Hepatic AMPK Protects Mice from hepatic steatosis and Insulin Resistance on a ketogenic diet

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# Abstract

# Introduction

# Methods

## Generating liver-specific AMPK 1/2 knockout mice

Mice were housed under controlled temperature and lighting (12-hour light/12-hour dark cycle) with unrestricted access to water and normal chow (Lab Diet; 2.91 kcal/g; 5% fat, 24% protein, 2.7% sucrose, 32% starch). Mice that harbored homozygous, floxed alleles for both AMPK 1 and 2 were generated by S. Morrison (UT Southwestern Medical Center) as described (11, 14). To produce liver-specific AMPK 1/2 knockout mice, at 70 days old these mice were injected through the tail vein with adeno-associated virus expressing either GFP (AAV8-TBG.PI.eGFP.WPRE. bGH) or Cre recombinase (AAV8.TBG.PPI.Cre.rBG) from a liver-specific TBG promoter (1.5 × 10 to 10 plaque-forming units per mouse). Mice were allowed to recover for two weeks prior to randomization onto experimental diets. We ordered custom control and ketogenic diets from Research Diets. These diets were matched for protein, choline and fiber levels. The diet compositions are described in Table 1. Animal use was performed in accordance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animalsand approved by the University Committee on Use and Care of Animals at the University of Michigan.

## Protein Lysates and Western Blotting

Mice were sacrificed using isoflurane and cervical dislocation. The tissues were harvested immediately upon sacrifice, weighed and placed in liquid nitrogen. The tissues were then stored at -80 ˚C. Frozen tissue samples were cut using dry ice to 20-50 mg of tissue per sample. 20 uL/mg of RIPA buffer (from where?) was added to each sample and they were homogenized using Qiagen Tissue Lyser (3 min at 25Hz). Samples were centrifuged at 14,000 RPM at 4C for 10 min. 160 uL of supernatant was removed and 40 uL of ThermoFisher Scientific NuPAGE sample reducing agent (10x) and 200 uL of Fisher Scientific Tris-Glycine SDS sample buffer (2X) were added. Samples were heated with loading buffer at 85C for 2 mins and then snap frozen to -80˚ C. Liver lysate samples were boiled for 3 minutes at 85˚C using a heating block. 10 uL of samples were placed in a Fisher Scientific 15 well, 4-12% Tris-glycine, 1.0 MM mini protein gel in 1x SDS running buffer at 125 Volts until samples and ladder reached the bottom of the gel. Protein was transferred to nitrocellulose paper from the gel using a Bio-Rad Transfer Apparatus in 1X transfer buffer diluted from 10X with water and 20% methanol. Transfer occurred overnight at 35V. Nitrocellulose paper was stained for total protein using LI-COR Revert Total Protein Stain for 5 minutes and rinsed twice in LI-COR revert wash solution. The blot was scanned for total protein using the LI-COR (model?). Nitrocellulose was then rinsed in LI-COR revert reversal solution for 5 minutes and then rinsed in 2% BSA (2g BSA in 100ml TBST) for 1 hour. The blots were incubated in primary antibody for greater than 1 hour and washed every 5 minutes for 15 minutes using TBST. The primary antibodies were: total AMPKalpha, AMPKalpha phosphorylated at Thr172, total ACC, ACC phosphorylated at Ser79, total FAS, total S6, S6 phosphorylated at \_\_\_\_, total S6K, S6K phosphorylated at \_\_\_, other antibodies? (All from Cell Signaling Technology). Blots were incubated with 10,000x secondary antibody (list both of them) for 45 minutes and washed every 5 minutes for 15 minutes with TBST and rinsed with double distilled water. Blots were scanned using LI-COR (model) and normalized using total revert stain.

## Serum Collection and Ketone body assays

Mice were anesthetized using isoflurane and blood was collected using retro-orbital bleeding. After collection, samples were centrifuged at 4˚C for 20 minutes. The top layer (serum) was transferred to another tube and frozen at -80 ˚C. Ketone bodies from serum were analyzed using the Wako Diagnostics Ketone Body Assay and quantified using a XXX plate reader.

## Food Intake and Body Composition Determinations

CD and KD were placed in shallow jars with holes in the lid allowing for ad libitum food access. The lid was essential to prevent the food from spilling out of the jar and for accurate food measurements. Jars with food were weighed and refilled every other day. The mice were weighed on a scale weekly to measure body weight. Fat and lean tissue mass was measured in vivo using Echo MRI 1100 weekly. Measurements were taken at approximately circadian time 8 hours (CT4) to limit fluctuations in body weight/composition throughout the day.

## Liver Histology

Liver cryosections were fixed in formalin overnight, then embedded in paraffin and stained with hematoxylin and eosin by the University of Michigan Tissue and Molecular Pathology Core. Histology was blindly scored by four trained researchers and these scores were used to select representative images for each animal, sex and treatment group.

## Insulin tolerance tests

Mice were fasted for 6 hours and then injected with 0.75 U/kg of lean body mass of insulin into the intraperitoneal cavity. Blood glucose was measured from the tail vein at the times indicated from a tail vein bleed using an Accuchek glucometer. These experiments were done at CT8.

## Statistical analyses

Statistical significance for this study was set at an alpha of p=0.05. All statistical tests and graphs were prepared using R v4.02 (15). For pairwise tests, normality and homoscedasticity were tested prior to appropriate pairwise tests. For 2x2 comparisons, interactions between factors were tested by ANOVA and reported or excluded from the adjusted models depending on whether they met statistical significance. Insulin tolerance tests were analyzed using mixed linear models using the time points as ordinate values to test for an interaction of the treatment/genotype. P-values were calculated using Sattherthwaite’s approximation using lmerTest v3.1-3 (12) and lme4 v1.1-26 (2). All raw and processed data, analysis code and graphs for this manuscript are available at [http://bridgeslab.github.io/TissueSpecificTscKnockouts/.](http://bridgeslab.github.io/TissueSpecificTscKnockouts/)

# Results

## Experimental Design

At the start of the experiment, mice were raised on a normal chow diet (Lab Diet, 5L0D, 2.91 kcal/g; 5% fat, 24% protein, 2.7% sucrose, 32% starch). At 70 days old, mice were injected with either AAV-TBG-GFP or AAV-TBG-CRE to produce liver-specific controls and knockouts, respectively. The mice continued to consume normal chow for two weeks post injection at which point they were placed on either a ketogenic (KD) (Research Diets, D17053002, 6.4 kcal/g; 85% fat, 15% protein, 0% sucrose, 0% starch) or matched control diet (CD) (Research Diets, D1053001, 3.8 kcal/g; 10% fat, 15 protein, 0% sucrose, 75% starch). One week later, blood samples were taken using retro-orbital bleeding. Another week later insulin tolerance tests were performed. Two weeks later mice were sacrificed and tissues were collected. Body composition (weight, fat mass and lean mass) and food intake was measured weekly from the start of injections until sacrifice.

We used western blots to ensure that AMPK 1/2 subunits were knocked out in the liver and to observe any changes in lipogenic and beta-oxidation genes. Western Blots of liver lysates shows efficient AMPK knock out in males that received AAV-TBG-CRE injections (Figure 2A).

pACC, a downstream target of AMPK and lipogenic gene, was reduced in males that received AAV-TBG-CRE injections compared to those that receives GFP injections (Figure 2A). This is another confirmation of knockout and also indicates there may be an increase in lipogenesis in the KO mice. pACC was also reduced on a KD relative to the CD for both treatment groups (Figure 2A). This indicates there may be an increase in lipogenesis on the ketogenic diet. Another lipogenic gene, FAS, was found to decrease on a ketogenic diet but was not different between treatment groups (Figure 2A) suggesting FAS was not driving any observed changes in the KO mice. ACLY, another lipogenic gene, was found to decrease on a ketogenic diet but again was not different between treatment groups (Figure 2A). pACC…..(Figure 2A)

Because of the known role of AMPK in regulating mTOR signaling and lipogenesis (citation) we observed downstream targets of mTOR such as total S6, pS6, total S6K and pS6K. We found that mTOR signaling was higher on a ketogenic diet but was not different between treatment groups (Figure 2B).

Overexpression of AMPK in the liver has been shown to increase beta-oxidation(5), so we next investigated changes in beta-oxidation genes.

(Need to look at these) The beta-oxidation genes, CPT1, MCAD and ACAD were found to \_\_\_\_\_\_\_ (Figure 2C)

(Need to look at this) Others have shown that sip7a1, a regulator of bile acid reuptake, decreases on a ketogenic diet. We found that sip7a1 \_\_\_\_\_\_\_\_\_ (Figure 2C?)

## Ketone Body Levels Increased on Ketogenic Diet and AMPK Ablation had minor Effects on Ketone Body Levels

Some have suggested the ketone production may be the key driver of physiological changes on a ketogenic diet (citation). Additionally, others have shown that overexpression of AMPK in the liver leads to increases in ketone production(5). Our data shows that serum ketone body levels increased in mice on a ketogenic diet but were not different between KO and WT mice (Figure 3A). This confirms any observed changes between treatment groups were not due to differences in ketone production. Both KO and WT males fed a control diet did not increase ketone body production (Figure 3A).

We looked at food intake to confirm any observed changes between KO and WT were not due to differences in caloric intake. We found there were no differences in caloric intake between KO and WT mice on either diet (Figure 3B). Mice on a KD, however, consumed more calories per day than mice on a CD (Figure 3B).

## Liver AMPK Knockout mice on had more gonadal and adipose tissue on a Ketogenic Diet

In agreement with previous findings(7), there was no difference in body weight, fat mass or lean mass between treatment groups on either diet (Figure 4 A, B and C). Both KO and WT mice on a ketogenic diet gained significantly more body weight than mice on a control diet (Figure 4A) which was due to an increase in fat mass (Figure 4B). There were no differences in fat mass between KO and WT mice on a ketogenic diet (Figure 4B). Consistent with no observed changes in lean mass between groups, the gastrocnemius and quadriceps weights were the same between KO and WT mice on both diet groups at the time of sacrifice (Figure 4D,E). Consistent with the change in fat mass over four weeks, inguinal adipose tissue weight at the time was sacrifice was higher in those on a ketogenic diet, but again not different between treatment groups (Figure 4F). Gonadal adipose tissues (GWAT), however, did show a treatment difference with KO males having an increase in GWAT compared to WT males on a ketogenic diet (Figure 4G). This difference was not seen between KO and WT on a control diet.

Consistent with the observed differences in GWAT between KO and WT mice on a ketogenic diet, KO mice on a ketogenic diet appeared to have increased liver fat accumulation compared to WT on a ketogenic diet (Figure 5A). Additionally, the observed increases in fat mass on a ketogenic diet were also seen in the male livers on a ketogenic diet relative to the control diet (Figure 5A). There were no differences in liver fat mass seen between treatment groups on a control diet (Figure 5A). Need to quantify liver TGs and put that here (Figure 5B).

## Knockout of AMPK 1/2 impairs glucose homeostasis in a sex-specific manner on a ketogenic diet.

After three weeks of ketogenic diet, insulin tolerance tests were performed on both male and female mice. As shown in Figure 6A, we observed a 24% increased fasting glucose after KD feeding (p=0.002 from a two-way ANOVA) and a further 20% increase male mice (p=0.008), irrespective of diet. Upon insulin injection, all mice had an approximately 50% reduction in blood glucose after 45 minutes, but mice on a ketogenic diet recovered past their fasting glucose levels (Figure 6B). This effect was enhanced in female liver AMPK 1/2 knockout mice. A sex by genotype interaction was identified by mixed linear models generated for female (p=0.004) but not the male ITT’s (p=0.613). As an example, at the 60-minute time point female knockout mice had 30% lower blood glucose than wild-type littermates under control diet conditions, but 36% higher blood glucose levels on a ketogenic diet. This represents a significant interaction between diet and knockout status on glucose levels in female (p=0.001) but not male (p=0.614) mice. Our interpretation of these data is that glucose production (either by gluconeogenesis or glycogenolysis) is more active under KD feeding, and that this is enhanced by the loss of AMPK signaling in female mice.

To estimate early-phase insulin sensitivity, we calculated the rate of glucose decline for each animal from injection to 30 minutes. Again, here we noted interesting sex-specific differences (Figure 6C). On our ketogenic diet, the GFP-injected female mice had double the rate of glucose decline (p=0.03) in comparison to control diet/GFP-injected mice during the insulin tolerance test. In contrast, for the male GFP-injected mice, insulin sensitivity was unaffected by the diet (p=0.80; psex x diet=0.063). In the AMPK 1/2 knockout mice, we observed modest impairments in insulin sensitivity for all but the control diet female mice. For mice on a ketogenic diet knockout caused a 23% decrease in insulin sensitivity, after adjusting for sex (p=0.17). These data are suggestive of modestly impaired insulin responsiveness in liver AMPK knockout mice on a ketogenic diet.

# Discussion

* Increased glucose on KD
* “Previously found, genetic deletion of alpha a1, a2, or b2 isoforms in mice has little effect on liver AMPK activity (18),(10),(17). By contrast, germline deletion of AMPK b1 [65] leads to a large reduction in liver AMPK activity (\_95%), indicating that the b1 subunit is essential for maintaining the AMPK heterotrimer in the liver. mice with a liver-specific knockout of AMPK a2 were hyperglycemic and had elevated HGP relative to controls (1). However, mice lacking both AMPK a1 and a2 subunits in the liver (which led to no detectable liver AMPK activity) did not result in hyperglycemia or increases in HGP. In addition, AICAR continued to suppress HGP in liver-specific AMPKa1a2 KO mice suggesting that the glucose lowering-effects of AICAR are independent of AMPK (6),(13),(7),(8). liver-specific AMPK a1a2 null mice have reduced mitochondrial content, mitochondrial respiration, and oxidative capacity, demonstrating that AMPK is required for maintenance of hepatic of mitochondrial function (7). Importantly, this appears to have functional consequences because, during fasting, a time of heavy ATP demand because of gluconeogenesis, there are large reductions in ATP levels in liver-specific AMPK a1a2 null mice (8). In addition to reductions in mitochondrial content, this impairment in mitochondrial function may also be attributed to defects in mitophagy and mitochondrial fission. Reductions in liver AMPK activity in AMPK b1-knockout hepatocytes reduces the phosphorylation of ACC and leads to reductions in FAO and increases in lipogenesis (4). Overexpression of AMPK a1 in the liver reduces lipogenic gene expression, liver triglyceride content, and hepatic steatosis in hyperlipidemic, type 2 diabetic rats in vivo (16), and reduces triglyceride and intracellular lipid accumulation while increasing CPT1 expression (increasing fatty acid clear-ance via mitochondrial FAO) when overexpressed in hepatocytes in vitro (19) “ (3)
* Sex differences

This study has several advantages, including the use of inducible knockout of both AMPK catalytic isoforms, well controlled dietary exposures and an evaluation of phenotypes in both sexes. Our conclusions are limited by the inter-related increases in insulin resistance, adiposity and hepatic steatosis, and we are unable to unambiguously separate causality between these findings. At a population level, it has been estimated that 16% of Americans followed a low-carbohydrate eating pattern in 2018 (9). Most individuals report following this pattern for glucose control or weight management, but our study used lean metabolically healthy mice, so we cannot generalize our findings to mice with pre-existing metabolic dysfunction, but are closer to a model of a metabolically healthy mammal under low carbohydrate conditions.

# Author Contributions

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# Figure Legends

**Figure 1. Experimental design of AMPK 1/2 KO mice on a ketogenic diet**. Mice (n=?) were raised on a normal chow diet (Lab Diet; 2.91 kcal/g; 5% fat, 24% protein, 2.7% sucrose, 32% starch). At 70 days old (week -2), mice were injected with either AAV-TBG-GFP or AAV-TBG-CRE to produce liver-specific controls and knockouts, respectively. The mice continued to consume normal chow for two weeks post injection at which point they were placed on either a ketogenic (KD) (6.4 kcal/g; 85% fat, 15% protein, 0% sucrose, 0% starch) or matched control diet (CD) (3.8 kcal/g; 10% fat, 15 protein, 0% sucrose, 75% starch) (week 0). One week later (week 1), blood samples were taken using retro-orbital bleeding. Another week later (week 2) insulin tolerance tests were performed. Two weeks later (week 4) mice were sacrificed and tissues were collected.

**Figure 2. Western Blots for 1/2 AMPK KO and WT mice on either a KD or CD (n=?).**

* + A)
  + B)

**Figure 3. KO and WT mice consume the same number of calories per day and produce the same quantities of ketone bodies (n=?).**

* + A. Ketone bodies were analyzed using the Wako Diagnostics ketone bodies R1 and R2 kit.
  + B. Food Intake was measured by weighing food containers every other day. KD = ketogenic diet (Research Diets, D17053002, 6.4 kcal/g; 85% fat, 15% protein, 0% sucrose, 0% starch). CD = matched control diet (Research Diets, D1053001, 3.8 kcal/g; 10% fat, 15 protein, 0% sucrose, 75% starch)

**Figure 4. AMPK 1/2 KO mice on a KD have more gonadal and liver adiposity but exhibit no other changes in body composition compared to wild type mice. A)** Changes in body weight of KO and WT male and female mice on a KD and CD from the time of injection (-2 weeks) to the time of sacrifice (4 weeks). At week 0 mice (n=?) were started on either the ketogenic or control matched diet. **B)** Changes in fat mass of KO and WT male and female mice on a KD or CD measured weekly in vivo using using Echo MRI 1100. **C)** Changes in lean mass of KO and WT male and female mice on a KD or CD measured in vivo weekly using Echo MRI 1100. **D)** Gastrocnemius weight of KO and WT male and female mice on a KD and CD measured at the time of sacrifice (week 4). **E)** Quadriceps weight of KO and WT male and female mice on a KD and CD measured at the time of sacrifice (week 4). KO = knockout a1a2lox/lox+Albcre mice, WT = Wild type (a1a2lox/lox) mice, KD = ketogenic diet, CD = control diet.

**Figure 5. AMPK 1/2 KO increases fat accumulation on a KD but not on a control diet (n=?).**

* + A. Cross section of liver (magnification?)
  + B. TGs

**Figure 6.** Insulin tolerance tests in liver AMPK 1/2 KO after 2 weeks of control or ketogenic diet feeding A) Fasting glucose. B) Glucose normalized to fasting glucose levels, dashed line indicates basal glucose levels, asterisks indicate a significant diet-genotype interaction from a 2x2 ANOVA. C) Glucose change rates by group, calculated during the first 30 minutes of the insulin tolerance test. Asterisk indicates significant difference between control and ketogenic diet fed females. n=7-12 mice/group.