THE ROLE OF KETONE SIGNALING IN THE HEPATIC RESPONSE TO FASTING

by

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STATEMENT BY AUTHOR

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ABSTRACT

Ketosis is a metabolic condition that occurs during heat stress, prolonged exercise, fasting, and in obese and diabetic individuals. The major ketone body, β-OH butyrate, affects cellular signaling pathways in a hormone-like manner through its receptor GPR109a. While physiological ketosis is often adaptive, chronic hyperketonemia may contribute to the metabolic dysfunction of diabetes. To understand how β-OH butyrate signaling affects hepatic metabolism we compared the fasting response in control and 3-hydroxy-3-methylglutaryl-CoA Synthase II (HMGCS2) knockdown mice that are unable to elevate β-OH butyrate production, and in GPR109a +/+ and -/- mice. To directly assess the response to ketone signaling we gave intraperitoneal injections of the GPR109a agonists niacin (0.8mmol/kg) and β-OH butyrate (5.7mmol/kg) to HMGCS2 knockdown mice over the final 9 hours of a 16 hour fast. Ketogenic deficient mice showed a more robust increase in hepatic carnitine palmitoyltransferase 1 (CPT1) and uncoupling protein 2 (UCP2) mRNA than control mice in response to fasting. Accordingly, niacin and β-OH butyrate administration decreased fasting CPT1 mRNA expression in knockdown mice, while fasting knockdown UCP2 mRNA and control HMGCS2 mRNA were lowered by β-OH butyrate**.** GPR109a knockout did not alter the hepatic response to fasting, although the effect of niacin on fasting HMGCS2 mRNA expression was dependent on GPR109a expression. While the role of GPR109a and how chronic hyperketonemia alters β-OH butyrate signaling require further study, these data suggest that endogenous β-OH butyrate signaling during a fast regulates the transcript levels of hepatic genes directly involved in its own synthesis.

Study Rationale

Transitions between the fed and fasted state require significant changes in metabolic activity to accommodate systemic nutrient partitioning. The principle activities in hepatic metabolism are shifting from glucose oxidation and lipid synthesis in the fed state to lipid oxidation, and glucose and ketone synthesis and export in the fasted state. Upon joining the Renquist lab, I began working on a project investigating the relationship between metabolic changes in the liver and duration of fasting in the mouse. While gluconeogenesis, glycogenolysis, β-oxidation, and ketogenesis are activated in the fasted liver, the temporal induction of these different pathways, as well as, their relationship to changes in serum metabolites remain unknown. To address the timing of the metabolic response to fasting, we measured serum and hepatic metabolites, hepatic mRNA expression, and activity of metabolic enzymes at 4, 8, 12, and 16 hours fasting.

One of the first hepatic manifestations of fasting is an increase in non-esterified fatty acids (NEFA) concentrations. In the absence of insulin signaling, breakdown of triacylglycerides (TAG) and release of NEFA from adipose tissue is stimulated. After only 4 hours food deprivation, a 20% increase in serum NEFA concentrations was associated with a more than 5 fold increase in hepatic NEFA concentrations (Figures 1A and 1B). Non-esterified fatty acids serve as endogenous ligands for the nuclear receptor peroxisome proliferator activated receptor alpha (PPARα), which when active upregulates target genes in gluconeogenesis, β-oxidation, and ketogenesis [1-5]. Phosphoenolpyruvate carboxykinase (PEPCK), glucose 6-phosphatase (G6Pase), carnitine palmitoyltransferase 1 (CPT1), uncoupling protein 2 (UCP2), and 3-hydroxy-3-methylglutaryl-CoA Synthase II (HMGCS2) all possess PPARα response elements in their promoter regions and are upregulated by PPARα [2, 5-8]. PPARα is considered a master regulator of fasting liver metabolism, and is required to initiate full induction of the hepatic fasting response. In fact, fasted PPARα null mice are hypoglycemic and fail to elevate serum β-OH butyrate despite abundant circulating NEFAs and hepatic lipid accumulation [3]. PPARα null mice also displayed markedly higher liver lipid concentrations with fasting than controls, a result of impaired β-oxidation [9].

The end product of β-oxidation, acetyl-CoA, is the beginning substrate for ketogenesis, and the rate of lipid oxidation is highly correlated with serum β-OH butyrate [10]. Furthermore, maximal gluconeogenesis requires an active β-oxidative pathway [11]. Thus, hepatic catabolism of lipids is essential for hepatic output of glucose and β-OH butyrate during fasting. We hypothesized that elevated hepatic NEFA concentrations, acting both as a direct substrate and PPARα agonist, initiate the metabolic transition from the fed to the fasted state and that impaired adipose lipolysis during fasting would hinder the hepatic adaptations to food deprivation.

Interestingly, liver NEFA concentrations fell at 8 hours fasting, which coincided with elevations in serum β-OH butyrate and hepatic UCP2 expression (Figures 1B, 1D, and 2C). To support high flux through β-oxidation, the liver upregulates mitochondrial UCP2 to uncouple electron transport chain activity from ATP synthesis, allowing for uninhibited NAD^+ regeneration. This is critical in allowing hepatic ketone production, as UCP2 knockout mice are only able to partially elevated serum β-OH butyrate in response to fasting [12]. Hepatic NEFA concentrations did not further increase until 16 hours fasting. Similarly, hepatic PPARα expression increased most between 12 and 16 hours fasting, and hepatic NEFA and PPARα were highly correlated ($R^2 = 0.62$; $P < 0.0001$; Fig. 3A).

Two patterns of expression were observed for PPARα target gene upregulation over the fasting duration time points. Hepatic PEPCK and G6Pase mRNA only significantly increased at 16 hours of fasting (Figures 2A and 2B). This more than doubling of PEPCK and G6Pase expression between 12 and 16 hours fasting correlated well with the change in PPARα expression over this time frame ($R^2 = 0.66$; $P = 0.0023$; Fig. 3B) and ($R^2 = 0.44$; $P = 0.0192$; Fig. 3C), respectively. Alternatively, hepatic UCP2 and CPT1 expression increased more gradually over the duration of fasting. UCP2 and CPT1 expression increased by 27% and 70% between hours 8 and 12, respectively, and again by 21% and by 46% between hours 12 and 16 of fasting, respectively (Figures 2C and 2D). These correlate less well, but still significantly, with PPARα expression between 12 and 16 hours fasting ($R^2 = 0.34$; $P = 0.0462$; Fig. 3D) for UCP2 and ($R^2 = 0.35$; $P = 0.0396$; Fig. 3E) for CPT1. Interestingly, HMGCS2 expression

increased over 3 fold between 8 and 12 hours fasting, yet declined by 33% at 16 hours fasting (Fig. 2E).

Expression of HMGCS2 appeared to have a negative regulation at 16 hours that was absent at 12 hours fasting. We hypothesized that β-OH butyrate, the terminal product of ketogenesis, was feeding back to suppress expression of the rate limiting enzyme in its own production pathway [13]. From 0 to 12 hours fasting, hepatic HMGCS2 and serum β-OH butyrate were highly correlated ($R^2 = 0.74$; $P < 0.0001$; Fig. 4A), yet at 16 hours there was no correlation between HMGCS2 expression and serum β-OH butyrate ($P = 0.3743$; Fig. 4D). This suggests to us that HMGCS2 is limiting production of β-OH butyrate as fasting is initiated, but once the ketogenic pathway is fully active with an extended fast, β-OH butyrate production is not affected by HMGCS2 mRNA expression. These data further support the hypothesis that β-OH butyrate may feedback to inhibit HMGCS2 gene transcription. Genes that directly support ketogenesis exhibit this same relationship with β-OH butyrate. UCP2 and CPT1 are highly correlated with serum β-OH butyrate from 0 to 12 hours fasting ($R^2 = 0.51$; $P < 0.0001$; Fig. 4B) and ($R^2 = 0.77$; $P < 0.0001$; Fig. 4C), respectively, yet at 16 hours fasted no correlation exists ($P = 0.5498$; Fig. 4E) for UCP2 and ($P = 0.8199$; Fig. 4F) for CPT1.

β-OH butyrate is already known to regulate its own synthesis at the level of substrate availability. β-OH butyrate is the endogenous ligand for the G-protein coupled receptor GPR109a. Agonism of GPR109a on adipose tissue inhibits lipolysis through $G_{\alpha i}$ signaling [14], decreasing the amount of NEFAs released from adipose and limiting further accumulation of hepatic NEFAs. This not only restricts flux through ketogenesis, but is suggested as a mechanism to prevent exhaustion of adipose lipid stores too early in a fast. Thus, the idea that β-OH butyrate may regulate β-oxidative and ketogenic activity at the transcriptional level would be a novel feedback mechanism in fasting metabolism that warrants further investigation.

To address the role of ketogenesis in the hepatic adaptations to fasting, we have several animal models at our disposal. First, we can utilize the fed versus fasted model, allowing us to compare alterations in the normal metabolic fasting response. This model has been integral to our initial work

and will be combined with 3 additional models to better understand the role of β-OH butyrate in metabolic shifts during a fast. Second, intraperitoneal injection of β-OH butyrate or niacin, a GPR109a agonist and β-OH butyrate analogue [15], will allow us to test whether increased stimulation of β-OH butyrate signaling pathways result in hepatic transcriptional regulation. Treatment with niacin will confirm that the consequences of ketone signaling are not due to metabolism of β-OH butyrate, as niacin does not promote ATP synthesis. Third, mice treated with a HMGCS2 targeted antisense oligonucleotide are unable to upregulate ketogenesis with fasting [16]. Application of this model will allow us to assess the hepatic response to fasting with no elevation in serum β-OH butyrate, revealing fasting induced alterations in transcript levels in the absence of β-OH butyrate feedback. Intraperitoneal injection of β-OH butyrate or niacin in these mice should then restore the consequences of β-OH butyrate signaling on hepatic fasting gene expression.

It is possible that β-OH butyrate regulation of hepatic gene expression is downstream of its action at adipose GPR109a. Inhibition of lipolysis and influx of NEFAs to the liver would decrease activation of PPARα and result in lower upregulation of its target genes. This seems unlikely, however, given hepatic NEFA concentrations, PPARα expression, and expression of its targets PEPCK and G6Pase are maximal at 16 hours of fasting. Our fourth model, GPR109a knockout mice, will determine the involvement of GPR109a signaling in β-OH butyrate's regulation of hepatic metabolism. As GPR109a null mice upregulate ketogenesis normally, fasting these mice will reveal which aspects of β-OH butyrate signaling at the hepatic level are mediated by GPR109a and which are GPR109a independent. Exogenous β-OH butyrate or niacin injections will further confirm if alterations in fasting hepatic gene expression driven by β-OH butyrate signaling are downstream of GPR109a, given only GPR109a independent signaling mechanisms should be affected in knockout mice. Furthermore, treatment with niacin in wildtype mice will address our first hypothesis, that impaired lipolysis from adipose tissue during a fast will prevent induction of a full hepatic response to fasting. Each of these models will provide unique information about the role of β-OH butyrate signaling in fasting metabolism, and when interpreted as a whole will highlight a novel regulatory mechanism of feedback

by a hepatic produced metabolite to modulate expression of enzymes critical to the hepatic adaptation to fasting.

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Figure Captions

Figure 1. Serum and liver metabolic parameters in response to fasting. Serum (A) non-esterified fatty acids (NEFA; mM), hepatic (B) non-esterified fatty acids (NEFA; μM/g liver tissue), (C) PPARα mRNA expression, and (D) serum β-OH butyrate (μM). [a,b,c,d] Bars that do not share a common letter differ significantly ($P < 0.05$).

Figure 2. PPARα target gene upregulation in response to fasting. Hepatic (A) G6Pase mRNA expression (B) PEPCK mRNA expression (C) UCP2 mRNA expression (D) CPT1 mRNA expression and (E) HMGCS2 mRNA expression. [a,b,c,d] Bars that do not share a common letter differ significantly ($P < 0.05$).

Figure 3. Fasted PPARα correlations with target genes. Hepatic (A) non-esterified fatty acids (NEFA; μM/g liver tissue) against PPARα mRNA expression across 0-16 hours fasting, (B) PPARα mRNA expression against PEPCK mRNA expression across 12-16 hours fasting, (C) PPARα mRNA expression against G6Pase mRNA expression across 12-16 hours fasting, (D) PPARα mRNA expression against UCP2 mRNA expression across 12-16 hours fasting, (E) PPARα mRNA expression against CPT1 mRNA expression across 12-16 hours fasting.

Figure 4. Fasted β-OH butyrate correlations with genes supporting hepatic ketone production. (A) Serum β-OH butyrate (μM) against hepatic HMGCS2 mRNA expression across 0-12 hours fasting, (B) Serum β-OH butyrate (μM) against hepatic UCP2 mRNA expression across 0-12 hours fasting, (C) Serum β-OH butyrate (μM) against hepatic CPT1 mRNA expression across 0-12 hours fasting, (D) Serum β-OH butyrate (μM) against hepatic HMGCS2 mRNA expression at 16 hours fasting, (E) Serum β-OH butyrate (μM) against hepatic UCP2 mRNA expression at 16 hours fasting, (F) Serum β-OH butyrate (μM) against hepatic CPT1 mRNA expression at 16 hours fasting.

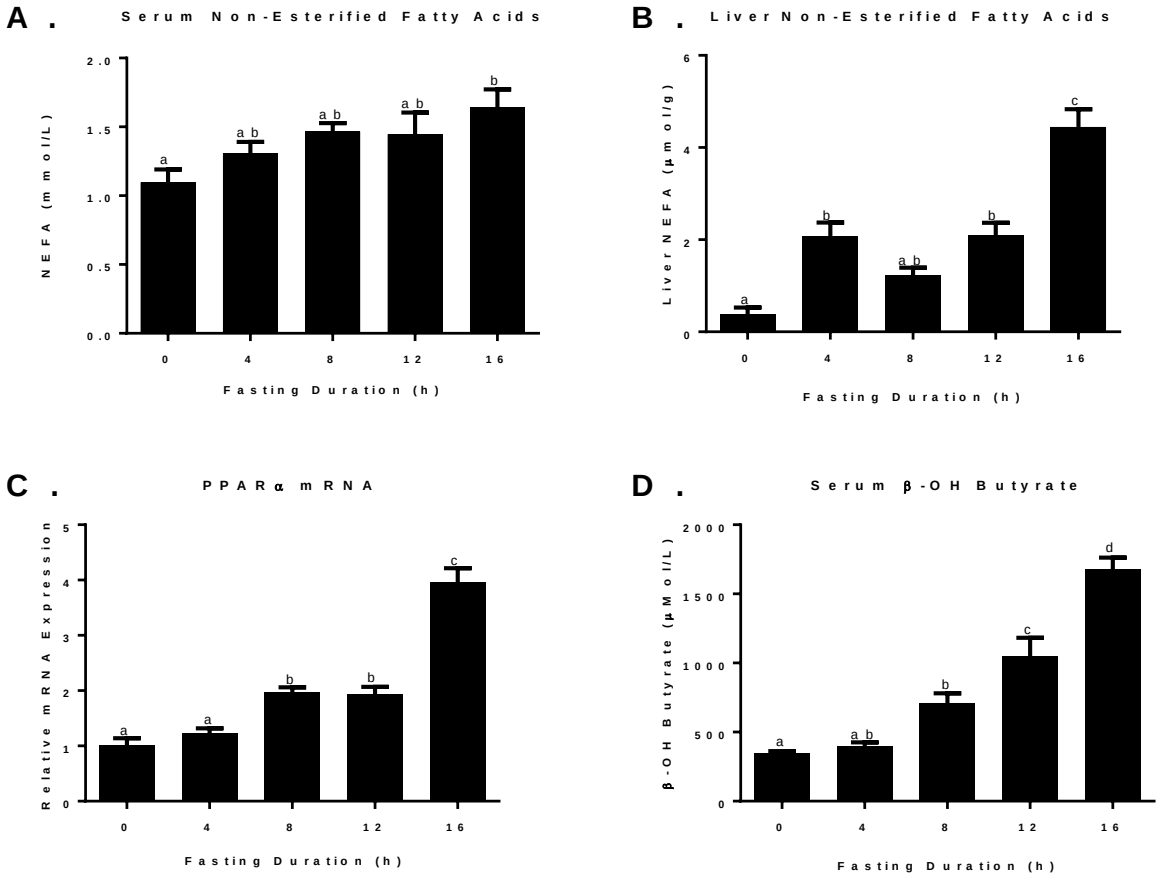
A .
Serum Non-Esterified Fatty Acids
NEFA (mmol/L)
2.0
1.5
1.0
0.5
0.0
a
a b
a b
a b
b
0
4
8
12
16
Fasting Duration (h)
B .
Liver Non-Esterified Fatty Acids
Liver NEFA (µmol/g)
6
4
2
0
a
b
a b
b
c
0
4
8
12
16
Fasting Duration (h)
C .
PPARα mRNA
Relative mRNA Expression
5
4
3
2
1
0
a
a
b
b
c
0
4
8
12
16
Fasting Duration (h)
D .
Serum β-OH Butyrate
β-OH Butyrate (µMol/L)
2000
1500
1000
500
0
a
a b
b
c
d
0
4
8
12
16
Fasting Duration (h)

Figure 1.

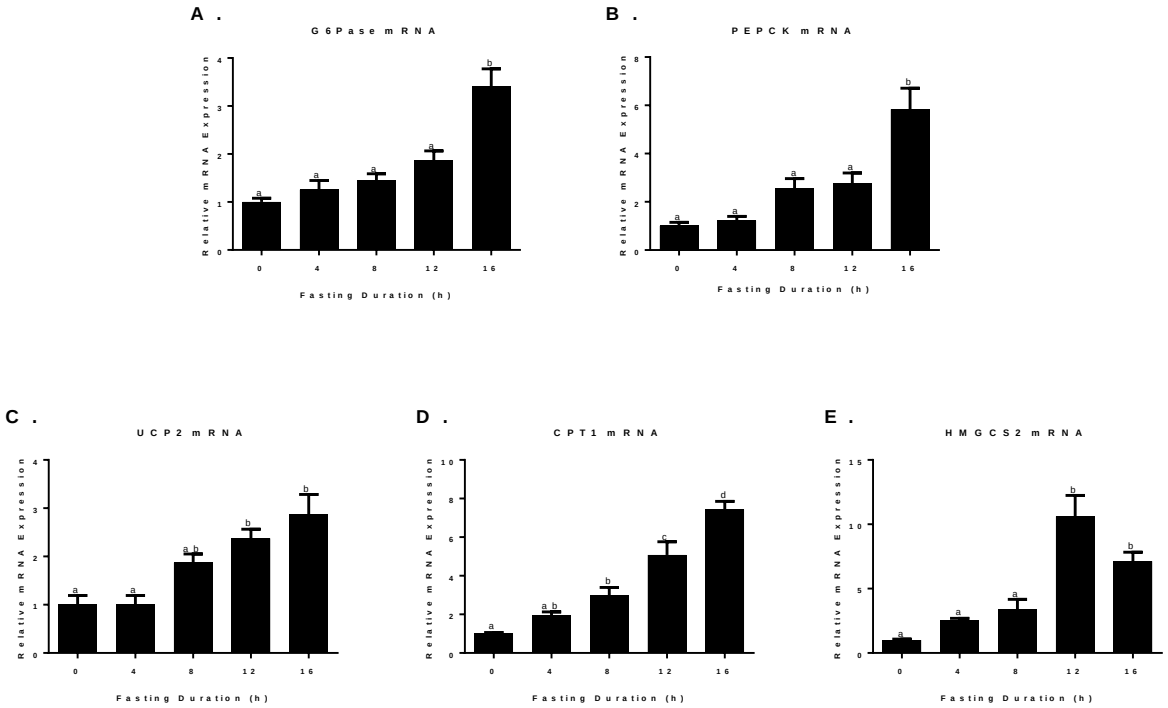
A .
G6Pase mRNA
Relative mRNA Expression
Fasting Duration (h)
B .
PEPCK mRNA
Relative mRNA Expression
Fasting Duration (h)
C .
UCP2 mRNA
Relative mRNA Expression
Fasting Duration (h)
D .
CPT1 mRNA
Relative mRNA Expression
Fasting Duration (h)
E .
HMGCS2 mRNA
Relative mRNA Expression
Fasting Duration (h)

Figure 2.

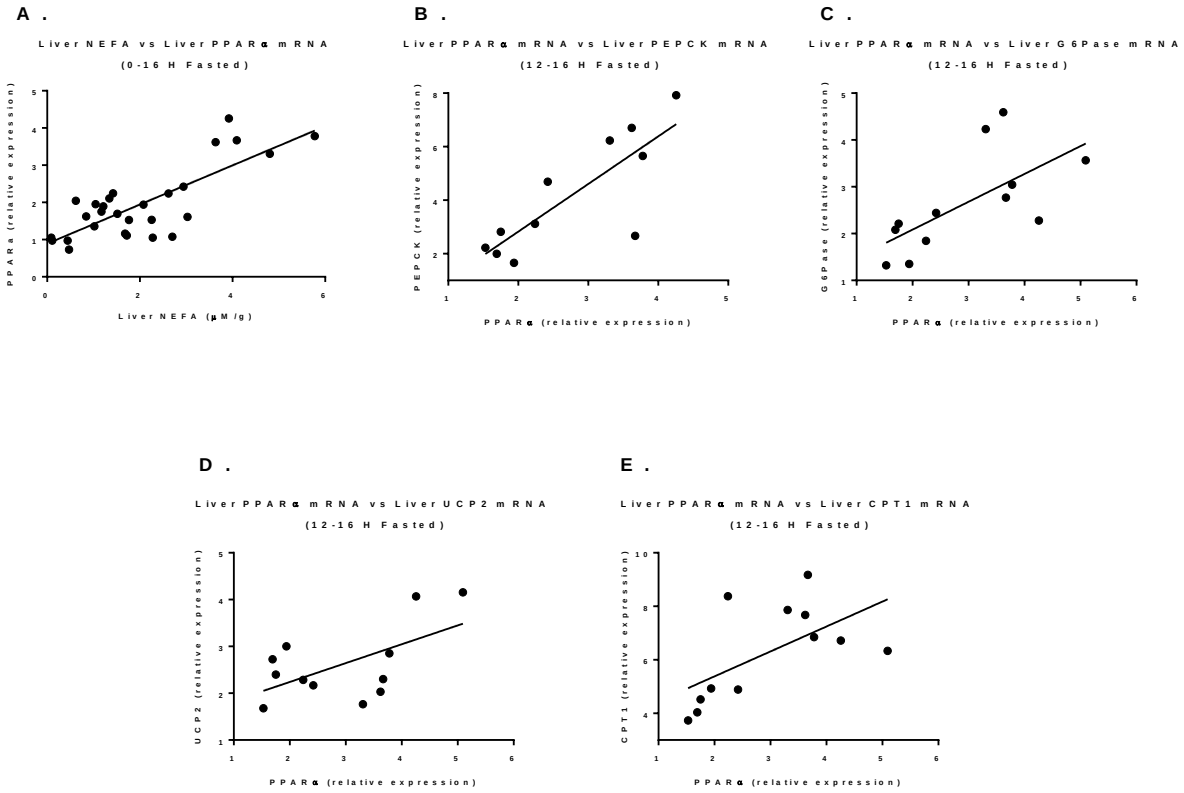
A .
Liver NEFA vs Liver PPARα mRNA
(0-16 H Fasted)
PPARα (relative expression)
Liver NEFA (μM/g)
B .
Liver PPARα mRNA vs Liver PEPCK mRNA
(12-16 H Fasted)
PEPCK (relative expression)
PPARα (relative expression)
C .
Liver PPARα mRNA vs Liver G6Pase mRNA
(12-16 H Fasted)
G6Pase (relative expression)
PPARα (relative expression)
D .
Liver PPARα mRNA vs Liver UCP2 mRNA
(12-16 H Fasted)
UCP2 (relative expression)
PPARα (relative expression)
E .
Liver PPARα mRNA vs Liver CPT1 mRNA
(12-16 H Fasted)
CPT1 (relative expression)
PPARα (relative expression)

Figure 3.

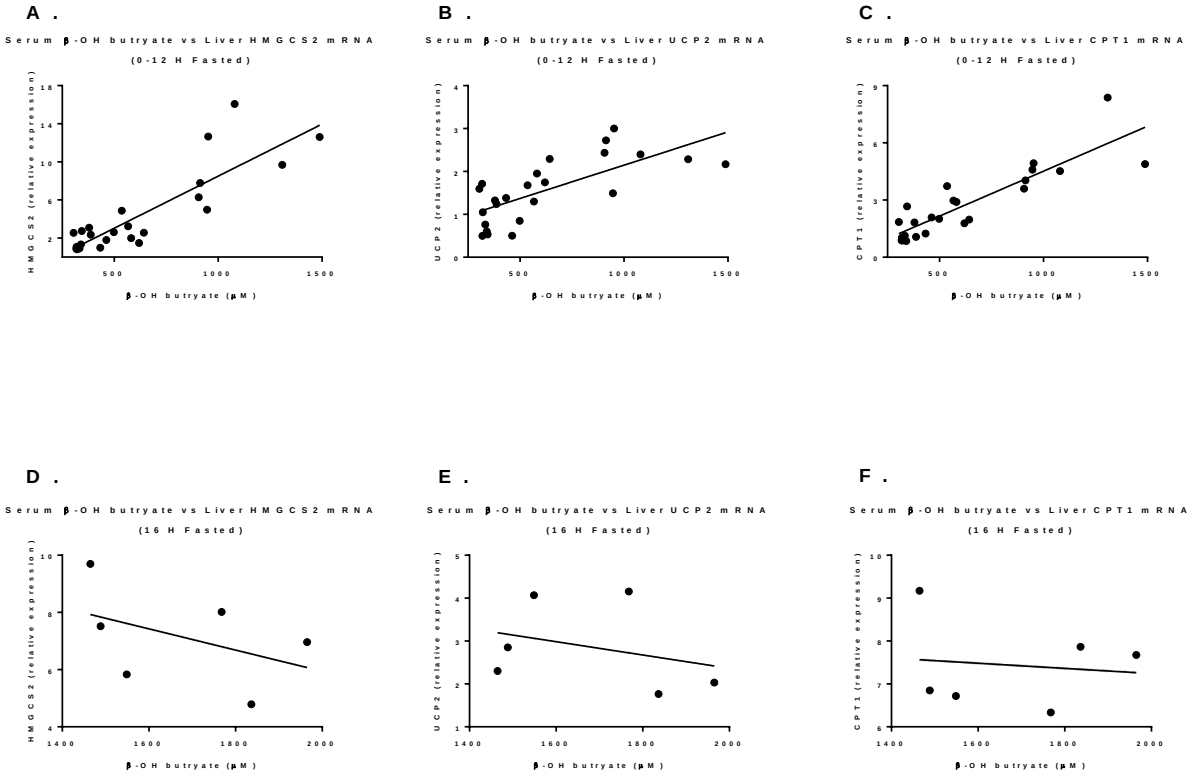
A .
Serum β-OH butryate vs Liver HMGCS2 mRNA
(0-12 H Fasted)
HMGCS2 (relative expression)
β-OH butryate (μM)
B .
Serum β-OH butryate vs Liver UCP2 mRNA
(0-12 H Fasted)
UCP2 (relative expression)
β-OH butryate (μM)
C .
Serum β-OH butryate vs Liver CPT1 mRNA
(0-12 H Fasted)
CPT1 (relative expression)
β-OH butryate (μM)
D .
Serum β-OH butryate vs Liver HMGCS2 mRNA
(16 H Fasted)
HMGCS2 (relative expression)
β-OH butryate (μM)
E .
Serum β-OH butryate vs Liver UCP2 mRNA
(16 H Fasted)
UCP2 (relative expression)
β-OH butryate (μM)
F .
Serum β-OH butryate vs Liver CPT1 mRNA
(16 H Fasted)
CPT1 (relative expression)
β-OH butryate (μM)

Figure 4.

The Role of Ketone Signaling in the Hepatic Response to Fasting

Introduction

Hepatocytes are stimulated to produce ketones under conditions dominated by lipid oxidation and low insulin signaling [1]. Ketosis is an evolutionarily conserved process, which normally occurs as an adaptive response to fasting, heat stress, and prolonged exercise [2]. Yet, with the rise in obesity and diabetes, conditions that promote ketogenesis, and the adoption of ketogenic diets, ketosis is a common metabolic state for many individuals. Given the broad incidence of ketosis it is essential to understand the consequence of this metabolic condition.

In the last decade, the role of metabolites has expanded from energy sources and pathway intermediates to allosteric regulators and signaling molecules that act like hormones to modulate fundamental cellular processes [3, 4]. G-protein coupled receptors and nuclear receptors sense glucose, fatty acids, and ketones to coordinate systemic metabolic function [5-8]. In the normal fed state, acetoacetate and β-OH butyrate exist in a 1:1 ratio and total circulating ketone concentrations rarely exceed 0.3 mM [9]. However, under conditions that favor ketogenesis, β-OH butyrate concentrations rise over six-fold above acetoacetate concentrations [1]. Interestingly, β-OH butyrate is the endogenous ligand for two G-protein coupled receptors, GPR109a and GPR41 [8], while acetoacetate has not been shown to have any "metabocrine" function. Thus, the enhanced production of β-OH butyrate under ketogenic conditions may serve to activate responses that allow for adaptation to an extended fast, prolonged exercise, or heat exposure.

The regulatory actions of β-OH butyrate require that concentrations are elevated above basal levels. The EC_{50} for β-OH butyrate agonism of GPR109a is approximately 0.7 mM, and while this receptor was first shown to inhibit adipose tissue lipolysis, it has since been identified in various other tissues with a broad range of physiological actions [10]. In fact, GPR109a signaling induces apoptosis and subcutaneous flushing, stimulates cholesterol efflux and adiponectin secretion, and inhibits inflammatory pathways and glucose stimulated insulin release [11-15]. Sympathetic ganglia express high levels of β-OH butyrate responsive GPR41, however the physiological consequences of this

signaling require further investigation [16, 17]. β-OH butyrate also regulates gene expression at concentrations above 1 mM by globally modifying transcription as an endogenous histone deacetylase (HDAC) inhibitor, sequestering carbohydrate responsive element binding protein (ChREBP) in the cytosol of hepatocytes, and likely acts directly at the butyrate response element in promoter regions of target genes [18-20].

Limiting white adipose tissue lipolysis and preventing nuclear ChREBP entry support the metabolic activity necessary to sustain a fast, likewise subcutaneous flushing is a beneficial cooling mechanism under heat stress. Likely many of β-OH butyrate's actions have etiological origins allowing for adaptation to conditions that result in physiological ketosis. Hepatic ketogenesis is stimulated in the absence of insulin signaling. Serum β-OH butyrate exceeds 1 mM at 12 hours food deprivation in the mouse [21] and after 2-3 days of fasting in humans [1]. Hyperketonemia also occurs in both type 1 and type 2 diabetes. Type 1 diabetics routinely display ketone levels between 1-2 mM and diabetic ketoacidosis can result in concentrations above 25 mM [22]. Interestingly, ketones impair insulin stimulated glucose uptake and have been implicated in the development of insulin resistance in diabetes [22-24]. The liver is a primary site of metabolic dysfunction in type 2 diabetics, commonly characterized by nonalcoholic steatohepatitis (NASH), mitochondrial uncoupling, ER stress, inflammation, and inappropriate hepatic glucose and ketone body output [25-29]. Furthermore, 98% of NASH patients are insulin resistant independent of body weight [30]. Ketogenic diets, while effective for weight loss, promote hepatic steatosis, ER stress, inflammation, and glucose intolerance [31]. β-OH butyrate signaling that is adaptive in the short term may become dysfunctional when chronically active and could contribute to the dysregulation of hepatic metabolism in conditions with persistent ketosis.

To understand the role of β-OH butyrate in the hepatic metabolic adaptations to ketogenic conditions, we used dsDNA to knockdown expression of hydroxy-3-methylglutaryl-CoA Synthase II (HMGCS2), the rate limiting enzyme in ketogenesis [32] and knocked out GPR109a to assess the role of this GPCR in the metabolic changes resulting from elevations in β-OH butyrate [33]. To directly assess the role of β-OH butyrate and GPR109a signaling in altering hepatic metabolism, we treated

HMGCS2 KD mice with intraperitoneal β-OH butyrate or niacin and GPR109a null mice with intraperitoneal niacin over the duration of fasting. Niacin is a potent GPR109a agonist that, importantly, cannot be metabolized to produce ATP [34]. While hepatocytes do not participate in ketolysis [1], the niacin treatment group substantiates that regulation by β-OH butyrate is not due to its use as an extrahepatic fuel source. Using a model that is unable to produce ketones and a model that lacks the GPR109a signaling, we aimed to understand the role of β-OH butyrate in the hepatic adaptation to ketogenic conditions.

Materials and Methods

Animals

All studies were conducted using 12-16 week old male WT C57BL/6J mice purchased from Jackson Laboratories (Bar Harbor, ME) or 12-14 week old male GPR109a +/+ or -/- littermates derived from in house crosses of GPR109a +/- mice. The founding GPR109a -/- mice were kindly provided by Dr. Klaus Pfeffer at the Institute of Medical Microbiology, Immunology and Hygeine at Heinrich Heine University [34]. Mice were kept on a 14 hour light/10 hour dark schedule and housed 3-4 mice per cage until 1 week prior to study initiation, at which point animals were individually housed. Ad libitum access to NIH-31 chow (Harlan Laboratories, Indianapolis, IN) and water was available. All studies were approved by the University of Arizona Institutional Animal Care and Use Committee.

3-hydroxy-3-methylglutaryl-CoA Synthase II knockdown

12 week old WT C57BL/6J mice received twice-weekly intraperitoneal injections of 25 mg/kg of murine Hmgcs2-targeted antisense oligonucleotides (ISIS 191229; 5′-CTGTTTGTCACTGCTGGATG-3′) or scramble control antisense oligonucleotide (ISIS 141923; 5′-CCTTCCCTGAAGGTTCCTCC -3′) for 4 weeks prior to experimentation. These oligonucleotides incorporate several chemical modifications to improve potency, duration of action, and tolerability. The internucleotide phosphates are modified with a phosphorothioate substitution, in which one of the nonbridging oxygen atoms is substituted with sulfur. Additionally the compound incorporates five 2′-*O*-(2-methoxyethyl) (2′-MOE) modified ribonucleosides at the 3′ and 5′ ends with ten

2'-*O*-deoxyribonucleosides in between to support RNaseH-1 mediated target mRNA degradation. These modifications improve the binding affinity for target mRNA as well as stability against exonuclease-mediated degradation. Treatment with this HMGCS2 targeted ASO reduces hepatic HMGCS2 mRNA by 90% in adult mice [33, 35].

Injection Studies

Mice were singly housed one week prior to experimentation. 16 hours before sacrifice all mice were switched to sani-chip bedding (Harlan Laboratories; Cat. # 7090 Sani-Chips) and food removed from mice in the fasted group. All mice had ad libitum access to water. Intraperitoneal injections of 0.8mmol/kg GPR109a agonist nicotinic acid (niacin), 5.7mmol/kg β-OH butyrate, or phosphate buffered saline (PBS) were given at 0.1mL/10g body weight 9, 7, 5, 3, and 1 hours before sacrifice. Sacrifice began at 10 am, 5 hours after lights on, and was completed within 1 hour.

Glucose Tolerance Test

Intraperitoneal glucose (2.5mg/kg; 0.1mL/10g body weight) was given to 4 hour fasted individually housed mice. All glucose tolerance tests began at 1 pm and glucose was measured by glucometer from blood collected from a nick of the tail vein (Manufacture # D2ASCCONKIT, Bayer, Leverkusen, Germany) at 0, 15, 30, 60, 90, and 120 minutes after glucose injection.

Tissue Collection

Mice were sacrificed by decapitation after isoflurane anesthesia using the bell jar method. We collected livers and snap froze them on dry ice and trunk blood, which was stored on ice. Within 2 hours of collection, blood was allowed to clot at room temperature for 30 minutes and serum was collected after centrifugation at 3,000xg for 30 minutes at 4°C. All tissues and serum were stored at -80°C. Prior to analysis, frozen livers were powdered using a liquid nitrogen cooled mortar and pestle to obtain homogenous liver samples.

Serum Assays

Serum triglycerides (Cat. # T7531, Ponte Scientific Inc., Canton, MI), glucose (Cat. # G7519, Pointe Scientific Inc., Canton MI), non-esterified fatty acids (HR Series NEFA-HR, Wako Diagnostics,

Richmond, VA), and β-OH butyrate (Cat. # 700190, Cayman Chemicals, Pittsburg, PA) were analyzed by colorimetric assay.

Liver Analyses

Whole liver mRNA was isolated from powered liver samples with TRI Reagent® (Life Technologies, Grand Island, NY) and purified using water-saturated butanol and ether to eliminate phenol contamination [36]. cDNA was synthesized by reverse transcription with Verso cDNA synthesis kit (Thermo Scientific, Inc., Waltham, MA), and qPCR performed using SYBR 2X mastermix (Bio-Rad Laboratories, Hercules, CA) and the Biorad iQ^{TM}5 iCycler (Bio-Rad Laboratories, Hercules, CA). Expression of β-actin (ACTβ), peroxisome-proliferator activated receptor α (PPARα), 3-hydroxy-3-methylglutaryl-CoA Synthase II (HMGCS2), phosphoenolpyruvate carboxykinase (PEPCK), glucose 6-phosphatase (G6Pase), uncoupling protein 2 (UCP2), carnitine palmitoyltransferase 1 (CPT1), and G-protein coupled receptor 109a (GPR109a) mRNA were measured using the primer pairs listed in table 1. LinReg PCR analysis software was used to determine the efficiency of amplification from raw output data [37]. ACTβ served as the reference gene for calculating fold change in gene expression using the efficiency$^{-\Delta\Delta Ct}$ method [38].

Total liver lipids were extracted from powdered liver samples. Briefly, 10-20 mg of sample was homogenized in 100µL PBS. 1 mL of 100% ethanol was added to each sample and agitated using a tube-holder vortex attachment for 10 minutes. Following 5 minutes of centrifugation at 16,000xg at 4°C, supernatant was transferred to a fresh tube for analysis of liver non-esterified fatty acids (HR Series NEFA-HR, Wako Diagnostics, Richmond, VA) and triglycerides (Cat. # T7531, Ponte Scientific Inc., Canton, MI). Triglyceride values obtained by this extraction method were compared against those obtained by the Folch method to confirm a complete extraction [39]. Values determined by the two methods were highly correlated ($R^2 = 0.8904$). Liver glycogen content was quantified by a colorimetric assay as previously described [40].

Statistical Analysis

All statistical analyses were completed in SAS Enterprise Guide 4.3 (SAS Institute Inc., Cary, NC). We performed a two-way mixed model analysis of variance, including genotype and diet, to assess the effect of GPR109a knockout in fed and fasted mice. An identical analysis was performed comparing the HMGCS2 and scramble control anti-sense oligonucleotide treated mice in the fed and fasted state. A two-way mixed model was used to assess the effect of GPR109a expression on the response to exogenous saline or niacin administration in the 16h fasted mouse. Again, an identical analysis was performed comparing the response to exogenous saline, niacin, or β-OH butyrate administration in HMGCS2 and scramble control anti-sense oligonucleotide treated mice. All independent variables were treated as classification variables. A Bonferroni correction was used to correct for multiple comparisons. Glucose tolerance tests were analyzed by repeated measures ANOVA. Figures were created in GraphPad PRISM® Version 6.0 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) and are displayed as Mean ± SEM. A significance level of $P \leq 0.05$ was used.

Results

The hepatic response to fasting differs in mice unable to upregulate ketogenesis

Successful systemic adaptation to fasting requires a dynamic change in liver metabolism, including upregulation of hepatic glucose and ketone output from increased flux through gluconeogenesis, glycogenolysis and ketogenesis. To investigate the role of ketone bodies in regulating the hepatic metabolic response to fasting, we used targeted antisense oligonucleotides (ASO) to knockdown HMGCS2. This completely prevented the fasting induced rise in HMGCS2 mRNA and serum β-OH butyrate. To understand how ketone body synthesis affected the hepatic metabolic adaptation to fasting, we measured serum and hepatic metabolites and the hepatic expression of mRNA for rate limiting enzymes that control flux through metabolic pathways during a fast.

Hepatic glucose output was assessed by hepatic glycogen content, PEPCK expression, G6Pase expression, and serum glucose. Fasting decreased hepatic glycogen levels independent of HMGCS2 expression (Fig. 1A; $P < 0.05$). PEPCK, an early enzyme in gluconeogenesis from TCA cycle derived

substrates, mRNA expression was elevated with fasting independent of HMGCS2 expression (Fig. 1B; $P < 0.05$). G6Pase, the enzyme that controls hepatic glucose output from gluconeogenesis and glycogenolysis, was elevated with fasting in KD mice, and tended to be elevated with fasting in control mice (Fig. 1C; $P < 0.1$). Serum glucose, which was decreased by fasting in control mice, was surprisingly lower in KD mice in the fed state compared to controls and did not further decrease with fasting (Fig. 1D). The lower serum glucose level in fed KD mice suggests that mice with impaired ketogenesis may be more glucose tolerant. Accordingly, we performed a glucose tolerance test, which revealed better glucose clearance in KD mice (Fig. 1E; $P < 0.05$).

Hepatic ketone synthesis depends on oxidation of fatty acids. To assess lipid metabolism and ketogenesis, serum and liver NEFA and TAG levels, hepatic PPARα, CPT1, UCP2 and HMGCS2 mRNA expression, and serum β-OH butyrate were measured. As expected, fasting elevated serum NEFA concentrations in control mice and tended to elevate serum NEFA concentrations in KD mice (Fig. 2A; $P < 0.1$). Serum TAG concentrations were not altered by fasting, although KD mice had significantly lower serum TAGs than control mice independent of nutritional state (Fig. 2B). NEFAs released from adipose tissue during fasting accumulate in the liver and either go through β-oxidation or are resynthesized into TAGs. In mice, hepatic NEFA concentrations begin to rise after only 4 hours of fasting, while hepatic TAGs don't accumulate until 12 hours of fasting [21]. 16 hours of fasting increased hepatic NEFA and TAG concentrations in both HMGCS2 KD and control mice (Figures 2C and 2D; $P < 0.05$). Unsaturated fatty acids bind to, activate, and upregulate expression of hepatic PPARα [41-43]. Since fasting induced a similar degree of lipid accumulation in control and KD ASO treated mice, we expected the similar rise in PPARα expression independent of HMGCS2 expression (Fig. 2E).

Long-chain fatty acid entry into the mitochondria and flux through β-oxidation is largely controlled by the activity of CPT1 [44]. Expression of hepatic CPT1 was increased with fasting in both treatments, yet significantly more so in HMGCS2 KD mice (Fig. 3A; $P < 0.05$). With fasting, the mitochondrial NAD^+:NADH ratio decreases [45] and flux through β-oxidation becomes limited by

mitochondrial NAD^+ availability. To sustain high β-oxidative capacity, the mitochondrial uncoupling protein, UCP2, is upregulated in the fasted liver. This PPARα target gene allows unrestricted NAD^+ regeneration and is required for maximal fasting lipid oxidation and ketone synthesis [46, 47]. Accordingly, hepatic UCP2 expression is elevated with fasting in control mice, and even more robustly elevated with fasting in mice that cannot make ketones (Fig. 3B; $P < 0.05$). The final product of β-oxidation, acetyl-CoA, can enter the ketogenic pathway. Hepatic expression of HMGCS2 and serum concentrations of β-OH butyrate were elevated with fasting in control mice. Mice treated with HMGCS2 targeted ASO had lower HMGCS2 mRNA expression than the control fed levels independent of nutritional state, and displayed fed state serum β-OH butyrate concentrations despite being fasted for 16 hours (Figures 3C and 3D).

Niacin or β-OH butyrate normalize the fasting response in KD mice

Mice that are unable to upregulate ketone synthesis in response to a fast showed a more robust increase in CPT1 and UCP2 mRNA than control mice and did not respond to a fast with a drop in serum glucose concentrations. To confirm that these derangements in the fasting response were due to a lack of β-OH butyrate signaling, we reintroduced either β-OH butyrate or niacin to KD and control mice over the final 9 hours of food deprivation.

Fasting hepatic glycogen content was unaltered by niacin or β-OH butyrate injections in control or KD mice (Fig. 4A). Neither niacin nor β-OH butyrate altered fasting PEPCK or G6Pase mRNA expression in control mice, but in KD mice IP injection of either lowered PEPCK mRNA expression and niacin administration lowered G6Pase mRNA expression (Fig. 4B and 4C). Niacin or β-OH butyrate administration did not affect fasting serum glucose levels in control mice, but in KD mice injections of either lowered serum glucose concentrations, resulting in serum glucose concentrations comparable to fasting controls (Fig. 4D).

Surprisingly, β-OH butyrate injections increased fasting serum NEFA concentrations in KD mice, while neither injection treatment affected fasting serum NEFA concentrations in control mice (Fig. 5A). Fasting serum TAG concentrations were unaltered by niacin or β-OH butyrate in either

control or KD mice (Fig. 5B). In control, but not KD mice, niacin injections decreased fasting hepatic NEFA and TAG concentrations while β-OH butyrate had no effect (Figures 5C and 5D). Niacin has been shown to dose dependently inhibit hepatic NEFA and TAG synthesis in vitro, and is known to noncompetitively inhibit hepatic diacylglycerol acyltransferase 2, an enzyme in the TAG synthesis pathway [48, 49]. Interestingly, these effects of niacin to limit fasting hepatic lipid accumulation seem to require an active ketogenic pathway. Niacin or β-OH butyrate injections did not significantly change fasting PPARα mRNA expression in control mice. In KD mice, niacin decreased fasting PPARα mRNA expression, while β-OH butyrate tended to depress PPARα expression (Fig. 5E; $P < 0.1$). Regulation of fasting PPARα mRNA expression by niacin appears independent of hepatic NEFA levels.

β-OH butyrate injections decreased fasting hepatic CPT1 and UCP2 mRNA expression in KD mice, returning transcript levels to those seen in fasted controls (Figures 6A and 6B; $P < 0.05$). Niacin treatment also lowered CPT1 mRNA expression in both control and KD mice ($P < 0.05$), while not significantly affecting UCP2 mRNA expression. Fasted HMGCS2 mRNA expression in KD mice of all injection groups remained over 20 fold lower than fasted controls. β-OH butyrate treatment in control mice lowered fasting HMGCS2 mRNA expression (Fig. 6C; $P < 0.05$). Serum β-OH butyrate concentrations increased with β-OH butyrate injections in both ASO treated groups (Fig. 6D; $P < 0.05$). There was a 35% and 62% increase in serum β-OH butyrate relative to the PBS injected group in control and KD mice, respectively.

The hepatic response to fasting does not differ in GPR109 null mice

To understand if the metabolic control of β-OH butyrate or niacin described in KD mice is dependent on signaling through GPR109a, we compared the fasting response in GPR109a null (-/-) and wildtype (+/+) mice. Fasting depleted hepatic glycogen content and lowered serum glucose, increased hepatic PEPCK and G6Pase mRNA similarly in both genotypes (Figures 7A, 7B, 7C and 7D; $P < 0.05$). Despite not altering the hepatic gluconeogenic and glycogenolytic potential, GPR109a deletion decreases whole body glucose clearance as assessed by IPGTT (Fig. 7E; $P < 0.05$).

By agonizing GPR109a, it has been postulated that β-OH butyrate regulates its own production through limiting NEFA efflux from adipose tissue and influx to the liver [10]. In WT mice, fasting increased serum NEFA concentrations (Fig. 8A; $P < 0.05$). Interestingly, serum NEFA concentrations in fed state GPR109a -/- mice were not significantly different from fasting serum NEFAs in either genotype, and did not further increase significantly with fasting. Serum TAG levels were unaffected by nutritional state or GPR109a expression (Fig. 8B). Both GPR109a +/+ and -/- mice displayed significant hepatic NEFA and TAG accumulation (Figures 8C and 8D; $P < 0.05$). Hepatic PPARα, CPT1, and HMGCS2 mRNA expression increased resulting in elevated serum β-OH butyrate concentrations in fasted animals independent of GPR109a expression (Figure 8E, Figures 9A, 9C and 9D; $P < 0.05$).

GPR109a expression affects fasting hepatic gene expression in response to niacin

To determine the role of GPR109a in the response to niacin injections, we injected GPR109a +/+ and -/- mice with intraperitoneal niacin. Niacin treatment did not alter fasting hepatic glycogen, serum glucose concentration, or hepatic PEPCK of G6Pase mRNA expression in either genotype (Figures 10A, 10B, 10C and 10D).

Niacin lowered fasting serum NEFA concentrations ($P = 0.0001$) independent of genotype, this resulted in significant differences in serum NEFA within GPR109a +/+ mice and tended toward significance in GPR109a -/- mice (Fig. 11A; $P < 0.1$). Consistent with previous reports [50], niacin treatment overall lowered serum TAG values independent of GPR109a expression ($P = 0.0038$), although this did not reach significance within either genotype (Fig. 11B). Fasting hepatic NEFA concentrations were lower with niacin treatment in KO mice while fasting hepatic TAG concentrations were lower in niacin treated mice of both genotypes (Figures 11C and 11D; $P < 0.05$). Together this data establishes that niacin diminishes fasting liver lipid accumulation independent of GPR109a. In accordance with the changes in hepatic lipid accumulation, niacin lowered fasting hepatic PPARα mRNA expression. This depression in PPARα mRNA expression was not significant in either genotype (Fig. 11E; $P < 0.05$).

Although baseline expression of CPT1 and HMGCS2 mRNA did not differ by genotype, within the niacin treated mice KO mice expressed more CPT1 and HMGCS2 mRNA than WT mice (Figures 12A and 12C; $P < 0.05$). Niacin did not significantly affect fasting hepatic UCP2 mRNA expression or serum β-OH butyrate concentrations in either genotype (Figures 12B and 12D).

Discussion

Understanding the regulation of metabolic activity in different physiological states is pertinent to enhancing our knowledge of basic science and providing insight into disease states. This hepatic switch from glucose import and fatty acid synthesis in the fed state to glucose and ketone export and fatty acid oxidation in the fasted state is critical to provide peripheral tissues with fuel during periods of food deprivation. Ketones are classically thought to serve as an alternative energy source during a fast, sparing glucose utilization for red blood cells. However, in the past ten years, the metabocrine functions of β-OH butyrate have been recognized. In fact, β-OH butyrate, a ligand for two G-protein coupled receptors, alters second messenger pathways to affect pathway flux and gene expression [8]. This research shows that β-OH butyrate regulates the normal hepatic fasting response.

Enhanced hepatic glucose output to prevent hypoglycemia is central to the metabolic adaptations of fasting. PEPCK mRNA expression represents the potential for flux through gluconeogenesis, while G6Pase mRNA provides a measure of potential hepatic glucose output from gluconeogenesis and glycogenolysis. In fact, overexpression of either G6Pase or PEPCK increases hepatic glucose output, while mice that are unable to upregulate PEPCK and G6Pase display fasting hypoglycemia [51-55]. Our data supports the proposition that that β-OH butyrate constantly suppresses hepatic gluconeogenic flux, as we were only able to observe a significant depression in hepatic PEPCK and G6Pase mRNA expression after eliminating hepatic production of ketones. Of note, niacin and β-OH butyrate both decreased fasting serum glucose concentrations in KD mice (Fig. 4D). While glucose uptake is improved by acute or intermittent niacin treatment [56, 57], chronic niacin treatment causes glucose intolerance [58, 59]. Ketones decrease peripheral glucose utilization and β-OH butyrate dose dependently inhibits insulin stimulated glucose uptake in oxidative skeletal muscle and cardiomyocytes

[23, 60, 61]. Thus, we propose that niacin and β-OH butyrate depressed serum glucose by both limiting hepatic glucose output and improving whole body glucose clearance. This fits with the well-established glucose sparing and insulin desensitizing effects of ketone body production. Of note, we were only able to observe this response in mice that were unable to produce ketones. Suggesting that basal ketone levels are enacting a mild depressive effect on hepatic glucose production and glucose clearance. Although non-significant both control ASO and GPR109a WT mice showed a similar pattern of depressed G6Pase and PEPCK mRNA expression in response to niacin that was not at all evident in GPR109a KO mice. Interestingly, glucose clearance appears to improve with GPR109a signaling as, glucose clearance was decreased in GPR109a null mice (Fig. 7E).

Hepatic glucose and lipid metabolism are intricately linked. Over 50% of fed state de novo lipogenesis is ChREBP dependent and conversely maximal gluconeogenesis with fasting requires adequate β-oxidation [6, 62, 63]. The absence of insulin signaling at adipose tissue during fasting promotes lipolysis and release of NEFAs into circulation, increasing substrate availability for hepatic lipid oxidation [64]. Interestingly, GPR109a -/- mice displayed fasting level serum NEFA concentrations in the fed state (Fig. 8A). Although β-OH butyrate concentrations in the fed state are well below the EC_{50} for GPR109a, this receptor may exhibit constitutive activity common to G-protein coupled receptors [65] and exert a constant basal suppression of lipolysis. Additionally, it was surprising that niacin tended to decrease serum NEFAs in GPR109a null mice (Fig. 11A) given that niacin's inhibition of adipose tissue lipolysis is dependent on GPR109a [10, 34, 50] It remains possible that niacin additionally affects serum NEFA concentrations by altering NEFA clearance.

NEFAs are the endogenous ligands for the nuclear receptor PPARα, inducing nuclear translocation and transcriptional regulation through peroxisome proliferator activated receptor response element (PPRE) binding [66]. PPARα upregulates its own expression and also has target genes involved in lipid catabolism, gluconeogenesis, and ketogenesis [43, 51, 67-70]. Accumulation of liver NEFAs during fasting initiate PPARα signaling, upregulating lipid oxidative genes to prevent hepatic lipotoxicity [71, 72]. Activation of PPARα is critical to the hepatic fasting response, as PPARα null

mice have impaired β-oxidation, fasting hypoglycemia, and do not elevate serum β-OH butyrate with fasting [71-75]. We anticipated that expression of fasting PPARα mRNA would depend on liver NEFA concentrations. Yet, in KD mice, niacin and β-OH butyrate lowered fasting PPARα mRNA expression without affecting hepatic NEFA concentrations (Figures 5C and 5E). While PPARα mRNA is upregulated by increased hepatic NEFA concentrations, additional factors also regulate expression [76]. We expect that regulation of PPARα by β-OH butyrate or niacin signaling is not through GPR109a, since genotype had no effect on PPARα mRNA expression in response to injection treatment (Fig. 8E). Therefore, β-OH butyrate control of fasting PPARα expression is independent of hepatic lipid accumulation and GPR109a expression.

Fatty acids directly and indirectly regulate expression and activity of genes involved in lipid metabolism. CPT1 and HMGCS2 control flux through β-oxidation and ketogenesis, and mRNA expression of these genes are indicative of pathway activity [77, 78]. Accordingly, HMGCS2 mRNA expression was highly correlated with serum β-OH butyrate concentrations in all mice (excluding β-OH butyrate injected groups; R^2 =0.72). Regulation of enzyme expression by pathway substrates or products is a common theme in metabolism. Carnitine increases transcription and activity of hepatic CPT1, while fatty acids increase CPT1 expression in both PPARα dependent and independent mechanisms and extend mRNA half-life [79, 80].We show that β-OH butyrate signaling also regulates CPT1 mRNA expression. Knockdown of HMGCS2 elevated fasting CPT1 expression (Fig. 3A), while β-OH butyrate and niacin injections returned expression to control levels in KD mice (Fig. 6A). This may be mediated by GPR109a as niacin injections did not decrease fasting hepatic CPT1 mRNA in GPR109a null mice and CPT1 expression was significantly lower in niacin treated WT mice than in niacin treated KO mice (Fig. 12A). UCP2 is critically involved in lipid metabolism and accordingly expression is increased by fatty acids via PPARα signaling. As such, UCP2 is upregulated with hepatic steatosis in part by the transcription factor SP1 [25, 81]. β-OH butyrate also has a regulatory role as hepatic UCP2 mRNA expression was more robustly elevated with fasting in KD than control mice, which in turn were decreased by exogenous β-OH butyrate (Figure 3B and 6B). However, this

suppressive effect of β-OH butyrate on UCP2 mRNA expression appears to be independent of GPR109a as niacin did not significantly alter mRNA expression in any group and GPR109a knockout had no impact on hepatic UCP2 mRNA (Figures 6B and 12B).

Interestingly, HMGCS2 functions as a coactivator for PPARα to self-upregulate expression at its own PPRE [82]. Palmitoylation of HMGCS2, which occurs spontaneously and dose dependently, is required for its interaction with PPARα [83]. Thus, conditions with abundant hepatic long chain fatty acids robustly induce HMGCS2 expression. We provide evidence that in addition to being upregulated by ketogenic substrates, HMGCS2 expression is downregulated by the terminal ketogenic product β-OH butyrate. In control ASO treated mice, β-OH butyrate injections decreased HMGCS2 mRNA concentrations (Fig. 12C). This regulation may be mediated by GPR109a as there was a significant treatment by genotype effect on fasting hepatic HMGCS2 mRNA expression where niacin decreased HMGCS2 mRNA in WT mice by 14% and increased HMGCS2 mRNA in KO mice by 28% (Fig. 12A). Control of CPT1, UCP2, and HMGCS2 expression by both fatty acids and β-OH butyrate allows for fine tuning of β-oxidative and ketogenic activity depending on the metabolic needs of the cell. Much like β-OH butyrate's regulation of its own synthesis through inhibition of adipose tissue lipolysis, β-OH butyrate signaling further regulates on its own production and protects against excess ketosis by limiting expression of enzymes directly involved in its synthesis.

GPR109a is expressed in adipocytes, macrophages, intestinal epithelium, retinal pigment epithelium, Langerhans cells, mammary tissue, pancreatic islets, and hepatocytes [12, 13, 15, 34, 84, 85]. Independent of treatment or nutritional state, hepatic GPR109a mRNA expression is upregulated by HMGCS2 knockdown ($P = 0.003$; data not shown). This suggests that β-OH butyrate downregulates expression of hepatic GPR109a, and that when β-OH butyrate is chronically low hepatocytes upregulate receptor expression in an attempt to increase sensitivity for its ligand. We acknowledge that while β-OH butyrate signaling likely affects GPR109a mRNA expression by decreasing transcription rates, it is also possible that lower transcript levels are a result of decreased mRNA stability. Since HMGCS2 knockdown increases hepatic GPR109a expression and simultaneously prevents basal signaling

through GPR109a, this created a highly sensitive model that allowed us to observe the suppression of PEPCK and G6Pase mRNA expression by niacin.

High serum ketone levels with fasting are part of the metabolic adaptation to this physiologic state. However, hyperketonemia is also pathologically observed in type 1 diabetes, type 2 diabetes, and is associated with obesity [22, 86]. Hepatic lipid accumulation promotes ketone production and serum β-OH butyrate is significantly correlated with lipid oxidation rates [86]. As with fasting, obesity and diabetes are characterized by fatty liver. In fact, 60-70% of type 2 diabetics and 65-85% of obese patients (BMI $\geq$ 30) are comorbid with non-alcoholic fatty liver disease [26, 87, 88]. Interestingly, obesity also increases hepatic CPT1, UCP2, G6Pase, and PEPCK mRNA expression [25, 89-91]. These elevations in gene expression inappropriately enhance gluconeogenic and ketogenic flux despite high serum glucose and β-OH butyrate concentrations. We propose that in obesity and diabetes, the constant elevation of β-OH butyrate, desensitizes the animal to normal feedback regulation by β-OH butyrate on hepatic gene expression observed during a fast. Chronic ligand stimulation induces GPR109a membrane internalization and obesity reduces adipose GPR109a expression [92, 93]. This follows with our observation that sensitivity to niacin and β-OH butyrate is increased with HMGCS2 knockdown. Decreased sensitivity to β-OH butyrate may contribute to the metabolic derangements of diabetes. Ketones are recognized to promote insulin resistance and alter β-cell metabolism, and have been implicated in glucose homeostasis disruption [22, 24]. Our data suggest that a loss of ketone signaling at the liver could contribute to the high hepatic glucose output of diabetes, and further validate the role ketone bodies as diabetogenic agents.

In summary, β-OH butyrate signaling during a fast inhibits hepatic PEPCK, G6Pase, CPT1, and HMGCS2 mRNA expression through a mechanism likely involving GPR109a, and inhibits hepatic PPARα and UCP2 mRNA expression through GPR109a independent mechanisms. The extent of GPR109a involvement in mediating β-OH butyrate regulation of fasting hepatic transcript levels requires further investigation. Almost half a century ago, ketones were first proposed to exert a fine regulatory control over metabolism in the fasted state [60]. We have demonstrated that β-OH butyrate

signaling at the liver affects hepatic expression of gluconeogenic, β-oxidative, and ketogenic enzymes. This feedback regulation of fasting hepatic metabolism may serve to prevent uncontrolled hepatic gluconeogenesis and ketogenesis.

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Figure Captions

Figure 1. Glucose homeostasis in response to fasting in control and KD mice. Hepatic (A) glycogen (mg/g liver tissue), (B) PEPCK mRNA expression, (C) G6Pase mRNA expression, and (D) serum glucose (mg/dL). [a,b,c] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group). (E) Glucose tolerance test in 4 hour fasted mice. (Control and KD n = 8) *Indicates significant difference between control and KD mice ($P < 0.05$).

Figure 2. Lipid homeostasis in response to fasting in control and KD mice. Serum (A) non-esterified fatty acids (NEFA; µM), (B) triacylglycerol (TAG; mg/dL), hepatic (C) non-esterified fatty acids (NEFA; µM/g liver tissue), (D) triacylglycerol (TAG; mg/g liver tissue), and (E) PPARα mRNA expression. [a,b,c] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

Figure 3. Changes in genes supporting hepatic ketone production in response to fasting in control and KD mice. Hepatic (A) CPT1 mRNA expression, (B) UCP2 mRNA expression, (C) HMGCS2 mRNA expression, and (D) serum β-OH butyrate (µM). [a,b,c] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

Figure 4. Effect of niacin and β-OH butyrate on fasting glucose homeostasis in control and KD mice. Hepatic (A) glycogen (mg/g liver tissue), (B) PEPCK mRNA expression, (C) G6Pase mRNA expression, and (D) serum glucose (mg/dL). [a,b,c] Bars that do not share a common letter differ significantly within ASO group ($P < 0.05$; number inside bar denotes n per group). *Indicates a significant difference between PBS treated control and KD groups ($P < 0.05$).

Figure 5. Effect of niacin and β-OH butyrate on fasting lipid homeostasis in control and KD mice. Serum (A) non-esterified fatty acids (NEFA; µM), (B) triacylglycerol (TAG; mg/dL), hepatic (C) non-esterified fatty acids (NEFA; µM/g liver tissue), (D) triacylglycerol (TAG; mg/g liver tissue), and (E) PPARα mRNA expression. [a,b] Bars that do not share a common letter differ significantly within ASO group ($P < 0.05$; number inside bar denotes n per group). *Indicates a significant difference between PBS treated control and KD groups ($P < 0.05$).

Figure 6. Effect of niacin and β-OH butyrate on fasting genes supporting hepatic ketone production in control and KD mice. Hepatic (A) CPT1 mRNA expression, (B) UCP2 mRNA expression, (C) HMGCS2 mRNA expression, and (D) serum β-OH butyrate (µM). [a,b] Bars that do not share a common letter differ significantly within ASO group ($P < 0.05$; number inside bar denotes n per group). *Indicates a significant difference between PBS treated control and KD groups ($P < 0.05$).

Figure 7. Glucose homeostasis in response to fasting in GPR109a +/+ and -/- mice. Hepatic (A) glycogen (mg/g liver tissue), (B) PEPCK mRNA expression, (C) G6Pase mRNA expression, and (D) serum glucose (mg/dL). [a,b,c] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group). (E) Glucose tolerance test in 4 hour fasted mice. (WT n = 5, KO n = 9).

Figure 8. Lipid homeostasis in response to fasting in GPR109a +/+ and -/- mice. Serum (A) non-esterified fatty acids (NEFA; µM), (B) triacylglycerol (TAG; mg/dL), hepatic (C) non-esterified fatty acids (NEFA; µM/g liver tissue), (D) triacylglycerol (TAG; mg/g liver tissue), and (E) PPARα mRNA expression. [a,b,] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

Figure 9. Changes in genes supporting hepatic ketone production in response to fasting in GPR109a +/+ and -/- mice. Hepatic (A) CPT1 mRNA expression, (B) UCP2 mRNA expression, (C) HMGCS2

mRNA expression, and (D) serum β-OH butyrate (μM). [a,b,] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

Figure 10. Effect of niacin and β-OH butyrate on fasting glucose homeostasis in GPR109a +/+ and -/- mice. Hepatic (A) glycogen (mg/g liver tissue), (B) PEPCK mRNA expression, (C) G6Pase mRNA expression, and (D) serum glucose (mg/dL). [a,b,] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

Figure 11. Effect of niacin and β-OH butyrate on fasting lipid homeostasis in GPR109a +/+ and -/- mice. Serum (A) non-esterified fatty acids (NEFA; μM), (B) triacylglycerol (TAG; mg/dL), hepatic (C) non-esterified fatty acids (NEFA; μM/g liver tissue), (D) triacylglycerol (TAG; mg/g liver tissue), and (E) PPARα mRNA expression. [a,b,c] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

Figure 12. Effect of niacin and β-OH butyrate on fasting genes supporting hepatic ketone production in GPR109a +/+ and -/- mice. Hepatic (A) CPT1 mRNA expression, (B) UCP2 mRNA expression, (C) HMGCS2 mRNA expression, and (D) serum β-OH butyrate (μM). [a,b] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

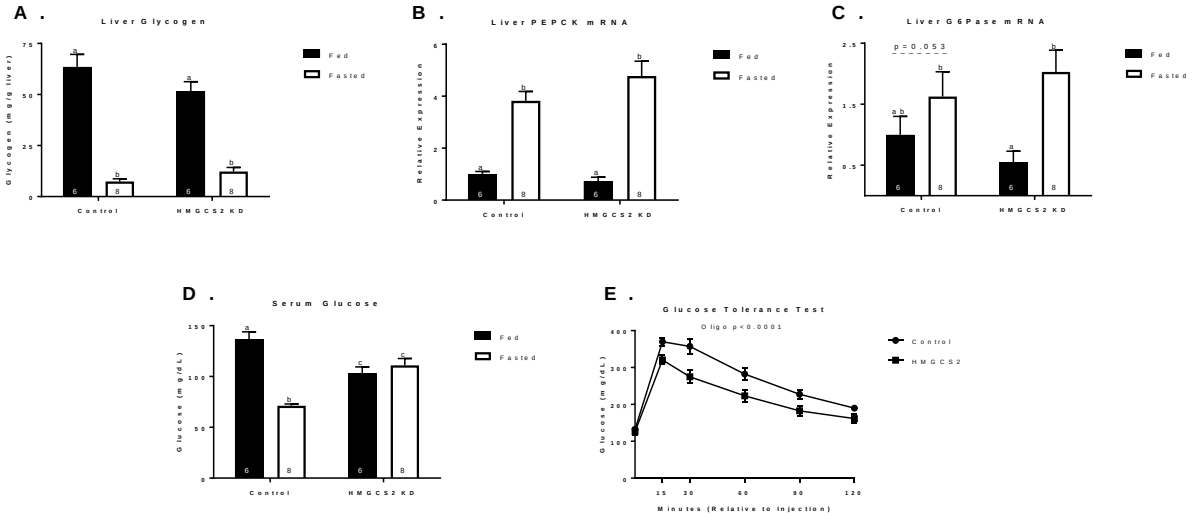
A .
Liver Glycogen
Glycogen (mg/g liver)
Fed
Fasted
Control
HMGCS2 KD
B .
Liver PEPCK mRNA
Relative Expression
C .
Liver G6Pase mRNA
p = 0.053
D .
Serum Glucose
Glucose (mg/dL)
E .
Glucose Tolerance Test
Oligo p < 0.0001
Control
HMGCS2
Minutes (Relative to Injection)

Figure 1.

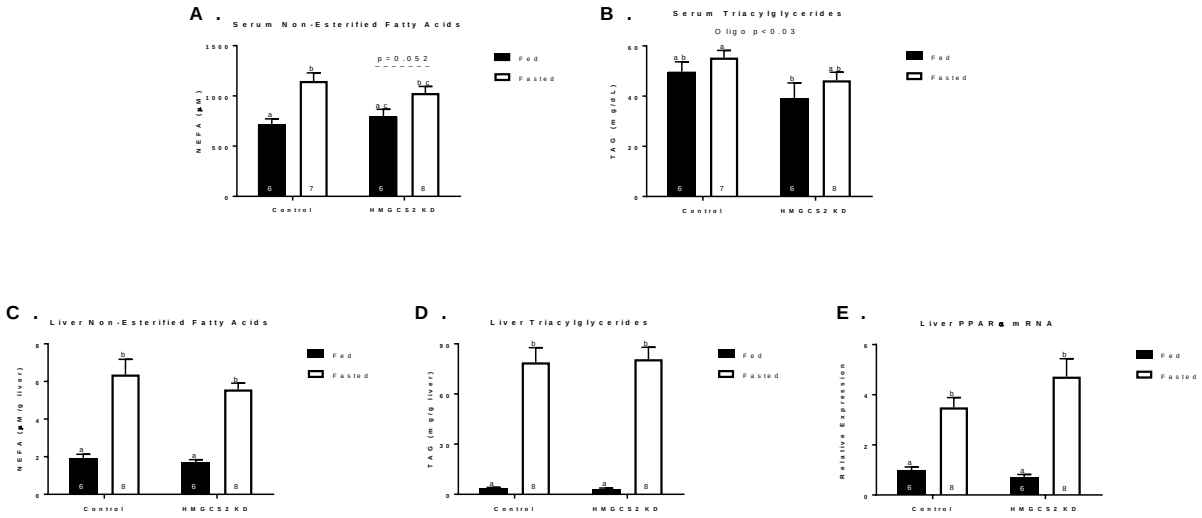
A .
Serum Non-Esterified Fatty Acids
p=0.052
NEFA (μM)
Fed
Fasted
Control
HMGCS2 KD
B .
Serum Triacylglycerides
Oligo p<0.03
TAG (mg/dL)
C .
Liver Non-Esterified Fatty Acids
NEFA (μM/g liver)
D .
Liver Triacylglycerides
TAG (mg/g liver)
E .
Liver PPARα mRNA
Relative Expression

Figure 2.

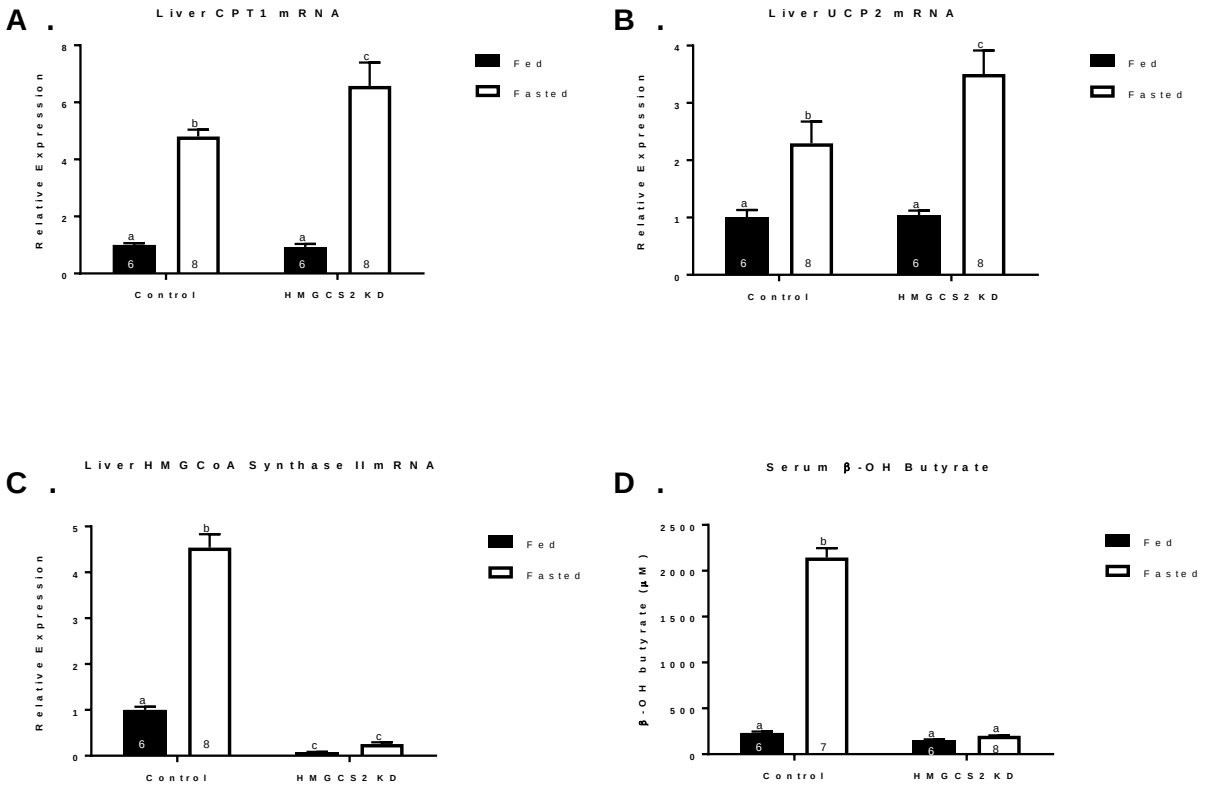
A .
Liver CPT1 mRNA
Relative Expression
Fed
Fasted
Control
HMGCS2 KD
B .
Liver UCP2 mRNA
Relative Expression
Fed
Fasted
Control
HMGCS2 KD
C .
Liver HMG CoA Synthase II mRNA
Relative Expression
Fed
Fasted
Control
HMGCS2 KD
D .
Serum β-OH Butyrate
β-OH butyrate (μM)
Fed
Fasted
Control
HMGCS2 KD

Figure 3.

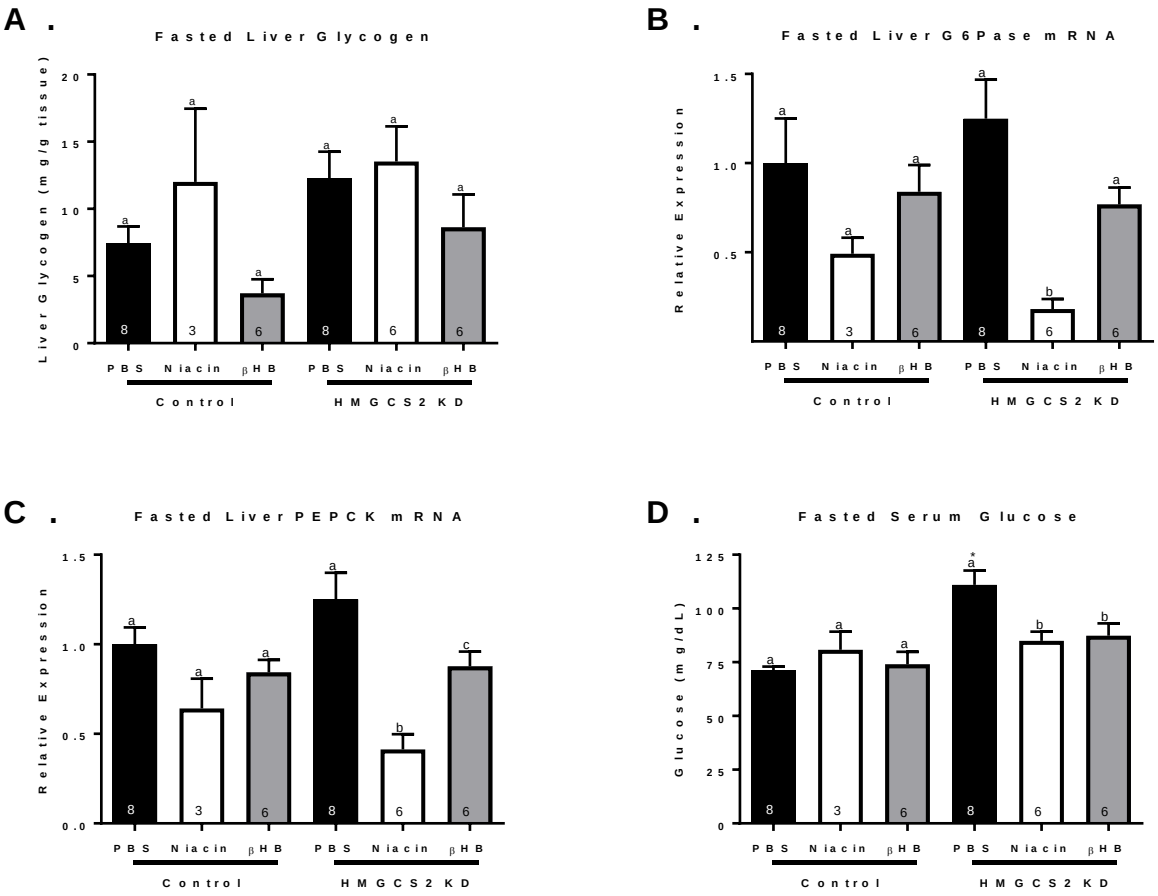
A .
Fasted Liver Glycogen
Liver Glycogen (mg/g tissue)
20
15
10
5
0
a
a
a
a
a
a
8
3
6
8
6
6
PBS
Niacin
βHB
PBS
Niacin
βHB
Control
HMGCS2 KD
B .
Fasted Liver G6Pase mRNA
Relative Expression
1.5
1.0
0.5
a
a
a
a
b
a
8
3
6
8
6
6
PBS
Niacin
βHB
PBS
Niacin
βHB
Control
HMGCS2 KD
C .
Fasted Liver PEPCK mRNA
Relative Expression
1.5
1.0
0.5
0.0
a
a
a
a
b
c
8
3
6
8
6
6
PBS
Niacin
βHB
PBS
Niacin
βHB
Control
HMGCS2 KD
D .
Fasted Serum Glucose
Glucose (mg/dL)
125
100
75
50
25
0
a
a
a
*
a
b
b
8
3
6
8
6
6
PBS
Niacin
βHB
PBS
Niacin
βHB
Control
HMGCS2 KD

Figure 4.

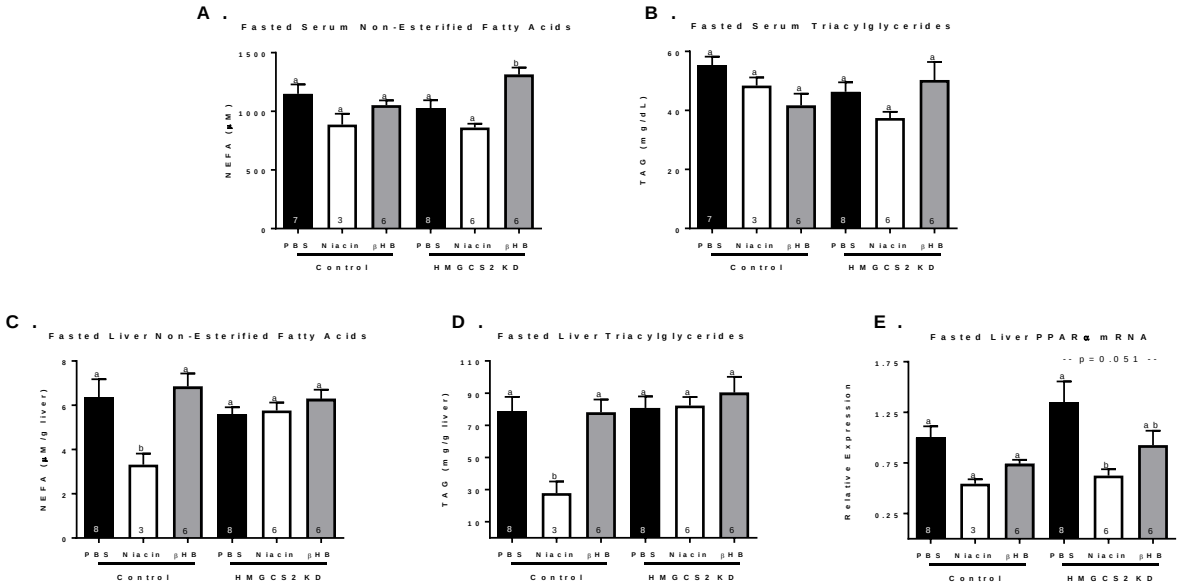
A .
Fasted Serum Non-Esterified Fatty Acids
NEFA (μM)
B .
Fasted Serum Triacylglycerides
TAG (mg/dL)
C .
Fasted Liver Non-Esterified Fatty Acids
NEFA (μM/g liver)
D .
Fasted Liver Triacylglycerides
TAG (mg/g liver)
E .
Fasted Liver PPARα mRNA
-- p=0.051 --
Relative Expression
PBS
Niacin
βHB
Control
HMGCS2 KD

Figure 5.

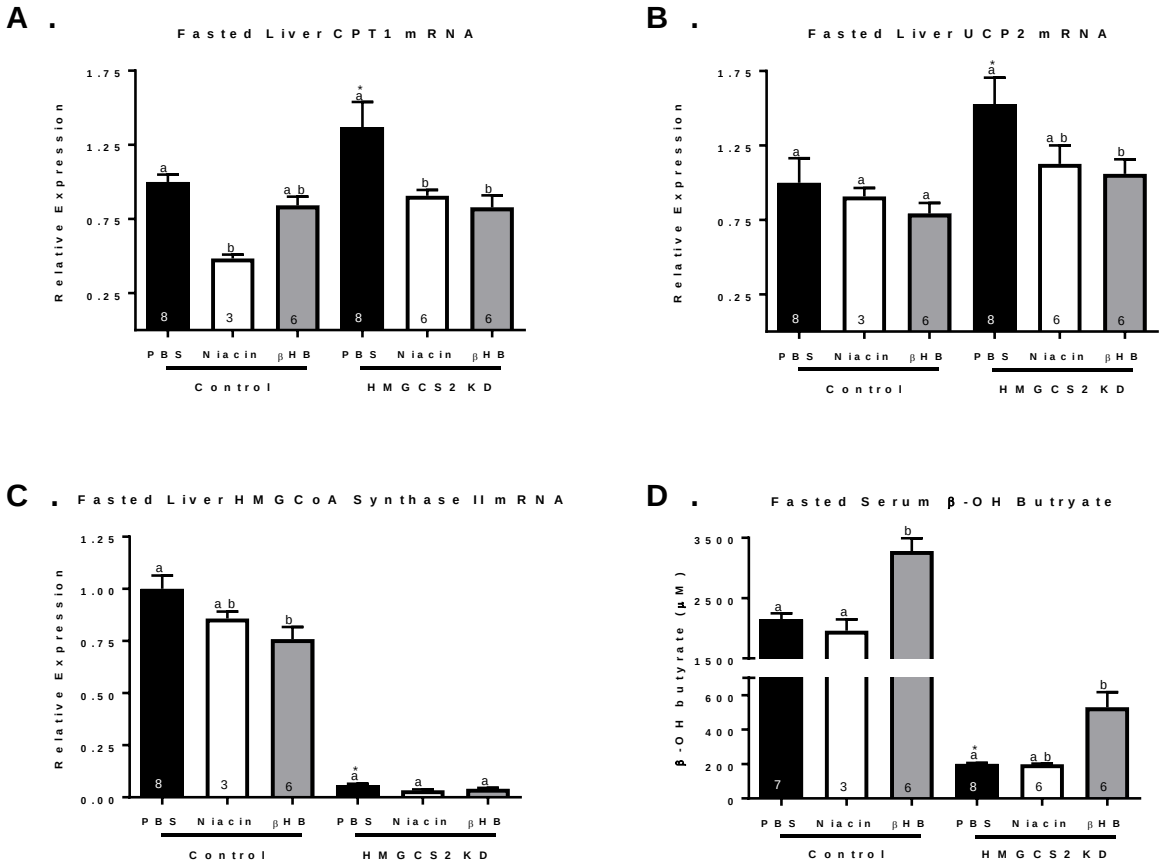
A .
Fasted Liver CPT1 mRNA
Relative Expression
1.75
1.25
0.75
0.25
a
b
a b
a*
b
b
8
3
6
8
6
6
PBS
Niacin
βHB
PBS
Niacin
βHB
Control
HMGCS2 KD
B .
Fasted Liver UCP2 mRNA
Relative Expression
1.75
1.25
0.75
0.25
a
a
a
a*
a b
b
8
3
6
8
6
6
PBS
Niacin
βHB
PBS
Niacin
βHB
Control
HMGCS2 KD
C .
Fasted Liver HMGCoA Synthase II mRNA
Relative Expression
1.25
1.00
0.75
0.50
0.25
0.00
a
a b
b
a*
a
a
8
3
6
PBS
Niacin
βHB
PBS
Niacin
βHB
Control
HMGCS2 KD
D .
Fasted Serum β-OH Butryate
β-OH butyrate (μM)
3500
2500
1500
600
400
200
0
a
a
b
a*
a b
b
7
3
6
8
6
6
PBS
Niacin
βHB
PBS
Niacin
βHB
Control
HMGCS2 KD

Figure 6.

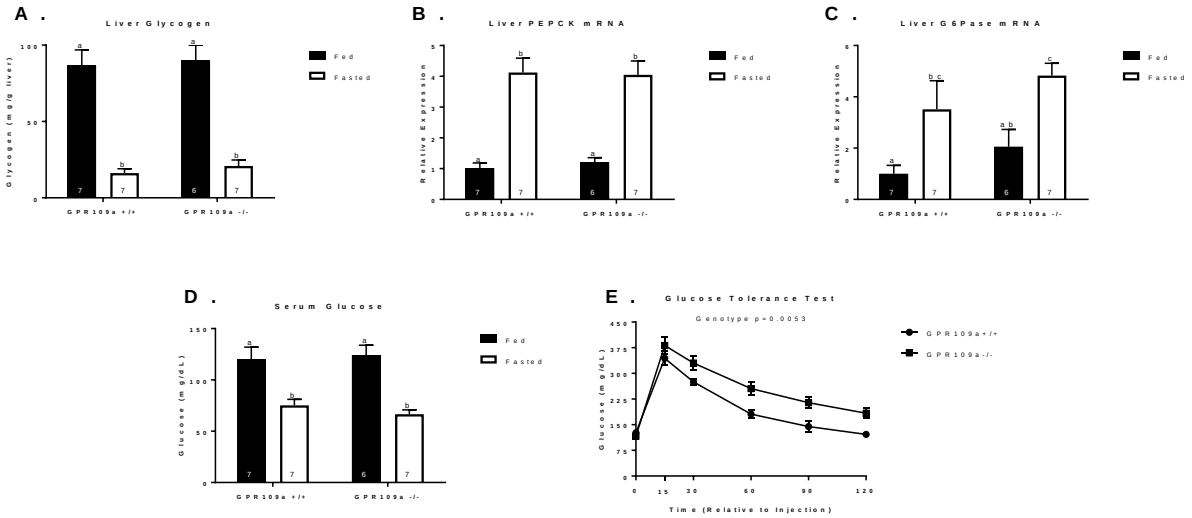
A .
Liver Glycogen
Glycogen (mg/g liver)
Fed
Fasted
GPR109a +/+
GPR109a -/-
B .
Liver PEPCK mRNA
Relative Expression
C .
Liver G6Pase mRNA
D .
Serum Glucose
Glucose (mg/dL)
E .
Glucose Tolerance Test
Genotype p=0.0053
GPR109a+/+
GPR109a-/-
Time (Relative to Injection)

Figure 7.

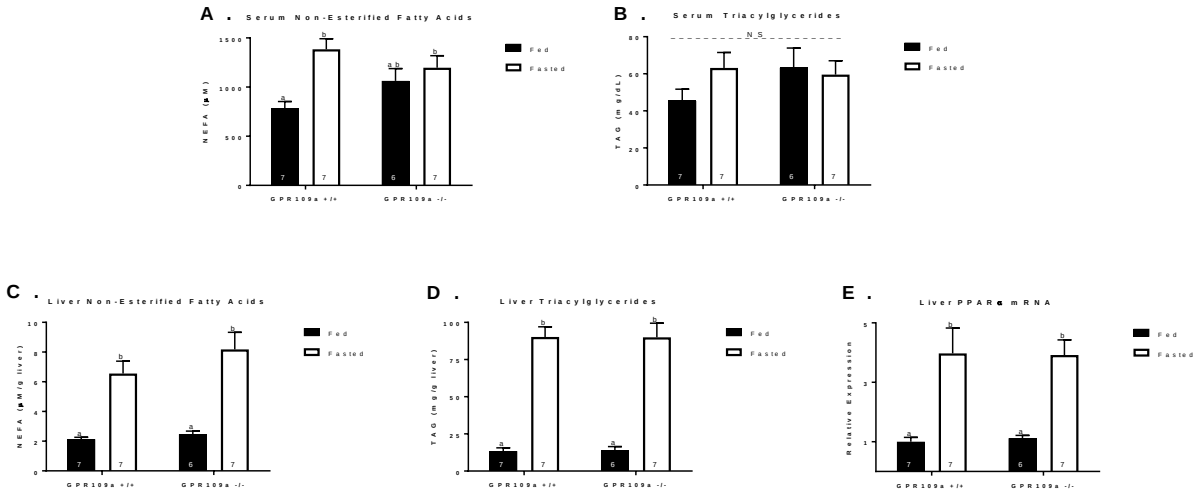
A .
Serum Non-Esterified Fatty Acids
NEFA (μM)
1500
1000
500
0
a
b
a,b
b
7
7
6
7
Fed
Fasted
GPR109a +/+
GPR109a -/-
B .
Serum Triacylglycerides
N.S.
TAG (mg/dL)
80
60
40
20
0
7
7
6
7
Fed
Fasted
GPR109a +/+
GPR109a -/-
C .
Liver Non-Esterified Fatty Acids
NEFA (μM/g liver)
10
8
6
4
2
0
a
b
a
b
7
7
6
7
Fed
Fasted
GPR109a +/+
GPR109a -/-
D .
Liver Triacylglycerides
TAG (mg/g liver)
100
75
50
25
0
a
b
a
b
7
7
6
7
Fed
Fasted
GPR109a +/+
GPR109a -/-
E .
Liver PPARα mRNA
Relative Expression
5
3
1
a
b
a
b
7
7
6
7
Fed
Fasted
GPR109a +/+
GPR109a -/-

Figure 8.

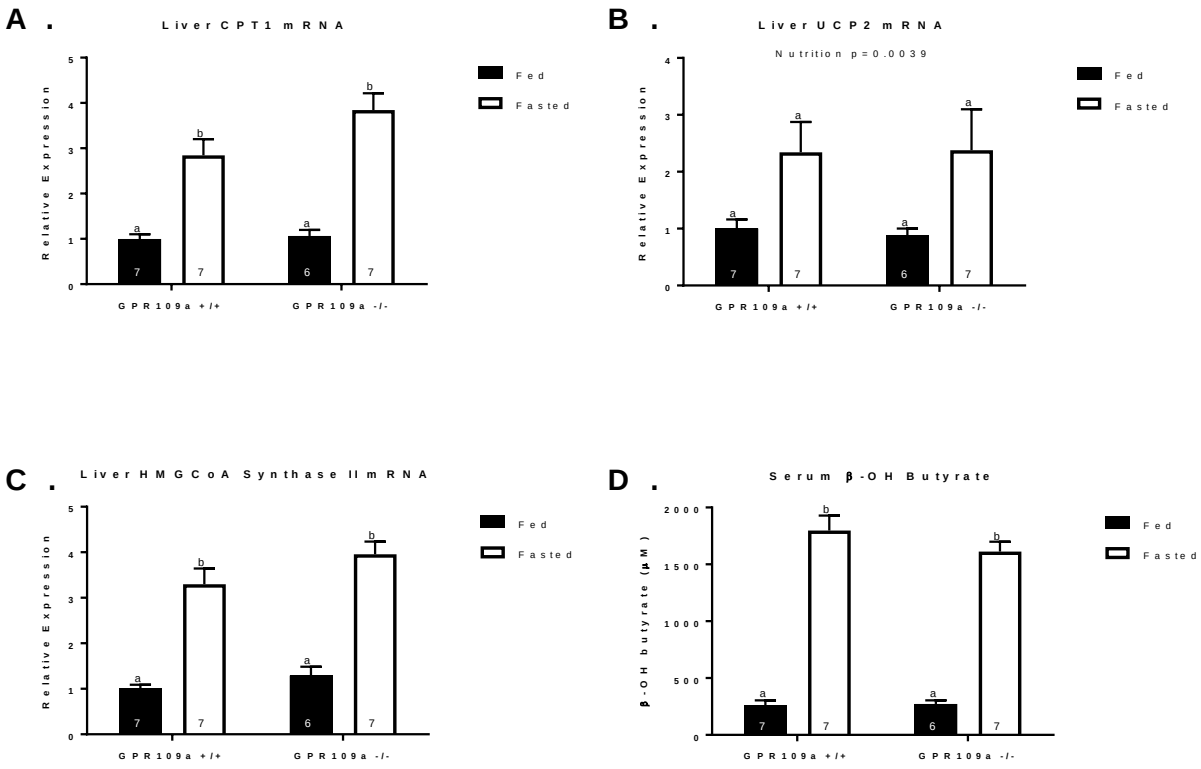
A .
Liver CPT1 mRNA
Relative Expression
Fed
Fasted
GPR109a +/+
GPR109a -/-
B .
Liver UCP2 mRNA
Nutrition p=0.0039
Relative Expression
Fed
Fasted
GPR109a +/+
GPR109a -/-
C .
Liver HMGCoA Synthase II mRNA
Relative Expression
Fed
Fasted
GPR109a +/+
GPR109a -/-
D .
Serum β-OH Butyrate
β-OH butyrate (μM)
Fed
Fasted
GPR109a +/+
GPR109a -/-

Figure 9.

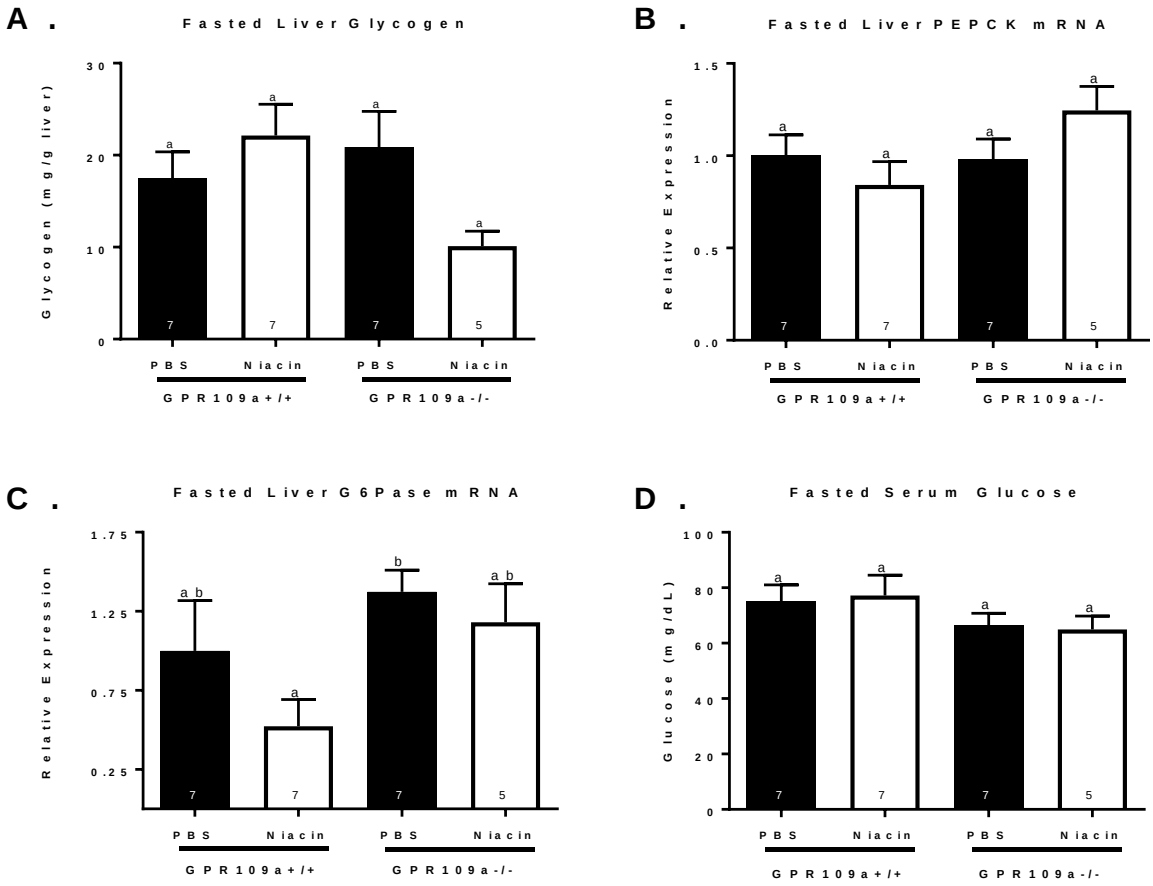
A .
Fasted Liver Glycogen
Glycogen (mg/g liver)
30
20
10
0
a
a
a
a
7
7
7
5
PBS
Niacin
PBS
Niacin
GPR109a+/+
GPR109a-/-
B .
Fasted Liver PEPCK mRNA
Relative Expression
1.5
1.0
0.5
0.0
a
a
a
a
7
7
7
5
PBS
Niacin
PBS
Niacin
GPR109a+/+
GPR109a-/-
C .
Fasted Liver G6Pase mRNA
Relative Expression
1.75
1.25
0.75
0.25
a b
a
b
a b
7
7
7
5
PBS
Niacin
PBS
Niacin
GPR109a+/+
GPR109a-/-
D .
Fasted Serum Glucose
Glucose (mg/dL)
100
80
60
40
20
0
a
a
a
a
7
7
7
5
PBS
Niacin
PBS
Niacin
GPR109a+/+
GPR109a-/-

Figure 10.

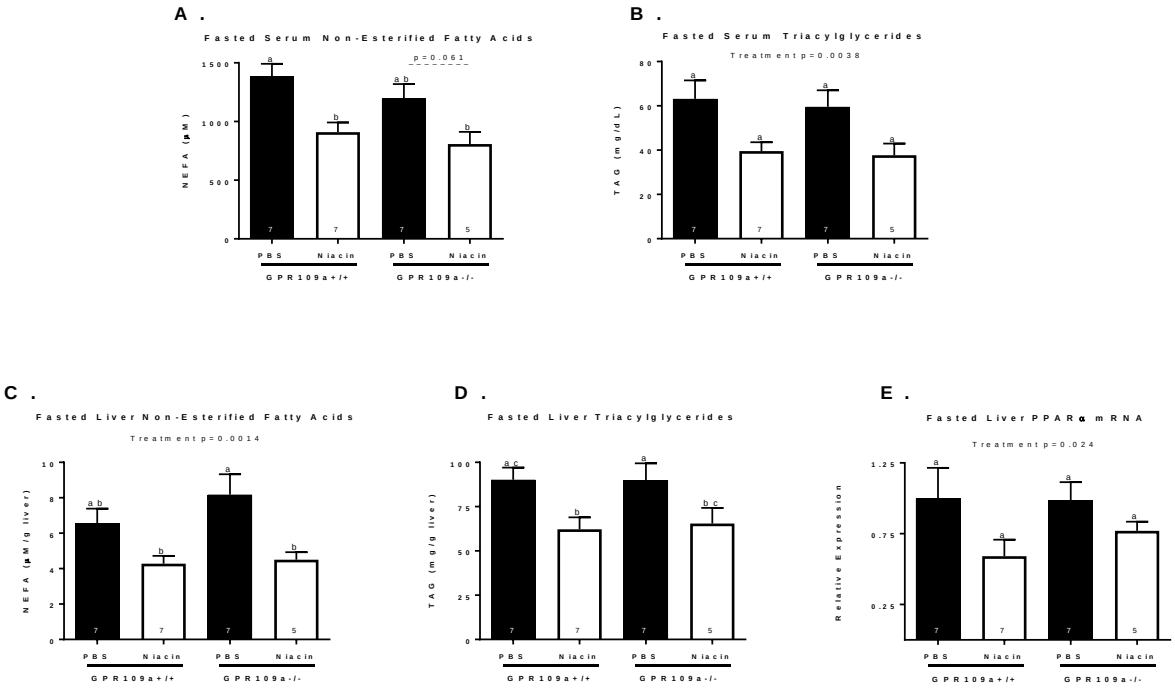
A .
Fasted Serum Non-Esterified Fatty Acids
p = 0.061
NEFA (μM)
1500
1000
500
0
a
b
a b
b
7
7
7
5
PBS
Niacin
PBS
Niacin
GPR109a+/+
GPR109a-/-
B .
Fasted Serum Triacylglycerides
Treatment p=0.0038
TAG (mg/dL)
80
60
40
20
0
a
a
a
a
7
7
7
5
PBS
Niacin
PBS
Niacin
GPR109a+/+
GPR109a-/-
C .
Fasted Liver Non-Esterified Fatty Acids
Treatment p=0.0014
NEFA (μM/g liver)
10
8
6
4
2
0
a b
b
a
b
7
7
7
5
PBS
Niacin
PBS
Niacin
GPR109a+/+
GPR109a-/-
D .
Fasted Liver Triacylglycerides
TAG (mg/g liver)
100
75
50
25
0
a c
b
a
b c
7
7
7
5
PBS
Niacin
PBS
Niacin
GPR109a+/+
GPR109a-/-
E .
Fasted Liver PPARα mRNA
Treatment p=0.024
Relative Expression
1.25
0.75
0.25
a
a
a
a
7
7
7
5
PBS
Niacin
PBS
Niacin
GPR109a+/+
GPR109a-/-

Figure 11.

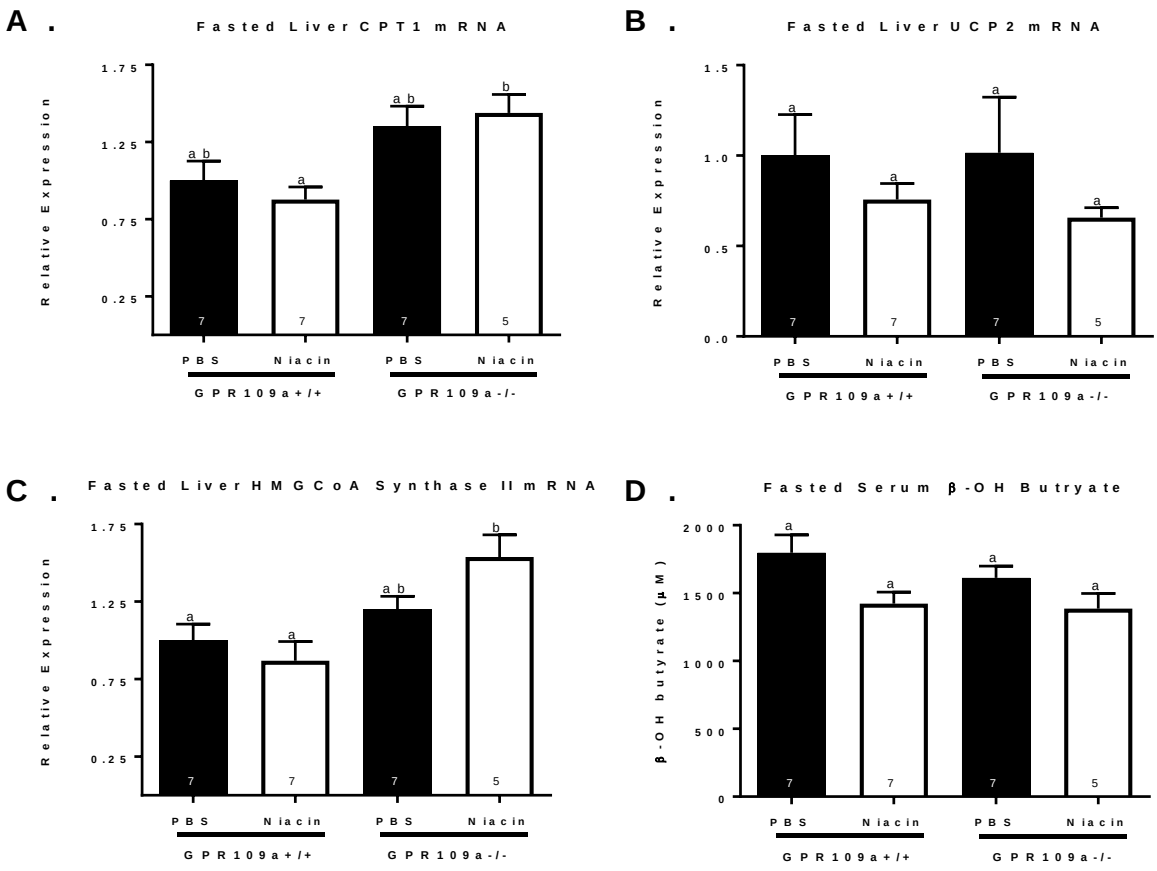
A .
Fasted Liver CPT1 mRNA
Relative Expression
1.75
1.25
0.75
0.25
a b
a
a b
b
7
7
7
5
PBS
Niacin
PBS
Niacin
GPR109a+/+
GPR109a-/-
B .
Fasted Liver UCP2 mRNA
Relative Expression
1.5
1.0
0.5
0.0
a
a
a
a
7
7
7
5
PBS
Niacin
PBS
Niacin
GPR109a+/+
GPR109a-/-
C .
Fasted Liver HMGCoA Synthase II mRNA
Relative Expression
1.75
1.25
0.75
0.25
a
a
a b
b
7
7
7
5
PBS
Niacin
PBS
Niacin
GPR109a+/+
GPR109a-/-
D .
Fasted Serum β-OH Butryate
β-OH butyrate (μM)
2000
1500
1000
500
0
a
a
a
a
7
7
7
5
PBS
Niacin
PBS
Niacin
GPR109a+/+
GPR109a-/-

Figure 12.

Future Directions

Having completed the work described in this manuscript, I want to address future research aims that would enhance our understanding into the role of β-OH butyrate in 1) regulating hepatic metabolism, 2) cholesterol metabolism, and 3) central nervous system function (cognition, central control of food intake, control of sympathetic tone, and reward signaling).

Further Work on the Role of Ketone Signaling in Hepatic Metabolism

We have shown that β-OH butyrate signaling feeds back to inhibit mRNA expression for genes critical to the hepatic fasting response in gluconeogenesis, β-oxidation, and ketogenesis. Although our metabolite assays agree with the use of mRNA expression as an accurate representation of metabolic flux, future research should focus on directly assessing ketogenic and gluconeogenic pathway flux. These studies could be performed either *in vivo* or *ex viv*o in perfused liver from control, HMGCS2 KD and GPR109a KO mice [1].

Of interest is the mechanism behind GPR109a mediated regulation of hepatic gene expression. Our whole body GPR109a knockout model does not allow us to determine if the GPR109a dependent effects of niacin or β-OH butyrate are due to direct responses at the liver or mediated through GPR109a action at other tissues. By using a cell culture model we can isolate the effects of niacin and β-OH butyrate to the hepatocyte. I propose 2 models to test for niacin and β-OH butyrate responses in hepatocytes. First, primary hepatocytes from WT and GPR109a KO mice. Second, an immortalized human hepatocyte cell line (HepG2 cells). Our primary experiments will test for a response in gene expression (PPARα, PEPCK, G6Pase, CPT1, UCP2, and HMGCS2) under basal conditions. However, we have previously observed that neither niacin nor β-OH butyrate treatment (48 h) significantly altered expression of PPARα, PEPCK, or ABCA1 mRNA (Appendix 2; Figures 2.1A, 2.1B, and 2.1C). We suspect that we will have to first induce expression of these genes to observe a down-regulation in response to niacin or β-OH butyrate treatment. I propose to simultaneously treat cultured cells with the PPARα agonist WY-14643 to up-regulate expression of these PPAR sensitive genes. This should allow us to observe down-regulation in response to niacin or β-OH butyrate exposure.

Upon establishing that GPR109a signaling in hepatocytes is essential for a response to niacin or β-OH butyrate, we can use these primary and immortalized cell culture models to identify the intracellular signaling pathways initiated by hepatic GPR109a agonism. Niacin treatment of primary hepatocytes and HepG2 cells decreases cAMP concentrations by 30% in a GPR109a dependent manner [2, 3]. This suggests hepatic GPR109a signaling is $G_{\alpha i}$ mediated and presents an appealing explanation for transcriptional regulation. PEPCK, G6Pase, CPT1, and HMGCS2 are all upregulated by cAMP, and a GPR109a dependent decrease in cAMP would oppose glucagon action and limit the level of CREB (cAMP response element binding protein) induced transcription. However, *in vivo* hepatic cAMP concentrations were not altered by HMGCS2 knockdown or niacin/β-OH butyrate treatment in fasting control or KD mice (Appendix 2; Figures 2.2A and 2.2B). Alternatively, the MAPK cascade, calcium mobilization, and β-arrestin signaling have recently been identified as downstream GPR109a signaling pathways in adipose and macrophage cells [4]. Further investigation into signaling pathways downstream of hepatic GPR109a and their role in transcriptional regulation may allow for a better understanding of β-OH butyrate mediated feedback.

Obesity is a state of constant hyperketonemia. Thus, I propose studies investigating β-OH butyrate signaling in obesity. These studies would first address the effect of obesity on the response to niacin or β-OH butyrate administration and next the role of hyperketonemia (HMGS2 KD) and GPR109a signaling in obesity induced insulin resistance, glucose stimulated insulin secretion, hepatic glucose output, and hepatic steatosis. I hypothesize that the constant hyperketonemia may result in β-OH butyrate resistance, preventing the negative feedback on hepatic ketogenesis and gluconeogenesis.

Niacin and Cholesterol

Research focused on the physiological response to niacin precedes the discovery of niacin and a GPR109a agonist in 2003. In fact, for nearly 60 years we have known that niacin improves serum cholesterol. It protects against atherosclerosis by raising serum HDL concentrations and simultaneously lowering LDL and VLDL concentrations [5]. Until very recently, it was believed that niacin's inhibition of adipose tissue lipolysis, which limited fatty acid availability in the liver for TAG

synthesis and export of VLDL, was responsible for these positive lipid profile modifying effects. However, this limitation of adipose tissue lipolysis and availability of free fatty acids, did not explain the elevation in HDL. Furthermore, long term niacin treatment commonly results in a rebound of serum free fatty acids to or above pre-treatment levels [6, 7]. Accordingly, niacin induced changes in circulating cholesterol are independent of GPR109a expression and serum free fatty acid levels [8].

The liver is the only site for HDL synthesis. The membrane protein ATP binding cassette transporter 1 (ABCA1) is required for hepatic HDL synthesis, esterifying cholesterol and phospholipids to the extracellular apolipoprotein apoA-1 to form nascent HDL particles [9]. Niacin upregulates ABCA1 mRNA and stimulates cholesterol efflux in adipocytes and macrophages [10-12] Niacin induced ABCA1 transcription depends on the nuclear receptor LXRα, expression of which is upregulated by PPARγ. Niacin stimulates production and release of prostaglandin D2 [13], an endogenous PPARγ agonist [14], offering a possible mechanism behind the upregulation of ABCA1. Niacin also promotes LXRα-dependent ABCA1 transcription and HDL biosynthesis in hepatocytes [15].

Although β-OH butyrate and niacin are both GPR109a ligands, no other possible shared mechanisms of action have been investigated. We have demonstrated that β-OH butyrate negatively regulates hepatic ABCA1 expression. β-OH butyrate injections markedly decreased ABCA1 mRNA in both control and KD mice (Appendix 3; Fig. 3.1C). Accordingly, fasting increased ABCA1 mRNA in HMGCS2 KD, but not WT mice, suggesting that endogenous β-OH butyrate limits ABCA1 mRNA during a fast (Appendix 3; Fig. 3.1A). There was an effect of niacin treatment to increase fed state ABCA1 expression in ASO treated mice, and niacin tended to increase fed state ABCA1 expression in GPR109a mice (Appendix 3; Figures 3.1B and 3.2B). An inhibitory role of hepatic GPR109a on cholesterol efflux has been suggested [3]. Our data supported this, as niacin treatment in fasting GPR109a null mice increased ABCA1 mRNA expression by 286% compared to the 17% increase with niacin treatment in fasting wildtype mice (Appendix 3; Fig. 3.2C).Thus, it appears niacin and β-OH

butyrate exert opposing regulation on hepatic ABCA1 expression. Niacin most effectively increased expression in fasting GPR109a KO and HMGCS2 KD mice, suggesting that β-OH butyrate signaling through GPR109a suppresses ABCA1 transcript levels. If the negative regulation by β-OH butyrate is GPR109a mediated, why niacin does not share this mechanism and instead upregulates expression remains to be determined.

Interestingly, a long term ketogenic diet lowers serum LDL and TAG levels while raising HDL cholesterol in both normal and hypercholesterolemic adults [16]. Significant reductions in total cholesterol, LDL, and TAG concentrations have additionally been demonstrated with numerous intermittent fasting programs [17]. The potential to positively affect serum cholesterol under conditions that result in elevated β-OH butyrate production in a similar manner as long term niacin treatment demands research into the mechanism by which ketogenic states alter hepatic cholesterol metabolism. In addition to increasing ABCA1 expression, niacin elevates serum HDL by decreasing HDL clearance through reduced surface expression of the HDL receptor, ATP synthase β chain [18, 19]. Niacin has been proposed to lower serum LDL by increasing post-translational degradation of apoB100, the primary apolipoprotein in VLDL and LDL particles [20]. Niacin's inhibition of DGAT2 activity and TAG synthesis is thought to be partially responsible for the lower serum LDL, as insufficient lipidation prevents cellular export of apoB100 [21, 22]. Our data suggest that β-OH butyrate does not inhibit DGAT2, as only niacin but not β-OH butyrate treatment suppressed fasting hepatic TAG concentrations. The role of β-OH butyrate in altering serum cholesterol concentrations is an intriguing research question with implications for dyslipidemia improvement through dietary manipulations alone.

<u>β-OH Butyrate in the Central Nervous System</u>

1. β-OH butyrate and Cognitive Function

Fasting, heat stress, exercise, a ketogenic diet, and diabetes are all characterized by elevated β-OH butyrate. However, the cognitive response to these ketogenic metabolic perturbations varies. Food deprivation, heat stress, and diabetes are associated with varied deficits in cognitive performance. Food deprivation has been shown to either have no effect or depress cognitive function [23, 24]. Heat stress induces memory impairment and mental fatigue [25, 26], while diabetes is associated with accelerated cognitive decline [27]. The response to ketogenic diets may depend on the developmental stage of the CNS. A ketogenic diet improves cognitive function in adult rats, and ketones are therapeutically protective in neurodegenerative conditions [28, 29]. However, in weanling rats a ketogenic diet negatively impacts visual-spatial learning and memory [30]. Finally, exercise improves executive function, concentration related cognition, and reasoning related cognition [24, 31]. B-OH butyrate is directly implicated in this response to prolonged exercise, as β-OH butyrate inhibits central histone deacetylase activity, increasing brain derived neurotrophic factor (BDNF) expression, thereby enhancing memory formation and mental health [32]. Although each of these conditions leads to increased circulating β-OH butyrate, the cognitive response is varied. I propose that in each of these states β-OH butyrate is acting to enhance BDNF expression to improve mental health and cognition. Accordingly, the ketogenic conditions that result in depressed cognitive function would more severely depress cognitive function in the absence of β-OH butyrate, while those conditions that improve cognitive function rely on β-OH butyrate action in the CNS to improve cognition.

The HMGCS2 knockdown mouse would allow for tests focused on the role of β-OH butyrate in the cognitive changes induced by these physiological conditions. GPR109a is expressed in both the human and mouse brain, and has been shown to elevate blood pressure through activation of the rostral ventrolateral medulla (RVML) initiated pressor response and is proposed to have anti-inflammatory roles in CNS pathology [33, 34]. The impact of central GPR109a signaling on cognition has yet to be explored. Studies with the GPR109a null mouse would allow for understanding the role of GPR109a

signaling in the response to ketogenic stimuli. Research directed toward determining how elevated ketones affect cognitive function and mental health is integral to assessing therapeutic potential.

2. β-OH butyrate and Food Intake

Control of food intake is regulated by a complex biological network integrating both orexigenic and anorexigenic signaling from macronutrients and hormones across multiple brain areas. Interestingly, ketogenic diets commonly reduce food intake and reported feelings of hunger [35, 36]. Still the site mediating this response to ketogenic diets remains in question. Ketogenic diets alter secretion of gut-derived hormones, suppressing secretion of ghrelin, an orexigenic hormone, and increasing secretion of cholescystokinin, an anorexigenic hormone [37]. Ketogenic diets elevate serum non-esterified fatty acids, which induce satiety by decreasing hypothalamic neuropeptide Y (NPY; orexigenic) expression [35]. Supporting the possibility that the phagic response to β-OH butyrate depends on nutritional status, β-OH butyrate decreases hypothalamic agouti related peptide (AgRP; orexigenic) expression only when serum glucose concentration is low [35, 38]. Intraperitoneal β-OH butyrate administration suppresses food intake in rodents dependent on an intact vagal nerve [39, 40]. Thus, ketogenic diets may depress phagic drive by altering gut hormone secretion, increasing adipose tissue lipolysis, altering hypothalamic neuropeptide expression or acting on the peripheral nervous system. Contrastingly, β-OH butyrate signaling has been proposed to encourage food intake by changing central GABA signaling, AMPK phosphorylation, ROS production, and GPR109a dependent stimulation of adiponectin release [35]. As ketone concentrations rise in starvation, it seems logical that β-OH butyrate would have a role in the control of appetite. However, the effect of ketogenesis in appetite regulation and its dependence on hormone and metabolic status remains unclear.

3. β-OH butyrate and Sympathetic Tone

GPR41 (Free fatty acid receptor 3; FFA3) is a recently identified β-OH butyrate responsive G-protein coupled receptor that is abundantly expressed in sympathetic ganglia. Originally, β-OH butyrate

was classified as an antagonist at GPR41, opposing the action of short chain fatty acids (SCFA) at this receptor [41]. However, more recent studies have suggested that SCFAs and β-OH butyrate are both GPR41 agonists and that only β-OH butyrate acts at physiological concentrations [42]. In a co-culture system of primary cardiomyocytes and sympathetic ganglia neurons from WT and GPR41 KO mice β-OH butyrate inhibited neuron firing frequency and cardiomyocyte beat rate. *In vivo*, β-OH butyrate decreased heart rate and oxygen consumption in WT but not GPR41 KO mice. Heart rate was strongly decreased by the ketogenic states of fasting (48 h) and diabetes (STZ induced) in WT mice, yet these same conditions only mildly reduced heart rate in GPR41 KO mice (only ~1/3 of reduction observed in WT control). This group concludes that in the fasted state β-OH butyrate decreases energy consumption by limiting sympathetic tone. In dissociated rat sympathetic neurons, β-OH butyrate activates sympathetic ganglia GPR41 to inhibit N-type voltage gated calcium channel currents [42]. Inhibition of sympathetic neuronal calcium currents decreases norepinephrine release from nerve terminals, thereby reducing sympathetic activity.

Although there is conflicting literature on the agonist/antagonist effects of β-OH butyrate action at GPR41, there is agreement that β-OH butyrate decreases sympathetic activity. β-OH butyrate also attenuate norepinephrine induced brown adipose tissue thermogenesis [43] and decreases adipocyte β-adrenergic receptor ligand affinity [44]. These findings support the hypothesis that β-OH butyrate regulates energy homeostasis to preserve cellular and systemic energy sources during periods of fasting. Interestingly, obesity is associated with depressed energy expenditure ($kJ/Kg^{0.75}$). I suggest that future studies focus on the role of β-OH butyrate in the dysregulation of energy expenditure and sympathetic activity in obesity and diabetes. These studies will require a more complete understanding of β-OH butyrate effects on energy balance in the lean mouse.

4. β-OH butyrate and Reward

Short term fasting is frequently reported to improve mood, enhance alertness, evoke feelings of tranquility, and even act as an antidepressant [45-47]. Indeed, many religions and rituals employ fasting

as a means to attain a higher state of mind. Similarly, ketogenic diets are proposed to stabilize mood [48]. This may coincide with decreased feelings of hunger [49]. β-OH butyrate shares a similar structure with γ-hydroxybutyrate (GHB), a recreationally used drug that acts as a CNS depressant by binding the $GABA_B$ receptor [50]. It has been hypothesized that β-OH butyrate in the brain may have a weak affinity for $GABA_B$ receptors, explaining the mild euphoria that can occur under ketogenic conditions [51]. $GABA_B$ stimulation is known to modulate reward and addiction processes [52, 53]. Food is strongly connected with feeling of reward, and interestingly in both anorexia nervosa and obesity reward processing is dysregulated [54-57]. Although on opposite spectrums of weight disorders, hyperketonemia occurs in both conditions and possible effects of β-OH butyrate on reward may contribute to alterations in reward neurobiology. Thus far, this connection is almost entirely speculative and anecdotal, although a series of experiments testing the similarities between β-OH butyrate and GHB on mood have previous been outlined [51]. This intriguing line of thought warrants further investigation given the possible benefits and risks across numerous hyperketonemic states.

Herein, I have proposed a range of research aimed at understanding the physiological consequences of elevated β-OH butyrate. This question is timely given the obesity epidemic, global warming, and high incidence of eating disorders. New aspects of β-OH butyrate signaling are being uncovered at a rapid rate and new animal models allow us to better identify the mechanisms that mediate physiological or psychological responses. Future work focused on hyperketonemia will encourage understanding of hepatic metabolism, cholesterol metabolism, control of food intake, energy expenditure, cognitive function, and reward signaling.

APPENDIX 1

Fed State Niacin Treatment in GPR109a Null and HMGCS2 Knockdown Mice

Figure Captions

Figure 1.1 Glucose homeostasis in response to niacin treatment in fed control and KD mice. Hepatic (A) glycogen (mg/g liver tissue), (B) PEPCK mRNA expression, (C) G6Pase mRNA expression, and (D) serum glucose (mg/dL). [a,b] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

Figure 1.2 Lipid homeostasis in response to niacin treatment in fed control and KD mice. Serum (A) non-esterified fatty acids (NEFA; μM), (B) triacylglycerol (TAG; mg/dL), hepatic (C) non-esterified fatty acids (NEFA; μM/g liver tissue), (D) triacylglycerol (TAG; mg/g liver tissue), and (E) PPARα mRNA expression. [a,b] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

Figure 1.3 Changes in genes supporting hepatic ketone production in response to niacin treatment in fed control and KD mice. Hepatic (A) CPT1 mRNA expression, (B) UCP2 mRNA expression, (C) HMGCS2 mRNA expression, and (D) serum β-OH butyrate (μM). [a,b] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

Figure 1.4 Glucose homeostasis in response to niacin treatment in fed GPR109a +/+ and -/- mice. Hepatic (A) glycogen (mg/g liver tissue), (B) PEPCK mRNA expression, (C) G6Pase mRNA expression, and (D) serum glucose (mg/dL). [a,b] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

Figure 1.5 Lipid homeostasis in response to niacin treatment in fed GPR109a +/+ and -/- mice. Serum (A) non-esterified fatty acids (NEFA; μM), (B) triacylglycerol (TAG; mg/dL), hepatic (C) non-esterified fatty acids (NEFA; μM/g liver tissue), (D) triacylglycerol (TAG; mg/g liver tissue), and (E) PPARα mRNA expression. [a,b] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

Figure 1.6 Changes in genes supporting hepatic ketone production in response to niacin treatment in fed GPR109a +/+ and -/- mice. Hepatic (A) CPT1 mRNA expression, (B) UCP2 mRNA expression, (C) HMGCS2 mRNA expression, and (D) serum β-OH butyrate (μM). [a,b] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

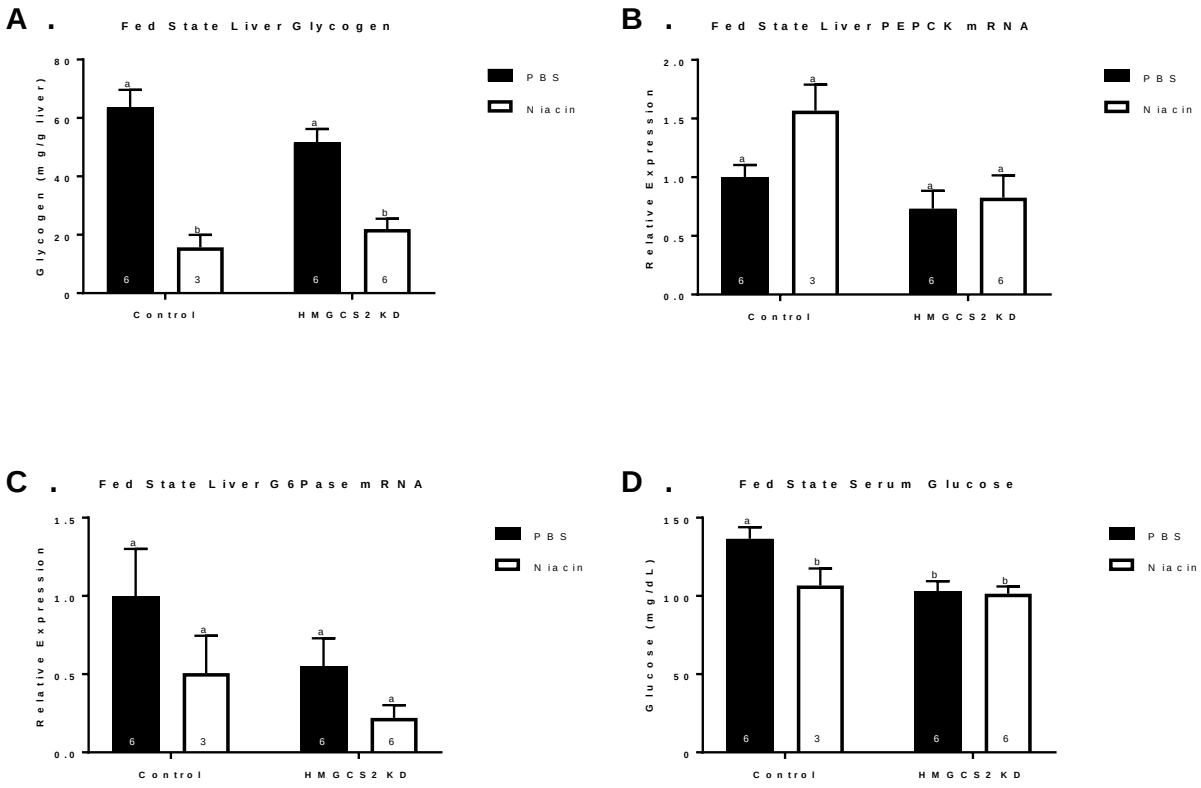
A .
Fed State Liver Glycogen
Glycogen (mg/g liver)
80
60
40
20
0
a
b
a
b
6
3
6
6
Control
HMGCS2 KD
PBS
Niacin
B .
Fed State Liver PEPCK mRNA
Relative Expression
2.0
1.5
1.0
0.5
0.0
a
a
a
a
6
3
6
6
Control
HMGCS2 KD
PBS
Niacin
C .
Fed State Liver G6Pase mRNA
Relative Expression
1.5
1.0
0.5
0.0
a
a
a
a
6
3
6
6
Control
HMGCS2 KD
PBS
Niacin
D .
Fed State Serum Glucose
Glucose (mg/dL)
150
100
50
0
a
b
b
b
6
3
6
6
Control
HMGCS2 KD
PBS
Niacin

Figure 1.1

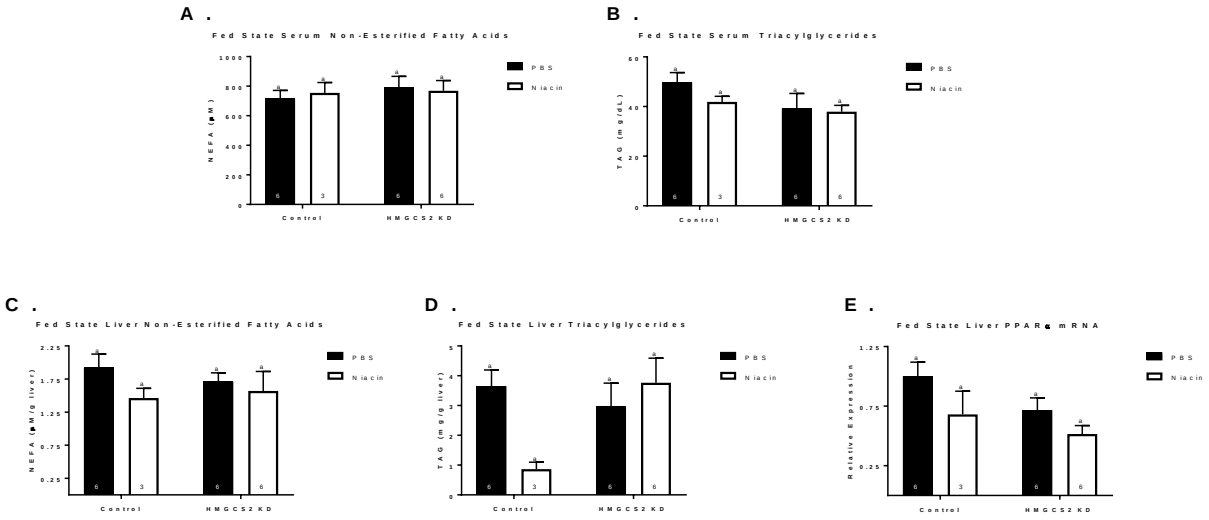
A .
Fed State Serum Non-Esterified Fatty Acids
NEFA (μM)
1000
800
600
400
200
0
a
a
a
a
6
3
6
6
Control
HMGCS2 KD
PBS
Niacin
B .
Fed State Serum Triacylglycerides
TAG (mg/dL)
60
40
20
0
a
a
a
a
6
3
6
6
Control
HMGCS2 KD
PBS
Niacin
C .
Fed State Liver Non-Esterified Fatty Acids
NEFA (μM/g liver)
2.25
1.75
1.25
0.75
0.25
a
a
a
a
6
3
6
6
Control
HMGCS2 KD
PBS
Niacin
D .
Fed State Liver Triacylglycerides
TAG (mg/g liver)
5
4
3
2
1
0
a
a
a
a
6
3
6
6
Control
HMGCS2 KD
PBS
Niacin
E .
Fed State Liver PPARα mRNA
Relative Expression
1.25
0.75
0.25
a
a
a
a
6
3
6
6
Control
HMGCS2 KD
PBS
Niacin

Figure 1.2

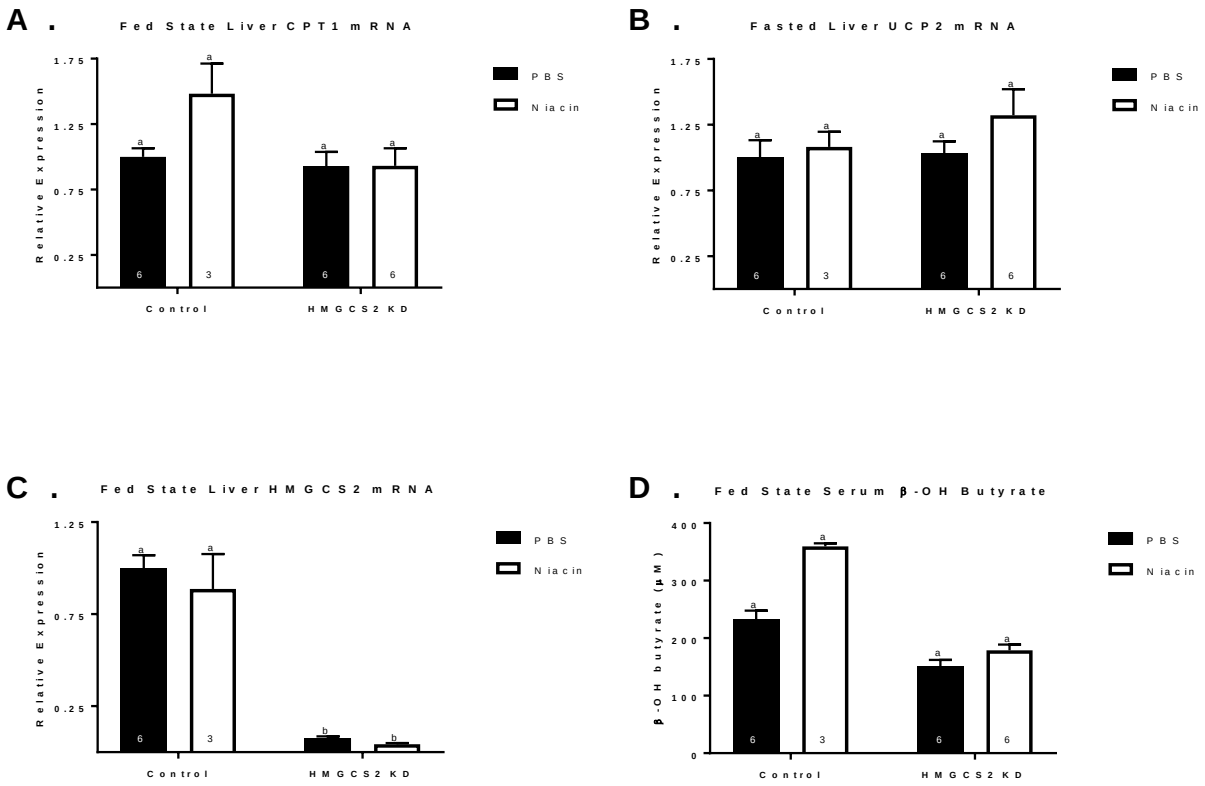
A .
Fed State Liver CPT1 mRNA
Relative Expression
1.75
1.25
0.75
0.25
a
a
a
a
6
3
6
6
Control
HMGCS2 KD
PBS
Niacin
B .
Fasted Liver UCP2 mRNA
Relative Expression
1.75
1.25
0.75
0.25
a
a
a
a
6
3
6
6
Control
HMGCS2 KD
PBS
Niacin
C .
Fed State Liver HMGCS2 mRNA
Relative Expression
1.25
0.75
0.25
a
a
b
b
6
3
Control
HMGCS2 KD
PBS
Niacin
D .
Fed State Serum β-OH Butyrate
β-OH butyrate (μM)
400
300
200
100
0
a
a
a
a
6
3
6
6
Control
HMGCS2 KD
PBS
Niacin

Figure 1.3

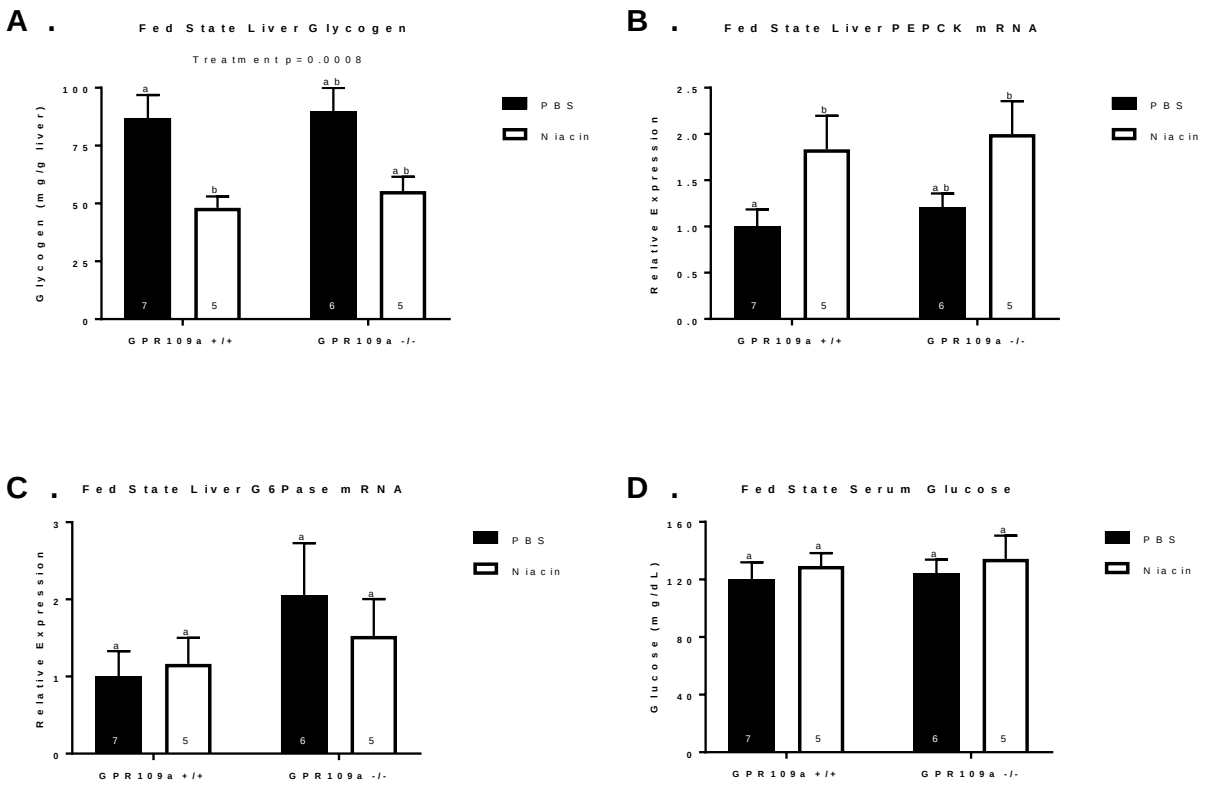
A .
Fed State Liver Glycogen
Treatment p=0.0008
Glycogen (mg/g liver)
100
75
50
25
0
a
b
a b
a b
7
5
6
5
GPR109a +/+
GPR109a -/-
PBS
Niacin
B .
Fed State Liver PEPCK mRNA
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
a
b
a b
b
7
5
6
5
GPR109a +/+
GPR109a -/-
PBS
Niacin
C .
Fed State Liver G6Pase mRNA
Relative Expression
3
2
1
0
a
a
a
a
7
5
6
5
GPR109a +/+
GPR109a -/-
PBS
Niacin
D .
Fed State Serum Glucose
Glucose (mg/dL)
160
120
80
40
0
a
a
a
a
7
5
6
5
GPR109a +/+
GPR109a -/-
PBS
Niacin

Figure 1.4

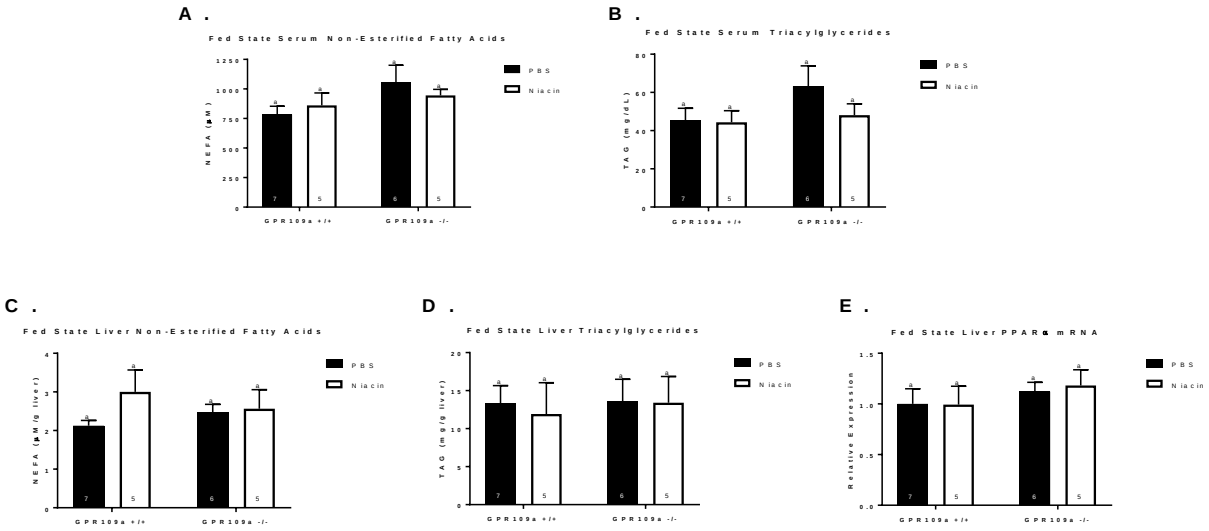
A .
Fed State Serum Non-Esterified Fatty Acids
NEFA (μM)
PBS
Niacin
GPR109a +/+
GPR109a -/-
B .
Fed State Serum Triacylglycerides
TAG (mg/dL)
C .
Fed State Liver Non-Esterified Fatty Acids
NEFA (μM/g liver)
D .
Fed State Liver Triacylglycerides
TAG (mg/g liver)
E .
Fed State Liver PPARα mRNA
Relative Expression

Figure 1.5

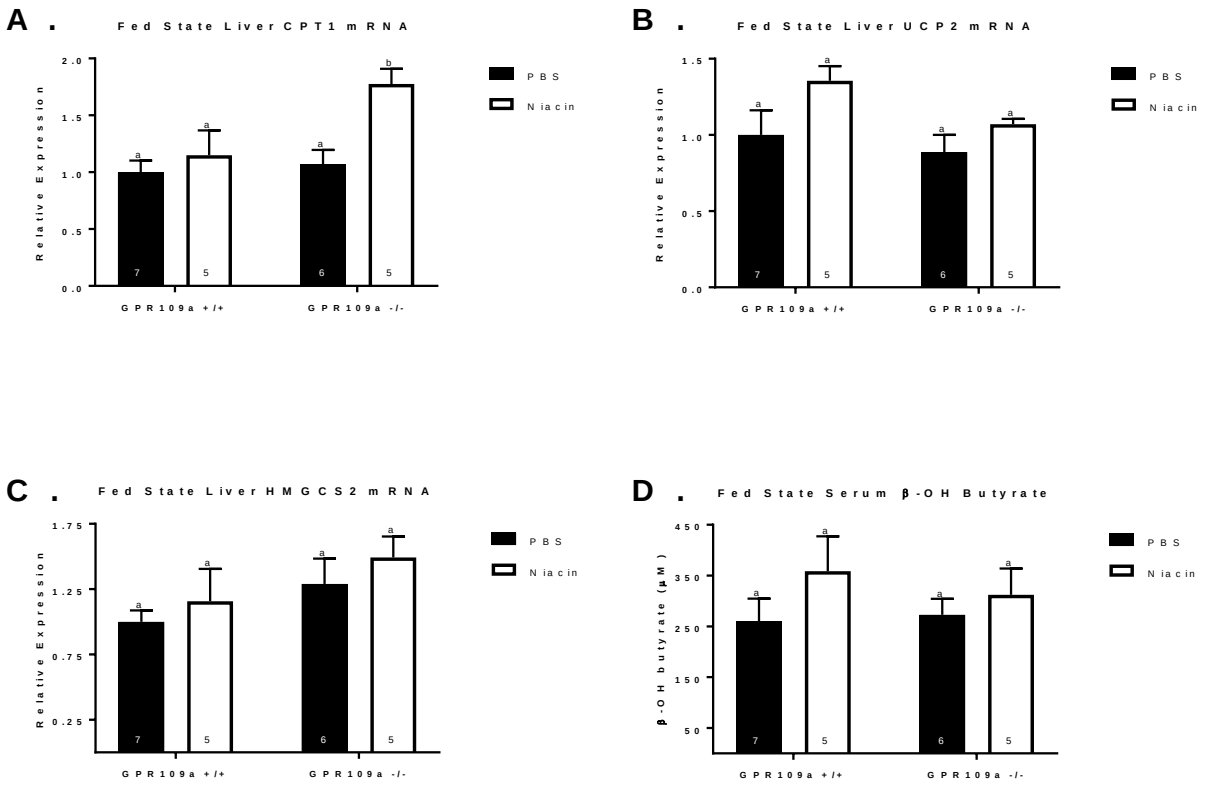
A .
Fed State Liver CPT1 mRNA
Relative Expression
2.0
1.5
1.0
0.5
0.0
a
a
a
b
7
5
6
5
GPR109a +/+
GPR109a -/-
PBS
Niacin
B .
Fed State Liver UCP2 mRNA
Relative Expression
1.5
1.0
0.5
0.0
a
a
a
a
7
5
6
5
GPR109a +/+
GPR109a -/-
PBS
Niacin
C .
Fed State Liver HMGCS2 mRNA
Relative Expression
1.75
1.25
0.75
0.25
a
a
a
a
7
5
6
5
GPR109a +/+
GPR109a -/-
PBS
Niacin
D .
Fed State Serum β-OH Butyrate
β-OH butyrate (μM)
450
350
250
150
50
a
a
a
a
7
5
6
5
GPR109a +/+
GPR109a -/-
PBS
Niacin

Figure 1.6

APPENDIX 2

Additional Experiments Performed to Assess the Role of Ketone Signaling in the Hepatic Response to Fasting

Figure Captions

Figure 2.1 HepG2 gene expression following 48 hours treatment with control media or media containing niacin (1mM) or β-OH Butyrate (15mM). (A) PPARα mRNA expression, (B) PEPCK mRNA expression, (C), ABCA1 mRNA expression. [a,b] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

Figure 2.2 Hepatic cAMP concentration in control and HMGCS2 KD mice. Hepatic (A) cAMP (pM/g liver) in PBS treated mice, and (B) cAMP (pM/g liver) in fasted mice. [a,b] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group).

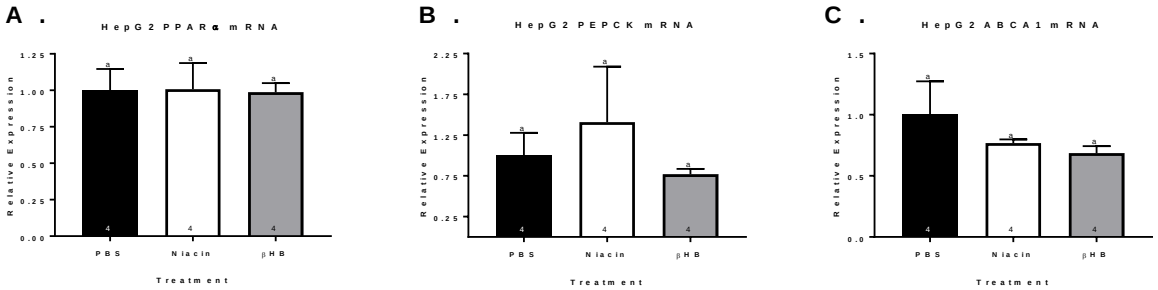
A .
HepG2 PPARα mRNA
Relative Expression
1.25
1.00
0.75
0.50
0.25
0.00
a
a
a
4
4
4
PBS
Niacin
βHB
Treatment
B .
HepG2 PEPCK mRNA
Relative Expression
2.25
1.75
1.25
0.75
0.25
a
a
a
4
4
4
PBS
Niacin
βHB
Treatment
C .
HepG2 ABCA1 mRNA
Relative Expression
1.5
1.0
0.5
0.0
a
a
a
4
4
4
PBS
Niacin
βHB
Treatment

Figure 2.1

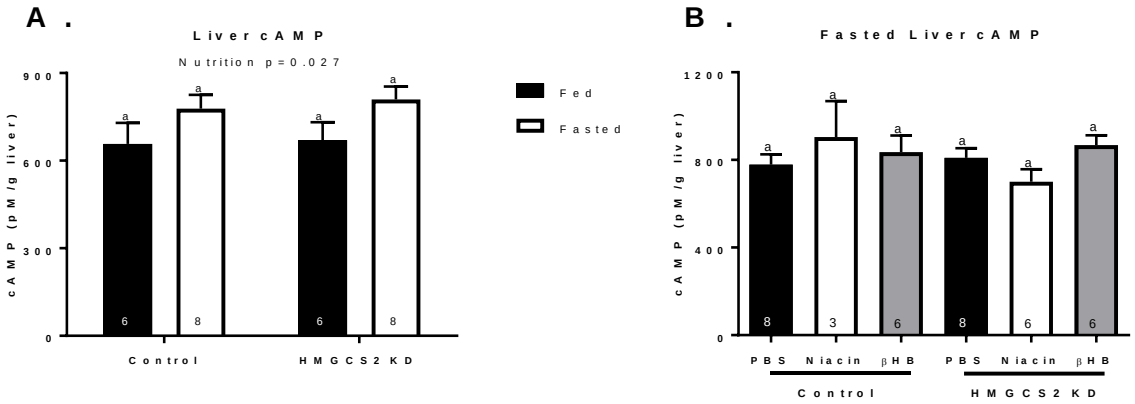
A .
Liver cAMP
Nutrition p=0.027
cAMP (pM/g liver)
900
600
300
0
a
a
a
a
6
8
6
8
Control
HMGCS2 KD
Fed
Fasted
B .
Fasted Liver cAMP
cAMP (pM/g liver)
1200
800
400
0
a
a
a
a
a
a
8
3
6
8
6
6
PBS
Niacin
βHB
PBS
Niacin
βHB
Control
HMGCS2 KD

Figure 2.1

APPENDIX 3

The Role of Niacin and β-OH Butyrate on *in vivo* Hepatic ABCA1 mRNA Expression

Figure Captions

Figure 3.1 Changes in hepatic ABCA1 mRNA expression with niacin or β-OH Butyrate treatment in control and HMGCS2 KD mice. Hepatic (A) ABCA1 mRNA expression in saline treated mice, (B) ABCA1 mRNA expression in fed state mice, and (C) ABCA1 mRNA expression in fasted mice. [a,b] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group). *Indicates a significant difference between PBS treated control and KD groups ($P < 0.05$).

Figure 3.2 Changes in hepatic ABCA1 mRNA expression with niacin or β-OH Butyrate treatment in GPR109a +/+ and -/- mice. Hepatic (A) ABCA1 mRNA expression in saline treated mice, (B) ABCA1 mRNA expression in fed state mice, and (C) ABCA1 mRNA expression in fasted mice. [a,b] Bars that do not share a common letter differ significantly ($P < 0.05$; number inside bar denotes n per group). *Indicates a significant difference between PBS treated WT and KO groups ($P < 0.05$).

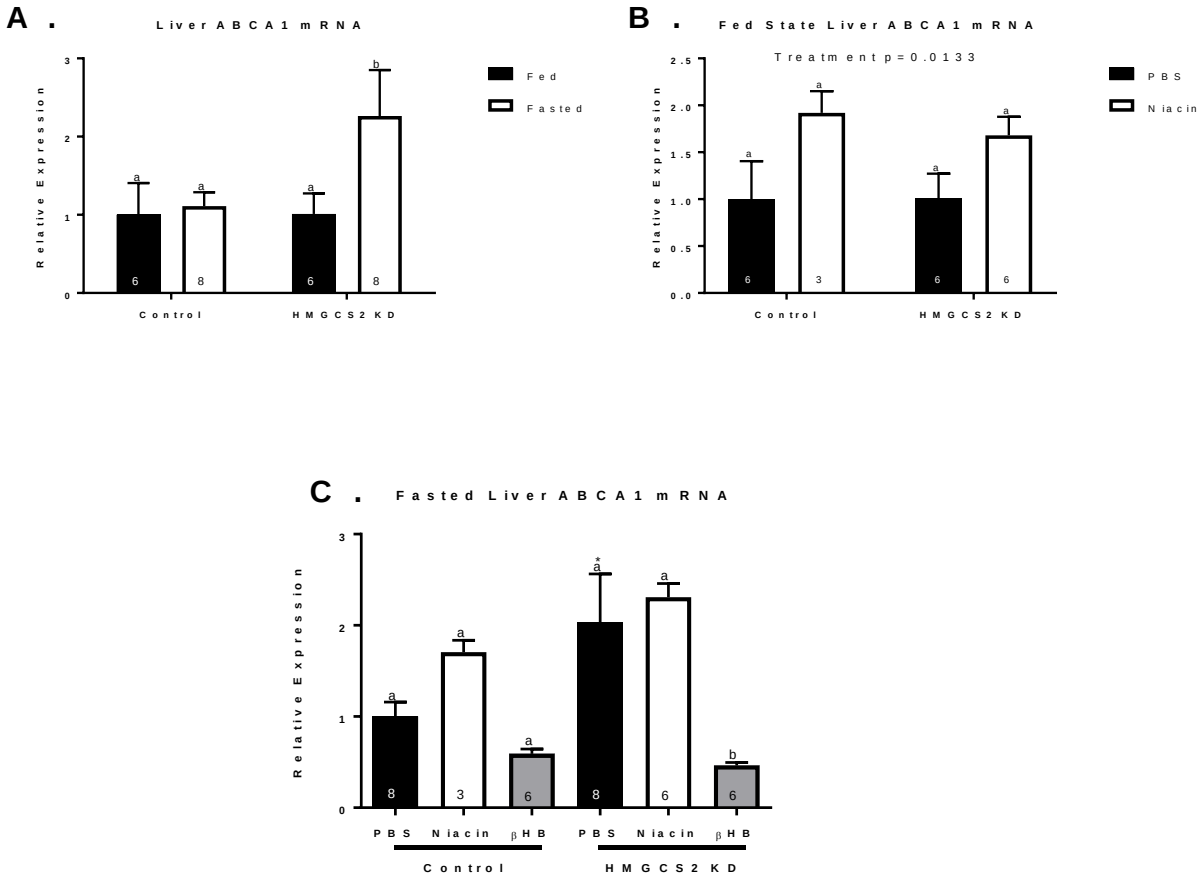
A .
Liver ABCA1 mRNA
Relative Expression
Fed
Fasted
Control
HMGCS2 KD
B .
Fed State Liver ABCA1 mRNA
Treatment p=0.0133
Relative Expression
PBS
Niacin
Control
HMGCS2 KD
C .
Fasted Liver ABCA1 mRNA
Relative Expression
PBS
Niacin
βHB
Control
HMGCS2 KD

Figure 3.1

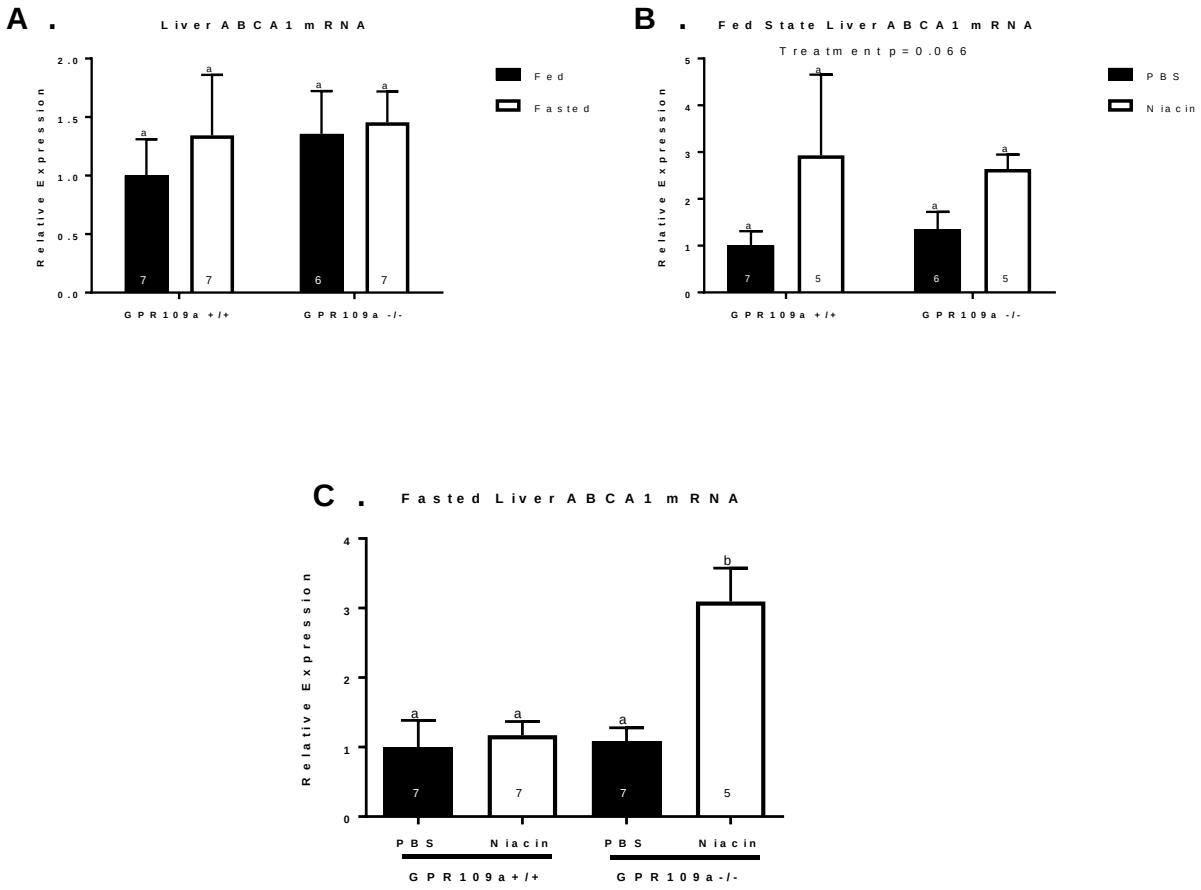
A .
Liver ABCA1 mRNA
Relative Expression
2.0
1.5
1.0
0.5
0.0
a
a
a
a
7
7
6
7
GPR109a +/+
GPR109a -/-
Fed
Fasted
B .
Fed State Liver ABCA1 mRNA
Treatment p=0.066
Relative Expression
5
4
3
2
1
0
a
a
a
a
7
5
6
5
GPR109a +/+
GPR109a -/-
PBS
Niacin
C .
Fasted Liver ABCA1 mRNA
Relative Expression
4
3
2
1
0
a
a
a
b
7
7
7
5
PBS
Niacin
PBS
Niacin
GPR109a+/+
GPR109a-/-

Figure 3.2

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