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Short and Long Chain Free Fatty Acids Differentially Regulate Glucagon-like Peptide-1 and Peptide YY Transcript Levels in Enteroendocrine Cells (STC-1)

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A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Physiology and Biophysics at Virginia Commonwealth University

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ABSTRACT

The regulation of glucagon-like peptide-1 and peptide YY hormone levels are regulated based on different influential factors, but primarily levels are dependent upon ingested food content. As meals today become more fat-enriched, there is greater requirement for evaluation of these hormones that regulate insulin and satiety levels within the body. We have shown that the gene expression transcript production of glucagon-like peptide-1 and peptide YY are modulated by different concentrations, and times of short-chain fatty acids and long-chain fatty acids. Although the peptide hormone levels have the influential physiological role on effector tissue, the regulation of these hormones begins at the transcript levels. Recent research indicates that glucagon-like peptide-1 and peptide YY hormones are altered in response to different free-fatty acids. The present investigation generally demonstrated an overall decrease in both hormones after chronic exposure to fatty acids. Intestinal secretin tumor cell line (STC-1 cells) was used as a representative for intestinal L-cells. Quantitative real-time PCR analysis was used to determine the changes in RNA transcripts. Overall, there was a decrease in the 3-hour timeline, which continued to decrease in the 16-hour and 24-hour timelines for glucagon-like peptide-1. Peptide YY transcript expression in 3-hours increased significantly after exposure to propionate, a significant decrease after exposure to acetate, and no significant increase or decrease after exposure to butyrate. However, there was a significant decrease in peptide YY once reaching 24-hour exposure. It was determined there is a threshold for different concentrations of free-fatty acids to influence glucagon-like peptide-1 and peptide YY production, which was present in the different concentrations of butyrate. Lastly, exposure to both concentrations of linolenic acid caused a significant decrease in glucagon-like peptide-1 and peptide YY.

I. INTRODUCTION

I. A) Diabetes Mellitus

Diabetes mellitus is a chronic disease that is characterized by either a decreased production (Type 1 DM) or increased insensitivity to insulin (Type 2 DM). Insulin plays a vital role in how cells within the body uptake glucose. As of 2014, there is an 8.5% prevalence of diabetes mellitus among adults alone, and the number of individuals with diabetes mellitus has risen to 422 million from 108 million in 1980 [World Health Organization]. This obesity epidemic has stimulated an increased research effort, since the numbers of individuals with this condition has steadily increased. Incretins, such as glucagon-like peptide-1 (GLP-1), play an important role in stimulating glucose-dependent insulin release from pancreatic beta-cells. Incretins are gut hormones produced and secreted into the blood rapidly after a meal ingestion from enteroendocrine cells. However, incretins' effects have been shown to be reduced in type-2 diabetic patients [Nauck 1986]. Meanwhile, peptide YY (PYY), another hormone that is influenced by food ingestion, also plays a key role in satiety, and food ingestion.

In western cultures, about 40% of the calories ingested are composed of lipids [Niot 2009]. This is clinically important because these factors create excess saturated fatty acids and cholesterol, which creates multiple health risks in addition to the development of obesity [Niot 2009]. As modern society expands the percentage of obese individuals, it is becoming more important to examine the factors influencing this epidemic and the resulting physiological changes.

I. B) Functions and Anatomy of the Gastrointestinal Tract

B1. Overview

The gastrointestinal (GI) tract is a tubular organ system with multiple components. There are two primary functions of the GI tract. First, through enzymes and water, ingested food is converted into water-soluble, aqueous solutions allowing it to be absorbed for other cellular functions. Mainly, these enzymes to aid in food digestion and absorption are capable of being recycled for the next meal consumed [Pandol 2009]. Second, it functions as an immune barrier as protective layer between the external environment and the internal conditions present in the body. There are two main divisions for the GI tract: the upper gastrointestinal tract, and the lower gastrointestinal tract. The upper gastrointestinal tract begins at the oral cavity in the mouth, which proceeds to form the esophagus as it moves down the throat and connects to the stomach. The stomach expels its contents into the small intestine. The small intestine consists of three different segments: the duodenum, the jejunum, and the ileum. Meanwhile, the lower gastrointestinal tract, which begins after the duodenum is the primary site where absorption of ingested material transpires. The small intestine connects to the large intestine-i.e., the colon, at the cecum. The colon transverses in and out the peritoneal and retroperitoneal cavities inside the abdomen. It finally ends at the rectum, which mediates the connection to the anus. Any undigested material is excreted through the alimentary canal as fecal matter-i.e., feces.

B2. Anatomy of the Small Intestine

The small intestine consists of the duodenum, jejunum, and ileum. The duodenum, the first part of the small intestine, receives the chyme from the stomach. It oscillates between the peritoneal and retroperitoneal abdominal cavities. Functionally, the duodenum is responsible for

the breakdown of the bolus in the small intestine, using enzymes, and it also provides mucus secretions through Brunner's glands. Meanwhile, L-cells are primarily found in the ileum which is the main source for peptide YY (PYY) secretion [Hand 2013].

B3. Characteristics of the Gut Microbiome

The gut microbiota-i.e. gut flora, found throughout the digestive tract contains trillions of microorganisms that live in a mutualistic relationship with its host. It is composed of bacteria, archaea, viruses, and eukaryotic microbes [Shreiner 2015]. In total, there are about 1,000 distinct species that compose the gut microbiome [Sommer 2013]. There is a gradient to the microbiome distribution. Microorganisms that make up the gut flora increases tremendously towards the colon, with the stomach, duodenum, jejunum, ileum, and colon containing 10^1, 10^3, 10^4, 10^7, and 10^{12} microbial cells per gram, respectively [Sommer 2013].

There are various functions for the microbiome. The primary function of the anaerobic bacteria present in the microbiome is to ferment indigestible carbohydrates various products. In the proximal colon, saccarolytic bacteria produce linear short-chain fatty acids (SCFA), H_2, CO_2 [McFarlane 2003, Roberfroid 2007, Wong 2006]. Meanwhile, when proteolytic bacteria ferments proteins and amino acids, branched SCFA, H_2, CO_2, CH_4, phenols, and amines are produced [Roberfroid 2007, Wong 2006]. Albeit fermentation of starches and other carbohydrates is a vital quality of the microbiome for bolus digestion. The other essential tasks of the microbiome include proliferating and differentiating the intestinal epithelium and immune system through induction of $CD4^+CD25^+FOXP3$ regulatory cells and T helper 17 cells, assisting in protection from opportunistic pathogens, and influencing tissue homeostasis [Smith 2007, Sommer 2013].

B4. Short-Chain Fatty Acids

Short-chain fatty acids (SCFA) are short carboxylic acids consisting of two to six carbons [Karaki 2006, Wong 2006]. The five SCFA that make up this category are acetate, propionate, butyrate, valerate, and caproate, respectively. Through the metabolic process of anaerobic bacterial fermentation in the colon, SCFA are produced from polysaccharides, oligosaccharides, protein, peptide, and glycoprotein precursors as the bolus moves through the colon [Wong 2006]. The most abundant SCFA present in order of decreasing quantity are acetate, propionate, and butyrate [Topping 2001]. However, this does not appear to be the case with the absorption of the SCFA. Colonic epithelium has the greatest molar concentrations of butyrate, while the liver contains the most propionate [Cummings 1987]. This indicates that the rate of production and absorption differs upon the location in the colon, the bacterial organisms present, and the SCFA present.

The SCFA are not consistently absorbed throughout the colon. The largest absorbance of SCFA is in the proximal colon. Meanwhile, other factors play a role in absorption of SCFA such as concentration, and the pH [Wong 2006]. Additionally, SCFA act on two specific receptors, GPR41 and GPR43 that are found in the GI tract [Karaki 2006]. The potency for the GPR41 based on SCFA decreases starting with propionate, butyrate, and acetate, while there is no discrimination in sensitivity between the different SCFA for GPR 43. [Karaki 2006].

B5. Long-Chain Fatty Acids

Triacyclglycerols (TAG) are typically 95% of the content of ingested lipids [Niot 2009]. These are composed of multiple subunits, one being long-chain fatty acids (LCFA). LCFA are characterized as being greater than 15 carbons. Digestion of LCFA is advanced from low pH

conditions of the stomach, which cause their breakdown, leading to lipid emulsification [Armand 1994]. Along with the production of diacylglycerols after TAG hydrolysis, free fatty acids (FFAs) can cross cellular membranes [Niot 2009]. LCFA crossing the membrane is necessary for them to be used as metabolic substrates. After LCFA are digested, they have multiple important effects. Some of these functions include: metabolic fuel, precursors of lipid mediators, regulators of ion channels and modulators of gene expression [Eyster 2007]. The concentrations of LCFA found after hydrolyzation of TAG results in μM concentrations of free-fatty acids (FFAs) [Eyster 2007]. Thus, the concentration of LCFA that are present are in smaller quantities compared to SCFA.

I. C) STC-1 Cells

Intestinal secretin tumor cell line (STC-1 Cells) have been shown to express a wide range of gut hormones involved with metabolism such as GLP-1, and PYY [Venema 2015]. Transcription for hormone secretion in STC-1 Cells is influenced by cyclic adenosine monophosphate (cAMP) and calcium (Ca^{2+}) concentrations within the cytosol of the cell [Venema 2015]. There are two possible situations where there can be increased cytosolic levels of Ca^{2+}. First, through voltage-gated calcium channels that can be activated within the plasma membrane causing an influx of calcium into the cell. Second, through inositol 1,4,5-triphosphate (IP_3) signals Ca^{2+} is released from intracellular stores. Production of GLP-1 and PYY hormones were shown to be present in the STC-1 cell line [Drucker 1994]. However, PYY production in STC-1 cells in vitro is not always representative of in vivo conditions because of the loss of luminal stimulation causing hormone release from vagal nerve reflexes. However, for a representative model, STC-1 cell line provides an excellent resource for being a reproducible enteroendocrine cell model.

I. D) Hormones

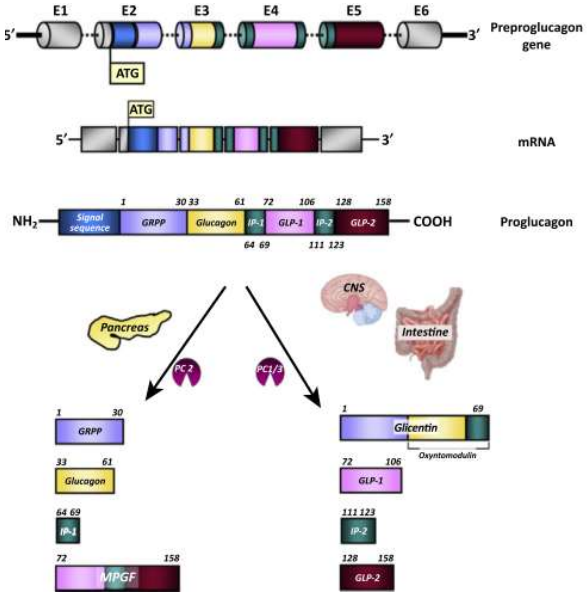
D1. GLP-1

Glucagon-like peptide-1 (GLP-1) was the second incretin hormone to be identified, after glucose-dependent insulinotropic polypeptide (GIP) [Drucker 2006]. It was shown that after ingestion of a meal, the concentration of GIP is significantly higher than GLP-1; however, the potency of GLP-1 is much greater than GIP [Nauck 1993]. The amino acid sequences of GLP-1 are similar across all species of mammals [Fehmann 1995]. There are two primary equipotent biologically active forms of GLP-1. There is the GLP-1(7-37) form, and the more abundant circulating form GLP-1(7-36)amide (NH_2) [Drucker 2006, Mojsov 1986, Mojsov 1990]. The production of these different forms of GLP-1 are the consequence of selective cleavage from the proglucagon precursor, preproglucagon [Fehmann 1995]. The RNA for GLP-1 is incorporated in the proglucagon RNA because it is transcribed from preproglucagon gene. Thus, it is important to distinguish that GLP-1 RNA refers to the segment of proglucagon RNA that is translated to form GLP-1 hormone. Meanwhile, there is limited difference between the two forms of peptide hormones with respect to their metabolic degradation and clearance and half-life [Orskov 1994]. Removal of the N-terminus histidine creating GLP-1(8-37) causes complete removal of biological effects of GLP-1(7-37) indicating that this residue is necessary for its function [Fehmann 1995, Gefel 1990]. Meanwhile, removal of the three amino acids located at the C-terminus causes GLP-1(7-34) to be 1000-fold less potent compared to GLP-1(7-37) (Figure 1) [Fehmann 1995, Suzuki 1989].

GLP-1 regulation is found to be mediated through G-Protein Coupled Receptor 120 (GPR120) in STC-1 cells. GLP-1's primary action is to increase insulin secretion from pancreatic beta-cells (Figure 2) [Drucker 2006]. GLP-1 has many impacts on different receptor organs. GLP-1 binding

to receptors in adipose tissue, muscle, and liver causes an increase in glucose uptake and an increase in glycogen synthesis [Drucker 2006]. In the brain and nervous system, there is decrease in appetite, food intake, and water intake, while an increase in satiety in the hypothalamus [Drucker 2006]. GLP-1 disruption with an antagonist demonstrated that there was reduced glucose clearance, increased production of glucagon, defective glucose-stimulated insulin secretion, and relatively faster gastric emptying [Drucker 2006, Schirra 1998]. GLP-1 binding to its receptor activates G-Protein Coupled Receptor (GPCR), specifically the stimulatory $G_{\alpha S}$ pathway. The trimeric GPCR $G_{\alpha S}$ pathway further leads to increase in adenylyl cyclase (AC) activity, which inherently increases cyclic adenosine monophosphate (cAMP) levels that can act as a second messenger within the cell [Chandarana 2013]. Regulation of GLP-1 comes the enzyme dipeptidyl-peptidase IV (DPP-IV), which rapidly metabolizes the hormone to render it inactive.

Figure 1. GLP-1 Amino Acid Sequence Formation from DNA. (LEFT) The preproglucagon gene, which contains six exons separated by five introns. The exons are transcribed to mRNA and translated to proglucagon, a precursor molecule. After post-translational manipulation by prohormone convertases, Glucagon, GLP-1, GLP-2, oxyntomodulin, glicentin, glicentin-related polypeptide (GRPP), a major glucagon fragment (MPGF), intervening peptide-1 (IP-1), and intervening peptide-2 (IP-2). (RIGHT) Multiple forms of GLP-1, which the first form GLP-1(1-37) is cleaved through post-translational modification of N-terminus provides the two active forms, GLP-1(7-37) and GLP-1(7-36). GLP-1 is subject to further post-translational modification to produce C-terminal amidated forms, GLP-1(7-37)amide and GLP-1(7-36)amide. Lastly, the cleavage site of dipeptidyl peptidase-IV (DPP-IV) activity is highlighted by the broken line [Cantini 2016].


E1
E2
E3
E4
E5
E6
5′
3′
Preproglucagon gene
ATG
ATG
5′
3′
mRNA
NH2
Signal sequence
GRPP
Glucagon
IP-1
GLP-1
IP-2
GLP-2
COOH
Proglucagon
1
30 33
61
72
106
128
158
64 69
111 123
Pancreas
CNS
Intestine
PC 2
PC1/3
GRPP
Glucagon
IP-1
MPGF
Glicentin
Oxyntomodulin
GLP-1
IP-2
GLP-2

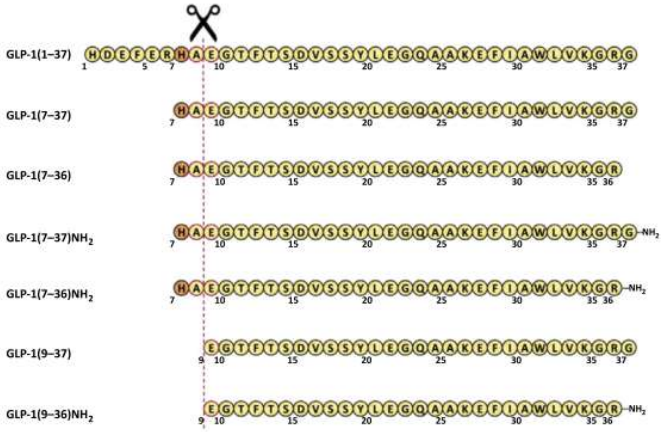
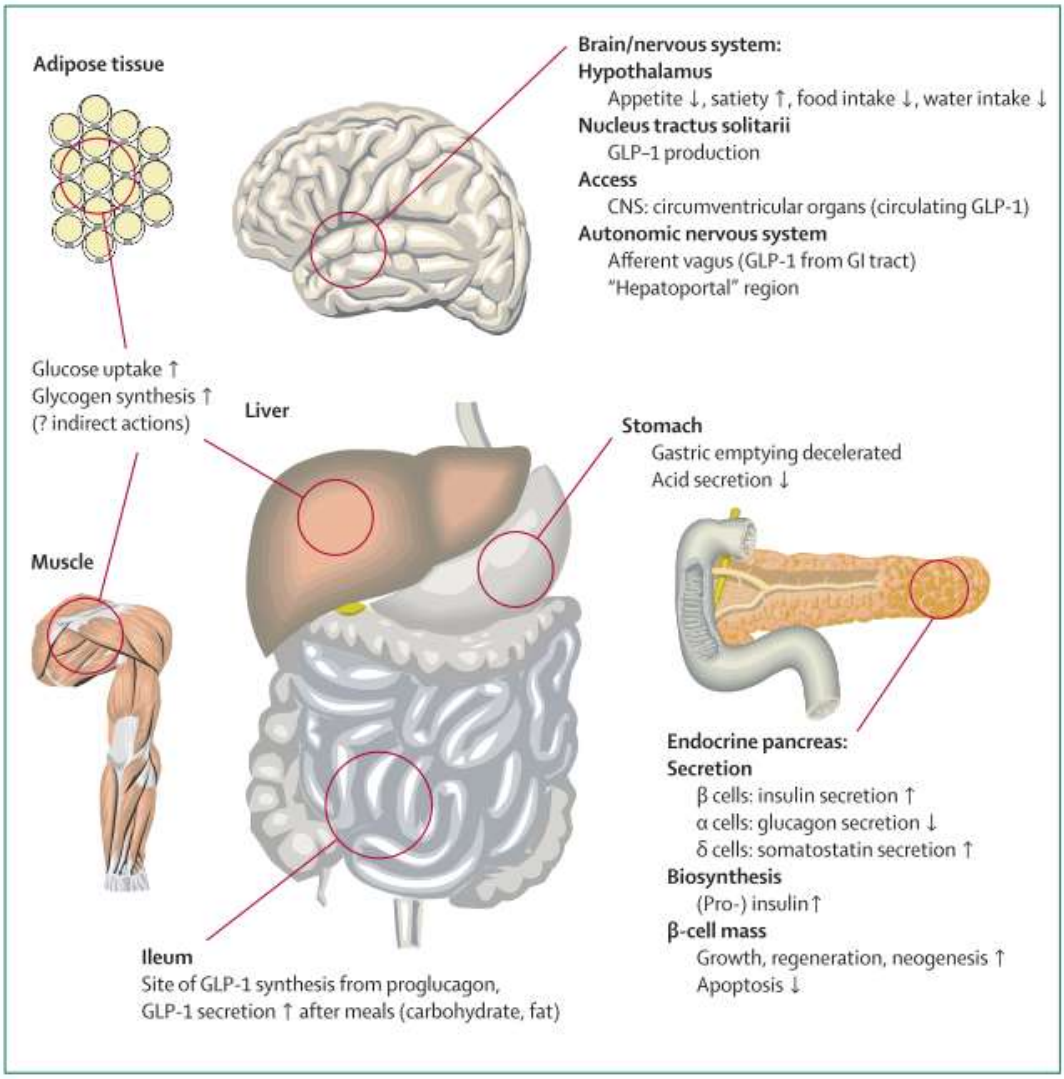

GLP-1(1–37)
GLP-1(7–37)
GLP-1(7–36)
GLP-1(7–37)NH2
GLP-1(7–36)NH2
GLP-1(9–37)
GLP-1(9–36)NH2

Figure 2. Physiology of GLP-1 Secretion and Action. GLP-1's effects on its receptors found on various organs and tissues [Drucker 2006]


Adipose tissue
Glucose uptake ↑
Glycogen synthesis ↑
(? indirect actions)
Muscle
Brain/nervous system:
Hypothalamus
Appetite ↓, satiety ↑, food intake ↓, water intake ↓
Nucleus tractus solitarii
GLP-1 production
Access
CNS: circumventricular organs (circulating GLP-1)
Autonomic nervous system
Afferent vagus (GLP-1 from GI tract)
"Hepatoportal" region
Liver
Stomach
Gastric emptying decelerated
Acid secretion ↓
Endocrine pancreas:
Secretion
β cells: insulin secretion ↑
α cells: glucagon secretion ↓
δ cells: somatostatin secretion ↑
Biosynthesis
(Pro-) insulin ↑
β-cell mass
Growth, regeneration, neogenesis ↑
Apoptosis ↓
Ileum
Site of GLP-1 synthesis from proglucagon,
GLP-1 secretion ↑ after meals (carbohydrate, fat)

D2. PYY

Peptide YY (PYY) is a member of the neuropeptide Y (NPY) family. Originating from the gene encoding PYY on chromosome 17q211.1, it is produced as a prepropeptide [Troke 2013]. The propeptide which includes a signal peptide on the N-terminus, a 36-amino acid active sequence, and a carboxy terminal extension, which undergoes amidation by a prohormone convertase to create an active C-terminal amide structure [Gefel 1999, Troke 2013]. Each end terminus consists of a single tyrosine (Y) residue, thus giving its name PYY, which is related to Pancreatic Polypeptide (PP) and to NPY (Figure 3) [Troke 2013]. However, the predominant from that is circulating is PYY(3-36), which is formed after N-terminal dipeptide cleavage by DPP-IV [Troke 2013]. It is important to note that PYY is produced by endocrine cells and neurons [Garaedts 2009]. PYY is abundantly expressed in endocrine cells within the mammalian gastro-intestinal tract, while in a few species have the peptide produced in systems of enteric neurons [Garaedts 2009]. Specifically, PYY has been shown to be present in enteroendocrine L-cells of the gastrointestinal tract, where it where it accumulates with GLP-1 [Stanley 2004, Troke 2013]. Meanwhile, the distribution of PYY mRNA is revealed to be increasing expression in the jejunum, cecum, and the colon, respectively.

PYY has important biological activity in both the intestine and the brain [Tatemoto 1980]. PYY acts on Y receptors, which are a subclass of the family G-protein coupled receptors (GPCRs). Receptors Y_1, Y_2, and Y5 are the primary target for PYY [Garaedts 2009]. Meanwhile, the predominant form, PYY(3-36), is more discriminatory for the Y2 and Y5 receptors [Keire 2000]. The Y receptors are considered inhibitory in nature. They are linked to pertussis toxin-sensitive G-proteins, which reduces cAMP accumulation, regulation of intracellular calcium concentrations through its channels, and potassium channels upon activation [Michel 1998, Troke 2013].

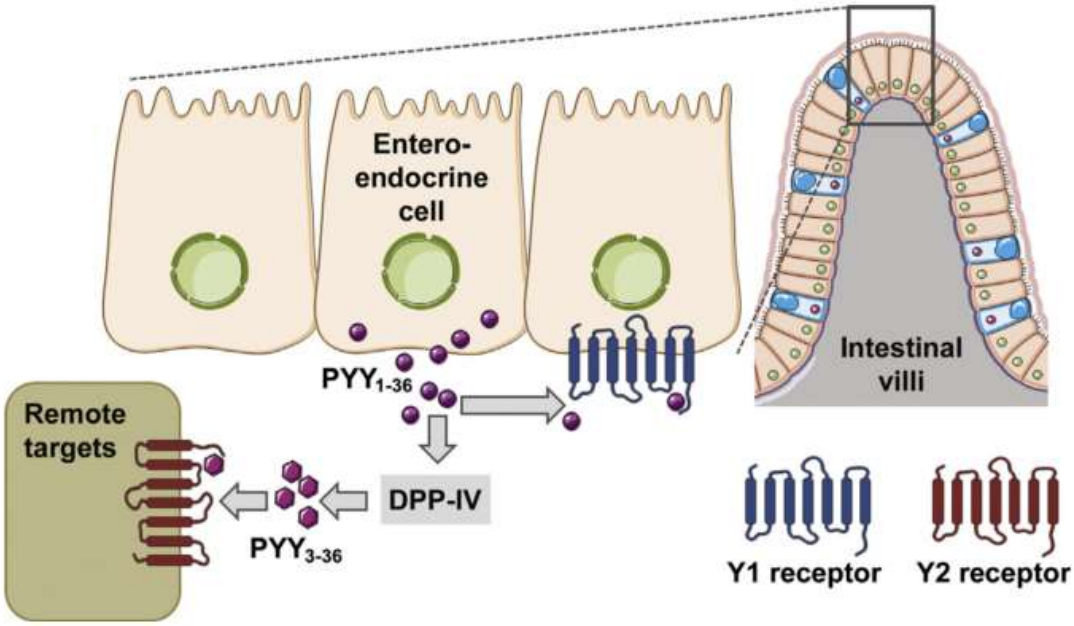
Regulation of PYY occurs through multiple factors. In the presence of oral nutrient load, L-cells of the distal gut secrete PYY [Stanley 2004]. After PYY secretion from an oral load stimulus, the plasma levels peak within two hours after a meal, and remain elevated for an additional four hours [Batterham 2003, Stanley 2004]. Meanwhile during a fasted state, PYY plasma concentrations are at its lowest [Troke 2013].

There are multiple functions for PYY. PYY influences gastric empty, slows intestinal transit time, influences gastric acid secretion, food intake and satiety, insulin sensitivity, and respiratory quotient [Ballantyne 2006, Batterham 2003, Guo 1987, Troke 2013, Stanley 2004, Yang 2002]. The slowing of the intestinal transit time is described as the "ileal brake" because it is designated when the ileum of the small intestine decreases gastric emptying [Ballantyne 2006, Troke 2013]. Specifically, PYY binding sites discovered in the dorsal vagal complex (DVC), which consists of the area postrema (AP), the nucleus tractus solitaris (NTS), and dorsal motor nucleus of the vagus (DMN), are suggested to be the cause of the "ileal brake" [Chen 1995]. For gastric acid, PYY injected peripherally inhibits gastric acid release; however, when PYY injected centrally into the DVC, there is increase secretion of gastric acid [Stanley 2004]. PYY may also interrelate with GLP-1 and secretin to produce an accompanying decrease in gastric acid out. [Yang 2002]. This interaction influences vagal innervation of cells, which acts through the DVC, to diminish gastric acid secretion [Yang 2002]. Food intake is decreased and satiety is increased in response to endogenous PYY(3-36) production, specifically on the Y2 present on the vagus nerve [Batterham 2003, Dockray 2009].

In obese individuals, PYY is decreased in both humans and rodents [Le Roux 2006, Xu 2011]. Specifically, secretion of PYY production is diminished in both the post prandial state and fasting state [Le Roux 2006]. Additionally, there is a decreased response of food intake even after

exogenous PYY(3-36) administration in Type 2 DM individuals [Le Roux 2006, Xu 2011]. This decreased response was matched with a reduced response in satiety as well [Le Roux 2006]. However, decreased PYY levels are thought to be a consequence of obesity, and not a source; thus, it is caused by moderation of satiety signals resulting in augmented food intake [Le Roux 2006].

Figure 3. **PYY illustration of Sites of Production and Action.** Diagram depicts PYY(1-36) being mainly released from distal enteroendocrine L-cells in the intestine. PYY is released in response to nutrient stimulation, which then acts as paracrine fashion on neighboring Y1 receptors. Meanwhile, PYY(1-36) experiences cleavage of N-terminal amino acids by dipeptidyl-peptidase IV (DPP-IV), which converts it to PYY(3-36). PYY(3-36) has increased affinity for Y2 receptor, which are inhibitory for G-protein coupled receptors [Stadlbauer 2015].


Entero-
endocrine
cell
Intestinal
villi
PYY1-36
Remote
targets
DPP-IV
PYY3-36
Y1 receptor
Y2 receptor

HYPOTHESIS

The hypothesis of this study was aimed to examine if glucagon-like peptide-1 and peptide YY transcripts are modulated by short-chain fatty acids and long-chain fatty acids during different exposure times and concentrations.

II. Materials and Methods

II. A) Reagents and Equipment

A1. Stock Solutions

Acetate ($C_2H_3O_2Na$, 82.03 g/mol, $pK_a = 4.76$), Butyrate ($C_4H_7O_2Na$, 110.1 g/mol, $pK_a = 4.82$), Alpha-Linolenic Acid, *cis,cis,cis*-9,12,15-Octadecatrienoic acid (18:3ω3), ($C_{18}H_{30}O_2$, 278.43 g/mol, $pK_a = 4.77$), and Propionate ($C_3H_5O_2Na$, 96.06 g/mol, $pK_a = 4.87$) were purchased from Sigma-Aldrich, St. Louis, MO. The free fatty acids (FFA) were mixed with double distilled H_2O (ddH_2O) as recommended by the manufacturer, and then the solutions were sonicated to ensure the FFA was dissolved within the solution. The solutions were also mixed prior to administration to cell cultures for its appropriate experimental conditions. Dulbecco's Phosphate Buffered Saline 1X without Calcium Chloride and Magnesium Chloride was used for the rinsing cell pellets to remove DMEM with Fetal Bovine Serum (FBS) and antibiotics (ABS).

A2. Medium

The medium for the STC-1 cells was Dulbecco's Modified Eagle Medium (DMEM). The DMEM was 1X with 4.5 g/L glucose, L-glutamine and sodium pyruvate. The medium included 100mL of filtered Fetal Bovine Serum (FBS) and antibiotics (ABS). The antibiotics included 20 mL Penicillin streptomycin, 2 mL of Gentamicin, were filtered and added to the DMEM solution. Finally, non-filtered Amphotericin B was added to the solution. For trypsin, a 100 mL bottle of 0.25% EDTA Trypsin (1X) from Gibco by Life Technologies from ThermoFisher Scientific, Grand Island, NY was stored at 4°C.

A3. Instruments

Multiple machines were used throughout the project. NanoDrop™ 8000 Spectrophotometer by Thermo Scientific was used to determine RNA purity and concentrations. For Reverse-Transcriptase polymerase chain reaction (RT-PCR), Eppendorf™ Mastercycler™ pro PCR System purchased from Fisher Scientific, Waltham, MA was used. Meanwhile, for quantitative real-time polymerase chain reaction (qRT-PCR), the StepOnePlus™ Real-Time PCR System created by Applied Biosystems was purchased from ThermoFisher Scientific, Grand Island, NY was used (CAT: 4376600).

The Fisher Scientific™ Model 705 Sonic Dismembrator was used to dissolve the FFA into solution. The samples were sonicated four separate occasions with each lasting 15 seconds each.

II. B) Cells

B1. Cell Culture

Intestinal secretin tumor cell line (STC-1) were used in the experiment because they are a strong representative of intestinal L-cells. The cells were grown on 10-cm culture dishes until 85-100% confluent. The STC-1 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) and incubated at 37°C and 10% CO_2. Cells were passaged between 5-50 times during their lifespan. Cells were transferred to new culture plates after they were lysed with 2.5 mL of 0.25% EDTA Trypsin and incubated at 37°C and 10% CO_2 for 3-5 minutes. Next the cells were transferred from the original petri dish to 15 mL falcon tubes and centrifuged at 2000 x g for 5-10 minutes. The supernatant was removed from the falcon tube. The pellet was re-suspended with fresh DMEM medium containing FBS and ABS. After re-suspension, the diluted cells were

transferred to a new 10-cm culture dish prefilled with 9 mL of DMEM medium containing FBS and ABS.

B2. Experimental Conditions

After 80-90% confluency to the cell plate, the cells medium was removed through suctioning and was replaced with Dulbecco's Modified Eagle Medium without antibiotics or fetal bovine serum (DMEM-0). The cells were left to incubate at 37°C and 10% CO_2 levels for a minimum of 2.5-hours. Once incubated for the pre-set amount of time, the solution concentrations were created by mixing DMEM-0 with the appropriate amount of reagent to create the dilutions. The 100-mM concentration of butyrate was created by mixing 1800 µL of DMEM-0 with 200 µL of the stock 1 M butyrate solution reagent to create 2 mL volume solution within a well. The 50 mM concentrations that are used for acetate, butyrate and propionate are crafted by mixing 1900 µL of DMEM-0 with 100 µL of the 1 M stock reagent solution for the appropriate reagent to create a total of 2 mL solution within the well. To create the 10 mM butyrate solution, 1980 µL of DMEM-0 was mixed with 20 µL of the 1 M stock butyrate solution. The butyrate 1 mM experimental solution was created by adding 1998 µL of DMEM-0 to each well of the 6-well plate, while only 2 µL the stock reagent was added to each.

Lastly, a 1 mM stock alpha-linolenic acid solution was created using the appropriate volume of dd-H_2O as recommended by the manufacturer. Next, the 100 µM experimental condition was created by mixing 1800 µL of DMEM-O solution with 200 µL of the 1 mM stock linolenic acid solution. The 10 µL experimental conditions was created by mixing 1980 µL of DMEM-0 with 20 µL of the 1 mM stock linolenic acid stock solution.

II. C) RNA

C1. RNA Isolation

Ribonucleic acid (RNA) isolation was done using two possible methods. First, the more utilized method, the RNA was isolated with the Invitrogen by Thermo Scientific RNAqueous™ Phenol-free total RNA Isolation Kit (REF: AM1912) from ThermoFisher Scientific, Grand Island, NY. After the cell cultures endured their designated period for that treatment within a well of a 6-well plate, the cell medium was suctioned off. Next, pre-warmed 0.25% trypsin-EDTA (1X) was added to each well and allowed to incubate for three to five minutes at 37°C and 10% CO_2. Next, the plates were removed and DMEM containing FBS and ABS was added to stop trypsin function. The cells within four separate wells of the 6-well plates that underwent the same experimental conditions were collected into a 15 mL Falcon tube on ice. The contents within each tube were centrifuged at 4000 rounds per minute (RPM) for six minutes at 12°C to achieve a pellet. After centrifugation and placing samples back in the ice bath, the DMEM medium and trypsin were removed through suction. Next, the pelleted samples were washed with 4°C Phosphate Buffered Saline 1X (PBS) three times. Lysis buffer, which contained guanidine thiocyanate and 2-mercaptoethanol, was added to the samples and mixed. Next, an equivalent amount of 64% ethanol was added to the sample and mixed. The solution was transferred to a RNase-free elution tube containing an RNA column. For entire procedure, the columns were spun at 12,200 rpm at 12°C for one minute. The samples were then washed with wash solution 1, two separate rinses with 500 µL of wash solution 2/3. After transfer to a new tube, RNase-free column underwent two sequential rinses of preheated 95°C elution buffer with 35 µL and 25 µL, respectively. Immediately following RNA isolation, the samples were placed on ice, and the purity of the

samples were tested with the NanoDrop 8000 spectrophotometer with ultrapure H_2O as the comparative blank.

The second method that was used for RNA isolation was through Invitrogen's TaqMan™ Gene Expression Cells-to-CT™ Kit purchased from ThermoFisher Scientific, Grand Island, NY. The cells were grown in the same previous manner as the first method, but only one well from the 6-well plate was needed. After the cell cultures endured their designated time frame for that treatment within a well of a 6-well plate, the cell medium was suctioned off. Next, pre-warmed 0.25% trypsin-EDTA (1X) was added to each well and allowed to incubate for three to five minutes at 37°C and 10% CO_2. Next, the plates were removed and DMEM containing FBS and ABS was added to stop trypsin function. Each well was placed into its own 1.5 mL RNase-free micro-centrifuge tube. The cells were pelleted at 4000 RPM for six minutes at 12°C. After centrifugation, the pellet was resuspended with DMEM with FBS and ABS in order to determine number of cells present. Using an automated cell counter, the cells were counted to ensure less than 100,000 cells were present in the sample. Dilutions were made if the cells present in the solution were greater than 100,000. Next, the cells were re-pelleted at 4000 RPM for six minutes at 12°C. After centrifugation and placing samples back in the ice bath, the DMEM medium and trypsin were removed through suction. Next, 4°C Phosphate Buffered Saline 1X (PBS) was added to the pelleted cells in the Falcon tubes, then spun at 4000 rpm for six minutes at 12°C. The RT-PCR process as recommended by the protocol of the kit was amplified using 20 µL reaction volumes.

C2. R*everse-Transcription (cDNA synthesis)*

The isolated RNA samples were standardized to 1000 ng by dilution with ultrapure nuclease-free H_2O. The isolated RNA product was transcribed to single strand complementary deoxyribonucleic acid (cDNA) using High Capacity cDNA reverse Transcription kit by Applied Biosystems from Thermo Fisher Scientific. The High Capacity cDNA reverse Transcription kit included 10X RT Buffer, 10X RT Random Primers, 25X dNTP Mix [100 mM], and Multiscribe™ Reverse Transcriptase that formed 2X reverse transcription (RT) master mix. The RT master mix was combined with the standardized, isolated RNA to create a total 20 µL reaction volume. The Eppendorf™ Mastercycler™ pro PCR System was used for the reverse transcription process. The cycle process consisted of initial holding temperature of 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and finished with an infinite hold at 4°C until the samples were removed and stored at -20°C.

The Cells-to-CT™ Kit required a RT sequence of 37°C for 60 minutes for reverse transcription, 95°C for 5 minutes for transcription termination, and finished with infinite hold at 4°C until the samples were removed for storage.

C3. Conventional PCR

Conventional PCR was used to amplify target gene GLP-1 and housekeeping gene GAPDH. For GAPDH and GLP-1, custom designed primers were used for amplification (Table 1). After the target sequences were amplified, the resulting PCR products were run on a 1.4% agarose gel.

Table 1. Forward and Reverse Primers for GLP-1 and GAPDH Used for Gel-Electrophoresis.

NAME:	Direction	Sequence
GLP-1	Forward	5'-TCA TCC CCA GCT TCC CAG ACA-3'
	Reverse	5'-TCT GGG AAG TCT CGC CTT CCT-3'
GAPDH	Forward	5'-AGA AAC CTG CCA AGT ATG ATG-3'
	Reverse	5'-GGA GTT GCT GTT GAA GTC G-3'

C4. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

The cDNA was utilized to perform single-plex Quantitative Real-Time PCR (qRT-PCR) to determine relative changes in RNA production. The specific primers were used for quantification exploited known mouse gene sequences for the GLP-1, PYY, and GAPDH sequences in mouse and TaqMan gene expression master mix. The reaction plate was created as a single-plex reaction to avoid issues encountered with GAPDH interfering with GLP-1 analysis. For each sample, there was a total of 20 μL reaction volume incorporating TaqMan^{TM} gene Expression Master Mix (Applied Biosystems, Foster City, CA), the genes specific probe assay, cDNA, and DNase/RNase-free H_2O. The temperature profile for the thermocycler used for quantification was two initial stages consisting of 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 40 seconds. The sequence was terminated with 4°C infinite hold. Annealing temperatures used for the thermocycler were established for each primer set. Relative comparative cycle threshold (Ct) was used to quantify the transcript expression. Cycle threshold is defined as the cycle number at which the fluorescence generated for a reaction becomes significantly larger than the passive reference used with the specific TaqMan gene expression master mix reagent, which was ROX. Quantification results were calculated with Microsoft Excel

using the $2^{-\Delta\Delta Ct}$ method. This method is a quantitative comparison between samples by standardizing the ΔCt to GAPDH values to determine the differences in experimental versus control changes in cycle threshold values (ΔΔCt). The following formula was used.

$$\Delta\Delta C_T = \left(Ct_{[Target]} - Ct_{[\,GAPDH]}\right)_{Experimental} - \left(Ct_{[Target]} - Ct_{[GAPDH]}\right)_{Control}$$

Eqn. 1. Formula used to calculate differences in the change of cycle threshold (ΔΔCt for comparison of the change in threshold of the experimental sample (sample that underwent manipulation) and the control sample (no-manipulation), where $Ct_{[Target]}$ is the cycle threshold for gene of interest for the sample, and $Ct_{[GAPDH]}$ is the cycle threshold for GAPDH of the same sample culture. The experimental condition was the tested sample that experienced variable manipulation- i.e. fatty acid addition, and control condition is the sample that did not have fatty acid added, but underwent the same time frame condition.

From the ΔΔCt, the fold change was calculated by the following equation.

$$FC = 2^{-\Delta\Delta Ct}$$

Eqn. 2. Formula used to calculate fold change. Involves comparing the differences in the cycle threshold for the experimental condition ΔCt values and control condition ΔCt values.

The primers used for the amplification during qRT-PCR quantitative analysis are listed below.

Table 2. The primers length and reporter dye used for qRT-PCR target sequence amplification and quantification.

Primer Set	Assay ID	Amplicon Length	Reporter Dye
GCG (GLP-1)	Mm00801714_m1	85 bp	FAM-MGB
PYY	Mm00520715_m1	87 bp	FAM-MGB
GAPDH	NM_008084.2	107 bp	VIC®

C5. Statistical Analysis

Statistical analysis of the results was completed using One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 7.03 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. The results were expressed as means ± SEM of *n* experiments.

III. RESULTS

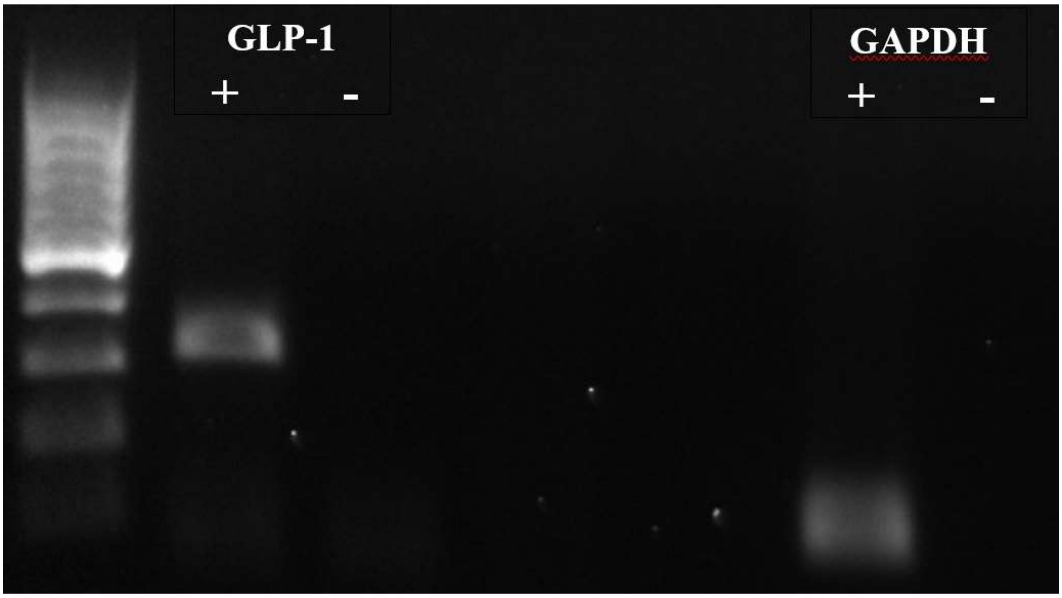
In the present study, the proglucagon mRNA was measured, but for functional implications, it is GLP-1 that produces the effect. Therefore, we have referred to our results as GLP-1 RNA rather than proglucagon mRNA. Changes in glucagon-like peptide-1 and peptide YY expression were investigated by qRT-PCR analysis. Meanwhile, GLP-1 was shown to be present through conventional PCR and gel-electrophoresis. For qRT-PCR analysis, changes in transcripts expressed were quantified using fold changes that was found with the STC-1 cell line model. The Materials and Methods section described the specific primers used for qRT-PCR analysis (Table 2). Again, the $^{2-\Delta\Delta Ct}$ quantification method used, which is previously discussed in the Materials and Methods section, standardized the expression of the targeted genes of interest, GLP-1 and PYY, to housekeeping gene expression, GAPDH. This was done by normalizing the ΔCt values to GAPDH expression. Thus, a quantitative comparison between fatty acid treated and non-treated cells was ascertained.

III. A) GLP-1 Analysis

A1. Gel-Electrophoresis

The presence of GLP-1 expression was verified through gel-electrophoresis (Figure 4). The presence of the housekeeping gene was used as a positive and negative control. The gel was created using 1.4% agarose solution with TAE Buffer as previously described in the Materials and Methods section. There was no presence of GAPDH in the negative control, along with no presence of GLP-1 in its negative control. Albeit, there were positive indications of GAPDH (122 bp) and GLP-1 (308 bp) in their respected wells.

Figure 4. Electrophoresis gel with GAPDH and GLP-1. A 1.5% agarose gel that examined presence of GLP-1 mRNA expression, while GAPDH served as positive and negative control. GAPDH length is 122 bp sequence, while GLP-1 is 308 bp length. There was positive indication of GLP-1 sequence present, while no indication in the negative condition. No PYY conventional PCR product was run.


GLP-1
+
-
GAPDH
+
-

A2. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

The effects of the SCFA: 50 mM acetate, 50 mM propionate, and 50 mM butyrate were examined over different time courses, while 1 mM butyrate, 10 mM butyrate, 50mM butyrate and 100 mM butyrate were examined for 24-hour treatments. For LCFA, 10 μM linolenic acid and 100 μM linolenic acid were examined at 24-hours only.

SCFA induced GLP-1 RNA expression examined at 3-hour time-frame indicated a decrease in RNA transcript following 50mM acetate and 50 mM propionate exposure (Figure 5). Meanwhile, no significant decrease in RNA transcript was present in 50 mM butyrate. For statistical significance, $P^* \leq 0.05$, comparisons of control to 50 mM acetate, control to 50 mM butyrate, 50 mM acetate to 50 mM butyrate, and 50 mM propionate to 50 mM butyrate were deemed significant, while control to 50 mM butyrate and 50 mM acetate to 50 mM propionate were not.

SCFA induced GLP-1 RNA expression examined at 16-hour timeframe indicated a decrease in RNA transcript following 50 mM acetate, 50 mM propionate, and 50 mM butyrate exposure (Figure 6). For statistical significance, $P^* \leq 0.05$, comparisons of control to 50 mM acetate, control to 50 mM propionate, control to 50 mM butyrate, and 50 mM acetate to 50 mM butyrate were deemed significant, while 50 mM acetate to 50 mM propionate, and 50 mM propionate to 50 mM butyrate were not.

SCFA induced GLP-1 RNA expression examined at 24-hour timeframe indicated a decrease RNA transcript following 50 mM acetate, 50 mM propionate, and 50 mM butyrate exposure (Figure 7). For statistical significance, $P^* \leq 0.05$, comparisons of control to 50 mM acetate, control to 50 mM propionate, control to 50 mM butyrate, and 50 mM propionate to 50 mM butyrate were deemed significant, while 50 mM acetate to 50 mM propionate, and 50 mM acetate to 50 mM butyrate were not.

Additionally, SCFA induced GLP-1 RNA expression was examined over three different time periods. It indicated that SCFA induced a decrease in GLP-1 RNA transcript following 50 mM acetate, 50 mM propionate, and 50 mM butyrate exposure as treatment time increased (Figure 8). For statistical significance, $P^* \leq 0.05$, comparisons of 50 mM acetate's 3-hour to 24-hour, 50 mM propionate's 3-hour to 24-hour, 50 mM butyrate's 3-hour to 16-hour, and 50 mM butyrate's 3-hour to 24-hours were deemed significant, while 50 mM acetate's 3-hour to 50 mM acetate's 16-hour, 50 mM acetate's 16-hour to 50 mM acetate's 24-hour, 50 mM propionate's 3-hour to 50 mM propionate's 16-hour, 50 mM propionate's 16-hour to 50 mM propionate's 24-hour, and 50 mM butyrate's 16-hour to 50 mM butyrate's 24-hour were not.

Butyrate induced GLP-1 RNA expression examined at 24-hour timeframe indicated a decrease in RNA transcript following 10 mM butyrate, 50 mM butyrate, and 100 mM butyrate exposure (Figure 9). Meanwhile, no significant decrease was present in GLP-1 RNA transcript following 1 mM butyrate exposure. For statistical significance, $P^* \leq 0.05$, comparisons of control to 10 mM butyrate, control to 50 mM butyrate, control to 100 mM butyrate, 1 mM butyrate to 10 mM butyrate, 1 mM butyrate to 50 mM butyrate, 1 mM butyrate to 100 mM butyrate, and 50 mM butyrate to 100 mM butyrate were deemed significant, while control to 1 mM butyrate and 10 mM butyrate to 100 mM butyrate were not.

Linolenic acid induced GLP-1 RNA expression examined at 24-hour timeframe indicated a decrease GLP-1 transcript following 10 μM linolenic acid and 100 μM linolenic acid exposure (Figure 10). For statistical significance, $P^* \leq 0.05$, comparisons of control to 10 μM, control to 100 μM, and 10 μM to 100 μM were deemed significant.

III. B) PYY Analysis

B1. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

SCFA induced PYY RNA expression examined at 3-hour timeframe indicated a decrease induced by 50 mM acetate only (Figure 11). Meanwhile, no significant decrease was present in PYY RNA transcript following 50 mM propionate, and 50 mM butyrate exposure. For statistical significance, $P^* \leq 0.05$, comparisons of control to 50mM acetate was deemed significant, while control to 50mM propionate, control to 50mM butyrate, and 50 mM propionate to 50 mM butyrate were not.

SCFA induced PYY RNA expression examined at 16-hour timeframe indicated an increase in RNA transcript induced by 50 mM propionate exposure (Figure 12). Meanwhile, no significant decrease in RNA transcript was present following 50 mM acetate and 50 mM butyrate exposure. For statistical significance, $P^* \leq 0.05$, comparisons of control to 50 mM propionate, 50 mM acetate to 50 mM propionate, and 50 mM propionate to 50 mM butyrate were deemed significant, while control to 50mM acetate, control to 50mM butyrate, and 50 mM acetate to 50 mM butyrate were not.

SCFA induced PYY RNA expression examined at 24-hour timeframe indicated a decrease in RNA transcript following 50 mM acetate, 50 mM propionate, and 50 mM butyrate exposure (Figure 13). For statistical significance, $P^* \leq 0.05$, comparisons of control to 50 mM acetate, control to 50 mM propionate, control to 50 mM butyrate were deemed significant, while 50 mM acetate to 50 mM propionate, and 50mM propionate to 50 mM butyrate were not.

Additionally, a time course of SCFA induced PYY RNA expression was examined over three different time periods. It indicated that SCFA induced an increase in PYY RNA transcript following 50 mM acetate, 50 mM propionate, and 50 mM butyrate treatment after 16-hour

exposure and a decrease in PYY RNA transcript following 50 mM acetate, 50 mM propionate, and 50 mM butyrate 24-hour exposure compared to both the 3-hour and 16-hour time periods. (Figure 14). For statistical significance, P*≤0.05, comparisons of 50 mM acetate's 3-hour to 16-hour, 50 mM acetate's 3-hour to 24-hour, 50 mM acetate's 16-hour to 24-hour, 50 mM propionate's 3-hour to 16-hour, 50 mM propionate's 3-hour to 24-hour, 50 mM propionate's 16-hour to 24-hour, 50mM butyrate's 3-hour to 16-hour, and 50 mM butyrate's 3-hour to 24-hour, 50 mM butyrate's 16-hour to 24-hour were deemed significant.

Butyrate induced PYY RNA expression examined at 24-hour timeframe indicated a decrease in 10 mM butyrate, 50 mM butyrate, and 100 mM butyrate (Figure 15). Meanwhile, no significant decrease in PYY RNA transcript was present following 1 mM butyrate exposure. For statistical significance, P*≤0.05, comparisons of control to 10 mM butyrate, control to 50 mM butyrate, control to 100 mM butyrate, 1 mM butyrate to 10 mM butyrate, 1 mM butyrate to 50 mM butyrate, 1 mM butyrate to 100 mM butyrate, 10 mM butyrate to 100 mM butyrate, and 50 mM butyrate to 100 mM butyrate were deemed significant, while control to 1 mM butyrate, 10 mM butyrate to 50 mM butyrate, 10 mM butyrate to 100 mM butyrate, and 50 mM butyrate to 100 mM butyrate were not.

Linolenic acid induced PYY RNA expression examined at 24-hour timeframe indicated a decrease following 10 μM linolenic acid, and 100 μM linolenic acid exposure (Figure 16). For statistical significance, P*≤0.05, comparisons of control to 10 μM, control to 100 μM, and 10 μM to 100 μM were deemed significant.

Figure 5. GLP-1 mRNA Expression (Mean ± SEM) in STC-1 Cells After Control, 50 mM Acetate, 50 mM Propionate, or 50 mM Butyrate Treatment for 3-hours ($n = 4$). Total RNA was isolated from STC-1 cells and was reverse transcribed to form single stranded cDNA. For each cDNA sample amplified, 20 µL reaction volume contained TaqMan^{TM} Master Mix and the specific target primer, either GAPDH or GLP-1. The qRT-PCR reactions were performed in either duplicates or triplicates depending on amount of cDNA present. Results were expressed in fold change in GLP-1 gene expression in comparison to control sample. All materials were obtained from ThermoFisher Scientific, Grand Island, NY. Values represent fold change mean from triplicate reactions ± SEM. One-way ANOVA with Tukey post-hoc test. P*≤0.05 were deemed significant. Comparisons of control to 50 mM acetate, control to 50 mM butyrate, 50 mM acetate to 50 mM butyrate, and 50 mM propionate to 50 mM butyrate were statistically significant. For clarity, * appear only over treatments that were statistically significant in comparison to the control sample.

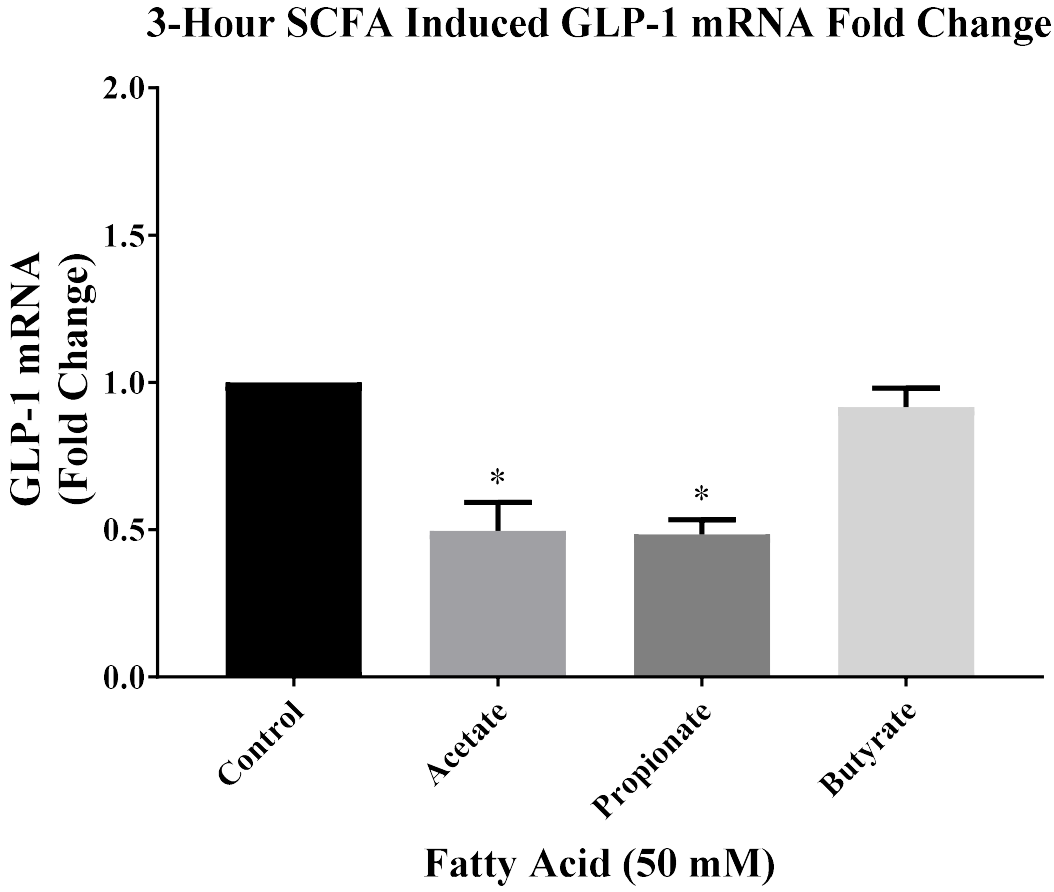

3-Hour SCFA Induced GLP-1 mRNA Fold Change
GLP-1 mRNA (Fold Change)
2.0
1.5
1.0
0.5
0.0
*
*
Control
Acetate
Propionate
Butyrate
Fatty Acid (50 mM)

Figure 6. GLP-1 mRNA Expression (Mean ± SEM) in STC-1 Cells After Control, 50 mM Acetate, 50 mM Propionate, or 50 mM Butyrate Treatment for 16-hours ($n = 3$). Total RNA was isolated from STC-1 cells and was reverse transcribed to form single stranded cDNA. For each cDNA sample amplified, 20 µL reaction volume contained TaqMan™ Master Mix and the specific target primer, either GAPDH or GLP-1. The qRT-PCR reactions were performed in either duplicates or triplicates depending on amount of cDNA present. Results were expressed in fold change in GLP-1 gene expression in comparison to control sample. All materials were obtained from ThermoFisher Scientific, Grand Island, NY. Values represent fold change mean from triplicate reactions ± SEM. One-way ANOVA with Tukey post-hoc test. P*≤0.05 were deemed significant. Comparisons of control to 50 mM acetate, control to 50 mM propionate, control to 50 mM butyrate, and 50 mM acetate to 50 mM butyrate were statistically significant. For clarity, * appear only over treatments that were statistically significant in comparison to the control sample.

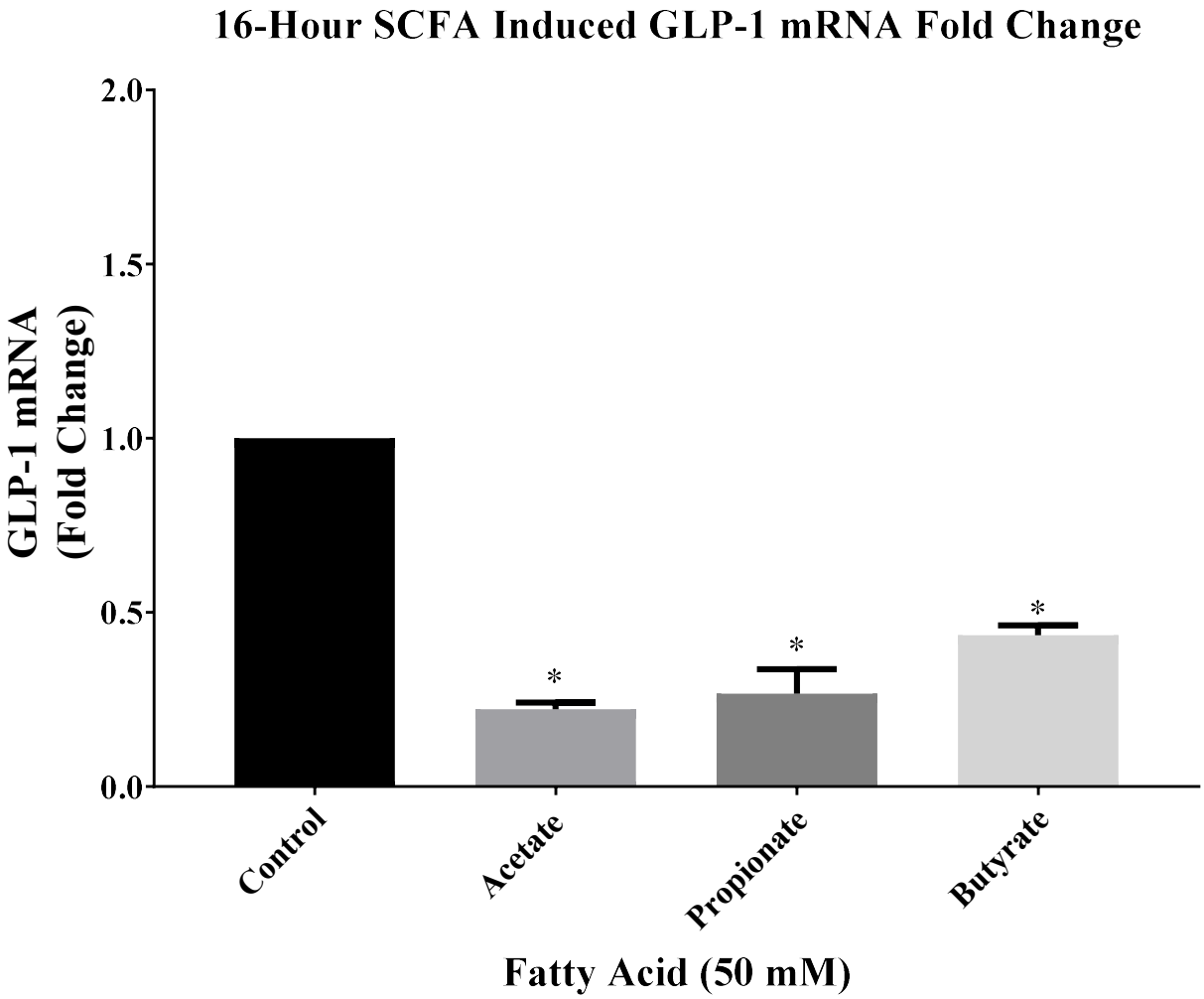

16-Hour SCFA Induced GLP-1 mRNA Fold Change
GLP-1 mRNA (Fold Change)
2.0
1.5
1.0
0.5
0.0
*
*
*
Control
Acetate
Propionate
Butyrate
Fatty Acid (50 mM)

Figure 7. GLP-1 mRNA Expression (Mean ± SEM) in STC-1 Cells After Control, 50 mM Acetate, 50 mM Propionate, or 50 mM Butyrate Treatment for 24-hours ($n = 3$). Total RNA was isolated from STC-1 cells and was reverse transcribed to form single stranded cDNA. For each cDNA sample amplified, 20 µL reaction volume contained TaqMan^{TM} Master Mix and the specific target primer, either GAPDH or GLP-1. The qRT-PCR reactions were performed in either duplicates or triplicates depending on amount of cDNA present. Results were expressed in fold change in GLP-1 gene expression in comparison to control sample. All materials were obtained from ThermoFisher Scientific, Grand Island, NY. Values represent fold change mean from triplicate reactions ± SEM. One-way ANOVA with Tukey post-hoc test. P*≤0.05 were deemed significant. Comparisons of control to 50 mM acetate, control to 50 mM propionate, control to 50 mM butyrate, and 50 mM propionate to 50 mM butyrate were statistically significant. For clarity, * appear only over treatments that were statistically significant in comparison to the control sample.

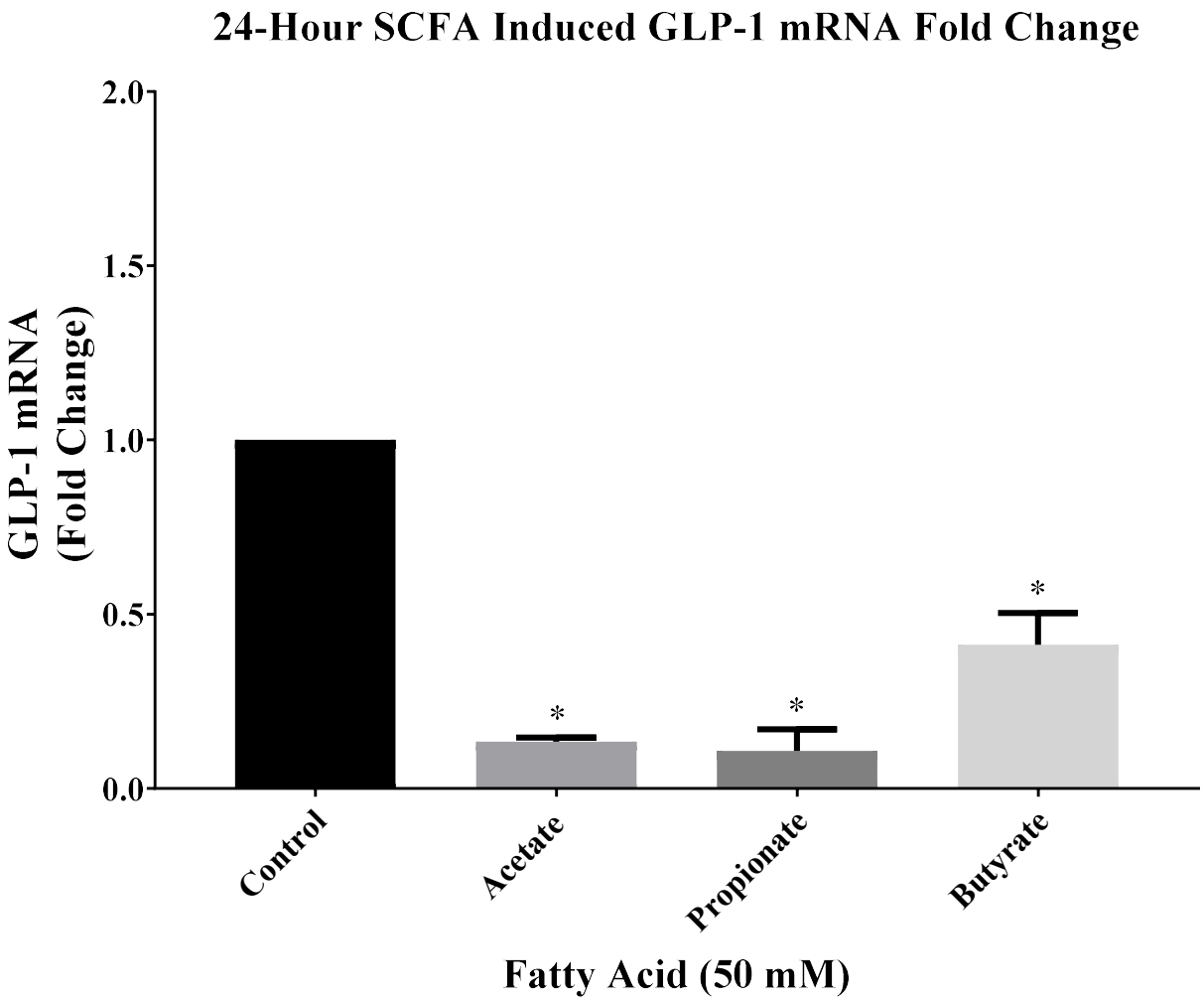

24-Hour SCFA Induced GLP-1 mRNA Fold Change
GLP-1 mRNA (Fold Change)
2.0
1.5
1.0
0.5
0.0
*
*
*
Control
Acetate
Propionate
Butyrate
Fatty Acid (50 mM)

Figure 8. GLP-1 mRNA Expression (Mean ± SEM) in STC-1 Cells After Control, 50 mM Acetate, 50 mM Propionate, or 50 mM Butyrate Treatment for 3-hours, 16-hours and 24-hours ($n = 3$). Total RNA was isolated from STC-1 cells and was reverse transcribed to form single stranded cDNA. For each cDNA sample amplified, 20 µL reaction volume contained TaqMan™ Master Mix and the specific target primer, either GAPDH or GLP-1. The qRT-PCR reactions were performed in either duplicates or triplicates depending on amount of cDNA present. Results were expressed in fold change in GLP-1 gene expression in comparison to control sample. All materials were obtained from ThermoFisher Scientific, Grand Island, NY. Values represent fold change mean from triplicate reactions ± SEM. One-way ANOVA with Tukey post-hoc test. P*≤0.05 were deemed significant. Comparisons of 50 mM acetate's 3-hour to 24-hour, 50 mM propionate's 3-hour to 24-hour, 50 mM butyrate's 3-hour to 16-hour, and 50 mM butyrate's 3-hour to 24-hour were statistically significant.

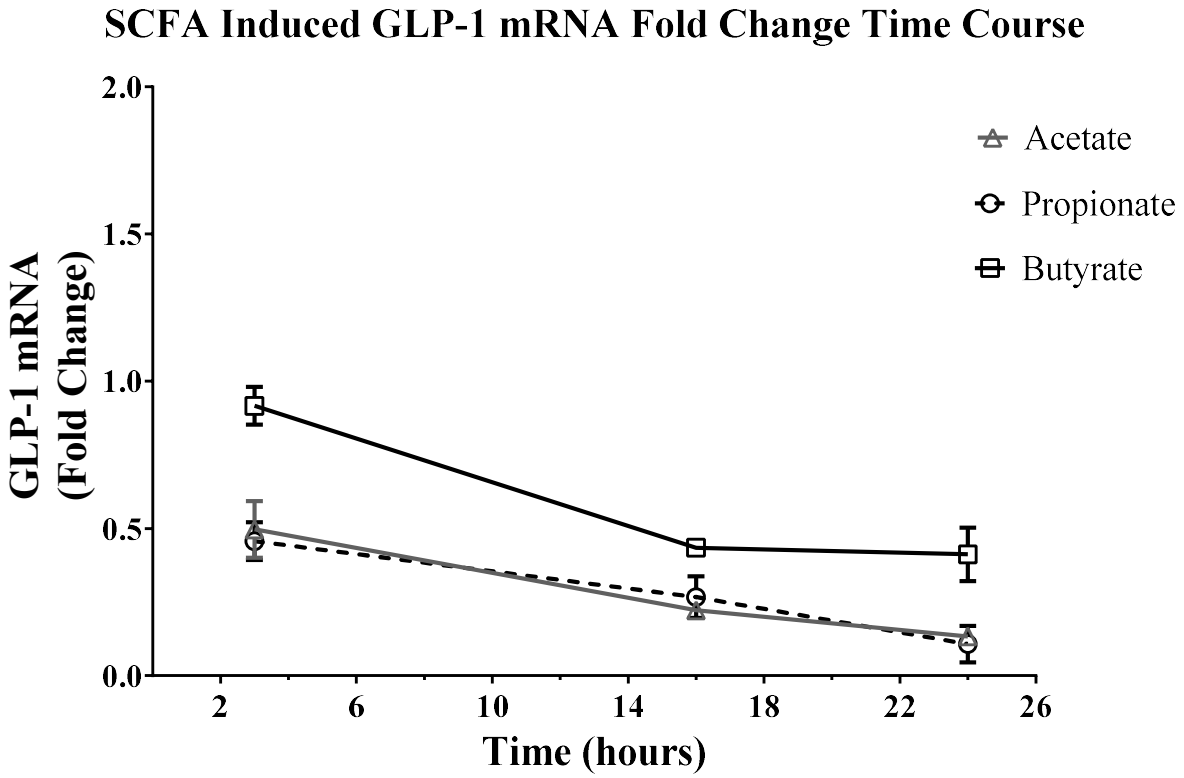

SCFA Induced GLP-1 mRNA Fold Change Time Course
GLP-1 mRNA
(Fold Change)
2.0
1.5
1.0
0.5
0.0
2
6
10
14
18
22
26
Time (hours)
Acetate
Propionate
Butyrate

Figure 9. GLP-1 mRNA Expression (Mean ± SEM) in STC-1 cells after Control and 1 mM, 10 mM, 50 mM, 100 mM Butyrate Treatment for 24-hours ($n = 3$). Total RNA was isolated from STC-1 cells and was reverse transcribed to form single stranded cDNA. For each cDNA sample amplified, 20 µL reaction volume contained TaqMan™ Master Mix and the specific target primer, either GAPDH or GLP-1. The qRT-PCR reactions were performed in either duplicates or triplicates depending on amount of cDNA present. Results were expressed in fold change in GLP-1 gene expression in comparison to control sample. These materials were obtained from ThermoFisher Scientific, Grand Island, NY, besides butyrate. Values represent fold change mean from triplicate reactions ± SEM. One-way ANOVA with Tukey post-hoc test. P*≤0.05 were deemed significant. Comparisons of control to 10 mM butyrate, control to 50 mM butyrate, control to 100 mM butyrate, 1 mM butyrate to 10 mM butyrate, 1 mM butyrate to 50 mM butyrate, 1 mM butyrate to 100 mM butyrate, 10 mM butyrate to 50 mM butyrate, and 50 mM butyrate to 100 mM butyrate were statistically significant. For clarity, * appear only over treatments that were statistically significant in comparison to the control sample.

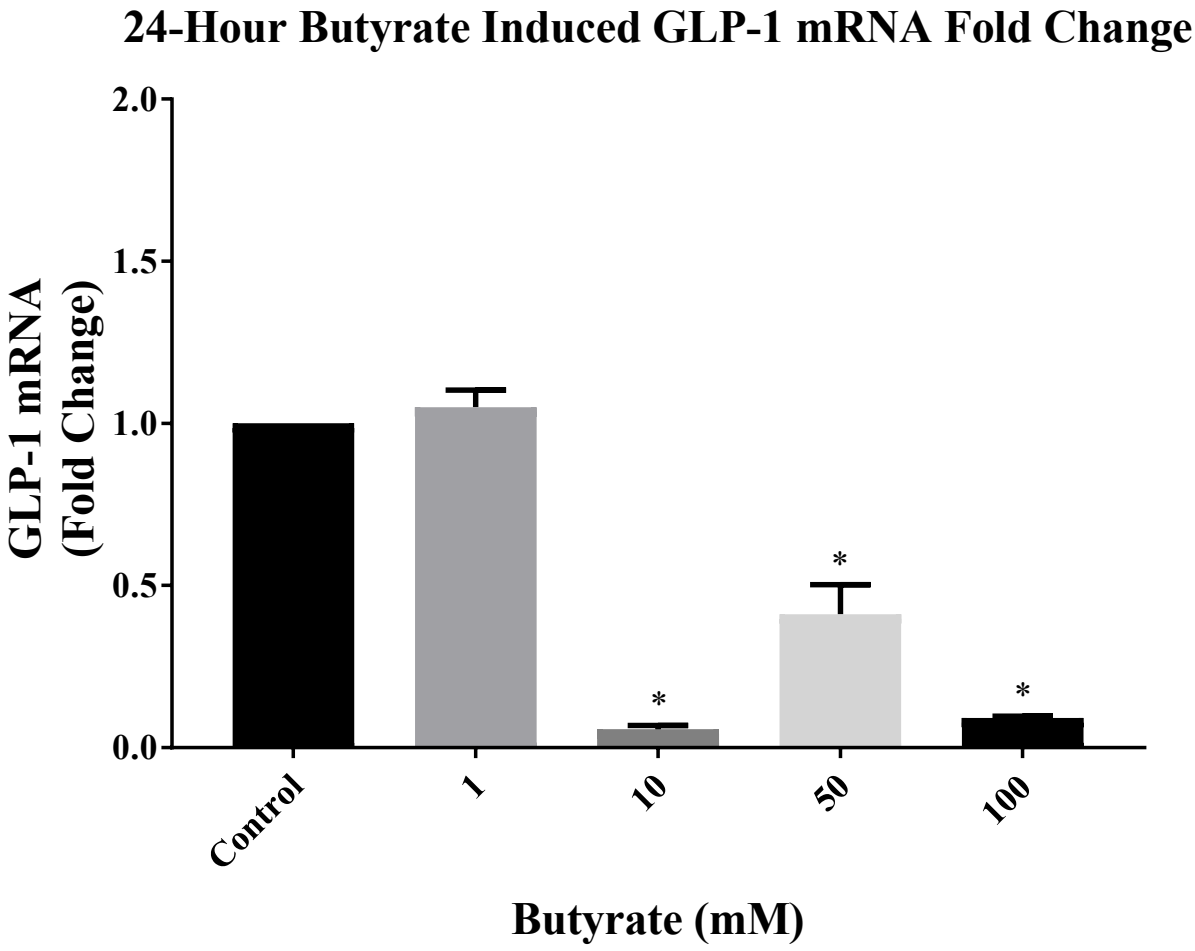

24-Hour Butyrate Induced GLP-1 mRNA Fold Change
GLP-1 mRNA (Fold Change)
2.0
1.5
1.0
0.5
0.0
*
*
*
Control
1
10
50
100
Butyrate (mM)

Figure 10. GLP-1 mRNA Expression (Mean ± SEM) in STC-1 Cells after Control, 10 µM Linolenic Acid, and 100 µM Linolenic Acid Treatment for 24-hours ($n = 3$). Total RNA was isolated from STC-1 cells and was reverse transcribed to form single stranded cDNA. For each cDNA sample amplified, 20 µL reaction volume contained TaqMan™ Master Mix and the specific target primer, either GAPDH or GLP-1. The qRT-PCR reactions were performed in either duplicates or triplicates depending on amount of cDNA present. Results were expressed in fold change in GLP-1 gene expression in comparison to control sample. All materials were obtained from ThermoFisher Scientific, Grand Island, NY. Values represent fold change mean from triplicate reactions ± SEM. One-way ANOVA with Tukey post-hoc test. P*≤0.05 were deemed significant. Comparisons of control to 10 µM, control to 100 µM, and 10 µM to 100 µM were statistically significant. Note: not all SEM are displayed due to sizing issues and fitting icons for each SCFA. For clarity, * appear only over treatments that were statistically significant in comparison to the control sample.

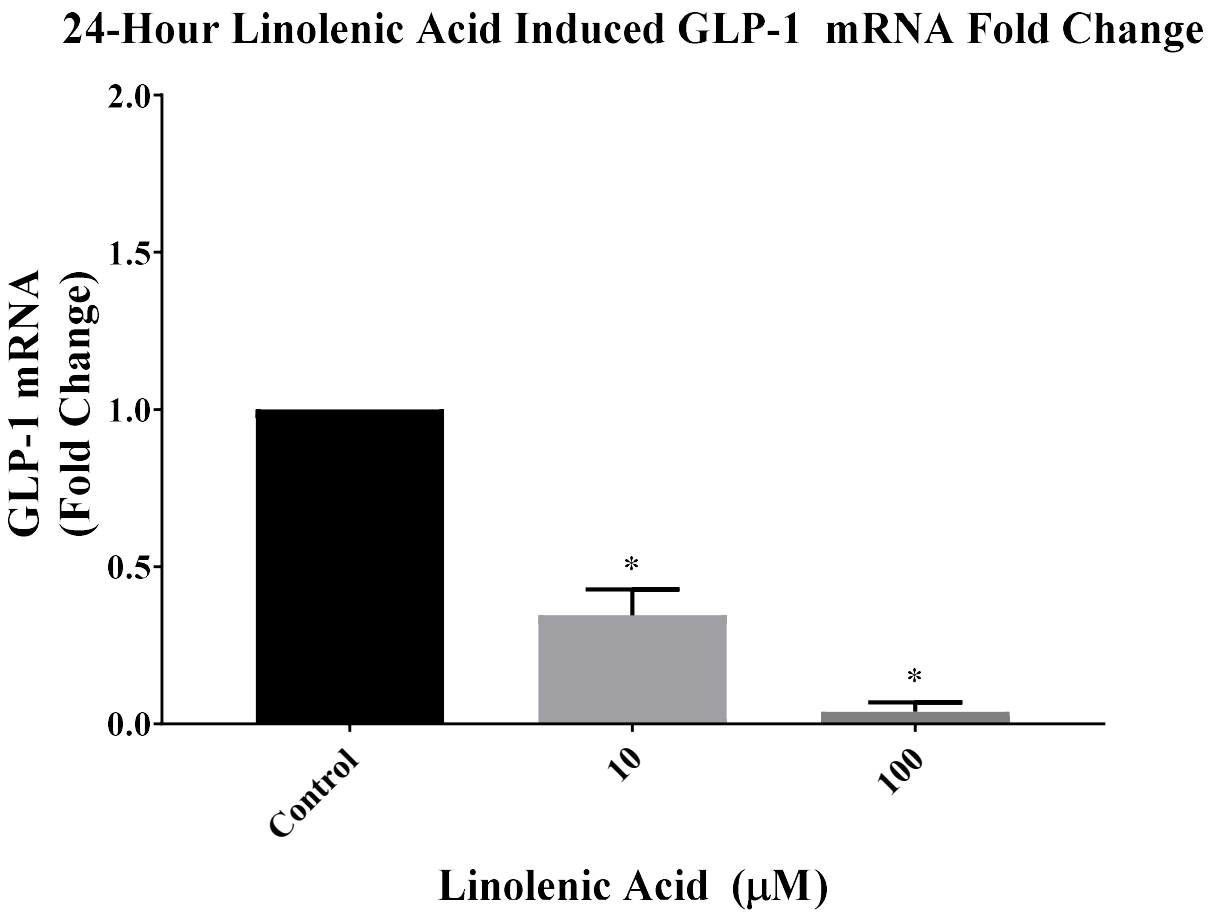

24-Hour Linolenic Acid Induced GLP-1 mRNA Fold Change
GLP-1 mRNA (Fold Change)
2.0
1.5
1.0
0.5
0.0
*
*
Control
10
100
Linolenic Acid (μM)

Figure 11. PYY mRNA Expression (Mean ± SEM) in STC-1 Cells After Control, 50 mM Acetate, 50 mM Propionate, or 50 mM Butyrate Treatment for 3-hours ($n = 4$). Total RNA was isolated from STC-1 cells and was reverse transcribed to form single stranded cDNA. For each cDNA sample amplified, 20 µL reaction volume contained TaqMan™ Master Mix and the specific target primer, either GAPDH or PYY. The qRT-PCR reactions were performed in either duplicates or triplicates depending on amount of cDNA present. Results were expressed in fold change in PYY gene expression in comparison to control sample. All materials were obtained from ThermoFisher Scientific, Grand Island, NY. Values represent fold change mean from triplicate reactions ± SEM. One-way ANOVA with Tukey post-hoc test. P*≤0.05 were deemed significant. Comparisons of control to 50 mM acetate, 50 mM acetate to 50 mM propionate, and 50 mM acetate to 50 mM butyrate were statistically significant. For clarity, * appear only over treatments that were statistically significant in comparison to the control sample.

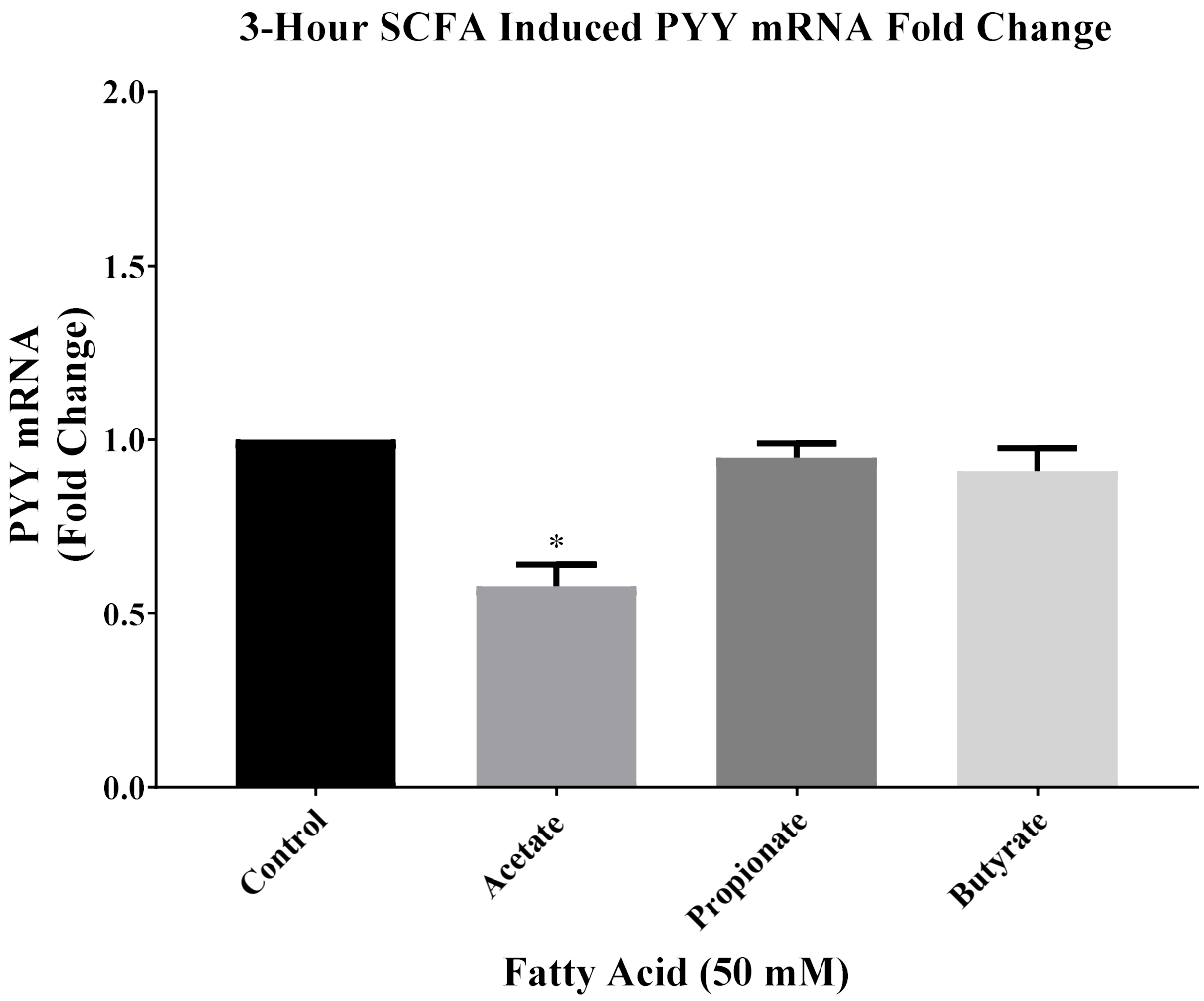

3-Hour SCFA Induced PYY mRNA Fold Change
PYY mRNA (Fold Change)
2.0
1.5
1.0
0.5
0.0
*
Control
Acetate
Propionate
Butyrate
Fatty Acid (50 mM)

Figure 12. PYY mRNA Expression (Mean ± SEM) in STC-1 Cells After Control, 50 mM Acetate, 50 mM Propionate, or 50 mM Butyrate Treatment for 16-hours ($n = 3$). Total RNA was isolated from STC-1 cells and was reverse transcribed to form single stranded cDNA. For each cDNA sample amplified, 20 µL reaction volume contained TaqMan™ Master Mix and the specific target primer, either GAPDH or PYY. The qRT-PCR reactions were performed in either duplicates or triplicates depending on amount of cDNA present. Results were expressed in fold change in PYY gene expression in comparison to control sample. All materials were obtained from ThermoFisher Scientific, Grand Island, NY. Values represent fold change mean from triplicate reactions ± SEM. One-way ANOVA with Tukey post-hoc test. P*≤0.05 were deemed significant. Comparisons of control to 50 mM propionate, 50 mM acetate to 50 mM propionate, and 50 mM propionate to 50 mM butyrate were statistically significant. For clarity, * appear only over treatments that were statistically significant in comparison to the control sample.

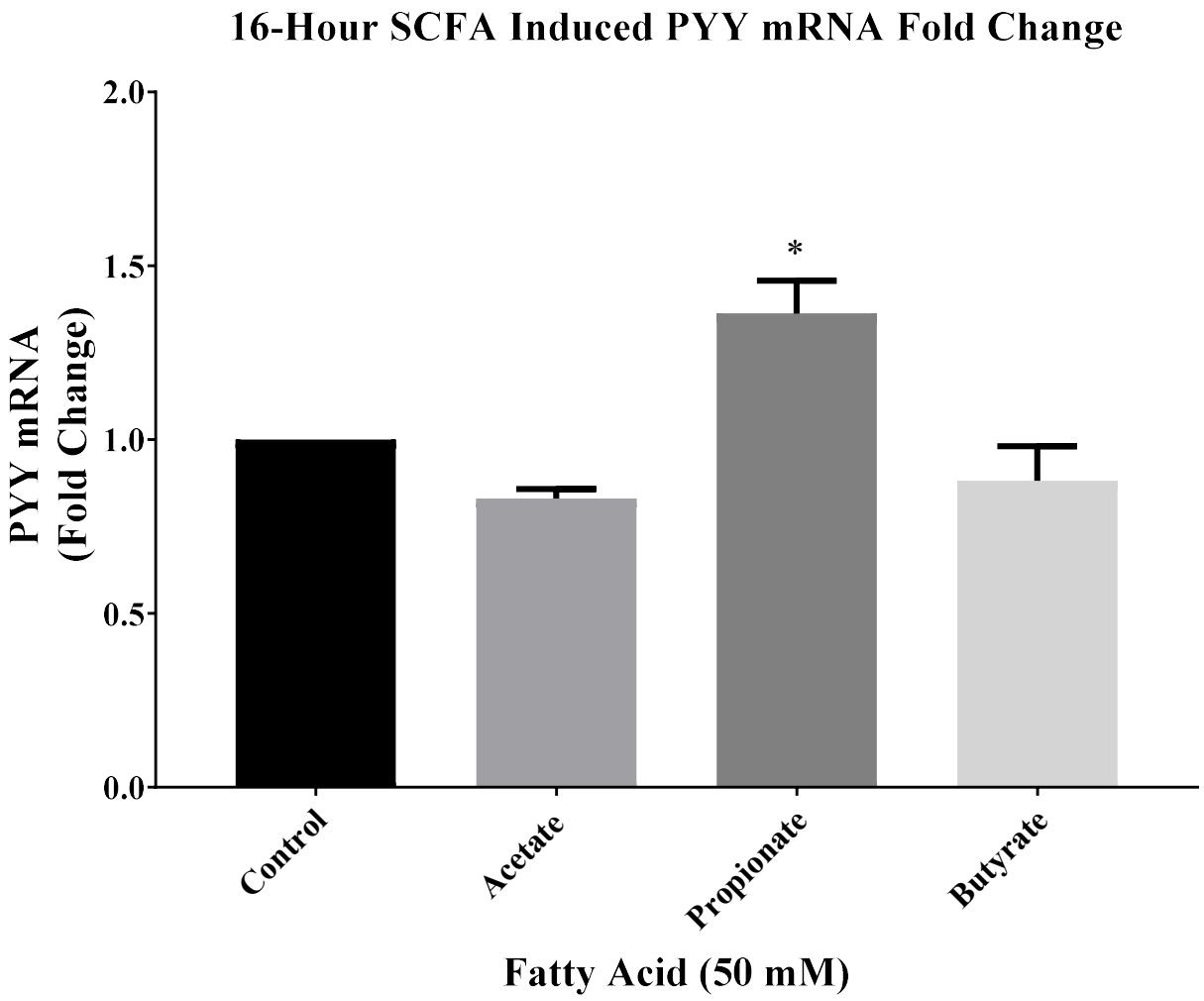

16-Hour SCFA Induced PYY mRNA Fold Change
PYY mRNA (Fold Change)
2.0
1.5
1.0
0.5
0.0
*
Control
Acetate
Propionate
Butyrate
Fatty Acid (50 mM)

Figure 13. PYY mRNA Expression (Mean ± SEM) in STC-1 Cells After Control, 50 mM Acetate, 50 mM Propionate, or 50 mM Butyrate Treatment for 24-hours ($n = 3$). Total RNA was isolated from STC-1 cells and was reverse transcribed to form single stranded cDNA. For each cDNA sample amplified, 20 µL reaction volume contained TaqMan™ Master Mix and the specific target primer, either GAPDH or PYY. The qRT-PCR reactions were performed in either duplicates or triplicates depending on amount of cDNA present. Results were expressed in fold change in PYY gene expression in comparison to control sample. All materials were obtained from ThermoFisher Scientific, Grand Island, NY. Values represent fold change mean from triplicate reactions ±. SEM. One-way ANOVA with Tukey post-hoc test. P*≤0.05 were deemed significant. Comparisons of control to 50 mM propionate, 50 mM acetate to 50 mM propionate, and 50 mM propionate to 50 mM butyrate were statistically significant. For clarity, * appear only over treatments that were statistically significant in comparison to the control sample.

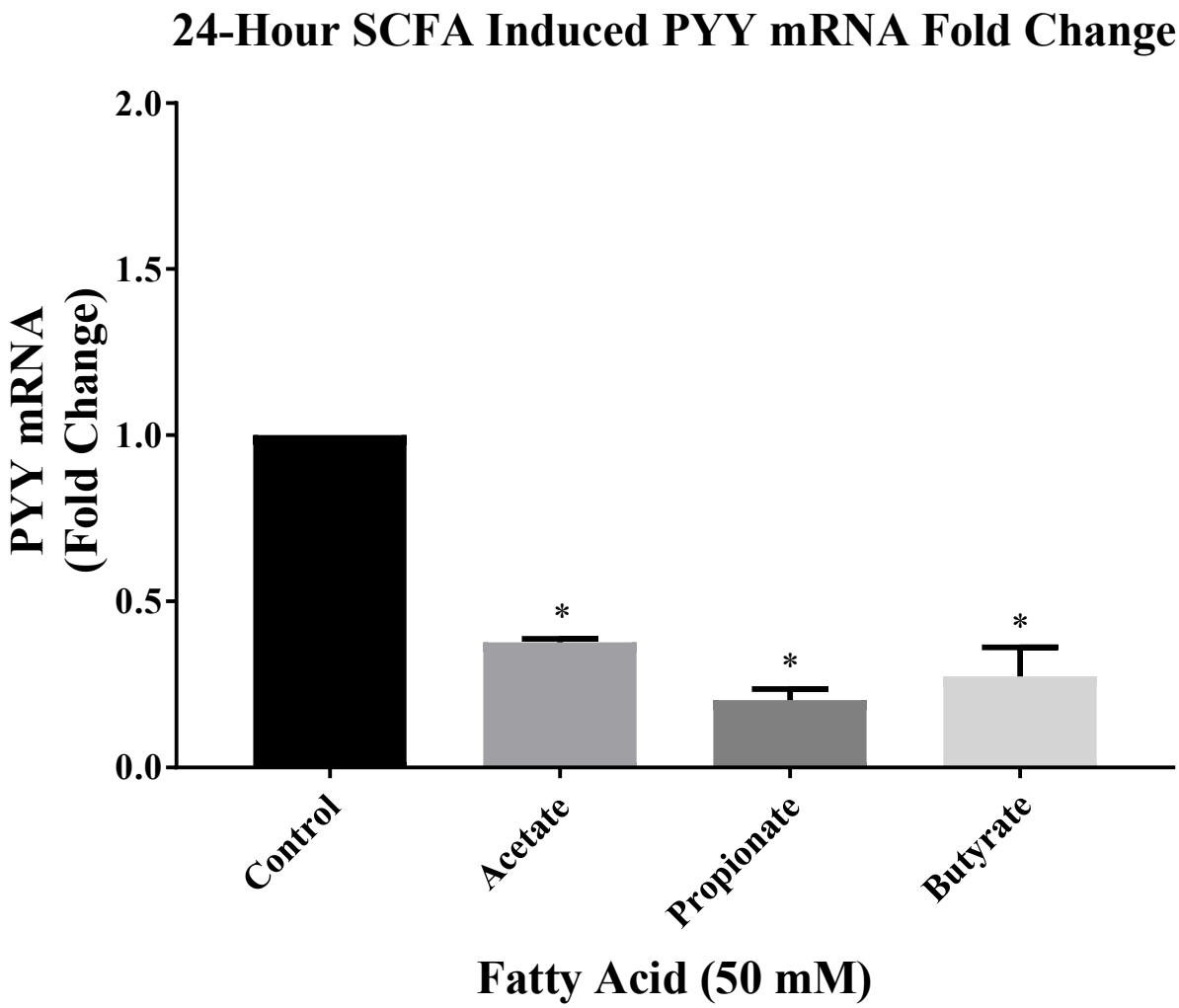

24-Hour SCFA Induced PYY mRNA Fold Change
PYY mRNA (Fold Change)
2.0
1.5
1.0
0.5
0.0
*
*
*
Control
Acetate
Propionate
Butyrate
Fatty Acid (50 mM)

Figure 14. PYY mRNA Expression (Mean ± SEM) in STC-1 Cells After Control, 50 mM Acetate, 50 mM Propionate, or 50 mM Butyrate Treatment for 3-hours, 16-hours and 24-hours ($n = 3$). Total RNA was isolated from STC-1 cells and was reverse transcribed to form single stranded cDNA. For each cDNA sample amplified, 20 µL reaction volume contained TaqMan™ Master Mix and the specific target primer, either GAPDH or PYY. The qRT-PCR reactions were performed in either duplicates or triplicates depending on amount of cDNA present. Results were expressed in fold change in PYY gene expression in comparison to control sample. All materials were obtained from ThermoFisher Scientific, Grand Island, NY. Values represent fold change mean from triplicate reactions ± SEM. One-way ANOVA with Tukey post-hoc test. P*≤0.05 were deemed significant. Comparisons of 50 mM acetate's 3-hour to 16-hour, 50 mM acetate's 3-hour to 24-hour, 50 mM acetate's 16-hour to 24-hour, 50 mM propionate's 3-hour to 16-hour, 50 mM propionate's 3-hour to 24-hour, 50 mM propionate's 16-hour to 24-hour, 50 mM butyrate's 3-hour to 24-hour, and 50 mM butyrate's 16-hour to 24-hour were statistically significant.

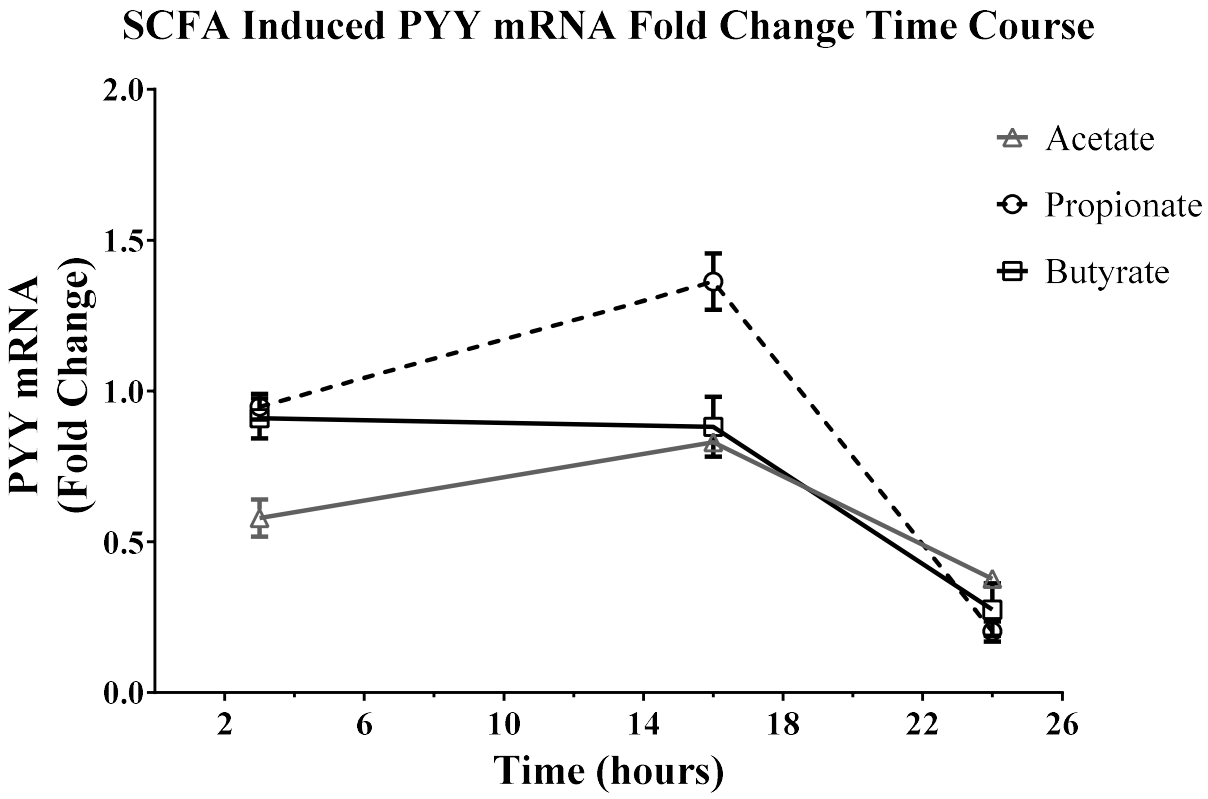

SCFA Induced PYY mRNA Fold Change Time Course
PYY mRNA (Fold Change)
2.0
1.5
1.0
0.5
0.0
2
6
10
14
18
22
26
Time (hours)
Acetate
Propionate
Butyrate

Figure 15. PYY mRNA Expression (Mean ± SEM) in STC-1 Cells After Control and 1 mM, 10 mM, 50 mM, 100 mM Butyrate Treatment for 24-hours ($n = 3$). Total RNA was isolated from STC-1 cells and was reverse transcribed to form single stranded cDNA. For each cDNA sample amplified, 20 µL reaction volume contained TaqMan™ Master Mix and the specific target primer, either GAPDH or PYY. The qRT-PCR reactions were performed in either duplicates or triplicates depending on amount of cDNA present. Results were expressed in fold change in GLP-1 gene expression in comparison to control sample. These materials were obtained from ThermoFisher Scientific, Grand Island, NY, besides butyrate. Values represent fold change mean from triplicate reactions ± SEM. One-way ANOVA with Tukey post-hoc test. P*≤0.05 were deemed significant. Comparisons of control to 10 mM butyrate, control to 50 mM butyrate, control to 100 mM butyrate, 1 mM butyrate to 10 mM butyrate, 1 mM butyrate to 50 mM butyrate, 1 mM butyrate to 100 mM butyrate were statistically significant. For clarity, * appear only over treatments that were statistically significant in comparison to the control sample.

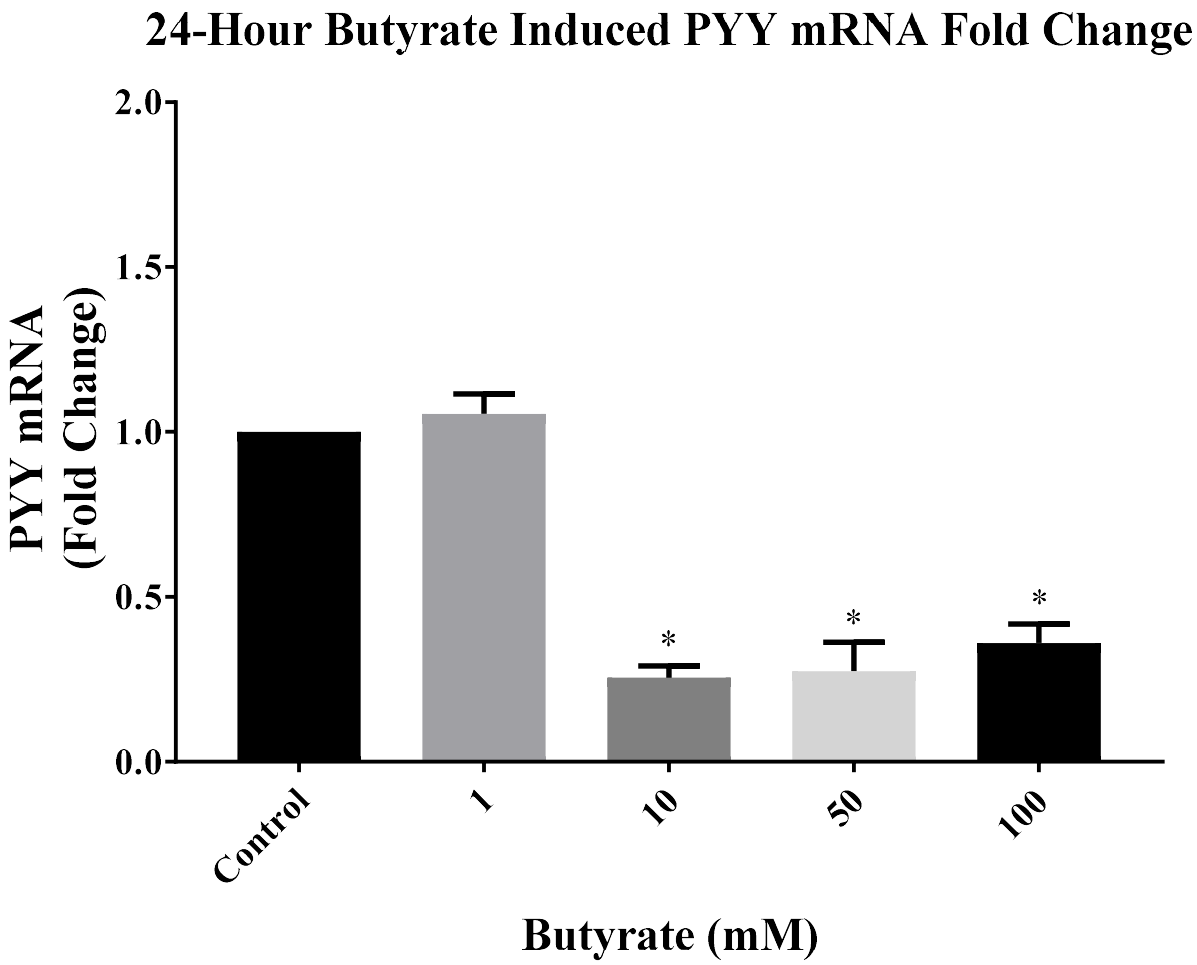
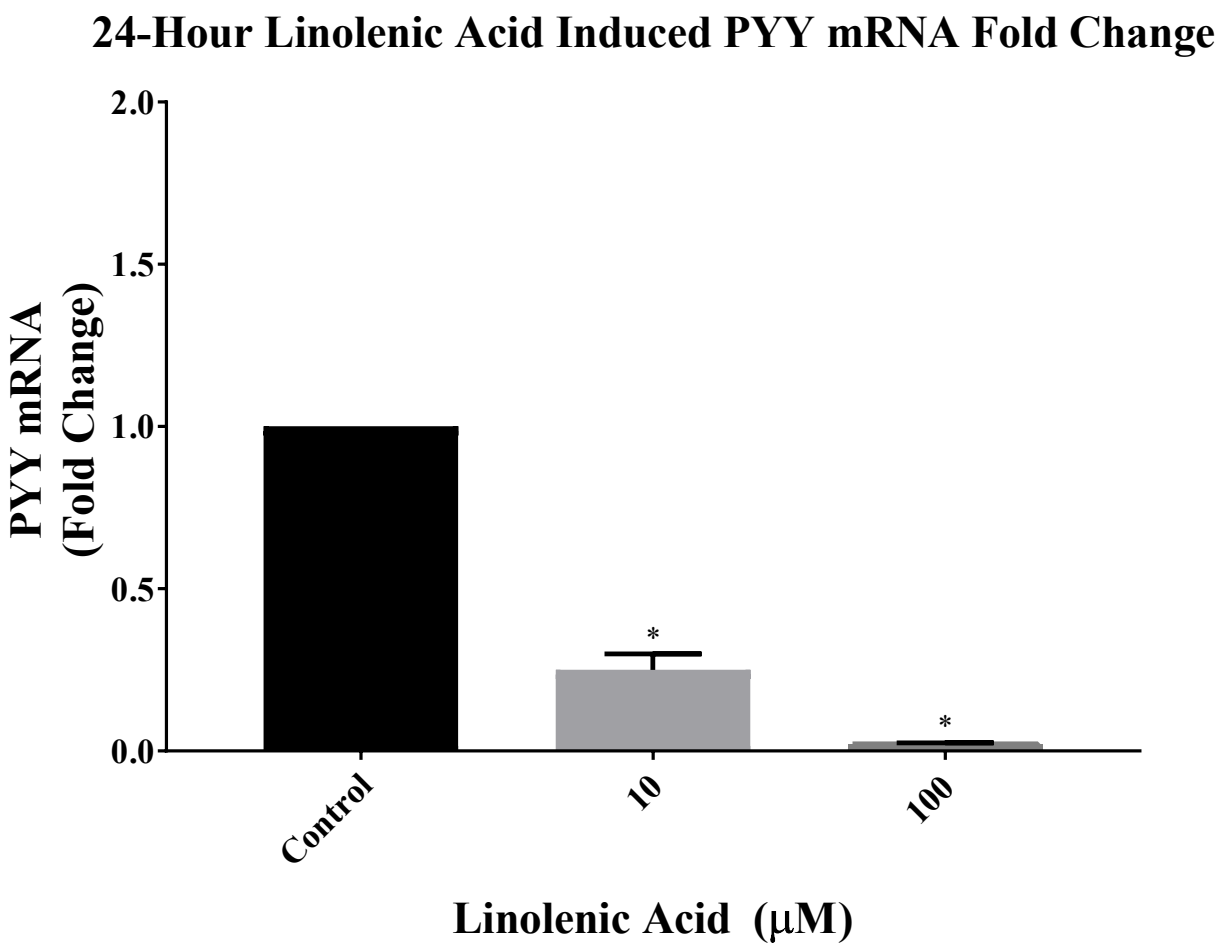

24-Hour Butyrate Induced PYY mRNA Fold Change
PYY mRNA (Fold Change)
2.0
1.5
1.0
0.5
0.0
*
*
*
Control
1
10
50
100
Butyrate (mM)

Figure 16. PYY mRNA (Mean ± SEM) in STC-1 Cells After Control, 10 µM, and 100 µM Linolenic Acid Treatment for 24-hours ($n = 3$). Total RNA was isolated from STC-1 cells and was reverse transcribed to form single stranded cDNA. For each cDNA sample amplified, 20 µL reaction volume contained TaqMan™ Master Mix and the specific target primer for GAPDH or PYY. The qRT-PCR reactions were performed in either duplicates or triplicates depending on amount of cDNA present. Results were expressed in fold change in PYY gene expression in comparison to control sample. All materials were obtained from ThermoFisher Scientific, Grand Island, NY. Values represent fold change mean from triplicate reactions ± SEM. One-way ANOVA with Tukey post-hoc test. P*≤0.05 were deemed significant. Comparisons of control to 10 µM, control to 100 µM, and 10 µM to 100 µM were statistically significant. For clarity, * appear only over treatments that were statistically significant in comparison to the control sample.


24-Hour Linolenic Acid Induced PYY mRNA Fold Change
PYY mRNA (Fold Change)
2.0
1.5
1.0
0.5
0.0
*
*
Control
10
100
Linolenic Acid (μM)

IV. DISCUSSION

IV. A) Overview

Hormone transcript regulation is dependent upon different SCFA with varying concentrations and varying LCFA concentrations. Prominent research shows that these concentrations and differing abundances are reliant on the microbiome present and the food materials ingested [Topping 2001, Cummings 1987, Wong 2006, McFarlane 2003, Roberfroid 2007, Sommer 2013, Smith 2007]. Across multiple species of mammals, the second incretin hormone discovered, GLP-1, has multiple effects that are due to its release and potency after food ingestion [Fehmann 1995, Drucker 2006, Mojsov 1986, Mosjov 1990, Nauck 1993]. Previously, GLP-1 and PYY hormone secretion has been suggested to be altered based on FFA present in the intestinal lumen [Hand 2013, Hirasawa 2005]. Thus, it is important to examine factors that regulate GLP-1 and PYY RNA expression. In the present study, changes in GLP-1 transcript and PYY transcript expression were examined in STC-1 cells that were subjected to various short-chain and long-chain FFAs concentrations with diverse time exposure through qRT-PCR analysis. Physiologically, STC-1 cells are a fair representative for in vitro experiments for humans and other mammal species [Hand 2013, McCarthy 2015]

IV. B) Findings

The main findings from the present investigation are: 1) SCFA and LCFA decrease GLP-1 transcript expression, 2) there is a minimum concentration needed for butyrate to induce GLP-1 mRNA transcript regulation, 3) SCFA do not regulate GLP-1 transcript expression equally, 4) differing linolenic acid concentrations change the degree GLP-1 transcript levels are inhibited, 5) SCFA increase PYY transcript expression overtime followed by a decrease, 6) there is a

therapeutic window for PYY transcript mRNA regulation that is dependent upon butyrate's concentration and SCFA time exposure, 7) different linolenic acid concentrations change the degree PYY transcript levels are inhibited. The present investigation of the regulation of GLP-1 and PYY transcript levels is in response to determining the presence of additional regulatory mechanisms for GLP-1 and PYY transcript levels outside of GPCRs. It further suggests that transcript regulation of GLP-1 and PYY are dependent upon fatty acid concentration and time exposure. Previously, research has demonstrated that there is decrease in PYY peptide expression with exposure to SCFA and LCFA over a 72-hour period [Hand 2013]. However, the present investigation's results indicate that regulation of these PYY peptides, along with GLP-1 are partially controlled through transcription factors that regulate gene transcription, prior to translation of the mRNA.

IV. C) GLP-1

Gut hormone concentrations have previously been shown to not be as abundant for GLP-1 for both secretion into the cell culture medium and intra-cellular content as PYY hormonal secretion from the STC-1 cell line [Hand 2013].

GLP-1 secretion is suggested to be mediated by G-protein coupled receptor 120 (GPR120) [Hirasawa 2005]. GPR120 has been previously identified as a sensor to fat, and its presence within the intestinal tract and on STC-1 cells is an indicator of regulation of GLP-1 secretion in response to FFAs [DiPatrizio 20014, Hirasawa 2005]. For the present investigation, the noticeable lagging of the 50 mM butyrate concentration following 3-hour treatment suggests that this receptor may have different affinities for different SCFAs. It is possible that the reason 50 mM butyrate exposure induced no significant change with regards to GLP-1 mRNA following 3-hour exposure

is because of its size and hydrophobic nature compared to acetate and propionate. Despite being a minor difference in structure, the extra CH_2 present in butyrate compared to propionate could be enough to cause a decrease in affinity for the receptor for the molecule. Despite the induced secretion of GLP-1 by alpha-linolenic acid, the present investigation indicates that mRNA is decreased over 24-hours [Hirasawa 2005]. However, there is the possibility of having dissimilar results during a shorter time frame. Over 24-hour timeframe or chronic exposure, GLP-1 and GIP positive cells, enteroendocrine hormones, nutrient sensitivity, and enteroendocrine transcription factors (TF) have a decreased secretion in response to fatty acids in enteroendocrine L-cells [Pais 2016, Richards 2016]. Thus, this change in activity by the transcription factors is a possible suggestion for the significant decrease in hormones, GLP-1 and PYY, observed in the present study after 24-hours. For the present study, it is important to suggest that these transcription factors modulate the secretion of the GLP-1 transcripts in STC-1 cells. It would be important to examine if SCFA and LCFA would induce an increase GLP-1 mRNA expression within a shorter time-i.e., shorter than 3-hour time exposure. Previously, GLP-1 hormone production is noticeable within 15 minutes of stimulation by fatty acids [Pais 2016]. Possibly, GLP-1 transcripts would demonstrate an increase in production within 15 minutes as well. It is possible that the 3-hour time course from the present investigation might have been already considered to be chronic exposure. Chronic exposure to the fatty acids could explain the downregulation resulting from the transcription factors actions on the proglucagon mRNA.

IV. D) PYY

PYY transcript expression did not show a significant change after 3-hour exposure to 50 mM propionate and 50 mM butyrate. Meanwhile, for 50 mM acetate, the transcript exposure was

decreased. It is possible the explanation of this occurrence is the length and hydrophobic nature of fatty acids as they increase in carbon length. As fatty acids increase in size and the number of carbons, the hydrophobic nature increases as well. This could possibly alter the affinity or specificity of the receptors to allow the substrate, SCFA, to bind to the STC-1 cells. However, this does not explain the increase in PYY transcript production after 16-hour exposure following exposure to 50 mM propionate. After the 16-hour exposure, there could be possible reduction in feedback mechanisms interacting with the transcription factors regulating PYY mRNA production causing an increase in transcript production after the 16-hour period following exposure to 50 mM propionate. Meanwhile, after 24-hour exposure, the transcription factors regulating the gene expression inhibit the production of the PYY transcript expression following all three 50 mM SCFA treatments tested in the present investigation.

It is vital to note that although STC-1 cells contain FFA receptors, it is important to consider that there are other factors that might mediate physiological PYY regulation. These other physiological factors that could impact PYY regulation involve vagal stimulation, humoral stimulation through bile release, pancreatic juice with gastric acid, mechanical stimulation, and multiple peptide hormone stimulation [Ballantyne 1993, Greeley 1989, Gomez 1996, Hara 2011, McFadden 1992, Rudnicki 1992, Zhang 1993]. It is important to consider other possible mechanisms to influence in vivo regulation of PYY. Thus, suggesting that PYY mRNA regulation only plays a part in its peptide production, and there are multiple mechanisms governing PYY regulation.

IV. E) Conclusion

In conclusion, the present study suggests that there is regulation of GLP-1 and PYY transcripts dependent upon time exposure in STC-1 cells. Specifically, these regulations are thought to occur through modulation of transcription factors for both GLP-1 and PYY mRNA production. As the STC-1 cell line was exposed to FFAs for longer periods of time, there was inhibition of GLP-1 mRNA production beginning after 3-hour exposure and continued to decrease transcripts as time increased. However, PYY mRNA production was not decreased until 24-hour time course, excluding 50 mM acetate, suggesting that PYY regulation by the transcription factors is not as sensitive to greater than 2 carbon fatty acids. In the future, it would be interesting to examine the time effects of mRNA production in STC-1 cells for GLP-1 and PYY within shorter time-frames, such as 15, 30, 60, and 90 minutes to see if these transcription factors regulate the transcripts for both GLP-1 and PYY in the same manner.

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