Article

The Role of GPR109a Signaling in Niacin Induced Effects on Fed and Fasted Hepatic Metabolism.

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Abstract: Signaling through GPR109a, the putative receptor for the endogenous ligand β-OH butyrate, inhibits adipose tissue lipolysis. Accordingly, this provides a feedback mechanism by which the liver can communicate to adipose tissue to limit lipolytic flux. Niacin, an anti-atherosclerotic drug, activates GPR109a at nM concentrations. However, the GPR109a mediated anti-lipolytic actions of niacin are not required for niacin to improve circulating triglyceride and lipoprotein concentrations. Niacin also modulates glucose metabolism and regulates transcription of gluconeogenic genes, although the role of GPR109a in these actions is unclear. To better understand the involvement of GPR109a signaling in regulating glucose and lipid metabolism, we treated GPR109a wildtype (+/+) or knockout (-/-) mice with repeated overnight injections of saline or niacin in physiological states characterized by low concentrations (ad libitum fed) of the endogenous ligand, β-OH butyrate, or in a ketogenic state (16 hour fast). Niacin decreased fasting serum non-esterified fatty acid concentrations in both GPR109a +/+ and -/- mice. Accordingly, independent of GPR109a expression, niacin blunted fast-induced hepatic triglyceride accumulation. Niacin decreased fasting hepatic mRNA expression of the lipid activated transcription factor peroxisome proliferator activated receptor α (PPARα). Still, hepatic expression of PPARα target genes in gluconeogenesis, β-oxidation, and ketogenesis during a fast was unaffected by GPR109a expression or niacin. Niacin did not alter serum or hepatic lipids in the fed state, suggesting the effects of acute niacin treatment during fasting are downstream of inhibiting lipolysis. Surprisingly, GPR109a knockout did not affect glucose or lipid homeostasis or hepatic gene expression in either fed or fasted mice. In turn, GPR109a does not appear to be essential for the metabolic response to the ketogenic state or the pharmacological benefits associated with niacin.

Keywords: GPR109a; β-OH butyrate; niacin; metabolic homeostasis; liver

Introduction

GPR109a was identified as the niacin receptor in 2003 [1]. Although niacin binds to GPR109a with high affinity (100 nM EC_{50}), this concentration is only reached in response to administration of pharmacological doses. In 2005, it was established that physiologically relevant concentrations of β-OH butyrate activated GPR109a [2]. With an EC_{50} of 700-800 μM, physiologically relevant changes in β-OH butyrate concentrations that accompany a fast can vary signaling at GPR109a [3]. While GPR109a was first shown to inhibit adipose tissue lipolysis, it has since been identified in various other tissues with a broad range of physiological actions [4]. The GPR109a agonist, niacin, regulates gene expression in liver, skeletal muscle, adipose tissue, and macrophages, although a direct role of GPR109a signaling has not been explored [5-8].

Niacin is a powerful anti-atherosclerotic lipid lowering drug whose clinical potential was first recognized over half a century ago [9]. Niacin effectively decreases circulating triglyceride (TAG) and very low density lipoprotein (VLDL) concentrations while raising high density lipoprotein (HDL) levels in patients with dyslipidemia [10,11]. While statins became the dominant therapy for

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hypercholesterolemia after their introduction in 1987, niacin is prescribed in statin resistant individuals and the benefits of statin/niacin combination treatment are under debate [12-14]. Interestingly, niacin was found to improve plasma cholesterol levels in GPR109a -/- mice, questioning the underlying role of GPR109a in niacin's lipid efficacy [15].

Studies investigating GPR109a dependent and independent components of niacin signaling are necessary to maximize the clinical applications of niacin therapy. Using HMGCS2 knockdown, we had previously established that ketones were important regulators of the metabolic response to a fast [3]. In the studies presented here, we expand upon those findings to focus on the role of GPR109a in this metabolic feedback. These studies focused on glucose and lipid homeostasis, hepatic metabolic enzyme mRNA expression, and serum lipid and ketogenic profiles, allow us to assess the on the role of GPR109a in the normal fasting response and pharmacological effects of niacin.

Results

We first investigated the metabolic response to niacin treatment in fed state wildtype and GPR109a null mice. Niacin decreased hepatic glycogen content in both genotypes but did not alter serum glucose concentrations (Figures 1A-1B). Niacin did not affect serum insulin or the glucose:insulin ratio (Figures 1C-1D). Additionally, glucose clearance during an IP glucose tolerance test and glucose stimulated serum insulin concentrations were not different between GPR109a +/+ and -/- mice (Figures 1E-1G). As niacin modulates cholesterol and triglyceride metabolism [9,10], we assessed the lipid profile in niacin treated GPR109a +/+ and -/- mice. Acute niacin treatment had no effect on serum or hepatic non-esterified fatty acid (NEFA) and triglyceride (TAG) concentrations in the fed state (Figures 2A-2D). Serum β-OH butyrate concentrations were not affected by niacin in either genotype (Figure 2E).

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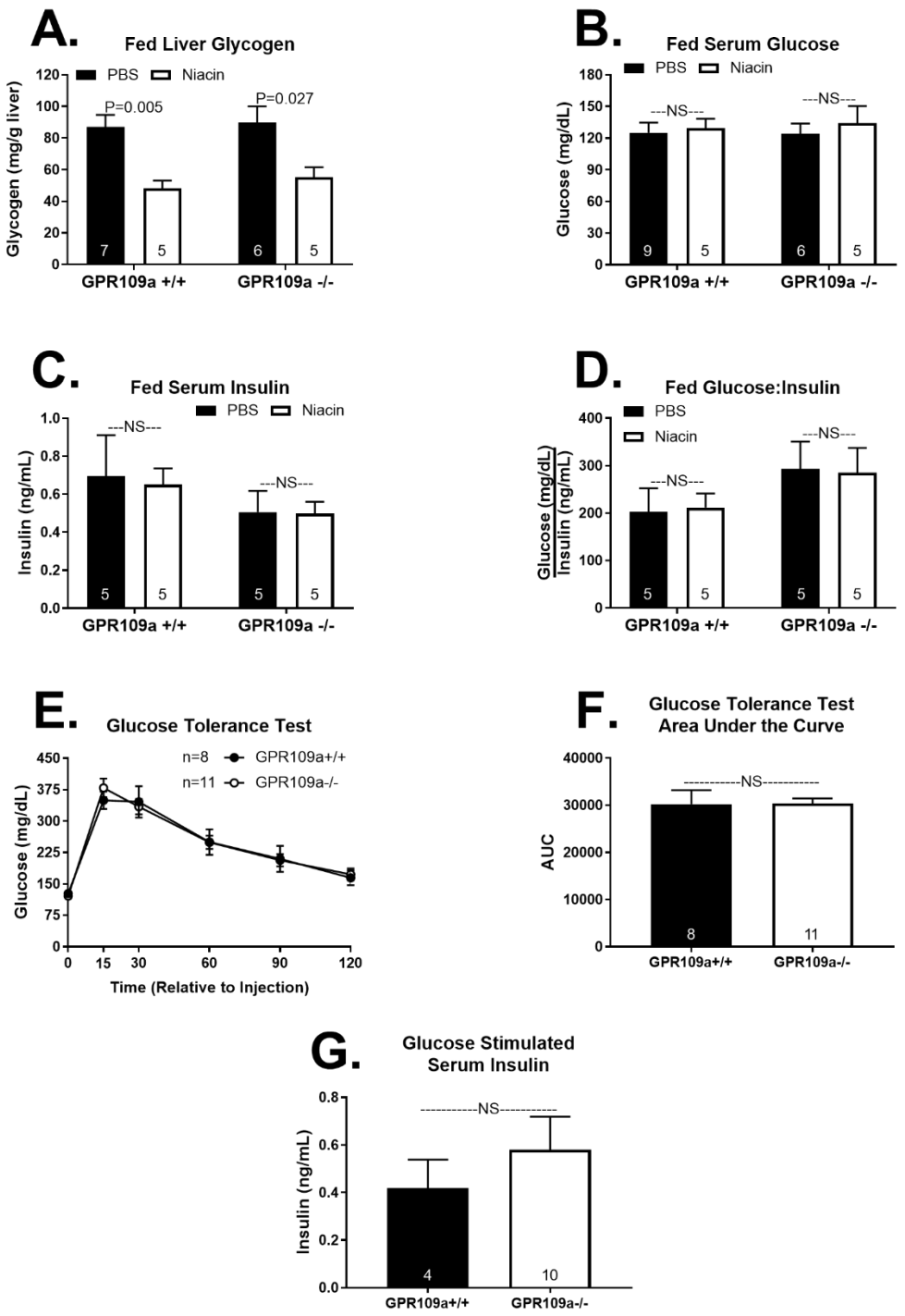

Figure 1. Effect of niacin on glucose homeostasis in fed GPR109a +/+ and -/- mice. Hepatic (A) glycogen (mg/g liver tissue), serum (B) glucose (mg/dL), (C) insulin (ng/mL), and (D) glucose:insulin ratio. Direct comparisons were made between injection treatment within genotype. (E) Glucose tolerance test in 4-hour fasted mice. (F) Glucose tolerance test area under the curve. (G) Glucose stimulated serum insulin. Bars were analyzed by a two-sided unpaired T-test. NS = non-significant; P > 0.05; PBS = phosphate buffered saline. Number inside bar denotes n per group.

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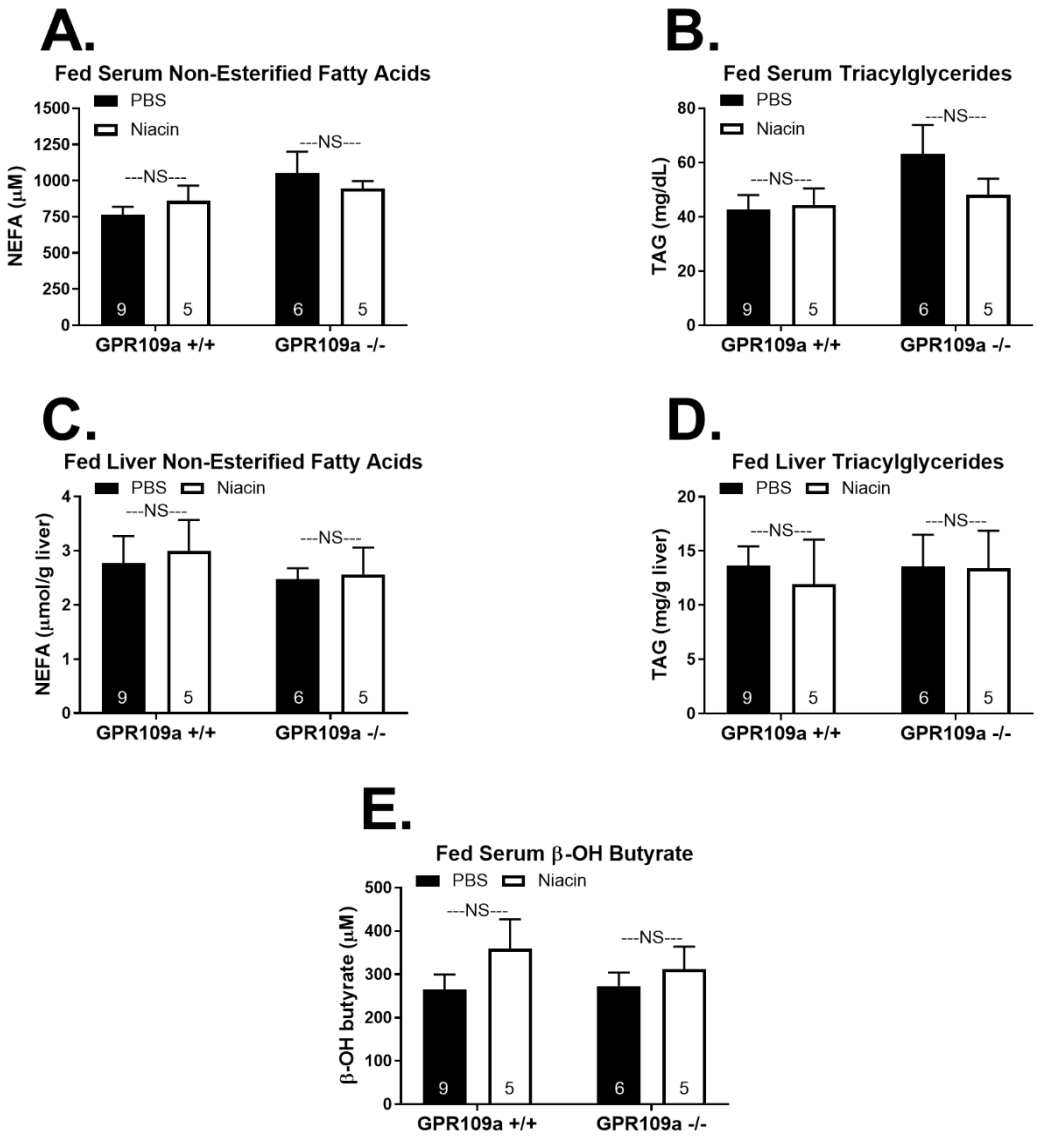

Figure 2. Effect of niacin on lipid homeostasis in fed GPR109a +/+ and -/- mice. Serum (A) non-esterified fatty acids (NEFA; μM) and (B) triacylglycerol (TAG; mg/dL). Hepatic (C) non-esterified fatty acids (NEFA; μmol/g liver tissue) and (D) triacylglycerol (TAG; mg/g liver tissue). (E) Serum β-OH butyrate (μM). Direct comparisons were made between injection treatment within genotype. NS = non-significant; $P > 0.05$; PBS = phosphate buffered saline. Number inside bar denotes n per group.

We expected that GPR109a signaling may exert physiologically relevant regulation of genes in pathways that are active when production of the endogenous GPR109a ligand, β-OH butyrate, is upregulated [4]. Accordingly, we examined hepatic mRNA expression of key genes in β-oxidation, ketogenesis, and gluconeogenesis. Fed state hepatic mRNA expression of the lipid activated transcription factor PPARα [16] was not affected by niacin treatment in either genotype (Figure 3A). The mitochondrial uncoupling protein 2 (UCP2) is essential for NAD^+ regeneration during fasting, to support high rates of lipid oxidation and ketone production [17,18]. Niacin did not alter fed hepatic UCP2 expression independent of GPR109a expression (Figure 3B). Hepatic mRNA expression of the mitochondrial long-chain fatty acid transporter that regulates lipid entry to β-oxidation, carnitine palmitoyltransferase 1 (CPT1), was increased by niacin in GPR109a -/- but not +/+ mice (Figure 3C). Niacin did not alter fed hepatic mRNA expression of the rate limiting enzyme in the ketogenesis pathway, hydroxy-methylglutaryl-CoA synthetase 2 (HMGCS2), in either genotype (Figure 3D). Niacin doubled fed hepatic mRNA expression of the early gluconeogenic gene, phosphoenolpyruvate carboxykinase (PEPCK), in GPR109a null mice (Figure 3E).

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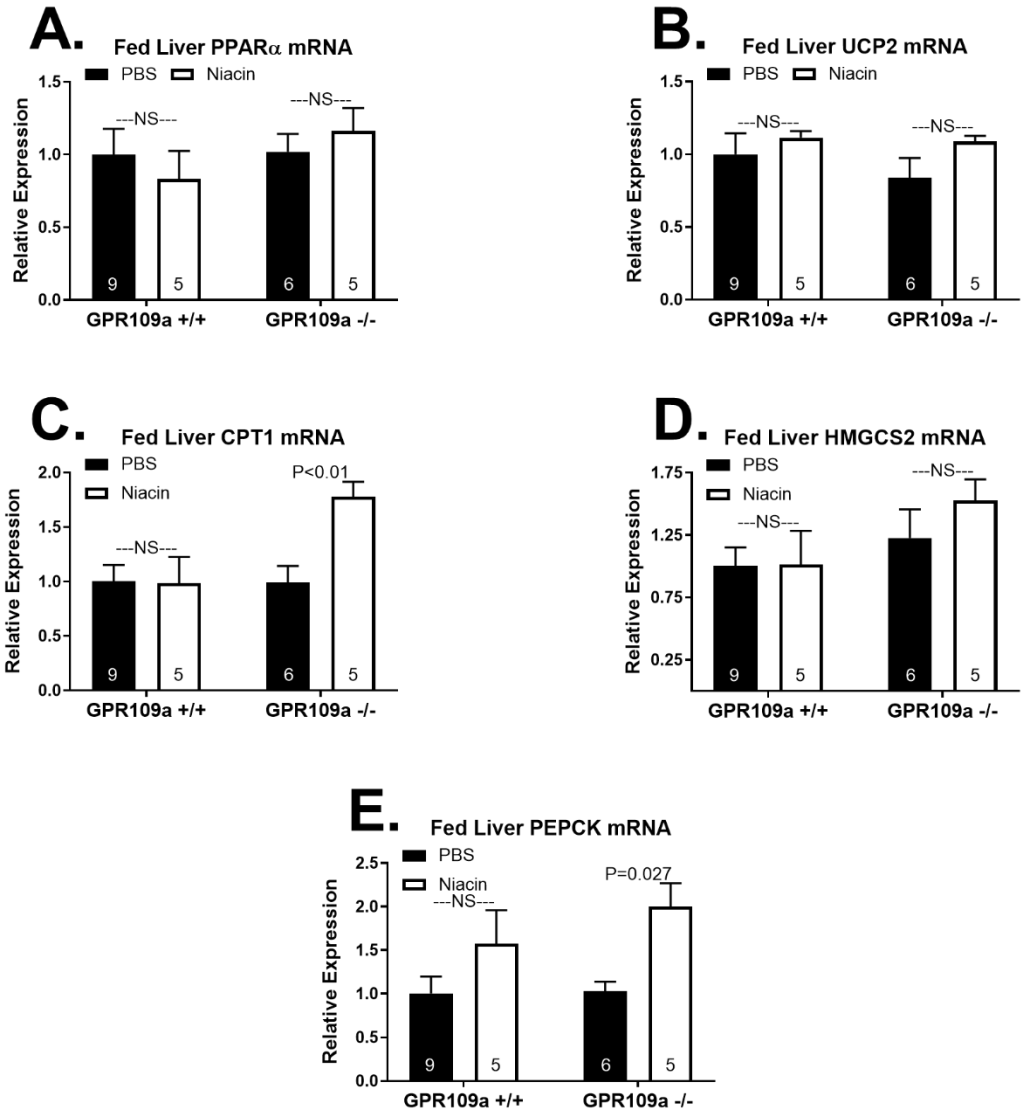

Figure 3. Effect of niacin on hepatic gene expression in fed GPR109a +/+ and -/- mice. Hepatic (A) PPARα mRNA expression, (B) UCP2 mRNA expression, (C) CPT1 mRNA expression, (D) HMGCS2 mRNA expression, and (E) PEPCK mRNA expression. Direct comparisons were made between injection treatment within genotype. NS = non-significant; P > 0.05; PBS = phosphate buffered saline. Number inside bar denotes n per group.

The lack of a robust metabolic phenotype in response to niacin treatment in the fed state independent of GPR109a expression prompted us to next examine the effect of niacin injections on hepatic metabolic homeostasis after a 16 hour fast. Niacin decreased fasted hepatic glycogen concentrations only in GPR109a -/- mice, and did not affect serum glucose, insulin, or the glucose:insulin ratio in either genotype (Figure 4A-4D). We report that niacin injections during the last 9 hours of a 16 hour fast decreased serum NEFA and TAG concentrations in both GPR109a +/+ and -/- mice (Figures 5A-5B). Niacin tended to decrease fasted hepatic NEFA concentrations in GPR109a null mice but had no effect in wildtype mice (Figure 5C). Niacin decreased liver TAG concentrations by ~25% in both genotypes (Figure 5D). Additionally, niacin treatment diminished serum β-OH butyrate concentrations independent of genotype (Figure 5E; P<0.005). Although this only reached significance in wildtype mice (22% decrease), niacin also decreased serum β-OH butyrate by 16% in GPR109a -/- mice (Figure 5E).

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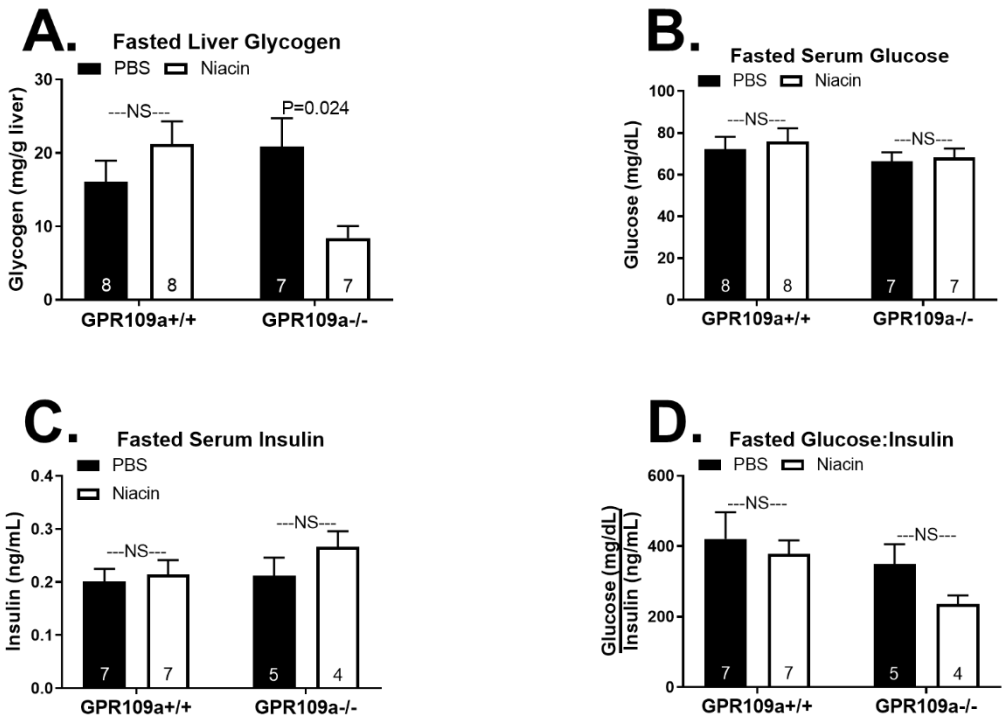

Figure 4. Effect of niacin on glucose homeostasis in 16h fasted GPR109a +/+ and -/- mice. Hepatic (A) glycogen (mg/g liver tissue), serum (B) glucose (mg/dL), (C) insulin (ng/mL), and (D) glucose:insulin ratio. Direct comparisons were made between injection treatment within genotype. NS = non-significant; $P > 0.05$; PBS = phosphate buffered saline. Number inside bar denotes n per group.

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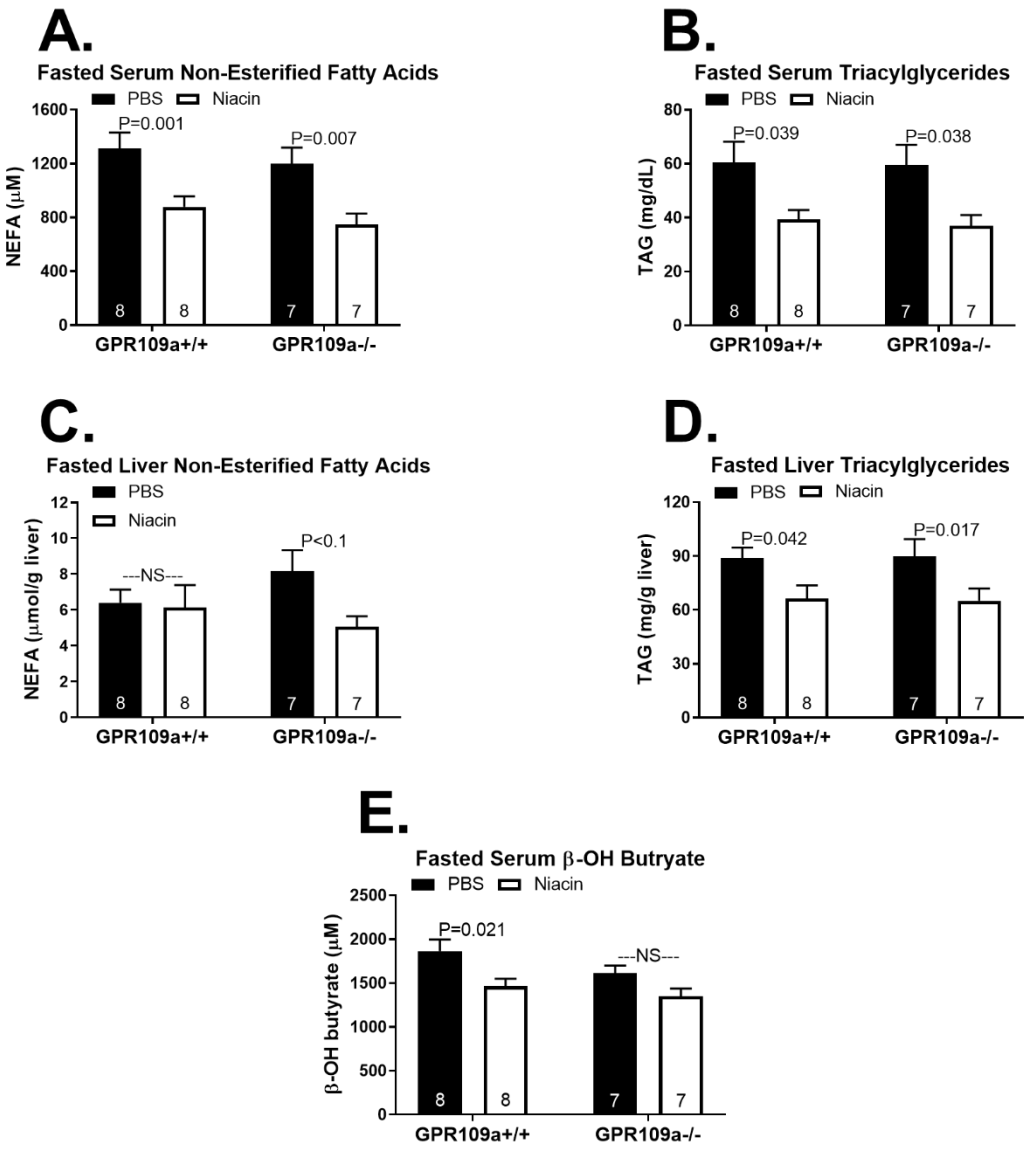

Figure 5. Effect of niacin on lipid homeostasis in 16h fasted GPR109a +/+ and -/- mice. Serum (A) non-esterified fatty acids (NEFA; μM) and (B) triacylglycerol (TAG; mg/dL). Hepatic (C) non-esterified fatty acids (NEFA; μmol/g liver tissue) and (D) triacylglycerol (TAG; mg/g liver tissue). (E) Serum β-OH butyrate (μM). Direct comparisons were made between injection treatment within genotype. NS = non-significant; P > 0.05; PBS = phosphate buffered saline. Number inside bar denotes n per group.

There was a strong effect for niacin to decrease fasted hepatic PPARα mRNA expression independent of GPR109a expression (Figure 6A; P=0.017). However, this decrease was only significant in wildtype mice (Figure 6A). Despite the muted expression of PPARα mRNA with niacin treatment, fasted expression of the PPARα target genes UCP2, CPT1, HMGCS2, and PEPCK [19-23] were not altered by niacin treatment in either genotype (Figures 6B-6E).

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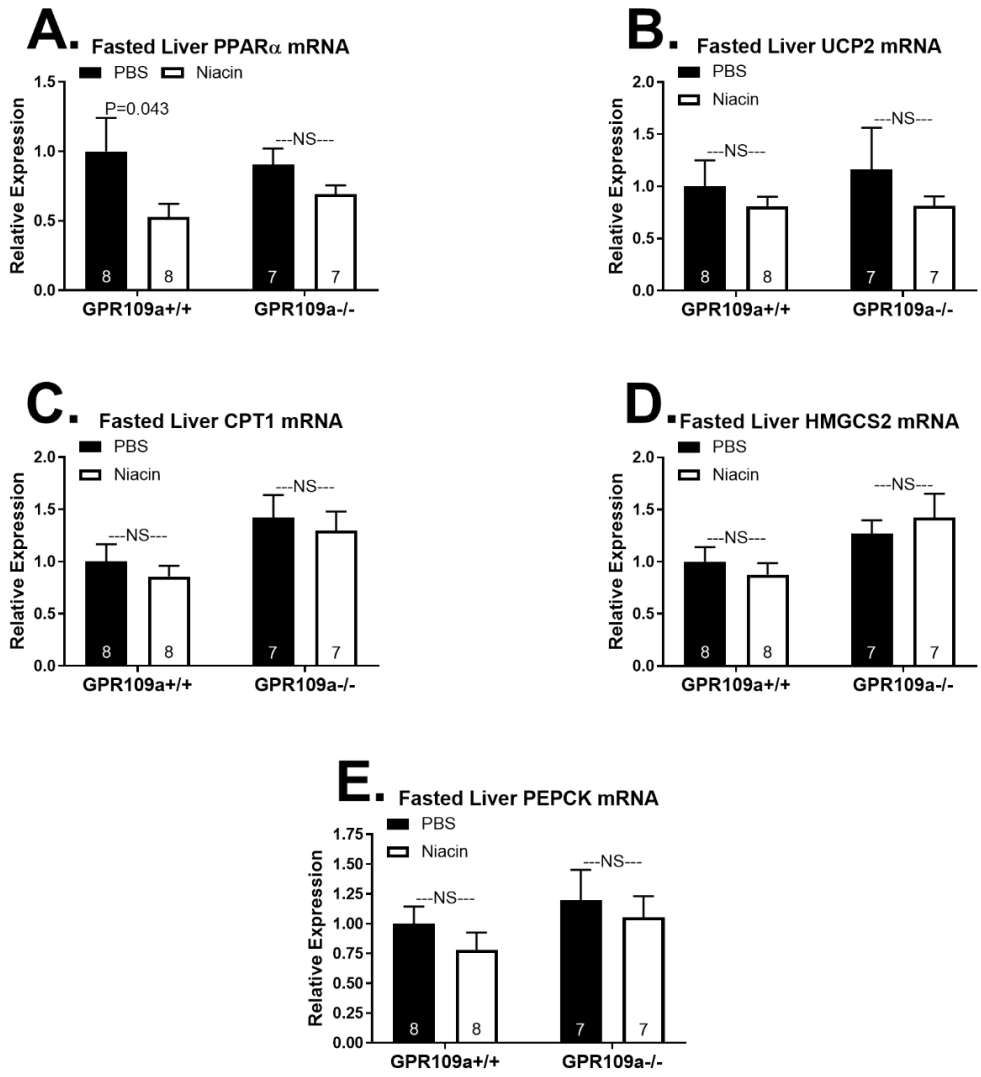

Figure 6. Effect of niacin on hepatic gene expression in 16h fasted GPR109a +/+ and -/- mice. Hepatic (A) PPARα mRNA expression, (B) UCP2 mRNA expression, (C) CPT1 mRNA expression, (D) HMGCS2 mRNA expression, and (E) PEPCK mRNA expression. Direct comparisons were made between injection treatment within genotype. NS = non-significant; P > 0.05; PBS = phosphate buffered saline. Number inside bar denotes n per group.

Discussion

Niacin has been used as a broad-spectrum lipid-lowering drug for over 60 years [9]. Although renowned clinically for its anti-atherosclerotic properties, niacin affects whole-body glucose and lipid homeostasis. Niacin's mechanism of action has been under investigation since it first uses in the clinic and recent research continues to reveal new complexities. We investigated the role of GPR109a expression in acute (9h) niacin mediated changes in serum and hepatic metabolites and hepatic gene expression in the fed and fasted state.

Niacin treatment can induce insulin resistance and fasting hyperglycemia [24,25]. In fact, niacin treatment for as little as one week decreases insulin stimulated glucose clearance [26]. One possible way niacin could cause insulin resistance is by altering skeletal muscle glucose utilization. Niacin increases the number of oxidative type 1 skeletal muscle fibers, a phenotype that favors lipid over glucose oxidation [7,27]. This decrease in glycolytic fibers could diminish muscle glucose utilization and impair insulin sensitivity. In support, niacin mediated increases in muscle lipid oxidation were correlated with niacin induced decreases in insulin sensitivity [28]. Although acute niacin signaling at adipocyte GPR109a inhibits lipolysis and decreases circulating NEFA concentrations, sustained niacin treatment causes NEFA levels to rebound to or above basal concentrations [1,29]. This NEFA

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rebound has been implicated in niacin induced insulin resistance [30-32]. We report that GPR109a knockout did not alter glucose tolerance (Figure 1E). Additionally, while niacin has been shown to decrease glucose stimulated insulin secretion through a GPR109a dependent mechanism [33,34], we found no effect of GPR109a knockout on glucose stimulated serum insulin concentrations (Figure 1G). While these results do not negate a role for GPR109a signaling in niacin induced insulin resistance, they support that endogenous GPR109a signaling does not affect glucose tolerance or glucose stimulated serum insulin.

Niacin treatment robustly decreased fed state hepatic glycogen concentrations independent of GPR109a expression (Figure 1A). This is consistent with evidence that niacin decreases hepatic glycogen in mammalian and avian species [35,36]. In the fasted state, the niacin induced decrease in glycogen was only evident in in GPR109a knockout mice. However, the physiological impact of this finding may be minimal as hepatic glycogen stores are almost entirely depleted following a 16h fast [3]. Three weeks of dietary niacin supplementation increased hepatic glycogen phosphorylase activity in basal fed, 48h fasted, and 24h refed turkey poults with no change in glycogen synthase activity [36]. This suggests niacin decreases glycogen concentrations by increasing glycogenolysis. Glycogen phosphorylase activity is negatively regulated by acetylation and SIRT1 increases glycogen phosphorylase activity [37]. Niacin is a substrate for NAD^+ synthesis and NAD^+ dependent activation of the deacetylase SIRT1 has been proposed to mediate some of niacin's effects [38-40]. However, repeated overnight injections of niacin but not nicotinamide, another NAD^+ precursor, decreased liver glycogen concentrations in rats [35,39]. Thus, the mechanism by which niacin decreases hepatic glycogen stores is not mediated by elevated NAD^+ levels or GPR109a signaling.

Niacin potently improves lipid metabolism by decreasing triglyceride, VLDL, and LDL concentrations and increasing HDL concentrations [10,15,41]. Central to the long standing free fatty acid hypothesis explanation for niacin's favorable lipoprotein effects is the notion that adipose derived NEFAs taken up by the liver can be re-esterified into TAGs which can then be incorporated into VLDL particles [42]. Accordingly, it was believed that niacin's action to inhibit adipose lipolysis decreased TAG and VLDL production by limiting substrate availability [43]. However, more recent findings that niacin acts through several mechanisms directly at the liver which decrease VLDL and increase HDL concentrations have questioned the free fatty acid hypothesis [44-48]. Moreover, it has been shown that while niacin's anti-lipolytic effects are GPR109a dependent, niacin still decreases plasma TAG and VLDL and increases HDL concentrations in GPR109a -/- mice [15]. We report that niacin decreased fasting serum NEFA and TAG concentrations in both GPR109a +/+ and -/- mice (Figures 5A-5B). This is in direct contrast to previous findings that niacin does not decrease plasma free fatty acids in GPR109a null mice [1,15]. One possible explanation for this discrepancy is the timing between niacin exposure and NEFA quantification. In these studies, plasma free fatty acids were measured within 60 minutes or less of niacin administration [1,15]. In fact, the study which concluded GPR109a dependent anti-lipolysis does not mediate niacin induced decreases in pro-atherosclerotic factors assessed plasma TAG and VLDL in GPR109a -/- mice after 4 days of niacin treatment but only reported plasma free fatty acids 15 minutes after niacin administration [15]. We assessed serum NEFAs 1-2 hours after the last niacin injection and following 9 hours of repeated niacin injections. To our knowledge we are the first to report serum free fatty acid concentrations in GPR109a null mice treated with niacin for more than one hour.

Supporting a physiological relevance of our findings, GPR109a expression did not affect the elevation in serum NEFA in response to a fast. If GPR109a was a significant regulator of serum NEFA, one would expect that fasting would result in a greater rise in serum NEFA, liver NEFA, liver TAG, or serum TAG in GPR109a null mice. It remains possible that niacin lowers serum NEFA concentrations by increasing non-hepatic NEFA clearance. However, an increase in NEFA clearance would be observable in the fed and fasted state, while the niacin induced decrease in serum NEFAs was specific to the fasted state. The mechanism by which niacin inhibits adipose tissue lipolysis and NEFA release through a GPR109a independent mechanism warrants further investigation. Diminished circulating NEFAs which provide substrate for hepatic TAG and ketone synthesis likely explain the niacin induced decrease in liver TAG and serum β-OH butyrate concentrations (Figures

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5D-5E). Although niacin can decrease TAG production by inhibiting diacylglycerol acyltransferase 2 (DGAT2) activity [45].

Niacin regulates gene expression and alters expression of lipoprotein transporters and receptors, accounting for some of niacin's anti-atherosclerotic effects [44,47-49]. Niacin has been shown to exert cAMP and liver x receptor α (LXRα) dependent transcriptional regulation which is proposed to be indirectly downstream of GPR109a activation [6,44,47,49-51]. We observed that niacin upregulated fed state hepatic CPT1 and PEPCK mRNA expression in GPR109a -/- mice (Figures 3C and 3E). One possible GPR109a independent mechanism of niacin regulated gene expression is through NAD^+ mediated activation of SIRT1 [40]. SIRT1 activates the transcriptional coactivator peroxisome proliferator-activated receptor γ-coactivator 1α (PGC-1α) and PGC-1α upregulates hepatic expression of PEPCK and CPT1 [52-54]. This does not explain why niacin mediated upregulation of CPT1 and PEPCK was only evident in the absence of GPR109a signaling. One might hypothesize that GPR109a signaling decreases expression of CPT1 and PEPCK, while niacin's GPR109a independent signaling increases expression of these same genes. Accordingly, these effects of niacin are offset in wildtype mice. CPT1 expression was 40% greater in fasted GPR109a null mice than in WT mice supporting a negative feedback role of CPT1 (Figure 6C).

PPARα is a master regulator of hepatic fasting metabolism which coordinates upregulation of gluconeogenesis, β-oxidation, and ketogenesis [20]. Hepatic PPARα is activated by unsaturated fatty acids and upregulates expression of itself through PPARα response elements in its promoter [16,55]. A decreased supply of NEFAs to the liver could explain the blunted fasting PPARα expression with niacin treatment (Figure 6A). Surprisingly, despite the niacin induced 47% reduction in fasted PPARα expression in wildtype mice and 23% reduction in GPR109a null mice, expression of the PPARα target genes UCP2, CPT1, HMGCS2, and PEPCK [19-23] were unaffected by niacin treatment (Figure 6).

Materials and Methods

Animals

All studies were conducted using 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 [1]. 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.

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) 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 (2.5g/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 in whole blood, collected from the tail vein, by glucometer (Manufacture # D2ASCCONKIT, Bayer, Leverkusen, Germany) at 0, 15, 30, 60, 90, and 120 minutes after glucose injection. At 15 minutes after glucose injection, a larger bleed (~50uL blood) was taken from the tail vein to measure glucose stimulated serum insulin. Blood was immediately stored on ice and 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. Serum was stored at -80°C.

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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 powered 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. Serum insulin was analyzed by enzyme-linked immunosorbent assay (ELISA; Cat. # 80-INSMSU-E01,E10, Alpco, Salem, NH).

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 [56]. 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™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), uncoupling protein 2 (UCP2), and carnitine palmitoyltransferase 1 (CPT1) mRNA were measured using the primer pairs previously published [3]. LinReg PCR analysis software was used to determine the efficiency of amplification from raw output data [57]. ACTβ served as the housekeeping gene for calculating fold change in gene expression using the efficiency$^{-\Delta\Delta Ct}$ method [58].

Total liver lipids were extracted from powered 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). Liver glycogen content was quantified by a colorimetric assay as previously described [59].

Statistical Analysis

All statistical analyses were completed in SAS Enterprise Guide 7.1 (SAS Institute Inc., Cary, NC). We used a mixed model ANOVA to assess the effect of genotype (GPR109a +/+ or -/-) and injection (saline or niacin) in mice that were fed or fasted. All independent variables were treated as classification variables. A Bonferroni correction was used to correct for multiple comparisons. There was no statistical difference between saline injected GPR109a +/+ and -/- mice for any variable in either nutrition state. Accordingly, post-hoc comparisons were focused on injection (niacin or saline) within genotype. Glucose tolerance tests were analyzed by repeated measures ANOVA. Figures were created in GraphPad PRISM® Version 8.2.1 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) and are displayed as Mean ± SEM.

Conclusions

GPR109a does not play a major role in regulating normal glucose or lipid homeostasis in either the fed or fasted state. Independent of GPR109a, niacin limits lipolysis and hepatic lipid accumulation without profound metabolic disturbances while fasting. Future work focused on understanding

GPR109a independent mechanisms of niacin action will be critical to enhance the therapeutic potential of niacin like derivatives.

Abbreviation

CPT1	Carnitine Palmitoyltransferase 1
HMGCS2/ HMG-CoA synthase II	3-hydroxy-3-methyl glutaryl CoenzymeA Synthase II
ACTβ	β-Actin
PPARα	Peroxisome Proliferator Activated Receptor α
UCP2	Uncoupling Protein 2
PEPCK	Phosphoenolypyruvate Carboxykinase
NEFA	Non-Esterified Fatty Acid
TAG	Triacylglyceride

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Author Contributions: CEG – Designed Studies, Performed Experiments, Completed Wet Lab Analyses, Conducted Statistical Analyses, and Wrote Manuscript. BJR- Designed Studies, Performed Experiments, Conducted Statistical Analyses, and Wrote Manuscript.

Conflicts of Interest: The authors have no conflicts of interest to report.

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