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Adaptive changes in amino acid metabolism permit normal longevity in mice consuming a low-carbohydrate ketogenic diet

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ABSTRACT

Ingestion of very low-carbohydrate ketogenic diets (KD) is associated with weight loss, lowering of glucose and insulin levels and improved systemic insulin sensitivity. However, the beneficial effects of long-term feeding have been the subject of debate. We therefore studied the effects of lifelong consumption of this diet in mice. Complete metabolic analyses were performed after 8 and 80 weeks on the diet. In addition we performed a serum metabolomic analysis and examined hepatic gene expression. Lifelong consumption of KD had no effect on morbidity or mortality (KD vs. Chow, 676 vs. 630 days) despite hepatic steatosis and inflammation in KD mice. The KD fed mice lost weight initially as previously reported (Kennnedy et al., 2007) and remained lighter and had less fat mass; KD consuming mice had higher levels of energy expenditure, improved glucose homeostasis and higher circulating levels of β-hydroxybutyrate and triglycerides than chow-fed controls. Hepatic expression of the critical metabolic regulators including fibroblast growth factor 21 were also higher in KD-fed mice while expression levels of lipogenic enzymes such as stearoyl-CoA desaturase-1 was reduced. Metabolomic analysis revealed compensatory changes in amino acid metabolism, primarily involving down-regulation of catabolic processes, demonstrating that mice eating KD can shift amino acid metabolism to conserve amino acid levels. Long-term KD feeding caused profound and persistent metabolic changes, the majority of which are seen as health promoting, and had no adverse effects on survival in mice.

1. Introduction

Ketogenic diets are of interest because in humans, they have been used for weight loss [2,3], and are also used to treat refractory epilepsy, particularly in pediatric populations [4,5]. Studies of KD have been extended to mice in an attempt to identify potential mediators of their metabolic effects. Despite the high-fat content of the diet, mice fed KD fail to gain excess weight and develop a unique metabolic state characterized by increased energy expenditure, increased systemic insulin sensitivity, and a distinct hepatic pattern of gene expression. Consumption of KD is also effective in inducing weight loss in wild-type mice with diet-induced obesity, as well as improving glucose tolerance without weight loss in the ob/ob mouse [1,6,7]. Additional studies demonstrated that diabetic nephropathy can be reversed through the use of KD intervention in both Type 1 (Akita) and Type 2 (db/db) mouse models [8]. These results show promise for the therapeutic use of KD; yet there is controversy regarding the potential adverse long term consequences. One study suggests that chronic KD consumption leads to pathological hepatic steatosis as well as insulin resistance and glucose intolerance [9,10]. However, to date the longest duration of a study of mice eating KD has been 12 weeks [10]. Since human consumption of KD for weight loss or refractory epilepsy can continue for months or years, the long-term health and lifetime effects of this dietary state are of clinical importance.

To fully understand the long-term physiologic consequences of KD on systemic health, longevity, and metabolism, we compared both the short-term effects of 8 weeks of KD (STKD) versus consuming a standard mouse "chow" diet, (STCH) and long-term effects after 66–80 weeks on the diets (LTCH and LTKD for chow and KD respectively). We compared physiologic parameters, gene expression profiles, and serum metabolomic profiles of each of the four cohorts. We found that the previously reported increase in energy expenditure and insulin sensitivity [1] persisted for over a year. We also compared changes in amino acid metabolism in mice fed the diet for 8 weeks to changes in mice fed the diet for 80 weeks. In both STKD and LTKD cohorts we found differences in amino acid metabolism as assessed both by metabolomics

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and gene expression, which were consistent with decreased amino acid catabolism. We also found increases in markers of oxidative stress, and inflammation in the liver in both STKD and LTKD mice. As there was no premature mortality over duration of the study, which continued until the death of the last mouse in the longevity cohort, we found no adverse effects of KD on life span. Thus we demonstrate that ketogenic diets are well tolerated over the lifespan of the animal, that animals maintain improved glucose homeostasis, and that the mice develop sustained compensatory changes in amino acid metabolism which may allow them to compensate for decreases in dietary protein intake and de novo amino acid biosynthesis.

2. Materials and methods

2.1. Animals

Animals were obtained from The Jackson Laboratory (C57Bl6J, 000664) in Bar Harbor, ME and maintained at 24 °C on a 12:12-h light-dark cycle. Animals were allowed ad libitum access to food, except where stated otherwise. All procedures were in accordance with NIH Guidelines for the Care and Use of Animals and were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee (IACUC). C57BL/6J mice were split into two groups at 8 weeks of age and fed standard chow or KD ad libitum. Within dietary groups, subsets of mice were randomly selected for physiologic tests or terminal harvest for tissue analysis. Physiologic tests included nuclear magnetic resonance (qNMR), indirect calorimetry, glucose tolerance test (GTT), and insulin tolerance test (ITT). Mice selected for tissue analysis and harvested at 16 weeks for the short-term study and 88 ± 1 week for the long-term study.

2.2. Diet studies

LabDiet 5008 (Pharmaserv, Framingham, MA) consisting of 6.5% fat, 23.5% protein, and 56% carbohydrate (2.5% sucrose) wt/wt was used as standard chow. The ketogenic diet (KD) was obtained from Bio-Serv (F3666, Frenchtown, NJ) and consisted of 78.9% fat, 9.5% protein, and 0.76% carbohydrate (0% sucrose) wt/wt. This formulation has been demonstrated to induce ketosis in rodents [11] and used by us in previous studies [1]. The macronutrient-calorie percent composition from each diet was as follows: standard chow: 16.7% fat, 26.8% protein, and 56.4% carbohydrate (6.5% sucrose); KD: 95% fat, 0% carbohydrate (0% sucrose), and 5% protein.

2.3. Glucose and insulin tolerance tests

Glucose tolerance test: mice on standard chow (CH) and KD were fasted for 16 h before an intraperitoneal (ip) injection D-glucose (2 g/kg body weight; Sigma, St. Louis, MO) was administered 4 h after light onset. Tail blood glucose levels were measured using a OneTouch Ultra glucometer (Lifescan, Milpitas, CA). To assess insulin tolerance, 7 CH and 5 KD ad libitum-fed-mice were injected ip, with insulin (0.75 U/kg) 8 h after onset of the light cycle (Lilly, Indianapolis, IN).

2.4. Indirect calorimetry

Mouse energy expenditure was measured by indirect calorimetry using the Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). Mice fed chow for 60 weeks were housed singly with *ad libitum* access to food and water. Analysis was performed at 24 °C under a 12:12-h light-dark cycle (light period 0600–1800). Mice were acclimated in the metabolic chambers for 48 h before collecting measurements used for data analysis. Ambulatory activity, oxygen consumption, and carbon dioxide production were simultaneously determined.

2.5. Body composition analysis

Body composition, including lean and fat mass, was assessed using an EchoMRI 3-in-1 quantitative nuclear magnetic resonance (qNMR) system (Echo Medical Systems, Houston, TX). This test is performed in conscious mice that are immobilized for one minute.

2.6. Quantitative RT-PCR

Ribonucleic acid (RNA) was isolated from tissue flash-frozen in liquid nitrogen using an RNAeasy Lipid mini kit (Qiagen, Germantown, MD) according to manufacturer's instructions. A DNase (Qiagen, Germantown, MD) step to digest the genomic DNA was included. Complementary DNA (cDNA) was made from isolated RNA using oligo(dt) and random hexamer primers and reverse transcriptase (QuantiTech RT Kit; Qiagen, Germantown, MD). Quantitative PCR was performed using the 7800HT (Applied Biosystems, Foster City, CA) thermal cycler and SYBR Green master mix (Applied Biosystems, Foster City, CA). Relative mRNA abundance was calculated and normalized to levels of the housekeeping gene *36B4*. Primers are included in Supplementary Table 1.

2.7. Survival analysis

C57BL/6J mice were split into two groups at 8 weeks of age and allowed ad libitum access to either standard chow or KD. The Kaplan–Meier survival curve was created with the log-rank test equal to the Mantel–Haenszel test. All statistical analyses were performed with GraphPad Prism v6.04 for Windows (GraphPad Software, San Diego, California, USA).

2.8. Serum and liver assays

Serum FGF21 concentrations were measured using Quantikine mouse FGF21 enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN), nonesterified free fatty acids (NEFAs) (NEFA C colorimetric assay; Wake Chemicals, Richmond, VA), triglycerides (Triglyceride Colorimetric Assay; Stanbio Laboratory, Boerne, TX), β-hydroxybutyrate (β-hydroxybutyrate colorimetric assay, Stanbio Laboratory), cholesterol (Cholesterol Liquicolor, colorimetric assay; Stanbio Laboratory), insulin (ultra-sensitive mouse insulin ELISA; Crystal Chem, Chicago, IL), and alanine aminotransferase (ALT Colorimetric Assay, Pointe Scientific, Canton, MI). For tissue cholesterol and triglyceride assays, a Folch extraction was first performed on 100 mg of frozen tissue [12] before colorimetric analysis (Cholesterol Liquicolor, colorimetric assay; Stanbio Laboratory), (Triglyceride Colorimetric assay; Stanbio Laboratory, Boerne, TX).

2.9. Targeted mass spectrometry and metabolomics

Serum (100 μL) was collected and isolated from mice 16 weeks (STCH and STKD) and 88 weeks (LTCH and LTKD) of age. Serum samples were extracted at dry ice temperatures using a 1:4 ratio of serum:methanol resulting in a total volume of 80% methanol. Samples were centrifuged and resulting supernatant was dried under vacuum. Samples were resuspended using 20 μL high-performance liquid chromatography (HPLC) grade water for mass spectrometry and 10 μL was injected and analyzed using a 5500 QTRAP triple quadrupole mass spectrometer (AB/Sciex) coupled to a Prominence UFLC HPLC system (Shimadzu) via selected reaction monitoring (SRM) of a total of 249 endogenous water soluble metabolites as previously described [13]. All mass spectrometry data were collected and processed as previous reported [13]. Data are presented as mean ± SEM. Comparisons between two groups were performed using an unpaired t test.