LIVER AMPK REGULATES TOTAL BODY LIPID ACCUMULATION ON A LCHF DIET BUT IS DISPENSABLE FOR INSULIN RESISTANCE

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Abstract

Introduction

Methods

* Generating liver-specific AMPKalpha1/2 knockout mice
  + Mice (C57BL6) were housed under controlled temperature and lighting (12-hour light/12-hour dark cycle) with access to water and normal chow (Lab Diet; 2.91 kcal/g; 5% fat, 24% protein, 2.7% sucrose, 32% starch). Animal use was performed in accordance with the Institute of Laboratory Animal Research ***Guide for the Care and Use of Laboratory Animals*** and approved by the University Committee on Use and Care of Animals at the University of Michigan. Mice that harbored homozygous, floxed alleles for both AMPK a1 and a2 were generated by S. Morrison (UT Southwestern Medical Center) as described 1 and shared by K. Inoki (University of Michigan Medical School)2. To produce liver-specific AMPKa1/2 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).
* Making Liver lysates
  + After harvesting liver from mice, livers were placed in an eppendorf tube in liquid nitrogen to freeze. 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˚ F.
* Western Blots
  + 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.
* Ketone body assay
  + Ketone bodies from serum were analyzed using the Wako Diagnostics ketone bodies R1 and R2 kit. 4 uL of each control sample and 1 uL of each ketogenic diet sample were pipet into a 96 well plate. 270 uL of R1 was added to each well and incubated for 5 minutes at 37˚C. Next, 90 uL of R2 were added to each well and then placed in the plate reader at 37˚C.
* Body composition measurements using MRI
  + The mice were weighed on a scale weekly to measure body weight. Fat and lean tissue mass was measured in vivo using MRI weekly. Measurements were taken at approximately the same time daily to account for fluctuations in body weight throughout the day.
* Serum collection
  + Mice were anesthetized using isoflurane and blood was collected using retro-orbital bleeding. After collection, samples were centrifuged at 4˚C for \_\_\_\_ minutes. The top layer (serum) was transferred to another tube and frozen at -80 ˚C.
* 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 interperitoneal cavity. Blood glucose was measured from the tail vein at time 0, 15, 30, 45, 60, 75, 90, 105 and 120 minutes after injection using a glucometer (brand?).
* Sacrifice mice
  + Mice were sacrificed using isoflurane and cervical dislocation. The
* Statistical analysis
  + Insulin tolerance tests were analyzed using a mixed linear model using the time points as ordinate values to test for an interaction of the treatment/genotype.

Results

* Experimental Design
  + Mice 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, mice were injected with either AAV-TBG-GFP or AAV-TBG-CRE to produce liver-specific knockouts and controls. 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). One week later, blood samples were taken using retro-orbital bleeding and ketone bodies were analyzed (do I include that here?). Another week later insulin tolerance tests was performed. Two weeks later mice were sacrificed, tissues were collected and ketone bodies were analyzed. Body composition (weight, fat mass and lean mass) and food intake was measured weekly from the start of injections until sacrifice.
* AMPK effectively knocked out and confirmed using Western Blots (AMPK alpha1/2 liver KO has no effect on mTOR pathway?)
  + Western Blots of liver lysates shows that males that received AAV-TBG-CRE injections had AMPK effectively knocked out in both diet groups (Figure 2A). pACC, a downstream target of AMPK, was also reduced in males that received AAV-TBG-CRE injections (Figure 2A). Surprisingly, females that received the received AAV-TBG-CRE injections did not show an efficient knockout of AMPK (Figure 2A). Similarly, pACC was not down regulated in the females that received the CRE injections (Figure 2A). For this reason, we investigated the males for the remainder of the experiments.
  + As expected FAS expression was decreased on a ketogenic diet (Figure 2B). However, FAS, pS6 and pS6K were all unchanged in the knocked out mice on either diet (Figure 2B). Would you expect mTOR to be changed on a ketogenic diet compared to control? AMPK is known to regulate mTOR so would you expect it to be decreased in the knockout?
* Ketone Levels Increase on Ketogenic Diet and AMPK had No Effect on Ketone Production
  + Ketone body levels increase in both KO and WT males fed a ketogenic diet with no difference between treatment groups (Figure 3). Both KO and WT males fed a control diet do not increase ketone body production (Figure 3).
* AMPK alpha1/2 KO lost less lean mass than WT on a ketogenic diet
  + There was no difference in change in body weight between treatment groups on either diet (Figure 4A). Both KO and WT mice on a ketogenic diet gained significantly more body weight than mice on a control diet (Figure 4A). The increase in body weight on a ketogenic diet was due to the increase in fat mass for both KO and WT mice but again there was no difference between treatment groups (Figure 4B). However, the KO mice on a ketogenic diet lost less lean mass than WT mice on a ketogenic diet (Figure 4C).
* AMPK alpha1/2 KO does not impact insulin sensitivity on a Ketogenic Diet
  + KO mice were equally insulin sensitive to WT mice fed either a ketogenic or control (Figure 5). Both treatment groups fed a ketogenic diet had a larger drop and subsequent increase in blood glucose compared to mice fed a control diet, however there was no difference between treatment groups (Figure 5).

Discussion

Author Contributions

Acknowledgements

References

1. Nakada D, Saunders TL, Morrison SJ. Lkb1 regulates cell cycle and energy metabolism in haematopoietic stem cells. *Nature*. 2010;468(7324):653-658. doi:10.1038/nature09571

2. Kazyken D, Magnuson B, Bodur C, et al. AMPK directly activates mTORC2 to promote cell survival during acute energetic stress. *Sci Signal*. 2019;12(585). doi:10.1126/scisignal.aav3249

Figure/Table Legends

* Figure 1. Experimental Design.
  + - Insert Figure 1 from illustrator?
* Figure 2. Western Blots
  + \*\*\*Figures not from illustrator\*\*
  + A)
  + B)
* Figure 3. Ketones
* Figure 4. Body Composition (should I show raw or change?)
  + A. Body Weight
  + B. Fat Mass
  + C. Lean Mass
* Figure 5. ITT