (whole tissue and mucosa) were also collected and stored in cryogenic vials on dry ice before transfer to -80º C freezer. Tissue thus treated contains less than ≤0.05 µCi /g bw, that was the below minimum concentration of methyl-D-glucose, 3-O-[methyl-^{14}C] that is considered an isotope containing sample.

Glucose absorption

The rate of glucose absorption was estimated through the appearance of methyl-D-glucose, 3-O-[methyl-^{14}C] in the blood samples drawn from the portal cannula over time. To measure the absorbance of methyl-D-glucose, 3-O-[methyl-^{14}C], the plasma samples were thawed on ice, then a 25 ul sample was diluted in scintillation fluid and counted in a scintillation counter. Methyl-D-glucose, 3-O-[methyl-^{14}C] counts were reported in DPM per ml plasma. Area under the curve (AUC) was integrated from the DPM per ml plasma using the trapezoidal rule macro, SAS software, version 9.3 (SAS Institute, Cary, NC).

Statistical analyses

Statistical computations were conducted using SAS software, version 9.3 (SAS Institute, Cary, NC). This experiment employed a 2 x 2 x 2 factorial design and was analyzed as a three way analysis of variance using the mixed procedure (Littell, 2006). The fixed effects were the meal (FED or FASTED), ileal treatment (Saline or Butyrate), and timing of ileal treatment (Pretreatment or Treatment). The rats were blocked by surgical date which was considered a random variable.

The normality of the data was confirmed with a univariate test. The homogeneity of residual variance was determined the Spearman's test and a visual examination of the plots of the residuals. When interactions existed, the interaction was broken down into the component parts. Least-squares means (lsmeans) were separated using least square differences. Subsequent multiple comparisons were tested using the Tukey-Kramer method. A significant difference was defined as $P \leq 0.05$. Values are shown as the least-squared mean ± SEM.

RESULTS

The appearance of methyl-D-glucose, 3-O-[methyl-^{14}C] in portal blood following administration of an intragastric glucose meal was used to determine if ileal infusion of butyrate increased the glucose absorptive capacity of the intestine.

Within the FASTED rats, the glucose absorptive capacity of the small intestine decreased significantly in rats that were pretreated with ileal saline for 1 h prior to the glucose meal (**Figure 4.4**). By 32 min after the meal, approximately 45% less glucose was absorbed in rats infused with saline for a longer period of time (156 min, Pretreatment) as demonstrated by the AUC of glucose absorption (4,640,245 ± 436,585 vs 2,567,799 ± 443,304 DPM* ml plasma; $P < 0.05$, **Figure 4.5**). The concentration of absorbed glucose as measured by methyl-D-glucose, 3-O-[methyl-^{14}C] was lower at 18, 16, 32 and 96 min ($P<0.05$), but not 64 min in rats treated with ileal saline infusions for a longer infusion (Saline*Pretreatment, **Figure 4.4**). In contrast, the FASTED rats receiving an ileal infusion of butyrate did not differ in glucose absorption from those treated with saline for the shorter infusion period (**Figures 4.4 and 4.5**). The duration of ileal butyrate infusion did not impact glucose absorption (**Figures 4.6**).

The FED rats did not demonstrate any impact of butyrate or saline infusion nor any change due to the duration of the infusion on glucose absorption (**Figure 4.6**). The AUC for glucose by 32 min was not changed by treatment. Overall, FED rats absorbed less methyl-D-3-O-[methyl-^{14}C] glucose into portal blood, than FASTED rats (AUC at 32 min 414,175 ± 207,321 vs 3,640,552 ± 222,162 DPM/ml plasma * min for FED and FASTED respectively; $P<0.0001$ **Figure 4.6**).

DISCUSSION

The absorption of glucose occurs mainly in the proximal small intestine. The influence exerted over the small intestine by the distal intestine is known to contribute to the mitigation of malabsorption, and to increased absorptive capacity. The role of butyrate derived from the fermentation of undigested nutrients could provide for the development of therapies for individuals suffering from malabsorption. Butyrate is known to stimulate L-cells within the distal intestine to release peptides that slow the rate of gastric emptying, increase glucose transporters and, therefore, the potential for absorption (Cheeseman and Tsang, 1996; Albin et al., 2003). This experiment examined the impact of butyrate delivered to the distal ileum on the absorption of glucose from the proximal intestine.

In this study, the largest change in glucose absorption was observed in the reduction of absorption of glucose by the FED rats compared to the FASTED rats as measured by the appearance of methyl-D-glucose, 3-O-[methyl-^{14}C] tracer in portal blood. Whereas the majority of the hexose molecules present and available for transport in the FED rats were unlabeled D-glucose the minority of the molecules were the methyl-D-glucose, 3-O-[methyl-^{14}C] tracer. This may have created a competition for transport between the methyl-D-glucose, 3-O-[methyl-^{14}C] tracer and the D-glucose, resulting in a greatly reduced appearance of methyl-D-glucose, 3-O-[methyl-^{14}C] tracer in the portal blood of FED rats compared to FASTED rats and lower calculations for the area under the curve at various time points. Within the FED group, the reported absorption of glucose and differences between treatments may underreport actual values because unlabeled D-glucose likely outcompeted the methyl-D-glucose, 3-O-[methyl-14C] tracer for transport.

In the FED rats, there was no significant change in glucose transport in response to the ileal perfusion of butyrate, saline, or duration of infusion. There may be greater actual differences in glucose transport than are reflected in the transport of the tracer methyl-D-glucose, 3-O-[methyl-^{14}C] due to the confounding effects of diminished transport of the tracer in the high glucose (FED) condition. The greatly reduced absorption of the methyl-D-glucose, 3-O-[methyl-14C] by the FED rats was an unexpected complication within the experimental model. Pretreatment with butyrate did not increase glucose transport capacity beyond control groups when intraluminal glucose concentration was high. Luminal glucose also influences glucose transport within the proximal intestine and these data may illustrate that proximal signals override distal signals.

In FASTED rats that received a longer ileal perfusion (156 min, Pretreatment), the perfused saline bathed the distal ileum and large intestine mucosa for 1 h prior to blood sampling, diluting any existing digesta that contained nutrients or SCFA within the ileum. Since there was an absence of, or greatly decreased, stimulation of the apical surface of the ileum due to saline treatment, it is likely that there were also fewer signals from the ileum to the proximal intestine. The result was that less glucose was transported by the intestine within 8 min of glucose administration. Since the lowest absorption was observed in the rats that were pretreated for 1 h, this infers that the change required time. This result, while unexpected, is in keeping with the decline in nutrient transport present during PN therapy where the intestinal function is lower when luminal nutrients are not present. These findings are also in concert with the lower jejunal absorption of glucose via active transport in rats following an ileal infusion of saline compared to glucose solution (52.49 ± 4.8 vs. 78.77 ± 4.78 μmol per 10 cm^{1} per 15 min^{-1} proximal absorption for saline or glucose distal infusion respectively) (Debnam, 1985). Four

hours of treatment were required to observe this difference. In this experiment, the rats received the protein synthesis inhibitor cycloheximide prior to the ileal infusion that ensured the observed differences were not due to changes in transporter proteins induced by the ileal perfusate. Notably, there was no difference in passive glucose transport when protein synthesis was disabled after cycloheximide treatment. Even excluding potential down-regulation in glucose transporters caused by ileal saline perfusion, there was a significant decrease in glucose transport after the infusion of saline to the ileum.

In our study, butyrate-treated rats maintained glucose absorption from the proximal intestine following pretreatment; thus, butyrate treatment prevented the down-regulation of glucose absorptive capacity. The provision of butyrate and SCFA is associated with stimulation of the L-cell to increase proglucagon mRNA abundance and with higher circulating levels of proglucagon derived peptides (Tappenden et al., 1998; Albin et al., 2003). These peptides are known for actions that contribute to increased nutrient absorption via slowing transit time and increasing glucose absorption (Cheeseman and Tsang, 1996; Cuche et al., 2000; Au et al., 2002). In contrast with our hypothesis, rats receiving ileal butyrate pretreatment did not increase glucose absorption above control treatments; rather, butyrate prevented the decrease in glucose transport capacity associated with prolonged ileal saline infusion.

The differences observed within the Pretreatment groups fit with previous reports of mRNA differences observed within 1 h of butyrate treatment and, therefore, include the possibility that butyrate administration impacts GLUT2 mRNA abundance (Cui et al., 2003; Mangian and Tappenden, 2009). Our window of timing may not have captured the full impact of the treatment and (or) allowed sufficient time for the upregulation of GLUT2 mRNA transcription and protein processing. In our *in vitro* experiments using Caco2-BBe monolayers,

GLUT2 mRNA was significantly increased following 1 h of treatment, but the 4 h time point had the greatest increase in mRNA abundance (Mangian and Tappenden, 2009). *In vivo* systems may require more time for translation to protein and processing.

Within the conditions selected to test our hypothesis, we demonstrated a sharp reduction in glucose transport from the proximal intestine when luminal stimulation is low as observed in the FASTED rats that were pretreated with a lengthy ileal saline infusion. Importantly, butyrate ameliorated this change and maintained the glucose absorptive capacity of the proximal intestine. This experiment demonstrated that the intestine responds very quickly to changes in intestinal contents. The capacity of the small intestine to absorb glucose changed within minutes of ileal treatment.

TABLES AND FIGURES

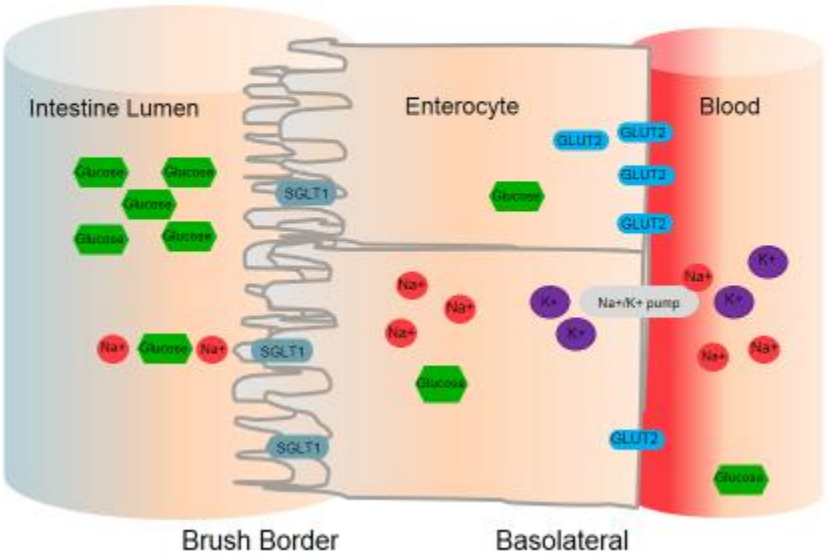

Figure 4.1 A model of glucose absorption from the lumen of the intestine via SGLT1 and into the portal blood via GLUT2.

Glucose enters the enterocyte via the SGLT1 transporter and exits via GLUT2. When glucose concentrations within the lumen are high, GLUT2 is inserted into the brush border and assists in the transport of glucose into the enterocyte.

Table 4.1 Nutrient composition of the rodent fiber-free elemental diet.[1]

Ingredient	gm%	kcal%
Protein		18
Carbohydrate		71
Fat		12
Total		**100**
L-Arginine	10	
L-Histidine-HCl-H2O	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-H2O	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	
Dextrose	675.5	
Soybean Oil	50	
Cellulose	0	
Mineral Mix[2]	35	
Sodium Bicarbonate	7.5	
Vitamin Mix[3]	10	
Choline Bitartrate	2	
Total	950	

[1] Research Diets® (New Brunswick, NJ 08901 US). Formulation A10021, Based on Hirakawa et al., Nutr. Res. 4:891-895, 1984. % Composition based on diet wet weight.
[2] Mineral Mix Research Diets® S1000, based on the AIN-76-A Rodent Diet, J. Nutr. 107:1340-1348, 1977; J. Nutr. 110:1726, 1980
[3]Vitamin Mix Research Diets® V10001, based on the AIN-76-A Rodent Diet

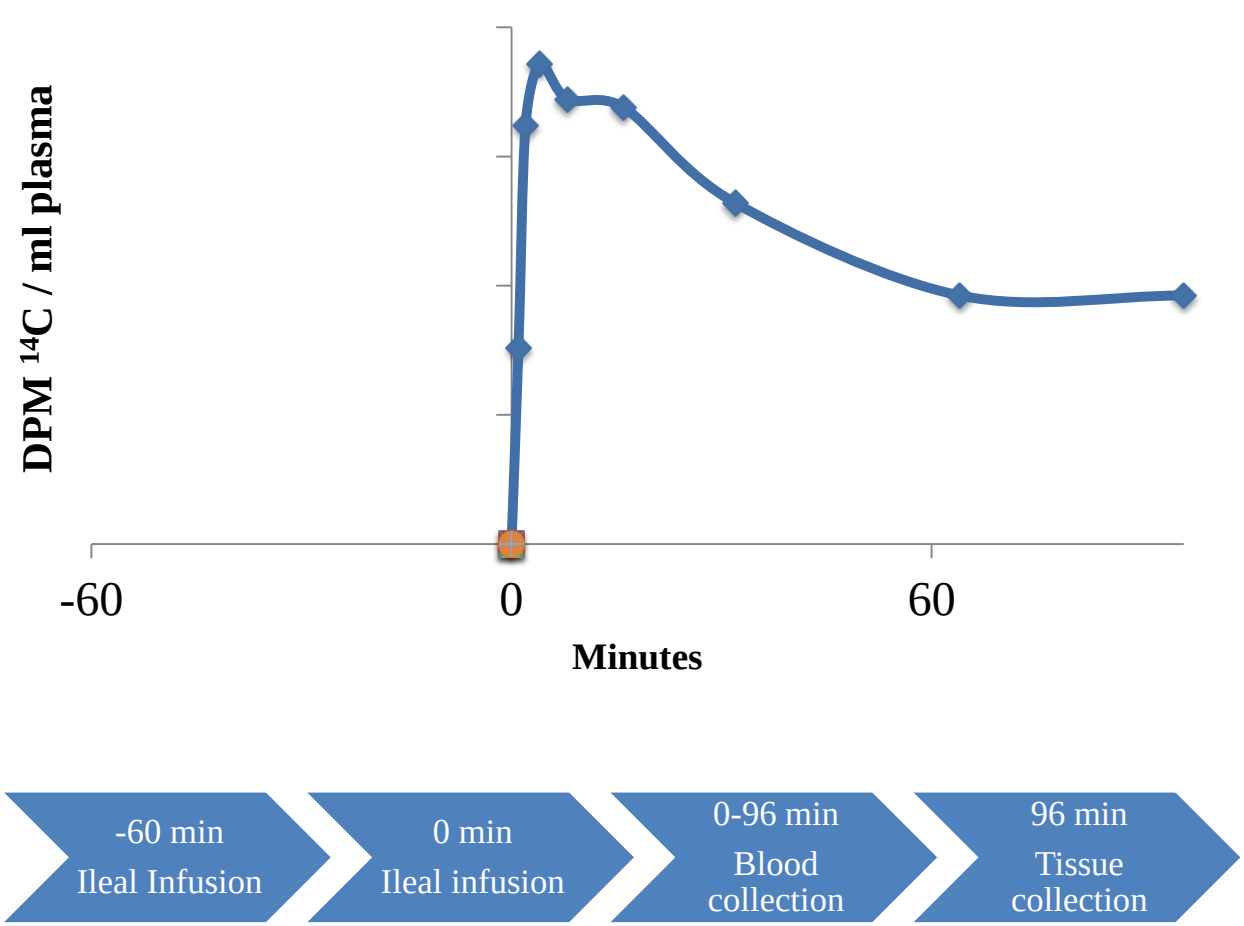

Figure 4.2. The experimental model.

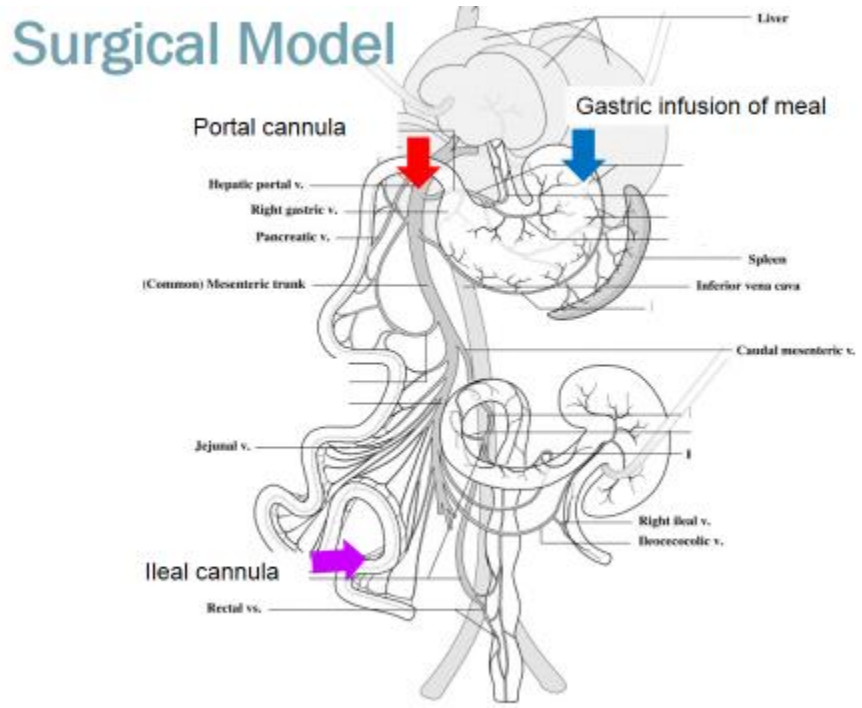

Figure 4.3 The surgical model.

Sprague Dawley rats were used to measure the absorption of glucose from the intestine and appearance in portal blood. They were fitted with an ileal cannula for the infusion of either saline or butyrate and a portal cannula for blood samples. A glucose meal was delivered directly into the stomach.

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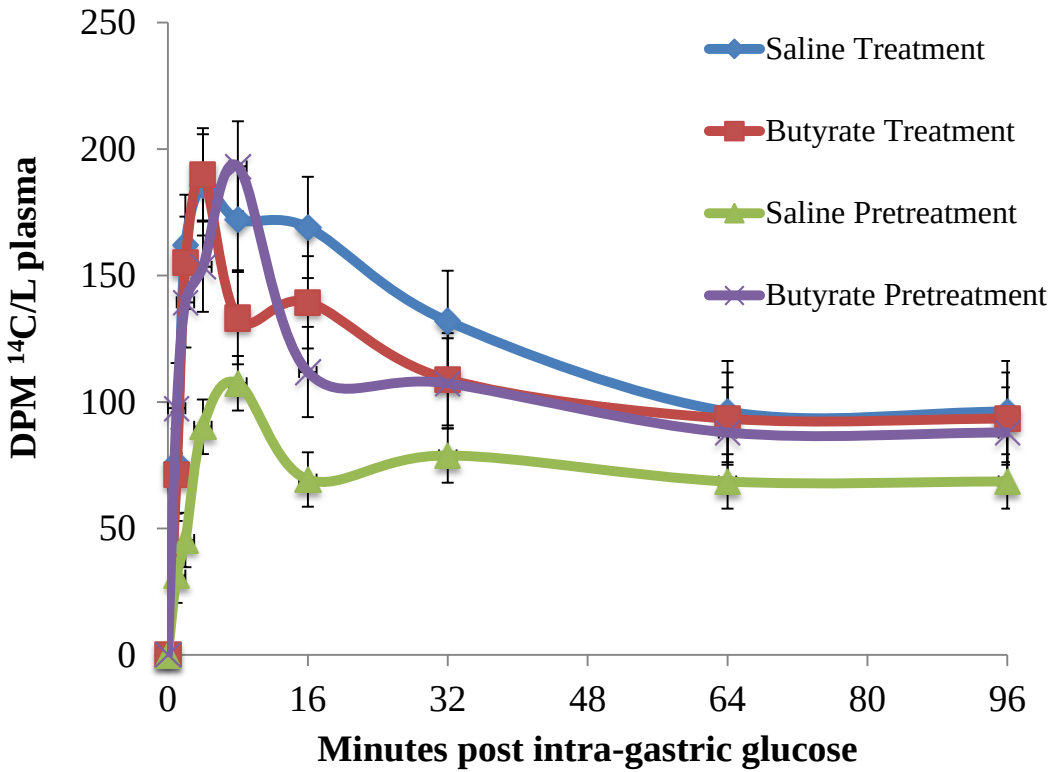

Figure 4.4 The appearance of methyl-D-glucose, 3-O-[methyl-^{14}C] in the plasma collected from the portal vein of FASTED rats.

Ileal infusion of FASTED rats with saline for a longer period of time (156 min, Pretreatment) decreased glucose absorption at 8, 16, 32, and 96 min ($P<0.05$, $n=6$).

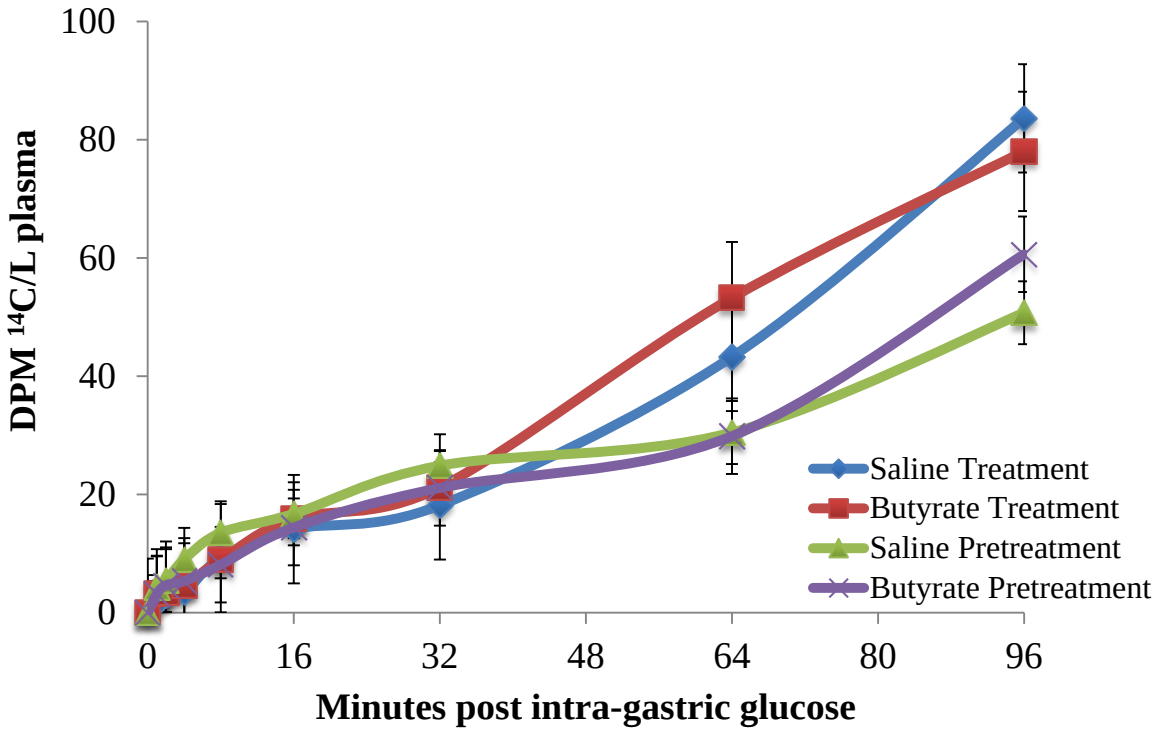

Figure 4.5. The appearance of methyl-D-glucose, 3-O-[methyl-^{14}C] in the plasma collected from the portal vein of FED rats.

The absorption of glucose as measured by methyl-D-glucose, 3-O-[methyl-^{14}C] was not changed by ileal butyrate infusion.

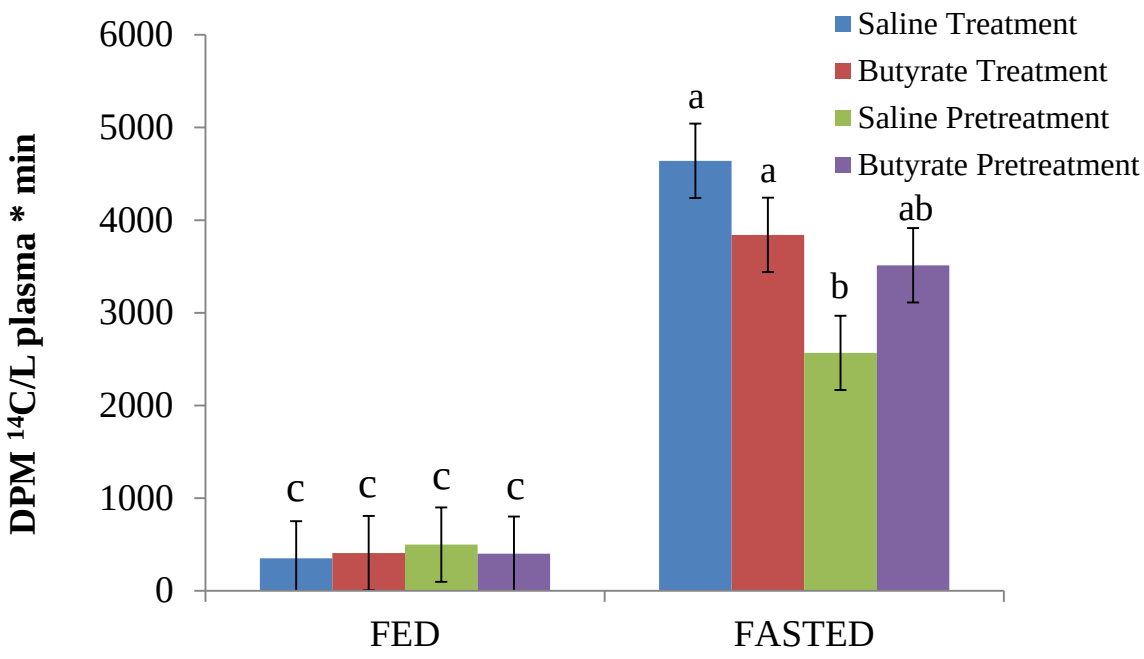

Figure 4.6 The AUC of methyl-D-glucose, 3-O-[methyl-^{14}C] absorbed by 32 min after intra-gastric infusion of the glucose meal.

The impact of ileal infusion on glucose absorption glucose transport was different in FED and FASTED rats (3-way interaction, $P< 0.05$). In FASTED rats, Pretreatment with a saline infusion into the ileum significantly decreased the glucose absorption from the jejunum. Butyrate treatment ameliorated the effects of Pretreatment with saline and maintained the glucose absorption rate. In FED rats, glucose transport was unchanged by ileal infusion. Data is expressed as lsmeans ± SEM; n=6.

Chapter 5 BUTYRATE-INDUCED SIGNALING PATHWAYS WITHIN THE L-CELLS OF THE DISTAL ILEUM AND LARGE INTESTINE IMPACT THE ABSORPTIVE CAPACITY OF THE PROXIMAL INTESTINE

ABSTRACT

Therapies focused on increasing the glucose absorptive capacity of the intestine are needed by individuals with malaborptive disorders. Butyrate demonstrates promise as a possible nutrient therapy. It maintains glucose transport from the proximal small intestine when provided as an infusion into distal ileum as compared with saline. This may be accomplished through increased glucose transporter abundance. Within *in vitro* and *in vivo* models for the small intestinal epithelium, butyrate increased the abundance of GLUT2 mRNA, an important glucose transporter within the mucosa of the intestine (Albin and Tappenden, 2001; Mangian and Tappenden, 2009). Thus the glucose absorptive capacity of the proximal small intestine may be maintained through upregulation of nutrient transporters in response to signals from the distal small and large intestine. Butyrate, a SCFA fermentation product, provides energy for the colonic mucosa, but has also been investigated as a signaling molecule. Identification of possible signals linking the butyrate produced within the distal ileum and large intestine to the upregulation of glucose transport by the proximal small intestine provides additional pathways involved in the upregulation of the glucose absorptive capacity of the intestine.

Objective: The overall objective of this study was to assess the impact of butyrate on glucose transporter abundance within the intestinal mucosa a support for increased glucose transport from the proximal intestine. The hypothesis that jejunal GLUT2 mRNA abundance would increase in response to ileal butyrate was examined. Further, intestinal L-cells were examined for a potential role in the coordination of the response of the proximal intestine to

butyrate infused into the distal ileum and large intestine. If butyrate upregulates proglucagon mRNA abundance, then the actions of GLP-1 or GLP-2 peptides derived from proglucagon could impact GLUT2 mRNA abundance within the mucosa of the proximal small intestine. The initiation of the response may begin when butyrate binds to FFAR2 and/or FFAR3 located on luminal facing membranes of L-cells of the distal ileum and large intestine. ***Method:*** Sprague Dawley rats were surgically modified with an ileal cannula for infusion of either 10 mM butyrate or saline solution (for 96 or 156 min) and a portal cannula for blood sampling. All rats received the tracer (3-O-[methyl-^{14}C] –D-glucose (8 uCi), but were randomized to be fed (3 mg/kg BW D-glucose) or fasted (0 mg/kg BW D-glucose). ***Results:*** Proglucagon mRNA abundance was 2-fold higher in the cecum of butyrate-treated rats (0.8154 vs. 0.4424 proglucagon mRNA/18S for rats receiving butyrate and saline infusions respectively; P=0.025). Butyrate also increased FFAR2 and FFAR3 mRNA abundance in the cecum. ***Conclusions:*** Treatment of the distal ileum and large intestine with 10 mM butyrate maintained the glucose absorptive capacity of the proximal intestine through increases in the hexose transporter, GLUT2, within its mucosa. Within the distal ileum and large intestine, butyrate may be sensed by the enteroendocrine intestinal L-cell through FFAR2 and FFAR3 embedded on their luminal facing membranes. FFAR2 and FFAR3 are GPCR that have SCFA as ligands and show increased mRNA abundance after butyrate treatment. In this study, butyrate increased cecal proglucagon mRNA abundance, the transcript for the intestinal peptides, GLP-1 and GLP-2. These peptides have exocrine and endocrine functions and may provide for changes in glucose transport by the proximal intestine.

INTRODUCTION

Glucose absorption occurs primarily within the proximal small intestine via the sodium dependent cotransporter 1 (SGLT1) and glucose transporter 2 (GLUT2). SGLT1 is not highly transcriptionally controlled by SCFA. GLUT2, the high capacity, low affinity passive glucose transporter, is transcriptionally regulated by luminal nutrients. The upregulation of glucose transporter mRNA abundance has not been established within the proximal intestinal mucosa after butyrate treatment of the distal ileum and large intestine. Increased transcription of glucose transporters, without direct stimulation of the enterocyte by luminal or systemic butyrate, may be important for control of glucose transport by the proximal intestine through signaling from the distal ileum and large intestine. Identifying the distal intestine as the site of the integration of luminal feedback signals from nutrients and the origination of signals to promote increased glucose transport capacity is important because it may lead to identification of targets able to conduct these activities and useful therapies for individuals with intestinal malabsorption.

Provision of SCFA or diets supplemented with fermentable substrates increases the absorptive capacity of the intestine through increases in mucosal surface and functional capacity. Consumption of diets that are high in fermentable fiber leads to the production of luminal SCFA. Acetate, propionate, and butyrate represent the majority of SCFA produced in the intestine. Of these, butyrate is the preferred fuel of colonocytes and used by the intestine rather than transported to other tissues. Recent studies have pointed to butyrate as a signaling molecule, giving the intestine a means of monitoring and responding to undigested enteral dietary components. Thus SCFA, and specifically butyrate, might have a role in providing for the increased absorptive capacity of the small intestine when a large amount of nutrients enter the distal ileum as substrates for fermentation. Increasing absorptive capacity to match luminal

nutrients could give an adaptive advantage in effectively maximizing nutrient use. Feedback from the distal intestine could help increase the efficiency of the intestine through up-regulating nutrient transporters when fermentation products are high in preparation for the next meal.

Fermentation occurs when intestinal microbes act on previously undigested nutrients, intestinal secretions, or sloughed cells to produce SCFA. Although microbes are found throughout the entire intestinal tract, they are present in the greatest number in the distal ileum through the proximal colon. Within this particular region of the intestine, the highest concentration of luminal SCFA and the greatest number of intestinal L-cells are located. L-cells are enteroendocrine cells that produce and secrete important peptides. Among these peptides are GLP-1 and GLP-2. These peptides are both encoded by the proglucagon mRNA. They have paracrine and exocrine activities that influence metabolism, intestinal health, and nutrient absorption. GLP-1 is an incretin that increases pancreatic insulin release and also acts within the intestine to slow gastric emptying, intestinal motility, and decrease gastrin release. GLP-2 is intestinotrophic and increases the abundance of the hexose transporter, GLUT2. These peptides are secreted from L-cells in a 1 to 1 ratio. Less than 25% of the secreted peptides enter the blood and once in circulation they have a very short half-life (only minutes) before they undergo extensive N-terminal degradation by the enzyme dipeptidyl peptidase IV (DPPIV). This is an important control point for the regulation of GLP-1 and GLP-2. A second major control point is the transcriptional control of proglucagon mRNA abundance. Thus, the L-cell, situated in close proximity to SCFA production, produces peptides that regulate the intestine. For these reasons, interest is focused on the response of the L-cell to butyrate.

Diets high in soluble fiber are associated with increased proglucagon mRNA abundance within the distal ileum and large intestine. Rats fed a a diet supplemented with a 30% fiber diet

for 14 d demonstrated a significant increase in ileal proglucagon mRNA, plasma GLP-1, and ileal SCFA (Reimer and McBurney, 1996). Increases in SCFA, and butyrate in particular, were measured within the large intestine (cecum and colon) of the rats consuming the high fiber diet compared to control diets. In earlier studies, plasma measures of enteroglucagon, a peptide with gut-glucagon like immunoreactivity, were used to measure L-cell peptide secretions and reported to be increased by dietary fermentable fiber. In conventional and germfree rats that were first starved and then re-fed an elemental diet with a highly fermentable fiber or cellulose, there was an approximate doubling in plasma enteroglucagon by conventional but not germfree rats consuming the highly fermentable fiber diet (Goodlad et al., 1983). The germfree rats did not show this response, so the products of fermentation were indicated as stimulants for an endocrine-type response of the distal intestine. Thus, the ingestion of a meal containing a substrate for fermentation by the intestinal microbiota led to increased SCFA production and the secretion of peptides by the L-cell with exocrine and endocrine capabilities.

The provision of SCFA within PN also increases proglucagon abundance in rats with a surgically shortened intestine (Tappenden et al., 1996; Tappenden et al., 1998). Specifically, butyrate is the SCFA responsible for the upregulation of proglucagon, and increased circulating plasma levels of GLP-1 and GLP-2 (Koruda et al., 1990; Tappenden et al., 1998; Bartholome et al., 2004). This was further demonstrated through significant increases in ileal and colonic proglucagon mRNA abundance in rats receiving thrice daily infusions of ileal infusions of butyrate (Woodard, 2010). In keeping with these results, Yadav showed higher GLP-1 secretion in mice fed a probiotic that increased butyrate within the intestine and blood (Yadav et al., 2013). The role of butyrate confirmed *in vitro* when human enteroendocrine L-cells (NCI-716) increased proglucagon mRNA, and GLP-1 secretion following butyrate treatment. Thus butyrate

treatment of intestinal L-cells is associated with increased of proglucagon mRNA abundance and also associated with GLP-1 secretion.

Although butyrate is shown to have a role in the upregulation of proglucagon abundance, the means of transmitting the information about luminal butyrate concentrations to L-cells has not been described. In recent studies, FFAR2 and FFAR3 have been located on intestinal L-cells and may provide the link for the L-cell to sense luminal butyrate concentrations (Kaji et al., 2011; Nohr et al., 2013). SCFA are the ligands for FFAR2 and FFAR3. Studies with $FFAR2^{-/-}$ and $FFAR3^{-/-}$ mice demonstrate that these GPCR are necessary for the increased secretion of GLP-1 in response to luminal butyrate (Lin et al.; Tolhurst et al., 2012). Butyrate treatment was associated with increased FFAR3 mRNA compared to controls within intestinal tissues harvested from the mice consuming a probiotic which elevated luminal and plasma butyrate and also from a cell culture model using human derived enteroendocrine NCI-H716 cells compared (Yadav et al., 2013).

This study was undertaken to examine the impact of a physiologically relevant dose of butyrate infused into the distal ileum and large intestine, on the glucose transport capacity of the proximal intestine as represented by the relative mRNA abundance of the glucose transporters SGLT1 and GLUT2. The potential mechanisms underlying butyrate induced changes in nutrient transporters, specifically within the enteroendocrine L-cell via the mRNA abundance of proglucagon, FFAR2 and FFAR3 were examined.

MATERIALS AND METHODS

The experimental design, and all methods for animal husbandry, surgery, assignment to treatments, sample collection, and storage are described in detail in Chapter 4. To summarize,

adult male Sprague Dawley rats were randomized to receive an intra-gastric infusion of either 0 or 3 g glucose/kg bw plus a radioisotope tracer (FASTED vs FED). The rats were further randomized to receive an ileal infusion of either saline or 10 mM butyrate that began 1 h prior to the meal (Pretreatment) or directly with meal (Treatment). Portal blood samples were collected over a 96 min period after the meal, whereupon the rats were euthanized. The intestines were removed and samples were taken and stored at -80º C for subsequent RNA analyses.

RNA isolation and measurement. The impact of butyrate on gene expression was measured through semi-quantitative real time reverse transcription-polymerase chain reaction (RT-PCR). Total mRNA was isolated from frozen intestinal tissue samples using the guanidium isothoicynate phenol cholorform based, TRIzol® method (Life Technologies, Gaithersburg, MD) as per the manufacturer protocol. The resulting total RNA sample was treated with an additional ethanol precipitation to remove any residual phenols. Briefly, the RNA sample produced from the TRIzol® technique isolation was precipitated with 0.1 volume of 3M sodium acetate solution (pH 5.2) plus 2.5 volumes of 100% ethanol and allowed to precipitate overnight at -20° C. Then the precipitated samples were spun for 15 min at 12,000 RPM and the supernant was decanted and discarded. The resulting RNA pellet was washed with 1 ml of 75% ethanol and 25% DEPC treated water solution, centrifuged for 5 min, and the supernant was decanted. Then the RNA pellet was air dried for 10 min. Finally, the RNA was reconstituted in 50 µl of sterile DEPC treated water that was also DNAase and RNAase free. The RNA concentration and quality of each sample was measured through the ratio of their absorbance at 260/280 and the 260/230 absorbance spectra (Nanodrop 1000 Spectrophotometer, Functional Genomics Unit of the W.M. Keck Center, Roy, J. Carver Biotechnology Center, University of Illinois UIUC). Samples with 260/280 ratios > 1.8 and minimal 230 absorbance were accepted for reverse transcription.

The RNA thus isolated was reverse transcribed by Superscript III ® Reverse Transcriptase (Invitrogen, Carlsbad, CA) in a GeneAMP® PCR 9700 System after priming the RNA with random hexamer primers (Promega Corporation, Madison, WI) within the manufacturer's protocol. The complimentary DNA (cDNA) that was produced was diluted and used for RT-PCR amplification reactions to measure the messenger RNA (mRNA) abundance of the glucose transporters (SGLT1 and GLUT2), proglucagon and the selected GPCR (FFAR2 and FFAR3). Gene specific Taqman® Minor Grove Binding (MGB) primer probes and TaqMan® Gene Expression Master Mix containing amplification reagents were used according to manufacturer's protocols using a Taqman ABI 7900 (**Table 5.1**, Applied Biosystems®, (ABI), Life Technologies, Foster City, CA). Sample amplification was conducted in a total volume of 10 ul with 2 ul of cDNA, 1X Universal Buffer, and 1X primer probe in a 384 well plate. The setting used for amplifications cycles for denaturing, annealing and extension included an initial cycle of 50º C for 2 min, 95º C for 10 min, then 40 cycles of 95º C for 15 sec followed by 60º C for 1 min.

The output was analyzed with Sequence Detection Systems 7900HT version 2.2.1 software (Applied Biosystems, Foster City, CA) to yield mRNA abundance/18S as estimated from the cDNA analyzed. The expression level of each gene was determined using the relative standard curve method to determine fold changes in gene expression. To summarize, a dilution series of a common pooled cDNA sample (standard) was created and measured for each target gene. This dilution series was tested with both the target gene of interest and the endogenous control gene, 18S rRNA (18S). The mRNA quantity for the gene of interest was determined by comparison of the Ct calculated from the amplification of the experimental cDNA sample to the

standard curve for the gene of interest and then normalized to the 18S endogenous control. A fold change in mRNA abundance was thus calculated.

Statistical Analyses. All statistical analyses were conducted using SAS version 9.3 software (SAS Institute, Cary, NC). The impact of luminal butyrate on mRNA abundance was determined using the mixed model as calculated by the SAS program (version 9; SAS Institute Inc., Cary, NC) followed by orthogonal comparison with $P \leq 0.05$ as the minimal criterion of statistical significance.

These experiments were designed as a 2 x 2 x 2 factorial arrangement with main effects of glucose meal status (FED vs FASTED), timing of exposure to ileal infusion (Treatment vs Pretreatment), and ileal treatment solution composition (Saline vs. Butyrate). The interactions examined were glucose meal status (FED or FASTED) * duration of exposure to ileal infusion (Treatment or Pretreatment) * solution composition (Saline or Butyrate). The rats were further blocked by the date of surgery, which was considered as the random block variable. Data were examined for normality according to the Shapiro-Wilks test and visual examination of all plots. Means were separated by least squared difference when main effect p-values were ≤ 0.05 according to a Tukey post hoc analysis. All data are presented as mean ± standard error.

RESULTS

Glucose transporters. The measures of glucose transporter mRNA within the jejunum showed that GLUT2, but not SGLT1, mRNA abundance was increased by ileal butyrate treatment (**Table 5.2**) Jejunal GLUT2 mRNA was increased 23% after butyrate treatment compared to saline (4.8 vs. 3.9, GLUT2 mRNA/18S rRNA for butyrate and control; respectively; P= 0.05, **Figure 5.1**).

Jejunal SGLT1 was lower in FED rats treated with ileal saline compared to those treated with butyrate in the Treatment (shorter) ileal perfusion ($P<0.05$, **Table 5.2**). This relationship was not observed in FASTED rats (**Figure 5.2**). Within the ileum, SGLT1 mRNA abundance was ~29% higher in FED compared with FASTED rats (0.8238 vs. 0.6446, SGLT1 mRNA/18S rRNA for FED and FASTED; respectively; $p= 0.04$, **Table 5.3, Figure 5.3**). Pretreatment of the distal ileum and large intestine with an infusion of any type increased ileal SGLT1 mRNA abundance (0.8822 vs 0.5953, SGLT1 mRNA/18S rRNA for Pretreatment and Treatment; respectively; $p= 0.004$, **Table 5.3**).

Enteroendocrine Cells and Signaling Pathways. The small intestine responded to the meal variable and duration of infusion rather than the type of ileal infusion. Within the jejunum, there were no changes in proglucagon mRNA abundance due to any treatment (**Table 5.2**). FED rats, had higher ileal proglucagon mRNA abundance compared to FASTED rats (**Table 5.3**). The proximal large intestine showed a larger response to treatment than the distal large intestine. Cecal proglucagon mRNA abundance rose 2- fold in butyrate treated rats compared to control rats (0.8154 ± 0.1260 vs 0.4424 ± 0.1370 proglucagon mRNA/18S rRNA for butyrate and control; respectively; $P=0.026$, **Table 5.4, Figure 5.4**). Proglucagon mRNA was unchanged by treatment in the colon (**Table 5.5**). Butyrate treatment also increased FFAR2 and FFAR3 mRNA abundance in the cecum (**Table 5.4**). FFAR2 mRNA abundance was higher in the cecum of Treatment rats, infused butyrate for a shorter period of time, compared to those infused with saline (1.9806 ± 0.212 vs 1.1454 ± 0.2124; FFAR2 mRNA/18S rRNA for butyrate infused for a shorter period (Treatment) vs. saline infused for a shorter period (Treatment) respectively; $P=0.035$**, Figure 5.5**). FFAR2 mRNA abundance was unchanged by butyrate in rats infused for a longer period of time. Ileal butyrate infusion increased FFAR3 abundance in the cecum of rats

compared to saline infusion regardless of the influences of duration of infusion or glucose meal (0.7435 ± 0.06704 vs 0.5736± 0.07201 FFAR3 mRNA/18S rRNA for butyrate and control; respectively; P=0.043, **Figure 5.6**).

DISCUSSION

Glucose transporters. Since glucose is a major form of energy used by most cells, increasing the glucose absorptive capacity of the intestine could provide significant benefit to individuals with malabsorptive disorders. In this experiment, ileal butyrate infusion increased the mRNA abundance of jejunal GLUT2, regardless of duration of butyrate infusion or parandial state (FASTED or FED). Higher GLUT2 mRNA abundance within the mucosa of the jejunum provides the potential for greater glucose transport. Others found significant increases in GLUT2 *in vivo* following intravenous butyrate or SCFA treatment (Tappenden et al., 1998; Albin et al., 2003; Bartholome et al., 2004). Butyrate also upregulated GLUT2 mRNA abundance through promoter activation *in vitro* (Mangian and Tappenden, 2009). While GLUT2 mRNA abundance responded to butyrate, SGLT1 mRNA abundance responded to the glucose meal. The lumen of the jejunum was directly exposed to a high concentration of glucose in the FED rats. Within the jejunum, SGLT1 mRNA abundance was lowest in FED rats with the shortest ileal infusion of saline. In contrast, in the distal ileum, SGLT1 was highest in FED rats and unaffected by any other treatment.

Proglucagon. In order to examine the role of the intestinal L-cell in the response of the rat to an ileal infusion of butyrate, we examined the mRNA abundance of proglucagon. Ileal infusions of either 10 mM butyrate or saline were administered for either 60 or 156 min under FED and FASTED conditions. The small intestine did not show any differences in the

production of proglucagon by the enteroendocrine cells present. The L-cell is the enteroendocrine cell that predominates in the distal ileum and large intestine. Within L-cells of the ileum and cecum, there were responses to butyrate infusion. The most robust response was observed in the cecum where proglucagon mRNA abundance was 2-fold higher after butyrate infusion. Since the cecum of the rat is a blind sac-like structure, the infusion solution was in contact with the luminal surface of the cecum for longer than occurred within the ileum or colon. The longer dwell time for the infusion solutions due to the sac-like shape of the cecum may facilitate a response within this portion of the intestine that is not present in the ileum or colon. Rats are cecal fermenters and, thus, this portion of the intestine is usually exposed to SCFA and may be primed to respond (Campbell et al., 1997). Butyrate upregulated cecal proglucagon mRNA abundance supports the hypothesis of a positive response to butyrate treatment and is also consistent with others reports of increased proglucagon mRNA abundance after exposure to butyrate, SCFA, and high fiber diets (Reimer and McBurney, 1996; Tappenden et al., 1998; Zhou et al., 2008; Woodard, 2010). Free living rats treated with an ileal infusion of 60 mM butyrate three times per day had significantly higher ileal and colon proglucagon mRNA after a week of treatment (Woodard, 2010). The study designs of Woodard (2010) and the present study have similarities in the animal model and diet, but there were differences in the butyrate concentration of the infusate (10 mM in the present study vs. 60 mM in Woodard, 2010) and the temporal aspects (156 min vs. 7 d for present study and Woodard 2010 respectively).

In the experiments described herein, butyrate was infused directly in to the distal ileum and large intestine, circumventing complications from the digestion of a meal and fermentation of fiber. Since the fermentation of the fiber of a meal occurs after the meal is digested, the impact from fermentation may impact following meals (Bach Knudsen et al., 2005). When pigs

were fed diets containing different fiber sources and blood glucose uptake was measured in the portal vein, there was no observed difference in glucose absorption from the meal during the first 3 hours. However, the absorption of SCFA into portal blood did not peak until 5h after the meal. Of the two fiber sources (wheat or rye), the consumption of diet containing rye resulted in higher butyrate production. However, the glucose from the meal was not absorbed when the production of butyrate was highest. Thus, experiments using SCFA produced from a fiber containing meal, may not capture the full extent of changes in glucose absorptive capacity unless a second meal is administered after fermentation of the first meal has taken place.

In this experiment, ileal butyrate stimulated proglucagon mRNA transcription within the cecum and was associated with greater jejunal GLUT2 mRNA abundance. This observation was in not concert with a previous experiment where GLUT2 mRNA abundance was not increased in the small intestine of rats following intravenously administered GLP-2, a peptide derived from proglucagon mRNA (Sangild et al., 2006). The observation that cecal proglucagon mRNA rises within minutes of contact with a relatively low concentration of butyrate demonstrates that the cecum is sensitive to luminal contents and able effect a quick response to butyrate stimulation and induce changes in proglucagon mRNA abundance in response to a meal.

The GPCR, FFAR2 and FFAR3, have SCFA as ligands and respond to butyrate by increasing mRNA abundance. There is little debate about their ligands, or potential for signaling within the intestine. In these studies, FFAR2 and FFAR3 were unchanged within the jejunum by butyrate infusion into the distal ileum and large intestine. The lack of regulation of mRNA abundance of FFAR2 within the proximal intestine in response to an ileal infusion of butyrate also was observed by Woodard (2010). In contrast, cecal FFAR3 and FFAR2 mRNA expression was significantly increased in response to ileal infusion of 10 mM butyrate

regardless of length of exposure, or glucose meal status, within the cecum in the experiments described. Butyrate stimulated upregulation of FFAR2 and FFAR3 may be important for the integration of luminal fermentation products and the response of the L-cell.

Taken together, our data support our hypothesis that butyrate stimulated an increase in mRNA abundance of proglucagon, and the GPCR, FFAR2 and FFAR3. Butyrate is a ligand of FFAR3 and, to a lesser extent FFAR2. FFAR2 and FFAR3 are GPCR that stimulate signaling cascades associated with the secretion of GLP-1 (Blad et al., 2012). The increase in proglucagon mRNA suggests increased secretion of GLP-1 and GLP-2. These peptides are likely to trigger mechanisms to increase the absorptive capacity of the proximal small intestine via increased jejunal GLUT2 (**Figure 5.7)**. Thus, butyrate-induced signaling from the distal ileum and large intestine impacts glucose absorption from the proximal intestine (**Figure 5.8**).

TABLES AND FIGURES

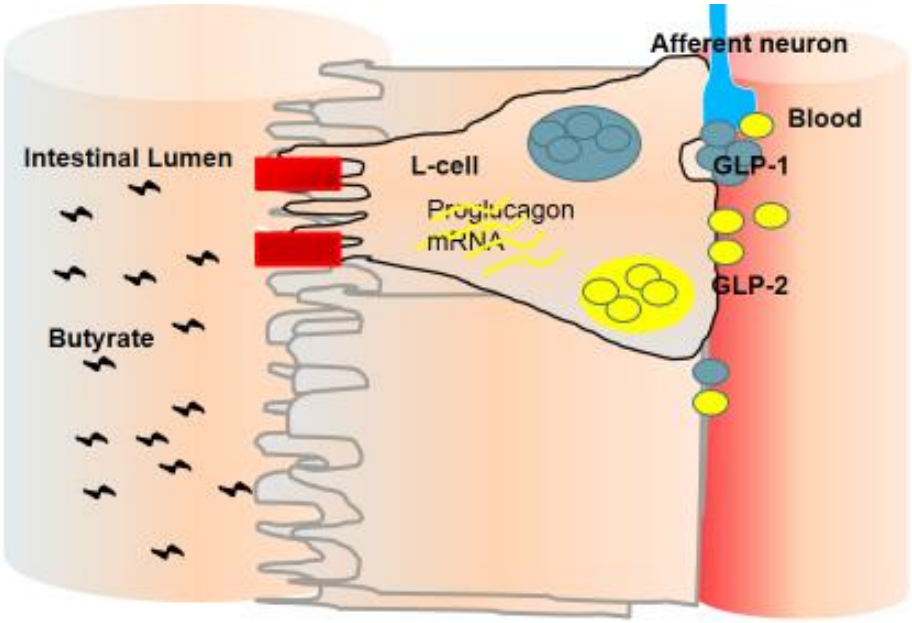

Figure 5.1. A model of the enteroendocrine L-cell production of proglucagon and proglucagon-derived peptides.

Luminal butyrate upregulates proglucagon mRNA abundance and the secretion of GLP-1 and GLP-2. FFAR2 and FFAR3 are located on the enterocyte and may facilitate sensing of luminal butyrate by the L cell and subsequent upregulation of proglucagon transcription.

Table 5.1. The primer and probes used to amplify genes of interest[1].

Gene	ID	ABI Taqman® Primer/Probe ID	Label	GenBank mRNA Interrogated Sequence	Exon Boundary	Assay Location	Amplicon Length
GLUT2	SLC2a3	Rn0056356_m1	FAM™-MGB	BC078875.1	4-5	527	76
SGLT1	SLC5a1	Rn0164634_m1	FAM™-MGB	BC081827.1	1	1328	64
Proglucagon	CGC/ GLP-1	Rn00562293_ml	FAM™-MGB	BP479145.1	2-3	154	65
FFAR2[2]	Ffar2	Rn02345824_s1	FAM™-MGB	AB106675.1	1	942	122
FFAR3[3]	Ffar3	Rn01457614_g1	FAM™-MGB	BC166522.1	1-2	170	62
Eukaryotic Endogenous Control[2]	18S rRNA	Hs99999901_s1	VIC® - MGB	X03205.1	—	—	187

[1] All primers, probes, and all reagents used for the RT-PCR reactions were purchased from Applied Biosystems, Inc.
[2] Both primers and probes lie within a single exon.
[3]An assay that may detect genomic DNA. The assay primers and probe may also be within a single exon.

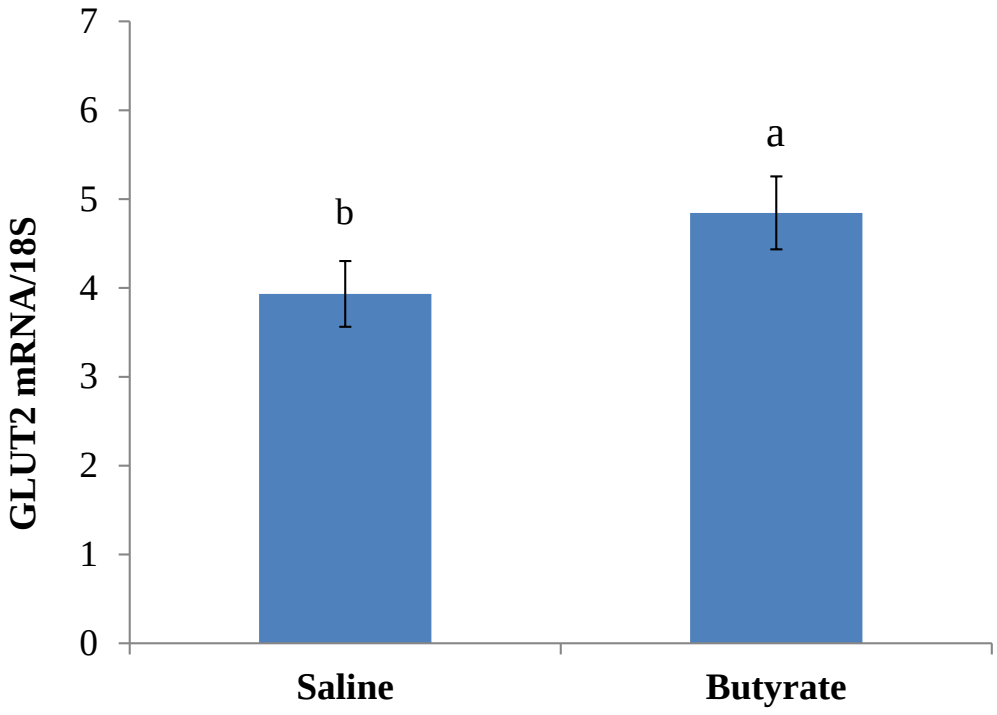

Figure 5.2. Jejunum GLUT2 mRNA was higher in rats treated with butyrate.

GLUT2 mRNA abundance was 23% higher in rats treated with ileal butyrate infusion regardless of the duration of ileal infusion or glucose meal status ($P < 0.05$; n=24).

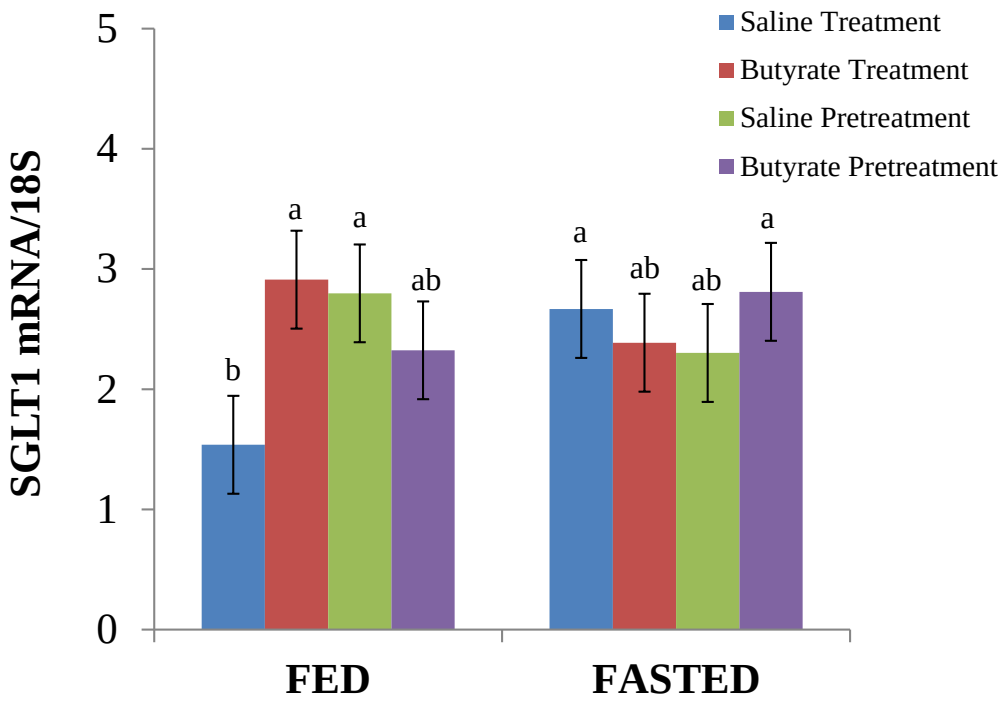

Figure 5.3. SGLT1 mRNA abundance within the jejunum.

In FED rats, a shorter duration of ileal butyrate infusion (96 min, Treatment) increased SGLT1 mRNA abundance over rats receiving a saline infusion. SGLT1 mRNA abundance is lowest in FED rats infused with saline for 96 min (Treatment) ($P<0.01$; $n=6$).

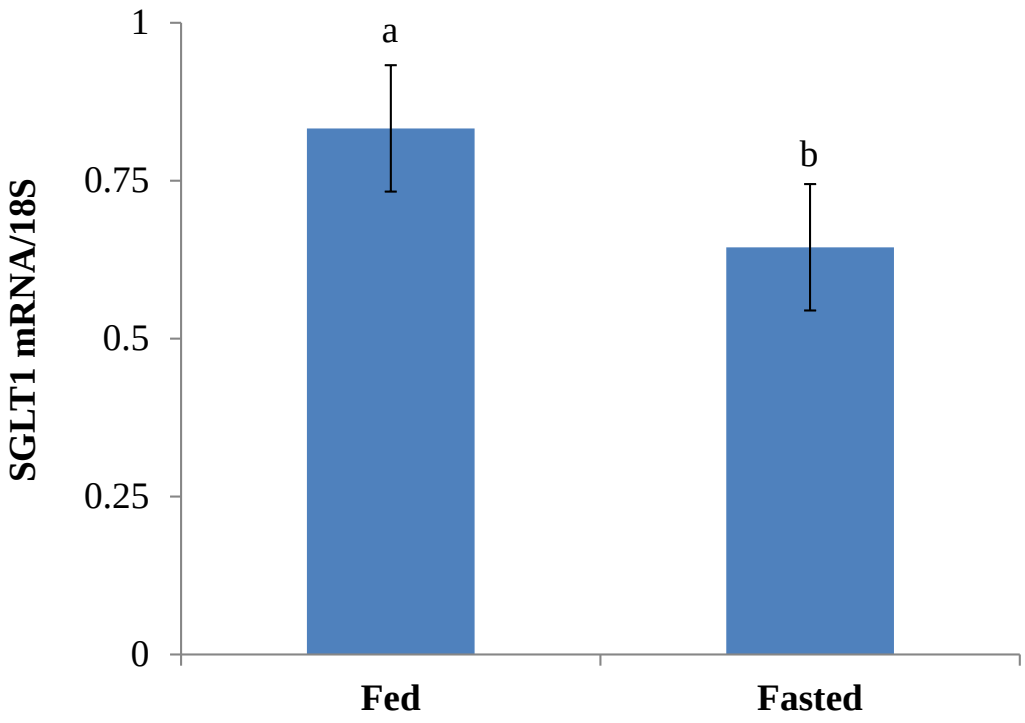

Figure 5.4. Ileal SGLT1 mRNA was higher in FED rats.

Ileal SGLT1 mRNA was significantly higher in FED rats following a high glucose meal compared to FASTED rats regardless of ileal infusion (P=0.04; n=24).

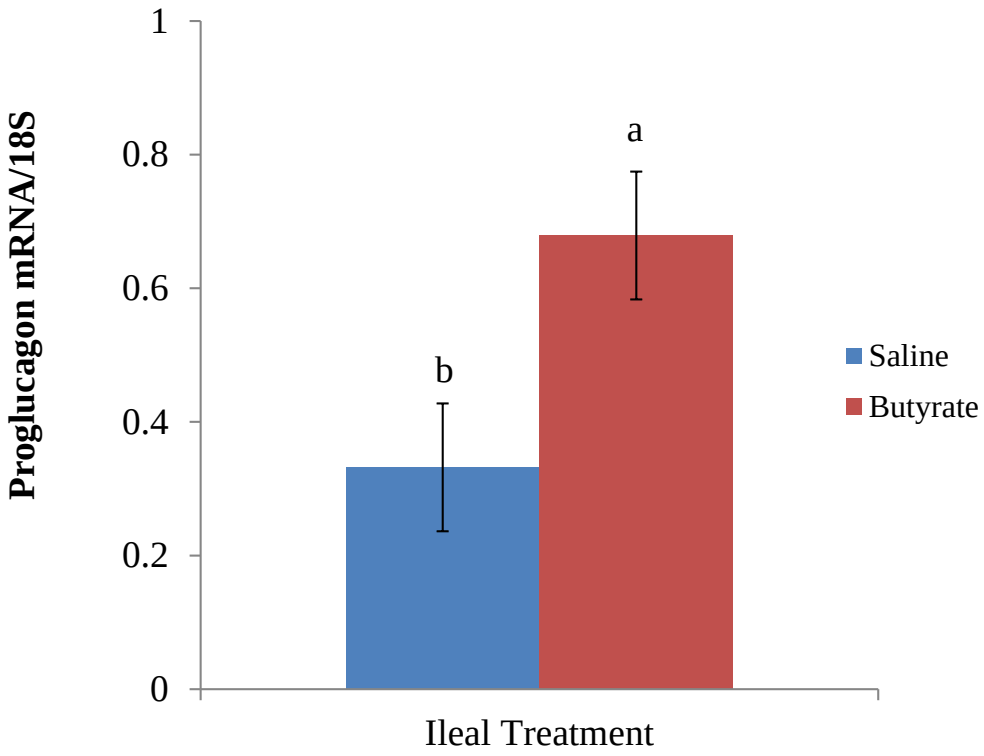

Figure 5.5. In the cecum, butyrate increased proglucagon mRNA abundance by 2-fold.

Cecal proglucagon mRNA mRNA abundance was significantly higher in rats receiving an ileal infusion of butyrate compared to saline (0.8154 ± 0.1260 vs 0.4424 ± 0.1370 proglucagon mRNA/18S rRNA for butyrate and saline control; respectively; P=0.026; n=24). Postprandial state did not impact cecal proglucagon.

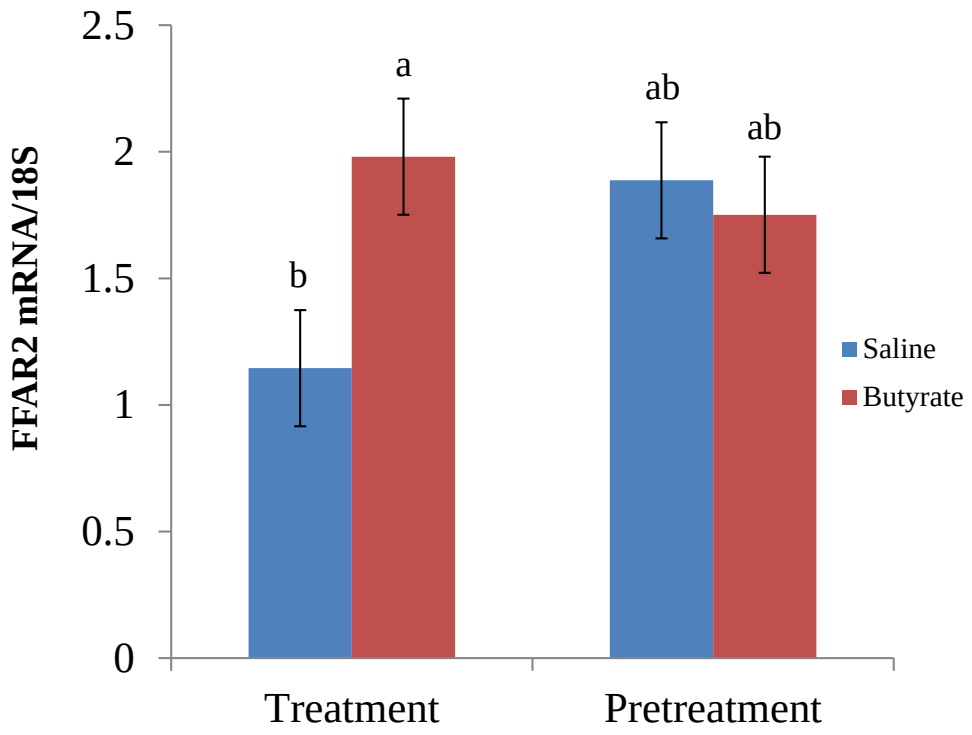

Figure 5.6. Butyrate increased cecal FFAR2 in rats with shorter ileal infusion.

A significant interaction between the duration and type of ileal infusion showed that FFAR2 mRNA abundance was acutely upregulated by a shorter ileal butyrate infusion (1.9806 ± 0.212 vs 1.1454 ± 0.2124; FFAR2 mRNA/18S rRNA for Butyrate * Treatment vs. Saline*Treatment respectively; P>0.035; n=12). However, FFAR2 expression was not changed in rats receiving a longer ileal butyrate infusion (Pretreatment).

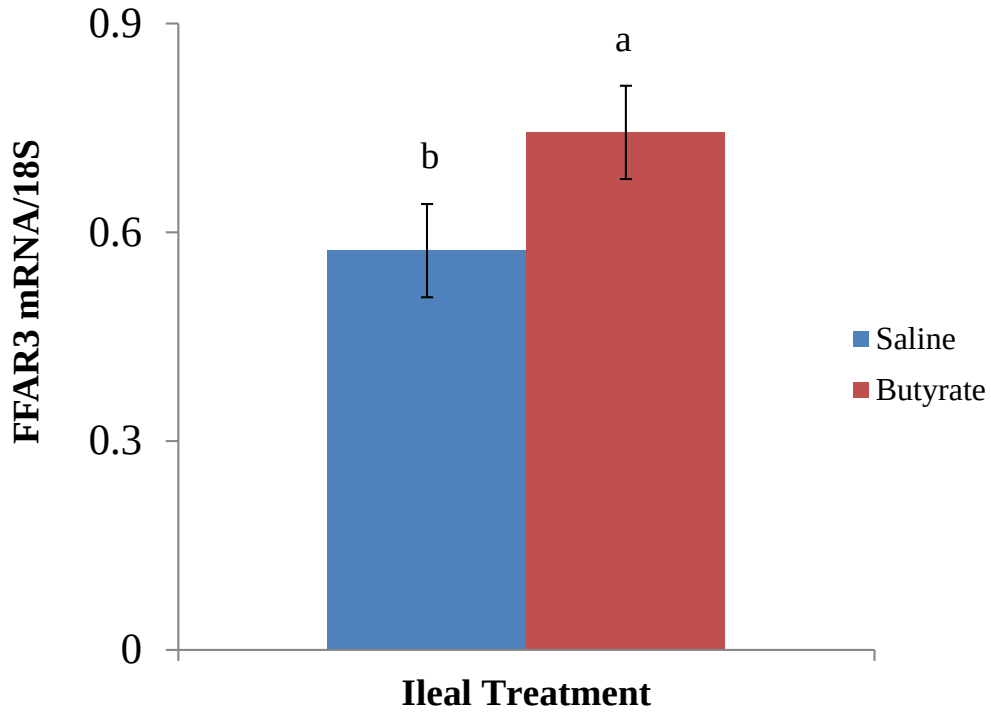

Figure 5.7. Butyrate increased cecal FFAR3 mRNA abundance**.**

FFAR3 mRNA abundance was higher in the cecum of rats receiving an ileal infusion of butyrate compared to those treated with a saline infusion regardless of the influences of duration of infusion or glucose meal (0.7435 ± 0.06704 vs 0.5736± 0.07201 FFAR3 mRNA/18S rRNA for butyrate and control; respectively; $P<0.04$; $n=24$).

Table 5.2. The effect of meal status, ileal infusion solution, and duration of infusion on the mRNA abundance of glucose transporters and other genes of interest in the jejunum of the rat[1].

Gene	Fed				Fasted					
	Treatment		Pretreatment		Treatment		Pretreatment		Effect	P-value
	Saline	Butyrate	Saline	Butyrate	Saline	Butyrate	Saline	Butyrate		
GLUT2	3.93 ± 0.84	4.56 ±0.71	3.60 ± 0.84	3.80 ± 0.77	3.74 ± 0.08	4.99 ± 0.77	4.46 ± 0.77	6.02 ± 0.71	Saline<Butyrate[2]	0.05
SGLT1	1.54[b] ± 0.46	2.91[a] ± 0.35	2.80[a] ± 0.46	2.32[ab] ± 0.38	2.67[a] ± 0.46	2.39[ab] ± 0.38	2.30[ab] ± 0.38	2.81[a] ± 0.35	3-way interaction	0.013
Proglucagon	0.429 ± 0.20	0.214 ± 0.21	0.168 ± 0.24	0.287 ± 0.22	0.282 ± 0.22	0.282 ± 0.22	0.444 ± 0.24	0.360 ± 0.24	ns	
FFAR2	1.222 ± 0.92	2.267 ± 0.98	0.635 ± 0.93	1.444 ± 1.12	2.780 ± 1.07	2.970 ± 1.01	1.971 ±1.26	2.415 ± 1.30	FED<FASTED[3]	0.006
									Treatment> Pretreatment[4]	0.049
FFAR3	0.761[a] ±0.16	0.460 [ab] ±0.14	0.355 [b] ±0.14	0.670 [ab] ± 0.16	0.465 [ab] ± 0.14	0.311 [b] ± 0.14	0.360 [b] ± 0.16	0.437 [ab] ± 0.16	3-way interaction	0.042

[1] Data are expressed as lsmean ± SEM. Within each dependent variable, means with different letters are statistically different from each other (P<0.05, n=6).
[2] Main effect of butyrate solution (P<0.05).
[3]Main effect of meal, (P<0.006)
[4] Main effect of the duration of infusion (P<0.05)

Table 5.3. The effect of meal status, ileal infusion solution, and duration of infusion on mRNA abundance of glucose transporters and other genes of interest in the ileum of the rat[1].

Gene	Fed				Fasted					
	Treatment		Pretreatment		Treatment		Pretreatment		Effect	P-value
	Saline	Butyrate	Saline	Butyrate	Saline	Butyrate	Saline	Butyrate		
GLUT2	0.582 ±0.20	0.866 ±0.18	0.982 ± 0.23	0.463 ± 0.19	0.615 ± 0.23	0.367 ± 0.19	0.626 ± 0.21	0.730 ± 0.20	ns	
SGLT1	0.567 ± 0.22	0.754 ± 0.24	1.180 ± 0.24	0.829 ± 0.24	0.555 ± 0.25	0.505 ± 0.24	0.745 ± 0.29	0.773 ± 0.29	FED>FASTED Pretreatment>Treatment	0.04 0.004
Proglucagon	0.576 ± 0.32	0.831 ± 0.37	0.832 ± 0.35	0.490 ± 0.36	0.897 ± 0.37	0.640 ± 0.36	1.092 ± 0.43	1.143 ± 0.43	FED<FASTED	0.03
FFAR2	1.106 ± 0.478	1.962 ± 0.44	1.333 ± 0.54	1.625 ± 0.44	2.107 ± 0.53	1.613 ± 0.44	1.652 ± 0.48	2.83 ± 0.40	FED<FASTED	0.050
FFAR3	1.062 ± 0.24	1.959 ±0.22	1.804 ± 0.25	1.592 ± 0.23	1.964 .± 0.25	1.374 ± 0.23	1.100 ± 0.25	1.220 ± 0.23	ns	

[1]Data are expressed as mean ± SEM. Within each dependent variable, means with different letters are statistically different from each other (P<0.05, n=6).

Table 5.4. The effect of meal status, ileal infusion solution, and duration of infusion on mRNA abundance of glucose transporters and other genes of interest in the cecum of rats.[1]

	Fed				Fasted					
Gene	Treatment		Pretreatment		Treatment		Pretreatment		Effect	P-value
	Saline	Butyrate	Saline	Butyrate	Saline	Butyrate	Saline	Butyrate		
GLUT2	0.0152 ± 0.025	0.00998 ± 0.024	0.0193 ± 0.026	0.0120 ±0.024	0.0667 ± 0.030	0.0641 ± 0.0242	0.0140 ±0.024	0.0100 ± 0.024	ns	
SGLT1	0.0343 ± 0.0075	0.0453 ± 0.0067	0.0411 ± 0.0083	0.0566 ± 0.0068	0.0436 ± 0.0097	0.0565 ± 0.0068	0.0594 ± 0.0068	0.0591 ± 0.0068	FED<FASTED Butyrate<Saline	0.048 0.053
Proglucagon	0.306 ± .26	0.690 ± 0.26	0.358 ± 0.29	0.723 ± 0.26	0.519 ± 0.29	1.214 ± 0.24	0.587 ± 0.24	0.634 ± 0.24	Butyrate>Saline	0.025
FFAR2	1.036 ± 0.31	1.841 ± 0.27	1.532 ± 0.31	1.532 ± 0.31	1.255 ± 0.35	2.120 ± 0.31	2.242 ± 0.29	1.951 ± 0.31	ns	
FFAR3	0.406 ± 0.14	0.616 ± 0.12	0.592 ± 0.13	0.798 ± 0.13	0.719 ± 0.14	1.016 ± 0.17	0.578 ± 0.12	0.543 ± 0.13	Butyrate>Saline	0.043

[1] Data are expressed as mean ± SEM. Within each dependent variable, means with different letters are statistically different from each other ($P<0.05$, $n=6$).

Table 5.5. The effects of meal status, ileal infusion solution, and duration of infusion on mRNA abundance of glucose transporters and other genes of interest in the colon of the rat[1].

	Fed				Fasted					
Gene	Treatment		Pretreatment		Treatment		Pretreatment		Effect	*p-value*
	Saline	Butyrate	Saline	Butyrate	Saline	Butyrate	Saline	Butyrate		
GLUT2	0.00727 ±0.0027	0.00304 ± 0.0025	0.00197 ± 0.0043	0.00251 ± 0.0031	0.00263 ± 0.0043	0.0125 ± 0.0035	0.00259 ± 0.0025	0.00289 ± 0.0027	ns	
SGLT1	0.0615 ± 0.013	0.0899 ± 0.0022	0.0889 ±0.027	0.106 ± 0.024	0.133 ± 0.024	0.0806 ± 0.022	0.077 ± 0.0022	0.0723 ± 0.0022	ns	
Proglucagon	0.882 ± 0.39	0.443 ± 0.42	0.492 ± 0.49	0.751 ± .053	1.004 ± 0.46	0.516 ± 0.44	0.390 ± 0.52	0.109 ± 0.56	ns	
FFAR2	2.286 ± 0.94	3.888 ±0.94	1.947 ±1.05	3.916 ±1.05	4.134 ±0.94	3.771 ±0.86	2.507 ±0.86	2.381 ±0.86	ns	
FFAR3	0.321 ±0.27	0.532 ±0.29	0.338 ±0.23	0.451 ±0.36	0.796 ±0.31	0.392 ±0.29	0.229 ±0.36	0.268 ±0.38	ns	

[1] Data are expressed as mean ± SEM. Within each dependent variable, means with different letters are statistically different from each other ($P<0.05$, $n=6$).

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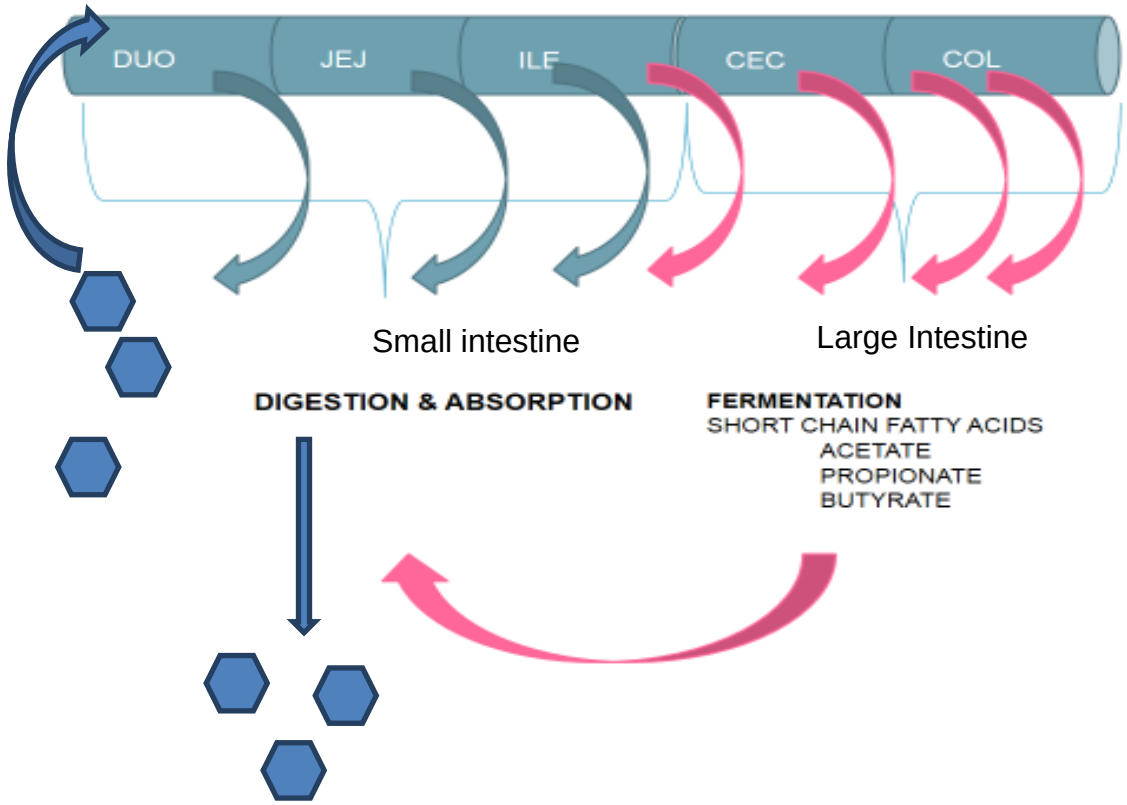

Figure 5.8. A model of glucose absorption.

Glucose is mainly absorbed by the proximal small intestine. SCFA are produced predominantly within the distal ileum and proximal large intestine through the fermentation of undigested nutrients. Signals from the distal intestine served to slow transit time, and may also increase the glucose absorptive capacity of the proximal intestine.

Chapter 6 General Discussion, Conclusions, and Future Directions

The nutrient absorptive capacity of the intestine is a complex orchestration of digestion, nutrient transporters, and sensing and signaling pathways. The distal ileum and large intestine have functions related to absorption, but also produce peptides that contribute to regulation of nutrient absorption and maintenance of the mucosa. Microbiota within the distal ileum and large intestine produce SCFA as the product of fermentation. SCFA may be used by the intestine for energy, but also may provide a feedback signal about nutrient availability. The goal of this research was to determine the impact of butyrate on the glucose absorptive capacity of the proximal intestine. This was investigated using both *in vivo* and *in vitro* models.

Hypothesis 1: Butyrate within the lumen of the ileum and distal large intestine acutely increases glucose absorption by the proximal small intestine through increased glucose transporter capacity.

a. *In vitro:* Butyrate alone up-regulates GLUT2 mRNA abundance through the activation of specific elements encoding transcription factor binding sites within the GLUT2 promoter.

b. *In vivo:* Glucose absorption is acutely increased by intestinal butyrate *in vivo*.

Using Caco2-BBe mature monolayers as a model for the small intestine, we found that butyrate is the SCFA capable of increasing GLUT2 mRNA abundance through promoter activation. Acetate, propionate, or a mixture of SCFA designed to mimic the physiologic ratios found within the intestine did not exert the same impact as butyrate. This demonstrates a direct response of the enterocyte-like Caco2-BBe monolayer cell to butyrate. Butyrate is preferentially used as a fuel source by colon cells, and Caco2-BBe monolayers are derived from colon cells, so

energetics may play an important role in this change. Butyrate is transported into the enterocyte and may act to stimulate transcription factors.

SCFA and butyrate increase intestinal GLUT2 mRNA *in vivo* as demonstrated in rats and piglets fed with PN solutions containing SCFA and butyrate (Tappenden et al., 1998; Drozdowski et al., 2002; Albin et al., 2003; Bartholome et al., 2004). Intravenous administration of butyrate allows the intestine access to butyrate via the bloodstream, and therefore, may provide direct contact between the enterocyte within the intestinal mucosa and the butyrate carried in the blood.

Butyrate activated the GLUT2 promoter in a dose-dependent manner. Various portions of the GLUT2 promoter were activated by butyrate indicating specific regions that might encode transcription factor binding sites. The +145/522 GLUT2 promoter subclone, with the largest 5' sequence deletion of the GLUT2 promoter, was activated. It may contain transcription factor binding sites that are sensitive to butyrate. The potentially indiscriminate effects of butyrate such as those exerted via the inhibition of histone deacetylase, were tested with enteroendocrine-like STC1 cells. They did not show a dramatic increase in GLUT2 promoter activation after butyrate stimulation, suggesting that cell type is an important factor in the response to butyrate. Enteroendocrine cells produce important peptides for the maintenance of the structural integrity and other functions of the intestine, while enterocytes primarily conduct nutrient transport.

Future experiments focused on the identification of the transcription factors involved in the butyrate-stimulated upregulation of GLUT2 transcription could yield greater detail about mechanisms of transcriptional control. Also, the abundance of mRNA is determined through a balance of mRNA transcription and degradation. Although butyrate induced transcription through promoter activation, it also may slow mRNA degradation. Experiments examining the

impact of butyrate on mRNA degradation would provide a full picture of the impact of butyrate on GLUT2 mRNA abundance.

Next, the role of butyrate in the upregulation of intestinal glucose absorptive capacity *in vivo* was examined. Since the majority of butyrate is produced within the distal ileum and proximal large intestine, downstream of the region of the greatest glucose absorption *in vivo*, an experiment was designed to see if a physiologically relevant concentration of butyrate within the distal ileum and large intestine would increase the glucose absorptive capacity of the proximal small intestine. In this experiment, a dramatic decrease in glucose absorption in FASTED animals after prolonged saline infusion into the ileum was demonstrated. Butyrate countered this effect and maintained the glucose absorptive capacity of the intestine, even during prolonged ileal infusion. This demonstrates that luminal stimulation, or lack thereof, rapidly changes the absorptive capacity of the intestine, emphasizing the dynamic environment of the intestine and constant integration of signals in response to luminal cues. Rapid communication between regions of the intestine and the means of the transmission of signals are not fully defined. Signals may arrive via chemical or nervous transmission, or an intricate combination of both. The sensing of the depletion of luminal contents rapidly decreased the glucose absorptive capacity.

In our experiment, 10 mM butyrate maintained glucose absorption. Future experiments using a higher concentration of butyrate may provoke a larger response. In the FED group, unlabeled D-glucose molecules were far more abundant than the 3-O-methyl glucose tracer and most likely limited the observed differences between treatment groups. In order to more fully explore glucose absorption in the postprandial condition, the high glucose meal should contain a

lower concentration of glucose to allow for better estimation of the true rate of glucose absorption.

Hypothesis 2: Exposure of the distal ileum and large intestine to butyrate increases GLUT2 mRNA abundance, while SGLT1 mRNA abundance remains unchanged. Thus, the glucose transporter mRNA abundance (GLUT2, SGLT1), as measured through RT-PCR, will reveal a positive impact of butyrate on the GLUT2 mRNA in mucosal cells from the site of administration in the distal intestine (ileum, cecum, and colon) and on the site of glucose absorption in the proximal intestine (duodenum, jejunum).

The upregulation of the transcription of GLUT2 mRNA in response to butyrate demonstrated *in vitro* was also present in an *in vivo* model. GLUT2 mRNA abundance, rather than GLUT2 protein, was measured because mRNA abundance is an indication of the immediate response of the intestine to butyrate. There is a vigorous ongoing discussion about the intricacies of measuring the GLUT2 protein, and the role of GLUT2 in glucose transport. Originally, GLUT2 was thought to reside only in the basolateral membrane as a high Km, low affinity transporter for glucose to exit the enterocyte into the bloodstream. Since the kinetics associated with active cotransport of glucose by SGLT1 by the enterocyte in high luminal glucose conditions could not be fully explained by SGLT1, other theories of transport were developed and tested. The Solvent Drag theory describing the paracellular transport of glucose when glucose concentrations are high within the lumen of the intestine was proposed by Pappenheimer (Pappenheimer and Reiss, 1987; Pappenheimer, 2001). Therefore, a hypothesis of GLUT2 within the brush border membrane to assist transport glucose down a concentration gradient was developed (Kellett and Helliwell, 2000) and widely accepted. A series of studies showed the

translocation and presence of GLUT2 in the brush border membrane in response to high glucose conditions (Kellett and Helliwell, 2000; Au et al., 2002; Affleck et al., 2003; Gouyon et al., 2003; Mace et al., 2007; Chaudhry et al., 2012). It was thought that limitations in detecting GLUT2 in the brush border were either due to the binding sites of particular antibodies used to measure GLUT2 that were raised different to epitopes (the end terminus or extracellular loops of GLUT2), or the speed at which GLUT2 exited the brush border membrane precluding accurate measurement (Affleck et al., 2003). A very recent study, however, supports the original hypothesis that the SGLT1 transporter is responsible for the vast majority of glucose transport from the lumen of the intestine into the enterocyte under high glucose conditions and negates the presence of GLUT2 in the brush border (Roder et al., 2014). In this study, wild type mice and mice lacking SGLT1 and GLUT2 were intubated with a high glucose meal containing D-glucose (4 mg/kg bw) plus ^{14}C (U)-D-glucose as a tracer. These experiments demonstrated that SGLT1 is the major glucose transporter within the brush border, and very little, if any GLUT2 was present.

Considering the ongoing debate, the dynamics, location, and abundance of GLUT2 within the enterocyte remains unresolved. When the various theories of the location of GLUT2 within the enterocyte membrane, brush border, basolateral, or cellular vesicles are fully tested, the precise role of GLUT2 in glucose transport will become clear. At present, the details of glucose transport by the enterocyte and the measure of the GLUT2 peptide do not have unanimous acceptance.

The focus of this research centered on the regulation of transcription of GLUT2 as an early indicator of increased glucose transport capacity. Transcriptional control of GLUT2 mRNA abundance represented the acute response of the intestine to butyrate and the appearance

of 3-O-[methyl-^{14}C] –D-glucose in the portal vein demonstrated intestinal absorptive capacity. Unlike the GLUT2 peptide, the measure of GLUT2 mRNA abundance through RT-PCR is widely accepted.

Previous experiments demonstrate an increase in GLUT2 mRNA abundance following intravenous butyrate or SCFA treatment in rats and neonatal piglets (Tappenden et al., 1998; Albin et al., 2003). In these experiments, rats treated with butyrate had greater abundance of GLUT2 mRNA in the jejunum, a site where the majority of glucose is absorbed.

In our experiment, a physiologically relevant concentration of butyrate (10 mM), infused into the distal ileum and large intestine, stimulated upregulation of GLUT2 mRNA abundance within the jejunum of a rat. This increase corresponded to the maintenance of the glucose absorptive capacity during prolonged ileal infusions. These results support the hypothesis that butyrate increases GLUT2 mRNA abundance.

The impact of butyrate on the upregulation of GLUT2 mRNA was greater than that of the glucose meal. Although others have observed that GLUT2 and not SGLT1 is increased by luminal fructose and glucose (Cui et al., 2003; Cui et al., 2004; Cui et al., 2005), the infusion of butyrate into the distal ileum and large intestine trumped any impact due to the meal status.

In our experimental model, butyrate was not directly applied to the enterocytes of the small intestine, yet still helped to facilitate increased glucose absorptive capacity through higher jejunal GLUT2 mRNA abundance. This suggests that the distal intestine detected the presence of luminal butyrate and provide a signal that is passed to the jejunum resulting in the upregulation GLUT2 mRNA. Within the distal ileum, cecum, and colon, GLUT2 mRNA abundance was far lower than within the jejunum and was unchanged by butyrate treatment.

In contrast to GLUT2, ileal SGLT1 was lowest in rats representing the postparandial state with the shorted duration of ileal saline infusion (FED * Treatment interaction). The time frame from the start of ileal infusion was short for tissue collection (96 min), so the down regulation of SGLT1 may represent a faster degradation of or decreased transcription of SGLT1 mRNA in these rats. This seems to contradict the high demand for glucose transport through the jejunal enterocytes. While SGLT1 was down regulated in the jejunum of the saline-treated FED rats, it was upregulated in the ileum of FED rats regardless of type of ileal treatment. Thus, the presence of glucose had a greater impact on the transcriptional control of SGLT1 than butyrate infusion. While SGLT1 was not upregulated by SCFA or butyrate in several previous studies (Tappenden et al., 1998; Albin et al., 2003), transcriptional up-regulation in response to sucrose and monosaccharides has been demonstrated (Yasutake et al., 1995; Kishi et al., 1999).

Kishi et al. (1999) examined the transcriptional control of SGLT1 in response to monosaccharide consumption using rats that were force-fed a liquid diet containing glucose, galactose, or fructose. Under these conditions, there was no change in jejunal SGLT1 transcription rate due to force feeding glucose, even though mRNA abundance was slightly higher after 12 h. SGLT1 protein and mRNA abundance was increased by high concentrations of carbohydrate in the diet of rats, horses, sheep, and pigs (Shirazi-Beechey et al., 1994; Dyer et al., 1997a; Dyer et al., 2009; Moran et al., 2010).

A limitation of our design is the lack of control for the fluctuation of the transcription of the glucose transporters due to circadian rhythm. All surgeries took place in the light cycle, but were otherwise at different times of the day. The mRNA abundance of glucose transporters is impacted by the diurnal rhythm (Balakrishnan et al., 2008; Stearns et al., 2009). Circadian rhythm may have contributed to variation within our model.

Future directions for this work involve exploring various concentrations of butyrate to find the maximal response point for the upregulation of GLUT2 mRNA abundance. This information will show if there is a tipping point of butyrate concentration beyond which GLUT2 mRNA is diminished, or if a plateau is reached. In the case of a tipping point, there may be an upper limit of butyrate supplementation, beyond which harmful consequences ensue. A plateau indicates a wide range of safety. Once a range is established, then the means of delivering the appropriate dose of butyrate to the distal ileum and large intestine can be developed.

Hypothesis 3: Butyrate up-regulates proglucagon mRNA abundance. This may be mediated through increased abundance of GPCR, FFAR2 or FFAR3 (also known as GPR43 and GPR41, respectively). There will be increased abundance of FFAR2 and FFAR3 mRNA in the ileum of rats treated with butyrate when compared to those treated with a saline control**.**

Within the intestine, proglucagon mRNA encodes two peptides capable of both paracrine and endocrine effects. The intestinal L-cells that produce proglucagon are located mainly in the distal intestine where they sample the intestinal milieu, produce and secrete GLP-1 and GLP-2. Proglucagon mRNA is increased in response to the consumption of a high fiber diet, or intravenous administration of SCFA and butyrate. We examined the acute response of the L-cell to a physiologically relevant infusion of butyrate in relation to the glucose absorptive capacity within a rat.

Infusing a defined concentration of butyrate directly into the distal intestine served to further define the response of the jejunum, ileum, cecum, and colon. In our experiments, ileal proglucagon mRNA rose in FASTED rats compared to FED rats, but there was no significant

effect of butyrate infusion. The impact of ileal infusion of butyrate into the distal ileum and large intestine were observed within the cecum where proglucagon mRNA abundance was doubled. Cecal proglucagon was not changed by glucose meal status. The colon, last to receive the butyrate infusate, showed a trend towards high proglucagon in rats treated with butyrate that did not achieve significance.

Our results are slightly different than others. In a previous experiment using chronic ileal butyrate infusions, Woodard (2010) saw increased ileal, but not colonic, proglucagon mRNA abundance after 7 d of thrice per day ileal infusion in rats. These infusions were administered as a bolus of butyrate in a much higher concentration (60 mM butyrate) than our experiments (10 mM butyrate) and were administered into either the ileum or colon but not in combination. Colonic infusions did not change either ileal or colonic proglucagon abundance. Cecal proglucagon was not measured. Perhaps 60 mM butyrate in the infusate was better able to stimulate the ileal L-cells than 10 mM butyrate, or the chronic time point (7 d) allowed more proliferative responses within the ileal mucosal surface than 96 to 156 min. Another study also showed an increase in ileal proglucagon abundance following butyrate treatment (9 mM) administered intravenously within PN delivered to neonatal piglets following massive small bowel resection (Bartholome et al., 2004). Ileal proglucagon mRNA abundance was approximately 2-fold higher in butyrate treated piglets from 24 h through 7 d post-surgery. Colonic proglucagon rose significantly regardless of time point (4 h – 7 d). Thus, the intestine is sensitive to butyrate in the blood as well as in the lumen. Although all of these experiments showed increases in proglucagon abundance, none measured cecal proglucagon. The time points and routes of administration for butyrate differ from our experiment. So while the Woodard and Bartholome studies concluded that butyrate raised proglucagon mRNA, they do not entirely

mirror our results. In our experiment, cecal but not ileal, proglucagon abundance responded to butyrate.

The intestinal L-cell produces GLP-1 and GLP-2 from translation of proglucagon mRNA into peptides that area processes by prohormone convertase 1/3 and secreted as GLP-1 and GLP-2. These peptides provides signals to the rest of the intestine and are released in response to luminal stimulation. GLP-1 and GLP-2 are secreted in a 1:1 ratio and have very short half-lives. Since the peptides have such short half-lives, experiments designed to test for GLP-1 or GLP-2 provide insight into production of the transcript encoding them. In humans, the importance of the ileum in production and secretion of GLP-2 is observed in surgical studies in the presence or absence of jejunum, ileum, and colon, and response to diets containing fermentable substrates, SCFA, or butyrate. In humans with an intact ileum, GLP-2 rose following a high fiber meal (50 pmol/L increase over baseline) compared to humans lacking an ileum due to ileal resection (5 pmol/L increase over baseline) (Jeppesen et al., 1999). Removing the ileum and cecum leaves rats less able to adapt through increasing plasma GLP-2, and intestinal growth compared to rats with sham resection (Koopmann et al., 2009). Taken together, these findings point to the ileum as having a unique and important role in the synthesis and release of GLP-2. In our experiment, the ileal proglucagon mRNA did not rise with butyrate treatment. It could be that 10 mM butyrate was too low to achieve an increase in ileal proglucagon mRNA. However, 10 mM butyrate was sufficient to stimulate an increase in cecal proglucagon mRNA and a trend within the colon ($P=0.09$) for upregulation by butyrate. The sac-like shape of the cecum may have contributed to retention of the butyrate solution and elicited a larger response in that tissue. In the rat, the cecum is the predominant site of fermentation and, thus, the cecal mucosa may be primed to sense and respond to luminal butyrate.

The GPCR FFAR2 and FFAR3 mRNA abundance within the cecum was increased by butyrate. Cecal FFAR3 mRNA abundance was higher in butyrate treated rats regardless of any other treatment. These results are in accordance with a study by Yadav et al. (2013) investigating the impact of a butyrate producing probiotic (probiotic VSL#3) on obesity and GLP-1. They confirmed higher levels of plasma butyrate and the DNA for the butyrate kinase gene (buk) in probiotic-treated mice and measured higher GLP-1 secretion, intestinal proglucagon, FFAR3, SGLT1, and prohormone convertase, the enzyme responsible for peptide processing for proglucagon to GLP-1 (Pcsk) mRNA in jejunum, ileum, and colon. In a follow-up *in vitro* experiment, Yadav et al. (2013) treated the human enteroendocrine cell line, NCI H716, with butyrate and measured increased GLP-1 secretion, and mRNA abundance of FFAR3, SLC5a1, and Pcsk over controls. A butyrate response was also demonstrated in neonatal piglets, surgically modified with a massive small bowel resection, that were fed a combination of PN plus a trophic partial enteral feeding containing the fiber scFOS, a probiotic (LGG) or both in combination resulting in greater ileal mRNA abundance of FFAR2 and FFAR3 (Barnes et al., 2012).

We found that cecal FFAR2 mRNA abundance was increased by a shorter duration of butyrate treatment. Experiments with FFAR2 $^{-/-}$ mice demonstrated that FFAR2, although increased by dietary butyrate, exerted very little control over the butyrate-induced secretion of GLP-1 (Lin et al., 2012). In contrast 60 mM butyrate infusions given as a bolus thrice per day did not impact FFAR2 levels in rats treated for 1 wk even when proglucagon mRNA was increased (Woodard, 2010). Therefore, it seems that the evidence for the role of FFAR2 in the upregulation of proglucagon mRNA transcription and subsequent release of the GLP-1 produced from proglucagon is not as strong as that for FFAR3.

Finally, these experiments support a role for luminal butyrate in up-regulating intestinal glucose transport capacity. Our data support our hypothesis that the L-cells located in distal ileum, cecum, and proximal colon may sense luminal contents, and signal the proximal intestine to increase the capacity to transport glucose. Further, FFAR3 seems more likely than FFAR2 to be involved in the sensing and signaling pathways related to the regulation of proglucagon transcription. As little as 10 mM butyrate is sufficient to accomplish these changes. Interestingly, FFAR3 is also found on afferent nerve endings within the distal intestine, while FFAR2 is not. This may point to additional potential for signaling through FFAR3 (Nohr et al., 2013). Ultimately, finding a delivery system to bring butyrate to the L-cells, such as probiotics, or designing FFAR3 agonists to stimulate the L-cell, may be interventions that hold promise for the potential treatment of individuals with malabsorption.

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