GDF15 Restricts Energy Intake on a Ketogenic Diet in Mice

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# Abstract

The ketogenic diet (KD), consisting of very-low-carbohydrate and high-fat content, is an emerging and effective method for weight loss and glucose control. In a previous study, mice were placed on a KD or matched controls (CD) for four weeks. Mice on a KD gained less fat mass and lean mass compared to those on CD despite consuming more calories. The satiety hormone, GDF15, was increased by the KD in both liver mRNA and blood. To further investigate the effects of GDF15 on appetite in relation to a KD, we used a GDF15-knockout model where mice do not have the ability to produce GDF15. We hypothesize that the GDF15-KO mice will consume more calories on a KD compared to their wildtype (WT) counterparts and gain more body mass on the CD. Another aspect of carbohydrate restriction is the activation of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in glucocorticoid elevation. We compared cortisol levels and GDF15 levels in human patients and found that these were tightly correlated. To understand the relationship between the HPA axis and GDF15 we investigated serum and liver mRNA expression from mice treated with the synthetic glucocorticoid dexamethasone and found elevations in GDF15. To look at this response *in vitro* we exposed liver hepatoma cells, either AML12 or Hepa1c, to the glucocorticoid dexamethasone and found downregulation of GDF15 indicating that the *in vivo* activation may not be cell autonomous. Our study aims to elucidate some of the mechanisms by which GDF15 affects appetite and metabolic regulation of weight loss.

# Introduction

Ketogenic or low carbohydrate diets, often accompanied by an increase in dietary fat are increasingly common in the population with 16% of Americans reporting that they are on a carbohydrate restricted diet (1). Several randomized controlled trials have demonstrated weight loss, improved glycemic control, and reduced energy intake (reviewed in 2–4). For those individuals who lose weight on a LCHF diet, there is broad agreement that much of this effect is due to energy restriction with either modest or insignificant changes in energy expenditure (5,6). A recent meta-analysis showed decreased hunger and increased satiety on LCHF diets, though the hormonal mediators of this increased satiation remain unclear (7).

GDF15 is a hormone and emerging drug target that signals through GFRAL receptors in the hindbrain to reduce food intake. In humans, elevations of this hormone are associated with pregnancy-related nausea and cancer-associated cachexia (8–11). Elevations in GDF15 suppress appetite in a GFRAL-dependent manner. In terms of specific macronutrients, when GDF15 causes a specific reduction in lipid consumption, not other macronutrients (12). GDF15 is generated in many tissues in response to a variety of stressors but the integrated stress response has emerged as an important pathway controlling GDF15 production (13,14). Prior studies have implicated the hepatic integrated stress response to ketogenic diets (13,15,16). In this study we investigate the role of GDF15 in moderating energy intake, body composition and insulin sensitivity on a ketogenic diet.

# Methods

## Animal Husbandry and Diets

Animals were either purchased from the Jackson Laboratory (A/J mice; stock 000646, or C57BL/6J stock 000664; all resource identifiers are provided in Table 1) or were previously described (*Gdf15* null; (12)). Diets were provided by Lab Diet (Normal Chow Diet; NCD, 5L0D) or Research Diets (Control Diet; CD; D1053001 or Ketogenic Diet; KD: D17053002). Mice were fed NCD until ten weeks of age and then transferred to CD or KD as described. Muscle *Tsc1* knockout mice were generated by crossing FVB-Tg(*Ckmm-Cre*)5Khn/J transgenic mice (Jackson Laboratory stock 006405) with floxed *Tsc1*tm1Djk/J mice (stock 005680). Wild-type mice are defined as *Tsc1*fl/fl, *Ckmm-Cre*+/+ while knockout mice were *Tsc1*fl/fl, *Ckmm-Cre*Tg/+. More details about these mice can be found in (17). Animals were euthanized via cervical dislocation under isoflurane anesthesia. All procedures were approved by the University of Michigan Institutional Animal Care and Use Committee.

## Food Preference Test

At nine weeks of age, mice were singly-housed for one week to acclimitize. Mice were given *ad libitum* access to two diets delivered in identical for 4 - 5 days in jars with custom lids to avoid spillage.  On day two of the experiment, if food was found in cage bedding, data was discarded. The physical position of each diet feeder was switched at least once during the experiment.  Data are presented as relative calories consumed from each jar over each period.

## Ketone Body and GDF15 Determination

Total ketone bodies were determined using the Wako Autokit Total Ketone Bodies: (Cat#'s 415-73301, 411-73401 and 412-7379) using mouse serum. Rates of changes in absorbance were determined using a Synergy HTX plate reader (Biotek). GDF15 levels were determined using a Quantikine ELISA Assay (Cat# MGD150) following the manufacturer’s instructions.

## Mouse Weight and Body Composition

Mice were weighed weekly on a digital scale. Body composition was determined using an echoMRI 2100 (echoMRI) using standard settings.

## AML12 and Ketogenic Media

AML12 cells were purchased from ATCC (Cat# CRL-2254) and grown in DMEM with 10% FBS and penicillin/streptomycin/glutamine. To treat the cells we followed the protocol described in (18). Briefly cells were treated with fresh DMEM/FBS or DMEM without glucose or serum, but supplemented with 50 M WY-14643 to activate PPAR and 2 mM sodium octanoate to supply lipids for conversion to ketones. After 48h cells were lysed and RNA was collected.

## Insulin Tolerance Tests

Mice were fasted for 6h starting at approximately ZT2 and blood was drawn via tail vein. Glucose was determined using an Accuchek glucometer. Insulin (Humulin HR from Lily) was injected at 0.75 mU/g of lean mass (as determined via echoMRI). Blood glucose was determined at 15 minute intervals.

## Statistics

Statistical significance for this study was set at p=0.05. All statistical analyses were performed using R version 3.6.2 (19). For longitudinal measurements, mixed linear models were constructed with random intercepts starting at the diet change., Significance was determined via Chi-squared tests between models with or without the genotype or diet terms using the lme4 package version 1.1-21 (20). For experiments using both sexes, a modifying effect of sex was tested for all outcomes and reported where significant. All raw data and analysis scripts can be found at <http://bridgeslab.github.io/TissueSpecificTscKnockouts/>.

# Results

## GDF15 Produced in Muscle Alters Preference Towards Fatty Foods

Our previous work, consistent with the work of others noted that activation of mTORC1 via tissue-specific *Tsc1* ablation results in lean mice that are resistant to diet-induced obesity (17,21,22). To identify potential factors secreted from muscle that may drive changes in systemic physiology we used transcriptomic data filtered to identify putatively secreted proteins based on the Human Protein Atlas annotated as secreted in blood (23). After converting to mouse identifiers, this nominated 1780 mouse genes with secreted peptides, of which 116 were significantly differentially expressed in *Tsc1* knockout muscles (Figure 1A, see Supplementary Table 1 for complete list). The most significantly altered genes was GDF15, which was upregulated 46 fold at the mRNA level (adjusted p-value 8.2 x 10-12). To test if this induction of muscle GDF15 transcription had an impact on circulating levels we performed ELISA assays on the blood from muscle *Tsc1* knockout animals and found an 8.7 fold increase in serum GDF15 (Figure 1B, p=4.7 x 10-5). This is consistent with a prior report of muscle *Tsc1* ablation causing induction of GDF15 in blood (24).

Based on our recent work, injection of GDF15 reduced the preference of mice to fat, but had little impact on protein or carbohydrate intake (12). We therefore reasoned that GDF15 inductions in muscle *Tsc1* knockout mice may result in an aversion to high fat, low carbohydrate diets relative to low fat high carbohydrate diets. We developed a custom pair of control and ketogenic diets, which were matched for fiber, protein and choline levels (see Table 2). We tested whether muscle *Tsc1* knockout mice preferred the high carbohydrate to the high fat diet when given the choice of both diets. Wild-type mice preferred the fat rich food by 11.7 fold over the carbohydrate rich food after adjusting for differing caloric content. Muscle *Tsc1* knockout mice had a much lower preference, only consuming 2.6 fold more of the fat rich diet. Together these data show that there is a functionally significant elevation of GDF15 in muscle *Tsc1* knockout mice that results in a reduced preference towards fat rich diets.

## GDF15 Is Induced on Mice Fed a Ketogenic Diet

Based on the inductions of GDF15 and the reduced preference towards diets high in fat, we next tested whether circulating GDF15 was affected when mice were placed on control or ketogenic diets. We randomized cages of A/J mice starting at 10 weeks of age on to one or other of these diets and measured their body weights and composition for three weeks. We observed that the mice on the ketogenic diets had reduced body weight and lean mass over the first two weeks of diet that recovered by week three (Figures 1A-B), and modest reductions in fat mass (Figure 1C). We confirmed elevations of blood ketone body levels after three weeks of ketogenic diet with 11.8 and 10.4-fold induction of total ketone bodies in male and female mice respectively relative to control diets (p<0.001, Figure 1D). To test whether GDF15 was altered in the blood of these mice, we performed ELISA assays from serum. After adjusting for sex, we found a 59% increase in GDF15 (p=0.03, Figure 1E) in mice fed a ketogenic diet for three weeks. To understand if this increase was the tapering of an acute effect, or a slowly increasing effect we fed male C57BL6/J mice a ketogenic diet and took blood samples at multiple time points over three weeks of ketogenic diet feeding. We found that there was a 2.9-fold increase in GDF15 after 8h after the diet change. GDF15 levels decreased from that peak, but stabilized over three weeks, but was maintained at approximately 57% higher than NCD levels, consistent with the findings in A/J mice (Figure 1F).

## Induction of Hepatic GDF15 Occurs with Activation of the Integrated Stress Response

While GDF15 is likely made in many tissues, due to the key role of the liver in responses to ketogenic diets, we examined liver mRNA expression and found a similar XXX in both male and female mice.

To test whether hepatocytes were able to produce GDF15 under ketogenic conditions we treated AML12 cells with control or ketogenic media as described in (18).

## Ablation of GDF15 Results in Fat Mass Gain and Increased Energy Intake on a Ketogenic Diet

While the above studies describe induction of GDF15 under ketogenic conditions, they do not evaluate if this hormone plays a physiological role. To test what happens to mice lacking GDF15 we fed male and female wild-type and *Gdf15* knockout normal chow diets until 10 weeks of age. At this point all mice were placed on KD for five weeks. We observed no significant differences in body weight and lean mass at baseline, or in response to diet (Figures 3A-B). but an increase in fat mass (Figures 3A-C). Consistent with increases in fat mass, we observed elevated fat mass accretion in both male and female mice (Figure 3D). While wild-type male mice gained 133% of their fat mass, male knockouts had 203% increased fat mass (p=0.002). Similarly, female wild-type mice gained 56% more fat mass, while *Gdf15* knockout females gained over 100% more fat mass relative to baseline body composition (p=0.031). There was a significant sex difference observed in terms of fat accretion with females gaining less fat mass upon ketogenic diet feeding (2.0 +/- 0.6g for males, 0.49 +/- 0.39g for females; pinteraction=0.0006).

To determine if there was any impact on insulin sensitivity in wild-type and *Gdf15* knockout mice on a ketogenic diet we performed insulin tolerance tests after two weeks of diet and monitored changes in blood glucose. After a 6h fast we noted a sex-dependent effect on fasting glucose in *Gdf15* knockout mice on a ketogenic diet (pinteraction=0.043). Female mice had a 19% reduction in fasting blood glucose while male mice had a 3% increase (Figure 3E). After insulin injection, there were no significant effects of *Gdf15* knockout in either sex (Figure 3F). Together these data support the hypothesis that a lack of GDF15 results in positive energy balance in mice on a ketogenic diet.

# Discussion

In this study, the observed increases in GDF15 are relatively modest, but similar increases in GDF15 in humans are associated with pregnancy-related outcomes such as pre-eclampsia, nausea, gestational diabetes and miscarriage (8,24–26). This is also the approximate magnitude of exercise-associated elevations in GDF15 (27–31)

There are mixed data on the effects of hypercaloric diets in *Gdf15* or *Gfral* knockout mice with some papers showing hyperphagia and weight gain (32–35), but several others showing no effect (12,36,37) potentially representing strain, timing or background differences. As such, it is plausible that GDF15 is only physiologically relevant when elevated, but when signaling is absent (especially from birth) it is either dispensable or made to seem so by other adaptations. It is also plausible that other hormones which affect LCHF-dependent feeding changes may partially or completely compensate in the absence of GDF15.

# Author Contributions

# Acknowledgements

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# Conflict of Interest

﻿RJS receives financial support from Novo Nordisk, Janssen, Zafgen, Kallyope, and Medimune. He has also served as a paid consultant for Novo Nordisk, Janssen, Kallyope, and Scohia.

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# Tables and Legends

Table 1: Composition of diets used in this study.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Control Diet** | **Ketogenic Diet** | **Normal Chow Diet** |
| Carbohydrate | 75% | 0% | 36% |
| Protein | 15% | 15% | 24% |
| Lipid | 10% | 85% | 5% |

Table 2: Resource identification information.

|  |  |  |
| --- | --- | --- |
| **Type** | **Resource** | **Identifier** |
| Mouse Line | *Ckmm-Cre* | RRID:IMSR\_JAX:006405 |
| Mouse Line | *Tsc1* Floxed | RRID:IMSR\_JAX:005680 |
| Mouse Line | A/J | RRID:IMSR\_JAX:000646 |
| Mouse Line | C57BL/6J | RRID:IMSR\_JAX:000664 |
| Mouse Line | *Gdf15* null |  |
| Diet | NCD |  |
| Diet | CD |  |
| Diet | KD |  |
| Cell Line | AML12 | RRID:CVCL\_0140 |
| Resource | Human Protein Atlas |  |
| Resource | Biomart | RRID:SCR\_010714 |
| Resource | Gene Expression Omnibus | RRID:SCR\_005012 |

# Figure Legends

**Figure 1: Muscle *Tsc1* knockout mice have elevated GDF15 and a lowered preference towards high fat foods.** A) Volcano plot of predicted secreted proteins, dark shading indicates statistical significance. B) GDF15 levels in male muscle *Tsc1* knockout mice. C) Preference of fat rich ketogenic diet relative to control diet in male muscle *Tsc1* knockout mice. Dashed line indicates equal caloric intake from both diets Asterisks indicate p<0.05 (n=6,9).

**Figure 2: GDF15 is induced upon feeding mice a ketogenic diet.** A) Body weight of male and female A/J mice on a control or ketogenic diet. B) Lean mass (B) and total fat mass (C). D) Ketone body levels at 3 weeks of age from fed serum (E) GDF15 levels in A/J mice after three weeks of diet. F) GDF15 levels over time in C57BL/6J. Dashed line indicates baseline GDF15 levels (on NCD). Asterisks indicate p<0.05, n=7-8/group (A-E) and 3-4 (F).

**Figure 4: Ablation of GDF15 causes increased fat accretion and energy intake on a ketogenic diet.** A) Body weight (B) lean mass and (C) fat mass of GDF15 knockout mice before and after four weeks of ketogenic diet. Dashed line indicates the start of the ketogenic diet. Asterisks indicate p<0.05, n=8-14/group.