

## A high-fat, ketogenic diet induces a unique metabolic state in mice

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Submitted 28 December 2006; accepted in final form 9 February 2007

**Kennedy AR, Pissios P, Otu H, Xue B, Asakura K, Furukawa N, Marino FE, Liu F, Kahn BB, Libermann TA, Maratos-Flier E.** A high-fat, ketogenic diet induces a unique metabolic state in mice. *Am J Physiol Endocrinol Metab* 292: E1724–E1739, 2007. First published February 13, 2007; doi:10.1152/ajpendo.00717.2006.—Ketogenic diets have been used as an approach to weight loss on the basis of the theoretical advantage of a low-carbohydrate, high-fat diet. To evaluate the physiological and metabolic effects of such diets on weight we studied mice consuming a very-low-carbohydrate, ketogenic diet (KD). This diet had profound effects on energy balance and gene expression. C57BL/6 mice animals were fed one of four diets: KD; a commonly used obesogenic high-fat, high-sucrose diet (HF); 66% caloric restriction (CR); and control chow (C). Mice on KD ate the same calories as mice on C and HF, but weight dropped and stabilized at 85% initial weight, similar to CR. This was consistent with increased energy expenditure seen in animals fed KD vs. those on C and CR. Microarray analysis of liver showed a unique pattern of gene expression in KD, with increased expression of genes in fatty acid oxidation pathways and reduction in lipid synthesis pathways. Animals made obese on HF and transitioned to KD lost all excess body weight, improved glucose tolerance, and increased energy expenditure. Analysis of key genes showed similar changes as those seen in lean animals placed directly on KD. Additionally, AMP kinase activity was increased, with a corresponding decrease in ACC activity. These data indicate that KD induces a unique metabolic state congruous with weight loss.

liver; gene expression

OVER THE PAST FEW DECADES the rates of obesity have risen substantially worldwide. Paradoxically, the increases in body weight, particularly in Western countries, occurred during a period of emphasis on diets low in fat as a means for avoiding weight gain. These dietary recommendations were based largely on the concept that high-fat diets were less satiating (39) and that reducing dietary fat reduced risk for cardiovascular disease by lowering circulating fat and cholesterol (5). As a result of the perceived failure of traditional dietary advice, attention shifted to alternative dietary regimes, including low-glycemic-index diets and very-low-carbohydrate ketogenic diets. Interest in these diets derives in part from the theoretical effects of dietary composition on energy expenditure. Although a small number of human studies have found such diets to be more effective in short-term weight loss and without adverse effects on glucose, insulin, lipids, or blood pressure (7, 14, 42, 49, 51), reports on metabolic effects remain inconclusive (8, 38). Thus the precise effects of macronutrient diet composition on energy balance remain controversial.

Studies of the physiological effects of dietary composition are intrinsically difficult in human populations because of problems achieving both compliance and accurate dietary reporting. Therefore, we developed a mouse model to examine the effect of diet composition on physiology, with particular reference to energy expenditure and metabolism. In the past, murine models of obesity have been instrumental in defining pathways important in mammalian energy homeostasis, and information from these models has been successfully translated into mechanisms of energy dysregulation in humans. For example, the leptin and melanocortin systems were found to be of critical importance in energy balance in mice (13, 18) and have since been found to be equally important in humans (9, 37, 47).

To examine the effects of gross dietary manipulation, we chose four distinct dietary conditions. First, we utilized a rodent ketogenic diet (KD) previously used extensively in studies of seizure susceptibility and known to induce consistent ketosis (6). We compared this diet to a high-fat (HF) diet that was also high in carbohydrate and commonly used in studies of mouse obesity. The effects of these diets were compared with the effects of ad libitum chow feeding as well as to the effects of restriction of calories to 66% of ad libitum chow (caloric restriction).

We found that mice tolerated the KD well, consuming at least as many calories as mice fed a high-fat diet. However, mice eating KD failed to gain weight despite the high caloric density of the diet. Compared with mice fed standard chow, mice fed KD transiently lost weight and then stabilized at a lower weight than chow-fed animals in a pattern that was the same as that seen calorie-restricted mice. KD fed mice had a unique metabolic and physiological profile, exhibiting increased energy expenditure and very low respiratory quotient. Insulin levels were extremely low compared with both animals fed chow and animals fed high-fat diet. Furthermore, despite the consumption of saturated fat, serum lipids did not increase. Analysis of gene expression using Affymetrix chips revealed that consumption of KD led to a pattern of expression in the liver distinct from all other diets. An interesting feature of gene expression was suppression of both transcription factors and enzymes involved in lipid synthesis. These included fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD-1), and sterol regulatory element-binding protein-1c (SREBP-1c).

In a second series of experiments we examined the effect of KD on obesity established through feeding of a HF diet. Mice transitioned from HF to KD rapidly lost weight and maintained lower body weights throughout the observation period. These

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effects were mediated largely through a substantial increase in energy expenditure and were associated with improved glucose tolerance and plasma lipid profiles as well as substantial lowering of plasma leptin and insulin levels. This change in metabolic state was accompanied by marked alterations in the expression and/or activity of molecules believed to be important in energy balance and nutrient sensing, including uncoupling protein-1 in brown adipose tissue and AMP kinase (AMPK) in muscle and liver. Interestingly, expression of several neuropeptides known to influence feeding and energy expenditure was consistent with the fall in leptin levels resulting from weight loss and was comparable with expression seen in the fasted state. Thus, energy balance in animals on a KD appears to be controlled by mechanisms outside of the normal hypothalamic pathways.

These data indicate that dietary manipulation is capable of altering energy balance and metabolic state. In these experiments a high-fat, ketogenic diet not only failed to cause obesity but was capable of reversing diet-induced obesity (DIO) in mice. These data suggest a more complex relationship between fat consumption and obesity than previously thought. Further investigation as to the mechanisms of energy balance in these animals may provide new targets in obesity research.

## MATERIALS AND METHODS

### Animals

Animals were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained at 24°C on a 14:10-h light-dark cycle. Animals were allowed ad libitum access to food, except where stated otherwise. All studies were approved by the Beth Israel Deaconess Medical center IACUC.

### 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. Diet D12451 (Research Diets, New Brunswick, NJ) consisting of 24% fat, 24% protein, and 41% carbohydrate (19% sucrose) wt/wt was used as high-fat food. A KD consisting of 78.9% fat, 9.5% protein, and 0.76% carbohydrate (0% sucrose) wt/wt (no. F3666) was obtained from Bio-Serv (Frenchtown, NJ). The KD was supplemented by the vendor with vitamin mix and mineral mix (AIN-76, formula nos. F8000 and F8505) intended to provide a diet nutritionally complete in micronutrients. The proportion of calories deriving from different nutrients was as follows: chow: 16.7% fat, 26.8% protein, 56.4% carbohydrate (6.5% sucrose); high fat: 45% fat, 24% protein, 35% carbohydrate (17% sucrose), 24% protein; KD: 95% fat, 0% carbohydrate (0% sucrose), 5% protein.

### Protocols

**Protocol 1: differential effects of Chow, KD, HF, and CR.** Thirty-two 8-wk-old C57BL/6 male mice were split into four groups of eight mice each. One group was maintained on ad libitum chow, the second group was fed ad libitum KD, a third was fed ad libitum HF, and a fourth group was calorically restricted (CR) to 65% of their average chow intake using Research Diet D12491G, formulated to provide normal minerals and vitamins for 65% of normal caloric intake. These diets were maintained throughout the experiment. This protocol was repeated in a second independent cohort using the same number of animals with the same results. The sources of carbohydrate and fat are described in Table 1.

**Protocol 2: effects of KD on obesity induced by HF.** Twenty-four 8-wk-old C57BL/6 male mice were split into three groups of eight

Table 1. Breakdown of diet macronutrient contents

|                      | Chow                              | KD    | HF   |
|----------------------|-----------------------------------|-------|------|
| Lard                 |                                   | 47.5  | 20.7 |
| Butter               |                                   | 19.95 |      |
| Vegetable oil        | 6.5                               | 11.4  | 3.3  |
| Protein              | 24.8                              | 9.5   | 24   |
| Sucrose/maltodextrin |                                   |       | 31.7 |
| Starch               |                                   | 0.76  | 8.1  |
| Total carbohydrate   | 44.2 (ground cereal and molasses) | 0.76  | 41   |
| Fiber                | 4.5                               | 5.0   | 5.2  |
| Ash                  | 7.75                              | 3.8   | NA   |

Values given as %content by weight. KD, ketogenic diet; HF, high fat; NA, not available.

mice each. Two groups were placed on the HF diet, whereas one group was maintained on chow (C). After a period of 12 wk, mice on the HF diet gained ~7 g of excess body weight compared with C animals and were deemed to have DIO. Of the two DIO groups, one remained on HF until the end of the study and one was fed KD ad libitum for the remainder of the study. The study continued for 5 wk after the introduction of KD, whereupon all mice were killed and tissues taken for analysis. This protocol was repeated in a total of three independent cohorts, and in each case a similar degree of DIO developed on HF, which was resolved when animals were transferred to KD.

### Glucose Tolerance Tests

Twenty-four mice were fasted overnight (1700–0800) and were subsequently injected with glucose (2 g/kg body wt ip). Tail blood was collected at 0, 15, 30, 60, and 120 min. Blood glucose concentrations were measured using a glucometer (Elite; Bayer, Mishawaka, IN).

### Indirect Calorimetry

Metabolic rate of mice was measured by indirect calorimetry in open-circuit oxymax chambers that are a component of the Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH). Mice were housed singly and maintained at ~24°C under a 12:12-h light-dark cycle (light period 0800–2000). Food and water were available ad libitum. All mice were acclimatized to monitoring cages for 48 h prior to the beginning of an additional 24 h of hourly automated recordings of physiological parameters. Mice were weighed prior to each trial. Sample air was passed through an oxygen (O<sub>2</sub>) sensor (Columbus Instruments) for determination of O<sub>2</sub> content. O<sub>2</sub> consumption was determined by measuring oxygen concentration in air entering the chamber compared with air leaving the chamber. The sensor was calibrated against a standard gas mix containing defined quantities of O<sub>2</sub>, CO<sub>2</sub>, and N<sub>2</sub>.

### Open Field Test

Individual mice were placed in a fresh cage and recorded by video camera for a period of 1 h. Total distance traveled was measured using tracking software (Ethovision Video Tracking, Noldus, The Netherlands).

### Body Fat Measurement

Fat and lean body mass were assessed in all animals using a dual-energy X-ray absorptiometry (DEXA) (Lunar PIXImus2 mouse densitometer; GE Medical Systems, Madison, WI) as instructed by the manufacturer. Mice were anesthetized by intraperitoneal injection of a (1:1) mixture of tribromoethanol and T-amyl alcohol, 0.015 ml/g body wt; scanned and total body fat, as well as lean body mass, were determined using the analysis program provided by the manufacturer.

After being scanned, mice were returned to their cages and held under a warming light until they awakened.

### Tissue Samples

Tissues were collected and snap frozen in liquid nitrogen before storage at  $-80^{\circ}\text{C}$ . Blood was collected by cardiac puncture into centrifuge tubes before centrifugation at 10,000 rpm for 10 min. Serum was separated and stored at  $-20^{\circ}\text{C}$ .

### Assays

Plasma was tested for content of nonesterified free fatty acids (NEFA) (NEFA C colorimetric assay, cat. no. 994-75409; Wako Chemicals, Richmond, VA), triglycerides (triglyceride colorimetric assay, cat. no. 2100-430; Stanbio Laboratory, Boerne, TX),  $\beta$ -hydroxybutyrate ( $\beta$ -hydroxybutyrate colorimetric assay, cat. no. 2440; Stanbio Laboratory), cholesterol (cholesterol liquicolor, colorimetric assay, cat. no. 1010-430; Stanbio Laboratory), leptin (cat. no. 90030; Crystal Chem, Chicago, IL), insulin (cat. no. Inskr020; Crystal Chem), glucagon (cat. no. GL-32K; Linco, St. Charles, MO), and TSH (ICN, Orangeburg, NY).

### Gene Expression

Gene expression was measured by real-time quantitative PCR. Total hypothalamic RNA was isolated using Ultraspec (Biotecx Laboratories, Houston, TX) and was stored at  $-80^{\circ}\text{C}$ . Quantity and purity were assessed by ultraviolet absorbance at 260 and 280 nm. Real-time quantitative RT-PCR was performed using One-Step RT-PCR method (PE Applied Biosystems, Foster City, CA) and measured in automated PE/AB 7700 sequence detector. Linear ranges and optimal RNA concentrations for each primer and probe set were previously determined. Each primer and probe set was designed to span an intron/exon splice junction to minimize amplification of genomic DNA, and each set had been previously tested for specificity using control reactions without addition of reverse transcriptase. The same concentration of RNA was used for all the samples for each neuro peptide determination. All the probes contained the fluorescent dye 6FAM at 5' end and the quencher TAMRA at 3' end. All of the samples were quantitated in triplicates with rodent VIC-labeled GAPDH (Applied Biosystems) as internal control. Primers and probes were used for amplification at the PCR reactions described in Table 2.

### Quantitative PCR II

RNA, from flash-frozen tissue, was extracted using an RNeasy minikit (Qiagen) according to instructions, and cDNA was generated from 1  $\mu\text{g}$  of RNA, using oligo(dt) primers and Moloney murine leukemia virus reverse transcriptase (advantage RT for PCR; Clontech), and diluted to 1 ml. Quantitative PCR was performed using the MX3000 thermal cycler and SYBR Green master mix (Applied Biosystems). Primers were obtained from Invitrogen, as detailed in Table 3.

### Microarray Analysis

The transcriptional profile of samples were probed using the Affymetrix MG-430 Plus 2.0 chips according to previously described

protocols for total RNA extraction and purification, cDNA synthesis, in vitro transcription reaction for production of biotin-labeled cRNA, hybridization of cRNA with Affymetrix gene chips, and scanning of image output files (26). The scanned array images were analyzed by dChip (31). A recent study has suggested that, regarding signal value calculation, dChip is more robust than Affymetrix software Microarray Analysis Suite 5.0 (1) for  $\sim 60\%$  of the genes (3). In the dChip analysis a smoothing spline normalization method was applied prior to obtaining model-based gene expression indexes, a.k.a., signal values. During this process, single, array, and probe outliers were interrogated as described in dChip, where image spikes are treated as single outliers. There were no outlier chips identified by dChip, so all samples were carried on for subsequent analysis.

### Differential Gene Expression Analysis and Clustering

When comparing two groups of samples to identify genes enriched in a given group, we used the lower confidence bound (LCB) of the fold change (FC) between the two groups as the cutoff criteria. If 90% LCB of FC between the two groups was above 1.2, the corresponding gene was considered to be differentially expressed. LCB is a stringent estimate of the FC and has been shown to be the better ranking statistic (30). Recently, dChip's LCB method for assessing differentially expressed genes has been shown to be superior to other commonly used approaches (24), such as Microarray Analysis Suite 5.0- and Robust Multiarray Average-based methods (43).

By use of LCB, we can be 90% confident that the actual FC is some value above the reported LCB. It was suggested by a study exploring the accuracy and calibration of Affymetrix chips using custom arrays and quantitative reverse transcriptase real-time PCR assays that the chip analyses underestimate differences in gene expression (52). In regard to their work, and that of Romalho-Santos et al. (40), a criterion of selecting genes that have an LCB  $>1.2$  most likely corresponds to genes with an "actual" FC of  $\geq 3$  in gene expression.

A hierarchical clustering technique utilizing average linkage was used to construct an unweighted pair group method with arithmetic-mean tree using Pearson's correlation as the metric of similarity (45). This tree represents the similarity between genes and/or samples based on the expression profile observed on the chips.

### Western Blots

Brown adipose tissue (BAT) from all eight animals was homogenized in lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) supplemented with various protease inhibitors. Protein concentration of the extracts was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Twenty-five micrograms of total protein were analyzed by SDS-PAGE on a 15% mini-protein gel (Biorad Laboratories) and transferred onto nitrocellulose (Protran; Schleicher and Schuell, Keene, NH). The primary [uncoupling protein (UCP)1] and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The blots were developed with Super Signal West Pico chemiluminescent reagent (Pierce, Rockford, IL).

Table 2. Primers and probes for hypothalamic qPCR

| Gene | Forward Primer           | Reverse Primer            | Probe                                    |
|------|--------------------------|---------------------------|--|
| NPY  | CAGACCTCTTAATGAAGGAAAGCA | GAGAACAAGTTTCATTTCCTCATCA | 6-FAM-CCAGAACAAGGCTTGAAGACCTTCCATTAMRA   |
| POMC | CTGCTTCAGACCTCCATAGATGTG | CAGCGAGAGGTCGAGTTTGC      | 6-FAM-CAACCTGCTGGCTTGCATCCGG-TAMRA       |
| AgRP | CAGAAGCTTTGGCGGAGGT      | AGGACTCGTGCAGCCTTACAC     | 6-FAMCTAGATCCACAGAACCAGGAGTCTCGTTTAMRA   |
| MCH  | ATTCAAAGAAGACAGGCTCCAAAC | CGGATCCTTTTCAGAGCAAGGTA   | 6FAM-AATCTTGTAACCTACGGGCTGCCACTGAGTTAMRA |

qPCR, quantitative PCR; NPY, neuropeptide Y; POMC, proopiomelanocortin; AgRP, agouti-related protein; MCH, melanin-concentrating hormone.



Table 3. Primers for hepatic qPCR

| Gene           | Forward Primer          | Reverse Primer          |
|----------------|-------------------------|-------------------------|
| mFAS           | ggttacactgtgctagggtgttg | tccaggcgcgatgaggctcagc  |
| mSCD-1         | gcgttccagaatgacgtgtac   | ctttcagggtcagacatgtccag |
| UCP2           | actttccctctggataccgc    | acggaggcaaaagctcatctg   |
| Hadhb          | actacatcaaaatgggctctcag | agcagaaatggaatgctggacc  |
| PEPCK          | catatgctgatcctgggcataac | caaaacttcatccaggcaatgtc |
| G6pase         | cgactcgtctatctccaaagtg  | ggtgaaccaggtctccgacca   |
| PFK-L          | tgacgectacaatctgctcc    | gtcaagtgtgctgtagttctga  |
| PGC-1 $\alpha$ | agccgtgaccactgacaacgag  | agccgtgaccactgacaacgag  |
| PGC-1 $\beta$  | cttgactactgtctgtgaggc   | ctccaggagactgaatccagag  |
| CD36           | gaaccactgctttcaaaaactgg | tgtgttcttttgcacagtc     |
| HBDH           | agtttggggtcgaggcttcc    | tggtagccgctatgaagttg    |
| SREBP-1c       | ggagccatggattgcacatt    | gcttccagagaggaggccag    |

FAS, fatty acid synthase; SCD-1, stearoyl-CoA desaturase-1; UCP2, uncoupling protein 2; HADHB, hydroxyacyl dehydrogenase- $\beta$ ; PEPCK, phosphoenolpyruvate carboxykinase; G6pase, glucose-6-phosphatase; PFK-L, phosphofructokinase liver isoform; CD36, fatty acid translocase; HBDH, hydroxybutyrate dehydrogenase; SREBP-1c, sterol regulatory element-binding protein-1c.

### AMPK

AMPK activity was measured by immune complex assay using isoform specific  $\alpha$ -subunit antibodies, with SAMS peptide as a substrate using the method previously described (33, 34). Briefly, 100  $\mu$ g of protein were immunoprecipitated with sheep polyclonal antibodies specific to  $\alpha$ 2-AMPK (kindly provided by Dr. David Carling, Imperial College, London, UK) bound to protein G-Sepharose overnight. Immunoprecipitates were washed twice in intraperitoneal buffer followed by twice in Assay buffer (240 mM HEPES and 480 mM NaCl). Kinase reactions were carried out in 40 mM HEPES (pH 7.0), 80 mM NaCl, 0.8 mM dithiothreitol (DTT), 5 mM MgCl<sub>2</sub>, 0.2 mM each of ATP and AMP, 0.1 mM SAMS peptide, and 2  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P] ATP (PerkinElmer Life and Analytical Sciences, Boston, MA) in a total volume of 50  $\mu$ l. Forty microliters of aliquot were spotted onto Whatman P81 paper washed four times in 1% phosphoric acid. <sup>32</sup>P incorporation was quantitated with a scintillation counter, and kinase activity was calculated as incorporated ATP (nmol) per gram of protein per minute (21).

### Acetyl-CoA Carboxylase Activity Measurement

Acetyl-CoA carboxylase (ACC) activity measurement was based on acetyl-CoA-dependent <sup>14</sup>CO<sub>2</sub> fixation (16, 34). Reactions were carried out with 100  $\mu$ g of tissue lysates in an assay buffer containing 80 mM HEPES (pH 7.5), 1.7 mM DTT, 4.2 mM ATP, 1 mM NaHCO<sub>3</sub>, 8.3 mg/ml BSA, 3.3 mM MgCl<sub>2</sub>, 0.42 mM acetyl CoA, and 6  $\mu$ Ci <sup>14</sup>CO<sub>2</sub> (Amersham Biosciences, Piscataway, NJ) in the presence or absence of 2 mM citrate. Reactions were stopped by adding 1N HCl and dried. <sup>14</sup>CO<sub>2</sub> radioactivity was determined with a scintillation counter. ACC activity was calculated as citrate-dependent incorporation of <sup>14</sup>CO<sub>2</sub> into acid-stable products (pmol) per milligram of protein per minute (16).

### Histology

Tissue was fixed in 4% formalin before being embedded in wax and sectioned on a microtome. Tissues were then deparaffinized in xylene and rehydrated through a series of alcohols. Staining for glycogen was performed using a periodic acid shift staining protocol before dehydration in alcohol and coverslipping in DePeX (Sigma, St. Louis, MO).

Mean differences were ascertained by unpaired *t*-test using SAS Statview (Cary, NC). A *P* value of <0.05 was considered statistically significant.

### RESULTS

#### Protocol 1: Profiling of Animals Fed C, KD, HF, and CR

**Food intake and energy expenditure.** Mice introduced to both KD and HF adapted immediately to the new diet. Over the course of 33 days, during which food intake was measured frequently (at 1- to 3-day intervals), there was no difference in the caloric intake of mice eating C, HF, or KD, except for the first day, when mice eating HF ate twice as many calories as mice eating chow or KD. Thus, on day 25 of the feeding study, mice in the chow group ate 13.2 calories, those on HF ate 12.6 calories, and those on KD ate 12.9 calories. CR mice received 9.2–9.5 calories daily (Fig. 1A). At the end of 33 days, mice fed standard C had consumed 463 calories, mice fed HF diet consumed 483 calories, and mice fed KD consumed 487 calories. CR animals consumed a total of 313 calories. The same pattern of food intake continued throughout the duration of the study, which lasted 60 days.

Mouse weights did not correlate with calories consumed. C animals had a small 2-g increase in weight (27.8–29.8 g) typical of C57BL/6 mice. As expected, animals placed on the CR diet lost weight and weighed 23.8 g at the end of the study, 3.5 g less than their initial weight, and 6.0 g less than animals fed C. Also as expected, C57BL/6 mice fed HF gained weight to a degree that has been previously described by a number of investigators (25, 28, 48). After 2 mo, these mice were 6 g heavier than mice fed C and weighed 35.9 g. In contrast, mice fed KD, although they ate as many calories as mice fed HF, demonstrated a pattern of weight change that paralleled that seen in the CR group, initially losing weight and then stabilizing at the same weight as CR, i.e., 23.8 g, a weight 6.0 g lower than that of C-fed animals (Fig. 1B).

**Metabolic analysis.** The finding of similar body weights in KD and CR mice in the context of substantial differences in calories consumed suggested that KD animals had increased metabolic rate. Metabolic profiles were compared in KD and CR animals using the CLAMS apparatus. C-fed animals were evaluated as a control group. Evaluation of heat production, which reflects total energy expenditure and is independent of animal weight, revealed that KD-fed animals had 11% higher energy expenditure than C-fed animals and 15% higher energy expenditure compared with the CR group, (24-h heat produc-

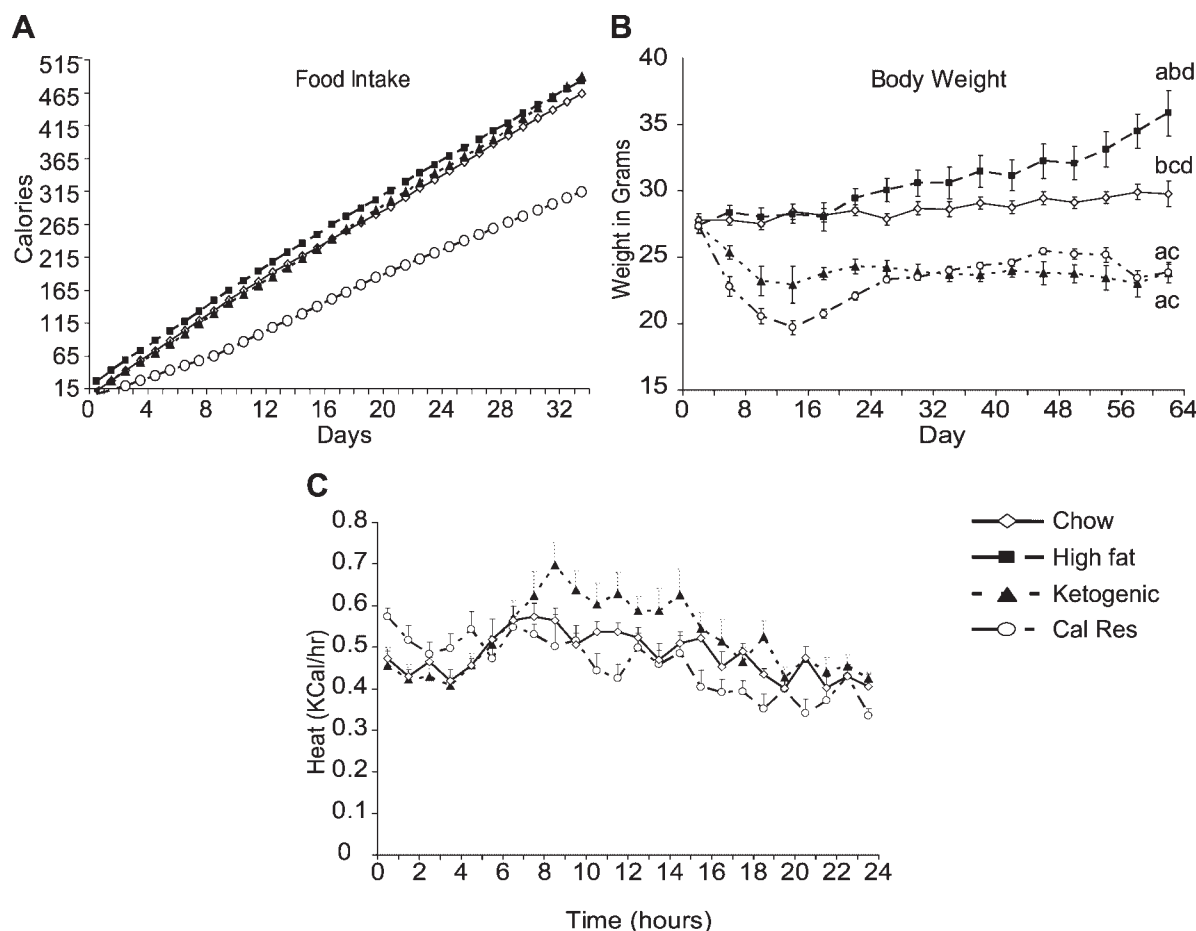


Fig. 1. Eight-week-old mice were placed on chow (◇), ketogenic (KD; ▲), high-fat (HF; ■), and calorie-restricted (CR; ○) diets. A: caloric intake was followed in all 4 groups. B: body weights were followed for 9 wk. C: at 5 wk, animals were tested for 24-h oxygen consumption. Graphs show means  $\pm$  SE. <sup>a</sup>Significantly different from chow; <sup>b</sup>significantly different from KD; <sup>c</sup>significantly different from HF; <sup>d</sup>significantly different from CR.

tion: KD,  $12.5 \pm 0.7$  kcal; C,  $11.6 \pm 0.3$  kcal; CR,  $10.9 \pm 0.5$  kcal). Because weight and body composition of KD fed animals were the same as that of the CR group (see below), it is unlikely that the increase in metabolic rate is secondary to differential  $O_2$  consumption by different tissue beds. Data shown are mean of eight animals from each group (Fig. 1C).

**Body composition.** At 5 wk, DEXA scan was performed to assess body composition. Consistent with the similarity in leptin levels, fat mass was the same in animals fed C, KD, and CR and averaged 3.4 g/animal. Fat mass of animals fed HF was increased twofold and averaged 6.7 g/animal. Lean mass ranged between 21 and 23 g in animals fed either C or HF. Lean mass of animals fed both KD and CR was the similar: 17.6 g for KD animals and 17.5 g for CR-fed animals (Fig. 2, A and B). Data represent mean of eight animals in each group.

**Hormonal and metabolic profiles.** The different diets were associated with dramatic differences in fed insulin levels (Table 4). As expected, mice fed HF gained weight and became hyperinsulinemic compared with C-fed animals, whereas insulin levels in lean CR animals were lower compared with the C-fed group. In KD animals, insulin levels were dramatically decreased at 1.0 pg/ml. This represents an 84% reduction compared with CR animals of the same weight and a 90% reduction compared with C-fed animals (Fig. 2C). Animals fed

KD maintained normal testosterone levels in contrast to CR animals, which showed a substantial decline in testosterone to 30% of initial values (Table 4). Furthermore, in one study of females fed KD, we found they continued to cycle normally through an 8-wk observation period, whereas females that were fed CR stopped cycling within 4 days of CR (data not shown).

In contrast, there was no significant difference in leptin levels in animals fed C, KD, or CR. Leptin levels in HF-fed animals were increased eightfold compared with C-fed animals (Fig. 2D and Table 4).

Mice fed KD achieved ketosis despite ad libitum feeding.  $\beta$ -hydroxybutyrate levels were fourfold higher than control animals. HF animals had significantly lower ketone levels compared with control animals, whereas CR animals had a slight decrease compared with ad libitum-fed animals (Table 4).

**Affymetrix analysis.** mRNA from liver from eight animals fed four different diets was used in the analysis. mRNA from two to three animals was pooled to generate three chips for each dietary group. Correlation values based on the expression of all genes on the chip revealed high similarities between the triplicate samples run for each diet. The means  $\pm$  SD correlation coefficients between replicates when each diet group was considered separately were  $0.98 \pm 0.006$  for C,  $0.98 \pm 0.01$  for CR,  $0.99 \pm 0.001$  for HF, and  $0.99 \pm 0.004$  for KD diets. When all of the replicate combinations were considered to-

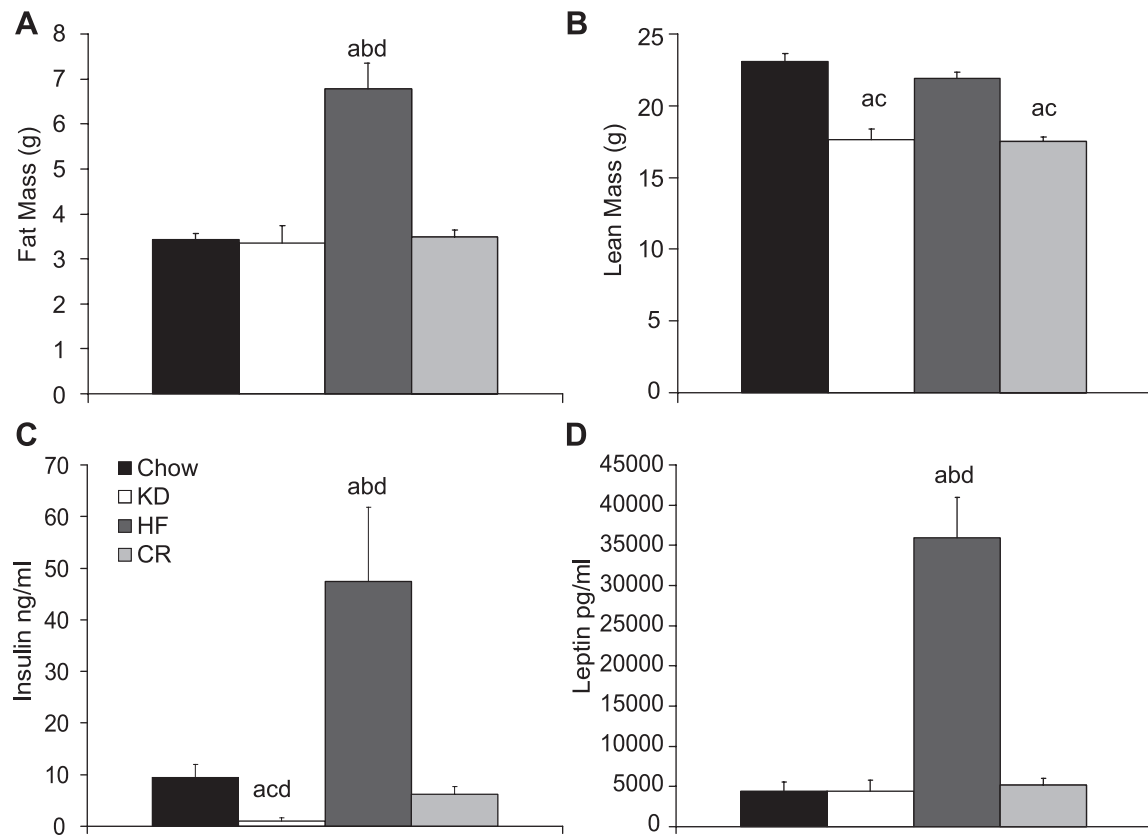


Fig. 2. Dual-energy X-ray absorptiometry analysis was performed 6 wk after initiation of the diet in chow, KD, HF, and CR groups to determine fat mass (A) and lean mass (B). Fed insulin (C) and leptin (D) in terminal serum were measured. Graphs show means  $\pm$  SE. \*Significantly different from chow; ^significantly different from KD; ^significantly different from HF; ^significantly different from CR. Significance =  $P < 0.05$  by ANOVA.

gether, the average correlation value was  $0.98 \pm 0.007$ , showing high reproducibility. Hierarchical cluster analysis using complete array expression values produced a cladogram reflecting global expression similarities across the samples (Fig. 3A). The resulting tree demonstrated high similarity between HF and C diets, with some distinction with the CR diet. On the other hand, samples that were fed with KD formed a separate branch, indicating substantial difference.

Recently, Lin et al. (32) analyzed livers from HF-fed mice and reported 14 genes to increase acutely in response to a HF diet. We then focused specifically on these 14 genes and found that, in our study, the pattern of gene expression in

animals chronically fed high-fat diet was very similar to that previously reported in the acute study. In contrast, animals fed KD had a distinctly different pattern. As is visually apparent from the heat map in Fig. 3B, expression of genes in KD was generally inverse to those seen on HF diet. For example, expression of genes such as FAS and SCD-1, which are essential for fatty acid synthesis, increased in animals fed HF. In contrast, in animals fed KD, expression of both of these genes was substantially suppressed. These data were confirmed by qPCR analysis, which revealed a twofold increase in FAS and a threefold increase in SCD-1 in animals on HF; FAS expression was reduced by twofold,

Table 4. Serum nutrient and hormonal profiles at 9 wk, including liver triglycerides

| Serum Concentration               | Chow                           | HF                                | KD                              | CR                             |
|-----------------------------------|--------------------------------|-----------------------------------|---------------------------------|--------------------------------|
| Triglycerides, mg/dl              | 111.2 $\pm$ 6.4                | 110.7 $\pm$ 9.0                   | 106.8 $\pm$ 5.2                 | 107.7 $\pm$ 3.6                |
| Cholesterol, mg/dl                | 132.2 $\pm$ 18.4               | 178.5 $\pm$ 12.9 <sup>d</sup>     | 153.9 $\pm$ 13.5                | 141.6 $\pm$ 10.4 <sup>b</sup>  |
| NEFA, mEq/l                       | 0.62 $\pm$ 0.06 <sup>c</sup>   | 0.63 $\pm$ 0.04 <sup>c</sup>      | 0.77 $\pm$ 0.07 <sup>abd</sup>  | 0.65 $\pm$ 0.03 <sup>c</sup>   |
| Liver triglycerides, mg/g         | 9.8 $\pm$ 0.7                  | 12.4 $\pm$ 1.8                    | 17.1 $\pm$ 1.2 <sup>abd</sup>   | 7.7 $\pm$ 0.5 <sup>abc</sup>   |
| $\beta$ -Hydroxybutyrate, mmol/dl | 0.39 $\pm$ 0.07 <sup>c</sup>   | 0.16 $\pm$ 0.03 <sup>c</sup>      | 1.62 $\pm$ 0.36 <sup>abd</sup>  | 0.30 $\pm$ 0.06 <sup>c</sup>   |
| Glucose, mg/dl                    | 336.6 $\pm$ 21.1 <sup>bc</sup> | 422.6 $\pm$ 37.9 <sup>acd</sup>   | 214.6 $\pm$ 29.4 <sup>ab</sup>  | 284.7 $\pm$ 31.7 <sup>b</sup>  |
| Insulin, ng/ml                    | 9.4 $\pm$ 2.6 <sup>bc</sup>    | 47.5 $\pm$ 14.3 <sup>acd</sup>    | 1.0 $\pm$ 0.6 <sup>abd</sup>    | 6.2 $\pm$ 1.6 <sup>bc</sup>    |
| Leptin, pg/ml                     | 4,390 $\pm$ 1,204 <sup>b</sup> | 35,976 $\pm$ 4,962 <sup>acd</sup> | 4,443 $\pm$ 1,361 <sup>b</sup>  | 5,161 $\pm$ 820 <sup>b</sup>   |
| Testosterone, ng/ml               | 0.36 $\pm$ 0.07 <sup>d</sup>   | 0.46 $\pm$ 0.08 <sup>d</sup>      | 0.38 $\pm$ 0.08 <sup>d</sup>    | 0.13 $\pm$ 0.03 <sup>abc</sup> |
| ALT, U/l                          | 17.98 $\pm$ 1.08 <sup>c</sup>  | 13.20 $\pm$ 1.67 <sup>c</sup>     | 26.17 $\pm$ 1.81 <sup>abd</sup> | 15.68 $\pm$ 2.45 <sup>c</sup>  |
| Urea nitrogen, mg/dl              | 31.45 $\pm$ 2.19 <sup>d</sup>  | 30.14 $\pm$ 2.34 <sup>cd</sup>    | 11.35 $\pm$ 0.73 <sup>abd</sup> | 26.01 $\pm$ 1.60 <sup>bc</sup> |

Data shown as means  $\pm$  SE. CR, calorie restricted; NEFA, nonesterified fatty acid; ALT, aminotransferase. Significance  $P < 0.05$  by ANOVA. <sup>a</sup>Significantly different from chow; <sup>b</sup>significantly different from HF; <sup>c</sup>significantly different from KD; <sup>d</sup>significantly different from CR.

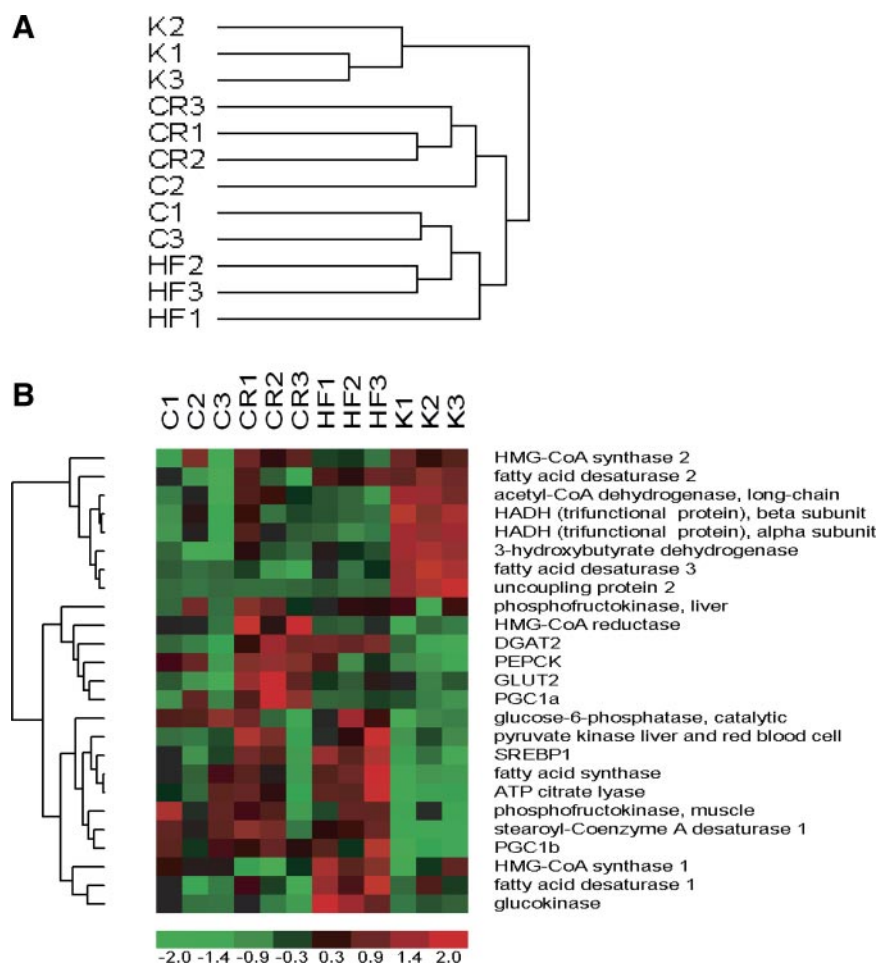


Fig. 3. **A**: hierarchical cluster tree showing relationship between microarray data sets from livers of mice after 9 wk on chow, KD, HF, and CR. **B**: heat map showing selected gene expression data sets from microarray performed on mouse liver at 9 wk after initiation of diet. Expression values are normalized to the  $[-2,2]$  interval, as shown in the bar below the figure. Red, high expression; green, low expression. HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HADH,  $\beta$ -hydroxyacyl-CoA dehydrogenase; DGAT, diacylglycerol acyltransferase 2; PEPCK, phosphoenolpyruvate carboxykinase; GLUT2, glucose transporter 2; PGC-1 $\alpha$  and - $\beta$ , peroxisome proliferator-activated receptor coactivator-1 $\alpha$  and - $\beta$ ; SREBP-1c, sterol regulatory element-binding protein-1c.

whereas SCD-1 expression was reduced 98% in animals fed KD (Fig. 4).

Analysis of the microarray data revealed unique expression of other genes involved in energy balance. For example, expression of UCP2 expression was the same in animals fed C, HF, and CR, whereas it was sixfold increased in animals fed KD.

Hypothalamic gene expression showed increased expression of orexigenic peptides and decreased expression of POMC in CR mice compared with controls. KD mice exhibited decreased proopiomelanocortin (POMC), with a nonsignificant increase in agouti-related protein (AgRP) expression (Fig. 5).

#### Protocol 2: Effects of KD in Mice Made Obese on HF

As expected, C57BL/6 animals placed on a HF diet gained weight significantly in excess of C-fed animals (Fig. 6A). At the time, when the ketogenic diet was introduced, C-fed animals weighed  $31.3 \pm 0.9$  g ( $n = 8$ ), whereas HF fed animals ( $n = 16$ ) weighed  $38.0 \pm 1.9$  g ( $P < 0.01$ ). The cohort of HF-fed animals transitioned to the KD rapidly lost all excess weight compared with those maintained on HF, reaching final body weights on average 1.4 g lower than C-fed animals (C  $31.3 \pm 0.85$  g vs. KD  $29.9 \pm 0.58$  g); after the period of acute weight loss, the weight of KD animals was stable until the end of the study. Animals on HF continued to gain weight, reaching final body weights of  $43.3 \pm 1.29$  g.

In the 4 days immediately following transition to KD, caloric intake was lower than both HF and C groups; this was followed by a transient increase in KD consumption in the following few days (Fig. 6B). Neither change reached statistical significance. Once body weights stabilized, food intake of KD fed animals was similar to that of the C-fed group and the HF group. Analysis of energy expenditure in the HF and KD groups by CLAMS revealed an increase in energy expenditure in KD animals. Thus, total heat output was 15% higher, averaged over 24 h, in KD animals ( $0.538 \pm 0.01$  kcal/h vs. HF  $480 \pm 0.01$  kcal/h,  $P < 0.05$ ,  $n = 4$ ). Oxygen consumption was increased by 34% averaged over 24 h (KD  $4.370 \pm 0.062$  ml $\cdot$ kg $^{-1}\cdot$ h $^{-1}$  vs. HF  $3.248 \pm 0.052$  ml $\cdot$ kg $^{-1}\cdot$ h $^{-1}$ ,  $P < 0.01$ ,  $n = 4$ ; Fig. 6C). Weight and CLAMS results were replicated in two additional independent cohorts using the same paradigm. CLAMS analysis also revealed that spontaneous dark-phase locomotor activity in KD animals was  $\sim 30\%$  lower than in HF animals.

To assess overall well-being of the animals, locomotor performance in an open field test was also assessed. This revealed no significant difference in locomotor activity of KD animals in a novel environment compared with C-fed animals. Total distance travelled was the same, and the extinction of the activity over time was the same (C  $11,600 \pm 400$  cm vs. KD  $11,100 \pm 300$  cm,  $P =$  not significant,  $n = 8$ ). Somewhat reduced exploratory activity was seen in HF animals compared



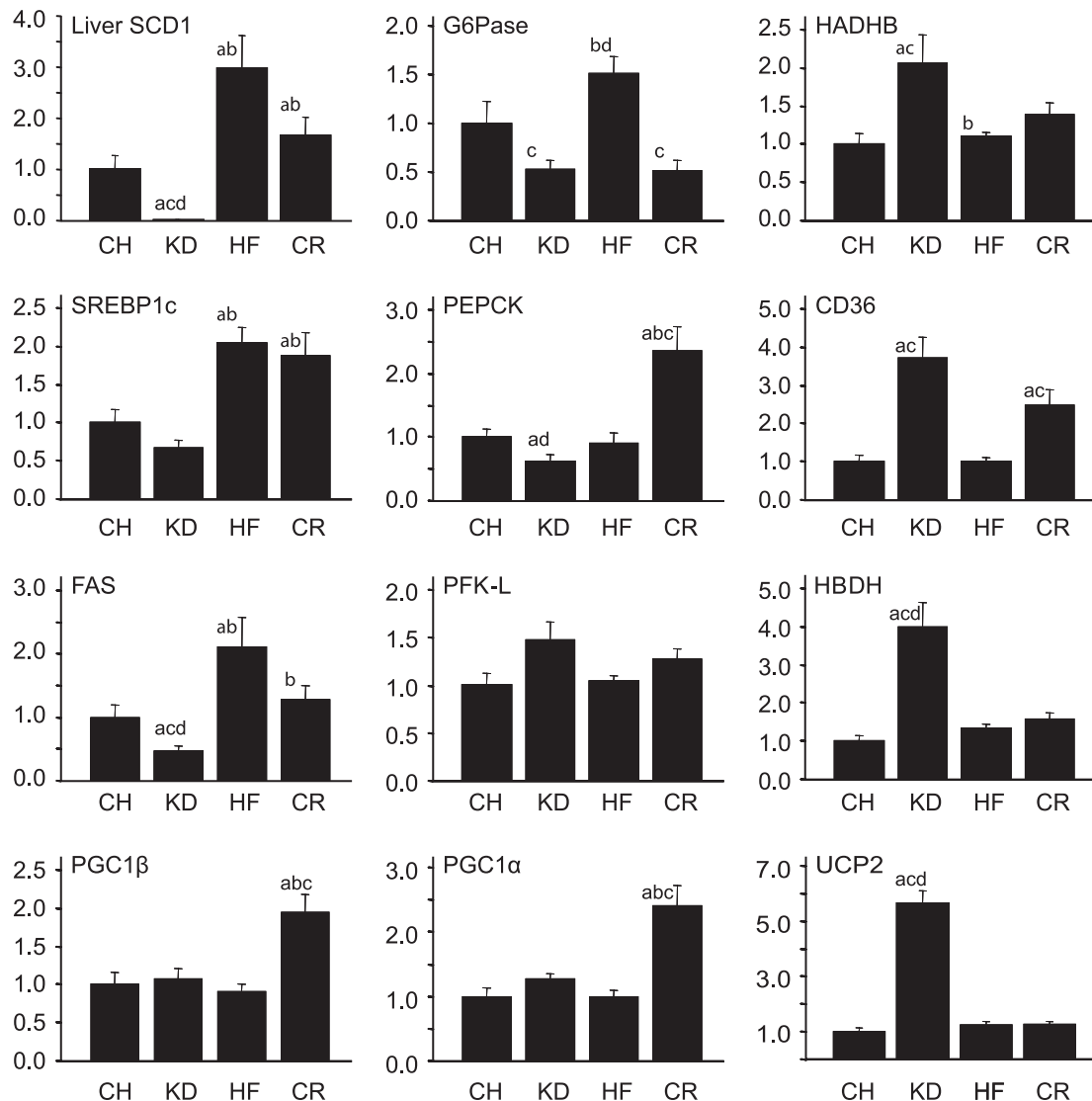


Fig. 4. Quantitative PCR (QPCR) expression data showing changes in gene expression in mouse liver after 9 wk of nutritional manipulation. SCD-1, stearoyl-CoA desaturase-1; FAS, fatty acid synthase; G6Pase, glucose-6-phosphatase; PFK-L, phosphofructokinase liver isoform; CD36, fatty acid translocase (FAT); butDH, butyrate dehydrogenase; UCP2, uncoupling protein-2. Graphs show means  $\pm$  SE. <sup>a</sup>Significantly different from chow; <sup>b</sup>significantly different from KD; <sup>c</sup>significantly different from HF; <sup>d</sup>significantly different from CR. Significance =  $P < 0.05$  by ANOVA.

with C-fed animals ( $10,100 \pm 500$  cm,  $P < 0.05$ ,  $n = 8$ ; not shown).

Analysis of body composition using DEXA analysis showed the expected increase in fat mass in animals fed HF; this was significantly decreased in the animals that had been transitioned to KD and was not statistically significant from the fat mass of animals fed C (Table 5). Whole body DEXA analysis also showed a decrease in total lean mass in KD-fed animals compared with both HF and C groups. To assess the possibility that the decrease in lean mass was secondary to muscle loss, DEXA analysis of the hind-limbs was performed. This revealed an increase in both fat and lean mass in the limbs of animals fed HF compared with both C-fed and KD-fed animals. There was no difference in either lean mass or fat mass between C-fed and KD animals (Table 5). Analysis of plasma lipids revealed improvement of both plasma triglyceride and plasma NEFA levels compared with HF as well as improved plasma cholesterol compared with HF (Table 5).

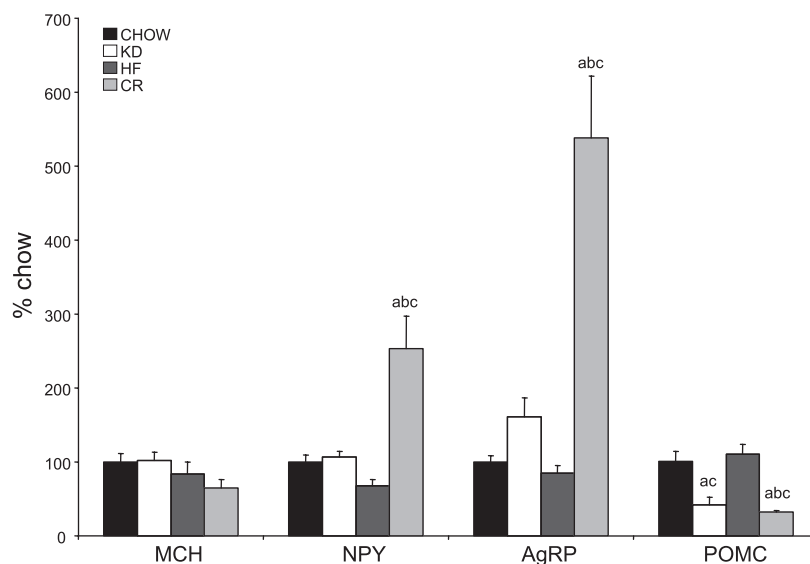
Histological analysis of the livers showed increased lipid vesicles in both HF and KD animals, and PAS staining showed decreased glycogen deposition in KD animals vs. both HF- and C-fed groups (data not shown).

In KD animals leptin levels were also lower than those seen in C- and HF-fed animals. HF animals had fivefold higher levels than C-fed animals, whereas KD animals had levels 50% lower than C-fed animals despite similar fat mass (Table 5).

As expected, insulin levels were high in animals fed HF. In animals transitioned to KD, hyperinsulinemia-resolved and fed insulin levels were dramatically lower than animals fed either chow or HF ( $>95\%$  decreased; Table 5). Fasting glucose in KD animals was significantly lower than both C and HF (C  $155.0 \pm 13.45$  mg/dl vs. HF  $133.25 \pm 13.45$  mg/dl vs. KD  $63.63 \pm 3.22$  mg/dl,  $P < 0.01$ ,  $n = 8$ ; Fig. 6D). Glucose tolerance testing revealed a significant increase in glucose tolerance in the KD group compared with HF-fed animals, with the plasma glucose area under the curve for KD being signif-



Fig. 5. Hypothalamic gene expression in fed mice on chow, KD, HF, and CR. Graphs show means  $\pm$  SE. <sup>a</sup>Significantly different from chow; <sup>b</sup>significantly different from KD; <sup>c</sup>significantly different from HF; <sup>d</sup>significantly different from CR. Significance =  $P < 0.05$  by ANOVA. MCH, melanin-concentrating hormone; NPY, neuropeptide Y; AgRP, agouti-related protein; POMC, proopiomelanocortin.



icantly lower (HF  $31,640 \pm 3,210$  vs. KD  $25,570 \pm 1,570$ ,  $P < 0.05$ ,  $n = 8$ ; Fig. 6D).

Paradoxically, the increase in glucose tolerance and correction of lipid profiles occurred in the context of increased hepatic triglyceride content compared with both HF and C

groups (C  $8.0 \pm 1.7$  mg/g vs. HF  $15.9 \pm 2.8$  mg/g vs. KD  $60.7 \pm 17.3$  mg/g,  $P < 0.01$ ,  $n = 8$ ).

Activity of the nutrient sensor, AMPK, was compared in liver and muscle from animals fed C, HF, and KD. Activity of the  $\alpha 2$ -subunit of AMPK in the liver of KD animals

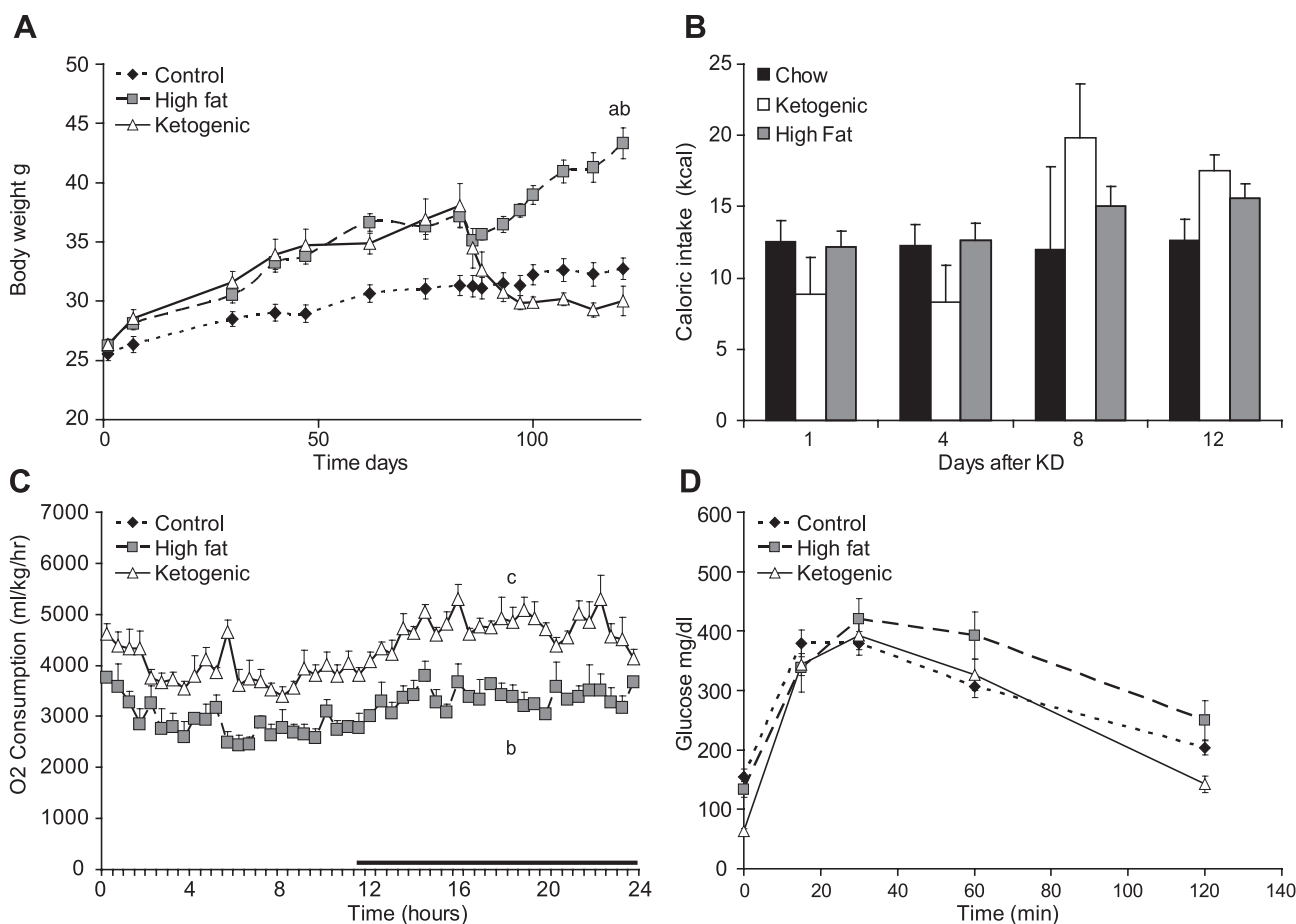


Fig. 6. Effect of KD on dietary-induced obesity. Animals were switched to KD on day 84. Body weight (A), 24-h caloric intake (B), O<sub>2</sub> consumption taken at 4 wk after KD initiation (C), and glucose tolerance test performed at 3 wk after KD initiation (D). Graphs show means  $\pm$  SE. <sup>a</sup>significantly different from chow; <sup>b</sup>significantly different from KD; <sup>c</sup>significantly different from HF. Significance =  $P < 0.05$  by ANOVA.

Table 5. Serum nutrient and hormonal profiles at 5 wk after initiation of KD, including DEXA analysis taken at 4 wk after KD

|                            | Chow                    | KD                       | HF                       |
|----------------------------|-------------------------|--------------------------|--------------------------|
| Triglycerides, mg/dl       | 118.3±2.2 <sup>c</sup>  | 96.2±1.6 <sup>a</sup>    | 106±3.6 <sup>ab</sup>    |
| Cholesterol, mg/dl         | 88.4±1.8 <sup>c</sup>   | 90.4±3.2 <sup>c</sup>    | 132.8±9.3 <sup>ab</sup>  |
| NEFA, mEq/dl               | 0.59±0.03 <sup>c</sup>  | 0.57±0.04 <sup>c</sup>   | 0.71±0.04 <sup>ab</sup>  |
| β-Hydroxybutyrate, mmol/dl | 0.39±0.05 <sup>b</sup>  | 1.59±0.3 <sup>ac</sup>   | 0.27±0.03 <sup>b</sup>   |
| Glucagon, ng/ml            | 22.13±2.84              | 28.8±9.9                 | 79.8±30.1                |
| Insulin, ng/ml             | 2.73±0.68 <sup>b</sup>  | 0.26±0.17 <sup>ac</sup>  | 3.25±0.40 <sup>b</sup>   |
| Leptin, ng/ml              | 5.2±1.34 <sup>c</sup>   | 2.3±0.27 <sup>c</sup>    | 24.2±4.15 <sup>ab</sup>  |
| TSH, ng/ml                 | 0.60±0.02 <sup>b</sup>  | 0.79±0.1 <sup>a</sup>    | 0.80±0.07 <sup>a</sup>   |
| DEXA (whole animal)        |                         |                          |                          |
| Fat mass                   | 4.54±0.34 <sup>c</sup>  | 5.16±0.50 <sup>c</sup>   | 13.27±5.16 <sup>ab</sup> |
| Lean mass                  | 28.98±0.73 <sup>b</sup> | 23.85±0.88 <sup>ac</sup> | 30.59±0.54 <sup>b</sup>  |
| DEXA (isolated limb)       |                         |                          |                          |
| Fat mass                   | 0.175±0.02 <sup>c</sup> | 0.187±0.01 <sup>c</sup>  | 0.275±0.03 <sup>ab</sup> |
| Lean mass                  | 0.96±0.04               | 0.912±0.04               | 1.04±0.05                |

Data shown as means ± SE. DEXA, dual-energy X-ray absorptiometry. Significance  $P < 0.05$  by ANOVA. <sup>a</sup>Significantly different from chow; <sup>b</sup>significantly different from KD; <sup>c</sup>significantly different from HF.

was twofold higher compared with both C and HF animals (C  $22.3 \pm 2.2$  nmol·g<sup>-1</sup>·min<sup>-1</sup> vs. HF  $30.8 \pm 3.5$  nmol·g<sup>-1</sup>·min<sup>-1</sup> vs. KD  $49.9 \pm 4.9$  nmol·g<sup>-1</sup>·min<sup>-1</sup>,  $P < 0.01$ ,  $n = 8$ ; Fig. 7A). AMPK phosphorylation paralleled the changes in AMPK activity. The changes in AMPK activity and phosphorylation in the liver of animals fed the KD were largely due to changes in the level of AMPKα2 subunit protein. This was associated with a 40% decrease in ACC activity in the livers of KD animals, whereas in contrast ACC activity was slightly increased in HF liver (C  $98.9 \pm 4.5$  pmol·g<sup>-1</sup>·min<sup>-1</sup> vs. HF  $122.8 \pm 10.9$  pmol·g<sup>-1</sup>·min<sup>-1</sup> vs. KD  $73.1 \pm 5.3$

pmol·g<sup>-1</sup>·min<sup>-1</sup>,  $P < 0.01$ ,  $n = 8$ ; Fig. 7B). Total ACC protein was increased fourfold in HF liver and was decreased >50% in KD. Changes in the phosphorylation of ACC mirrored changes in total protein. An even more dramatic increase was seen in soleus muscle, where, in KD animals, AMPK activity was more than threefold increased above C-fed animals and more than twofold above HF animals (C  $3.48 \pm 0.41$  nmol·g<sup>-1</sup>·min<sup>-1</sup> vs. HF  $6.11 \pm 0.54$  nmol·g<sup>-1</sup>·min<sup>-1</sup> vs. KD  $13.37 \pm 1.71$  nmol·g<sup>-1</sup>·min<sup>-1</sup>,  $P < 0.001$ , KD vs. HF and C; Fig. 7C). This change appeared to be due to increased AMPKα2 phosphorylation, as levels of total AMPKα2 subunit were unchanged. Consistent with increased AMPK activity, ACC activity in muscle was decreased by 60% (Fig. 7D). This could be explained by an increase in the phosphorylated and therefore inactive form of ACC.

Hepatic gene expression showed similar changes to those seen in *protocol 1*. However, a significant reduction in SREBP-1c and peroxisome proliferator-activated receptor-γ coactivator-1β was seen in KD-fed animals in this instance, whereas reductions in phosphofructokinase liver isoform and phosphoenolpyruvate carboxykinase (PEPCK) were lost in this cohort (Fig. 8).

Expression of hypothalamic neuropeptides known to regulate feeding and energy expenditure revealed that expression was consistent with the low levels of circulating leptin seen in KD animals. Compared with C-fed animals, expression of orexigenic neuropeptides was increased significantly; AgRP increased fourfold, neuropeptide Y (NPY) increased twofold, and melanin-concentrating hormone increased 1.5-fold. In contrast, there was a significant reduction in the expression of POMC (50%) in KD animals compared with HF and C groups (Fig. 9A).

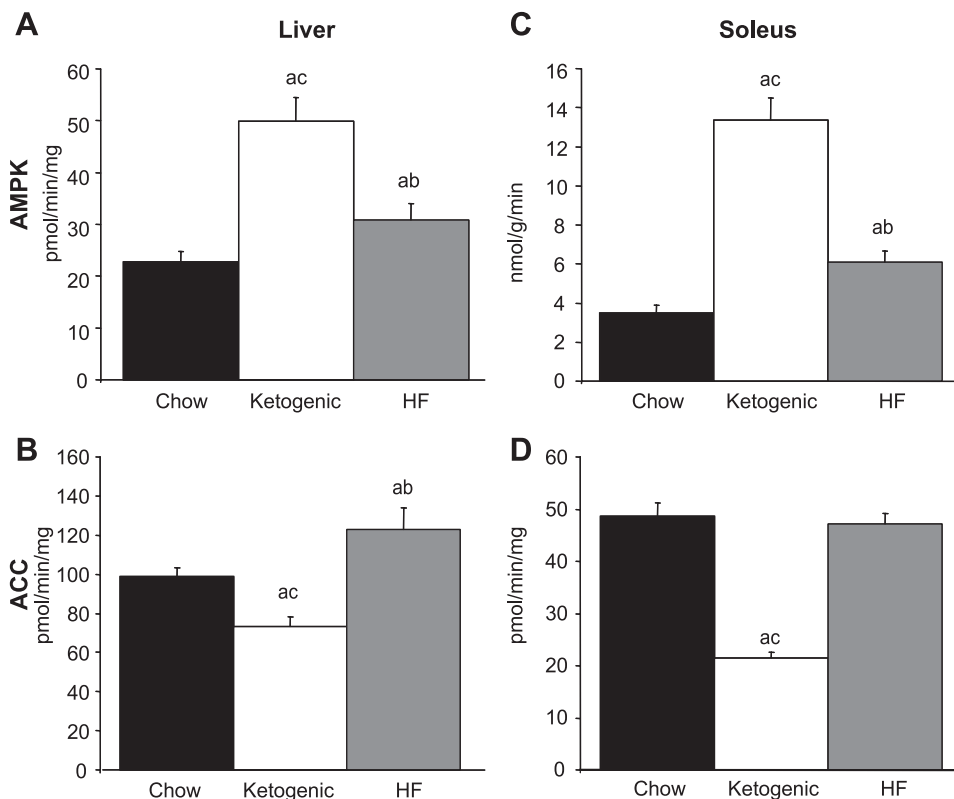


Fig. 7. Activity of AMP kinase (AMPK) and acetyl-CoA (ACC) in liver (A and B, respectively) and in soleus (C and D, respectively) 5 wk after initiation of KD. Graphs show means ± SE. <sup>a</sup>Significantly different from chow; <sup>b</sup>significantly different from KD; <sup>c</sup>significantly different from HF. Significance =  $P < 0.05$  by ANOVA.

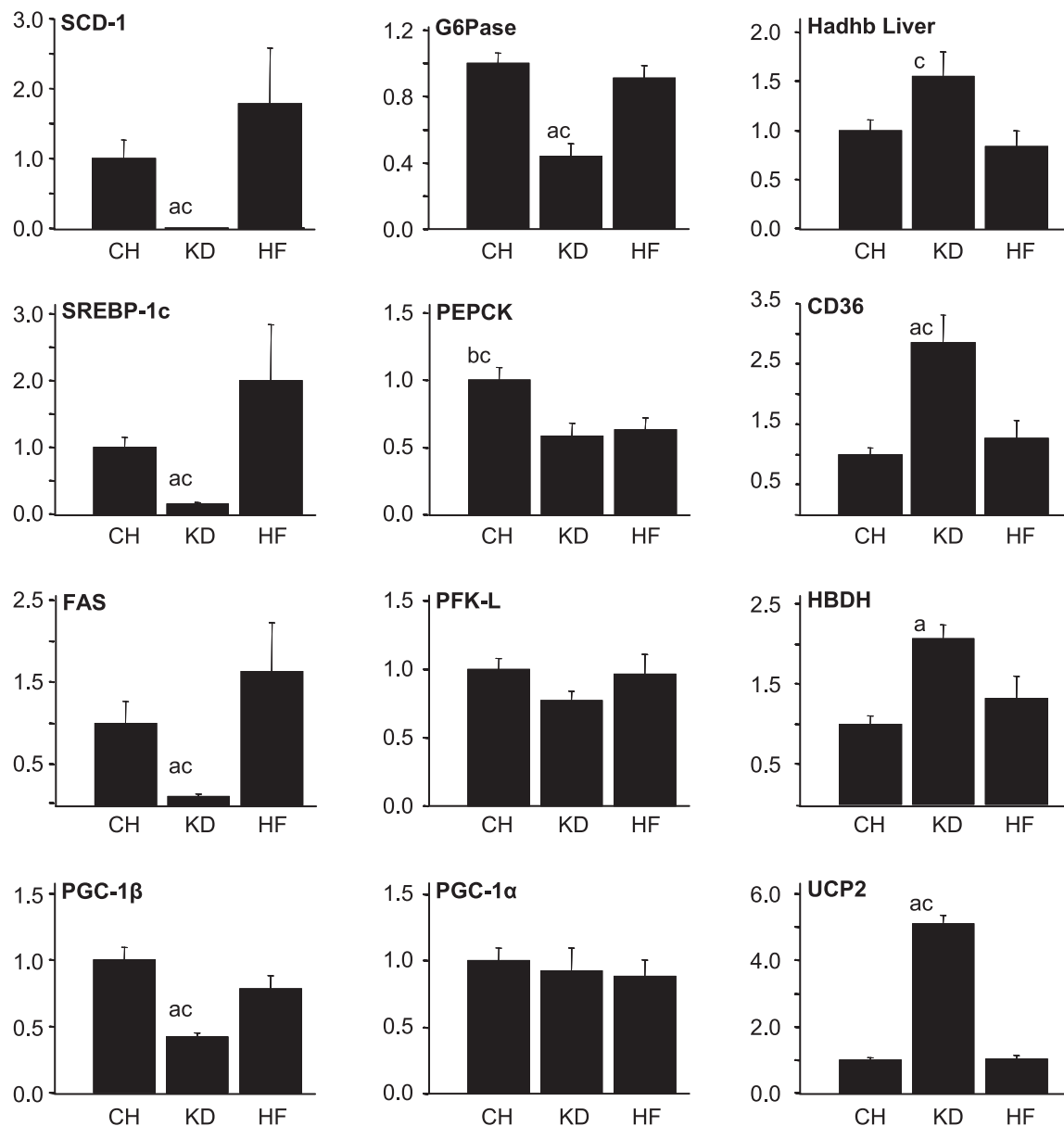


Fig. 8. QPCR expression analysis in mouse liver 5 wk after initiation of KD. Genes are matched to those in Fig. 4. Graphs show means  $\pm$  SE. \*Significantly different from chow (CH); <sup>b</sup>significantly different from KD; <sup>c</sup>significantly different from HF. Significance =  $P < 0.05$  by ANOVA.

Weight of BAT was not significantly different in KD animals compared with C-fed animals (KD  $0.11 \pm 0.015$  g, C  $0.15 \pm 0.021$  g) and was increased in HF animals (HF  $0.33 \pm 0.04$  g). Total protein content of BAT was unchanged between C and KD, whereas HF was increased by  $\sim 27\%$  (C  $4.42 \pm 0.54$  mg, KD  $3.68 \pm 0.54$  mg, HF  $5.61 \pm 0.84$  mg). Gels for Western blot were loaded on the basis of total protein. In the BAT of KD animals, levels of UCP1 were increased almost fivefold compared with both HF and C groups. Thus the dramatic increase of UCP1 KD animals reflects a real increase relative to total protein rather than an increase in BAT depot size (Fig. 9B).

## DISCUSSION

The potential effects of dietary macronutrient composition on both weight gain and weight loss remain the topic of much

debate. A number of studies (10, 11, 51) have shown that dieting subjects eating a high-fat, low-carbohydrate diet tend to lose more weight more rapidly than subjects eating a low-fat, high-carbohydrate diet, leading to the speculation that high-fat diets might enhance weight loss by attenuating the decrease in energy expenditure typically seen with dieting. However, such an effect has not been demonstrated in either humans or rodents. In this study we examined the effects that diet composition can have on metabolism and found that diets high in fat and low in carbohydrate do in fact lead to weight loss by increasing energy expenditure.

Animals fed ketogenic diet ate the same number of calories as animals that were fed either chow or a high-fat diet but nevertheless failed to gain weight. Remarkably, animals eating ketogenic diet lost a small amount of weight and achieved the same weight and body composition as animals that were

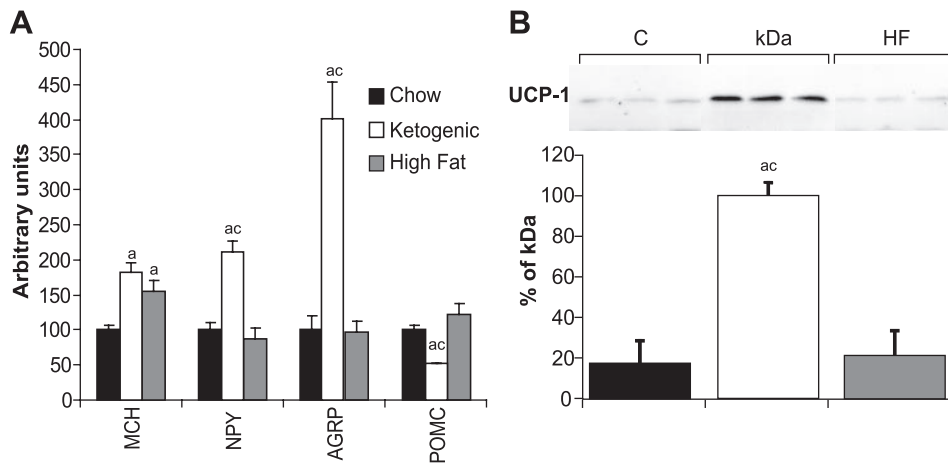


Fig. 9. *A*: expression of hypothalamic neuropeptides in fed animals. *B*: expression of UCP1 protein in brown adipose tissue 5 wk after initiation of KD. Graphs show means  $\pm$  SE. <sup>a</sup>Significantly different from chow (C); <sup>ac</sup>significantly different from HF. Significance =  $P < 0.05$  by ANOVA.

calorie restricted to 66% of usual daily intake. Fat mass, lean body mass, levels of leptin, and glucose were the same in ketogenic diet-fed and calorie-restricted animals. The somewhat surprising preservation of fat mass in calorie-restricted animals has been described previously (19). Thus, although severe caloric restriction is known to cause fat mass loss in rodents, metabolic adaptations prevent fat mass loss during moderate CR in mice and even permit a small weight gain as described for 20% calorie restriction. Insulin levels were somewhat reduced in calorie-restricted animals compared with the chow-fed group, whereas insulin levels in ketogenic diet-fed animals were dramatically lower to a level that was only 10% of that seen in the calorie-restricted group. The difference in feed efficiency between chow and conventional high-fat diets has been previously reported by us (28) and by others (2); however, this is the first time that the remarkable effect of ketogenic diet on energy expenditure has been documented.

Despite the limited, 9.5% protein content of the diet, animals tolerated the ketogenic diet well. In contrast to calorie-restricted animals, in which testosterone levels dropped to 30% of those of ad libitum chow-fed animals, ketogenic diet-fed animals maintained normal testosterone levels and females continued to cycle normally. In addition, they showed normal exploratory activity, which is an index of overall mouse well-being. Hindlimb analysis revealed no evidence of muscle wasting. This finding is consistent with previous reports (12) examining the effects of protein restriction in rodents. In this study, rats fed a diet with 10% protein by weight had only slightly lower body weight than rats fed 20% protein (234 vs. 246 g) and no change in body composition. Furthermore, although many rodent diets consist of 20% protein by weight, the recommended level of dietary protein for adult rodents is only 13% (41). For purposes of comparison, humans consuming ketogenic diets for seizure suppression consume diets formulated to provide 80% of calories from fat, with additional calories coming from protein based on a minimal requirement of 1 g/kg body wt (29). Protein content by weight ranges between 14 and 16% of the total diet while additional calories derive from carbohydrate, which usually contributes less than 5% by weight to the diet.

Hypothalamic neuropeptides are known to regulate energy expenditure; increased levels of the orexigenic peptides NPY and AgRP are associated with decreased energy expenditure,

whereas increased POMC is associated with increased energy expenditure (22, 23, 44). We speculated that consumption of ketogenic diet might drive decreased expression of NPY and AgRP and increased POMC expression and thus explain aspects of the phenotype of animals fed this diet. However, we found that the pattern of expression of these neuropeptides in ketogenic diet animals was not remarkably different from that seen in calorie-restricted animals. Compared with chow-fed mice, the MSH precursor POMC was similarly decreased in both ketogenic diet and calorie-restricted animals, consistent with reduced body weight. AgRP was increased in both groups of animals, although the increase in AgRP was significantly higher in CR animals. NPY expression was substantially increased in calorie-restricted animals but unchanged in ketogenic diet animals compared with chow. Thus, the pattern of expression of hypothalamic peptides in ketogenic diet did not explain the increase in metabolic rate.

Another organ worthy of investigation is the liver. The liver is known to play a key role in substrate availability and alters its metabolic profile in response to both nutrients and hormonal factors. In the immediate postprandial state it stores excess nutrients as glycogen and exports fat as lipoproteins. During fasting it synthesizes glucose and metabolizes fatty acids into ketones, providing the brain with a utilizable energy source. Hepatic metabolism is therefore likely to play an important part in the adaptation and metabolic responses to a high-fat ketogenic diet. These adaptations may be involved in mediating the systemic effects of such a diet. We used Affymetrix analysis to evaluate hepatic gene expression in all four diets. Compared with chow, all three diets (ketogenic diet, calorie restricted, and high fat high carbohydrate) led to distinct changes in the pattern of hepatic gene expression. Hierarchical clustering indicated that overall differences in gene expression in ketogenic diet animals compared with chow-fed animals were significantly greater than differences between HF and chow-fed animals and even calorie-restricted and chow-fed animals.

These differences were highlighted by analysis of distinct metabolic pathways. In ketogenic diet animals, fatty acid oxidation and ketogenic pathways were increased, as may be expected. However, UCP2 was also increased sixfold compared with the other diets. This may have been a response to increased availability of fatty acids and increased mitochondrial fatty acid oxidation. The uncoupling and associated



changes in metabolic efficiency may underlie some of the changes in energy expenditure seen in these animals (17). Furthermore, fatty acid synthesis, cholesterol synthesis, and glucose-handling pathways were reduced in ketogenic diet-fed animals. Additionally, critical enzymes of gluconeogenesis, PEPCK, and G-6-Pase were suppressed, consistent with the

reliance of these animals on ketones and fatty acids as energy substrates.

Since feeding ketogenic diet is associated with weight loss and increased energy expenditure, we also examined the possibility that feeding ketogenic diet to animals with diet-induced obesity (from feeding of high-fat chow) would lead to weight

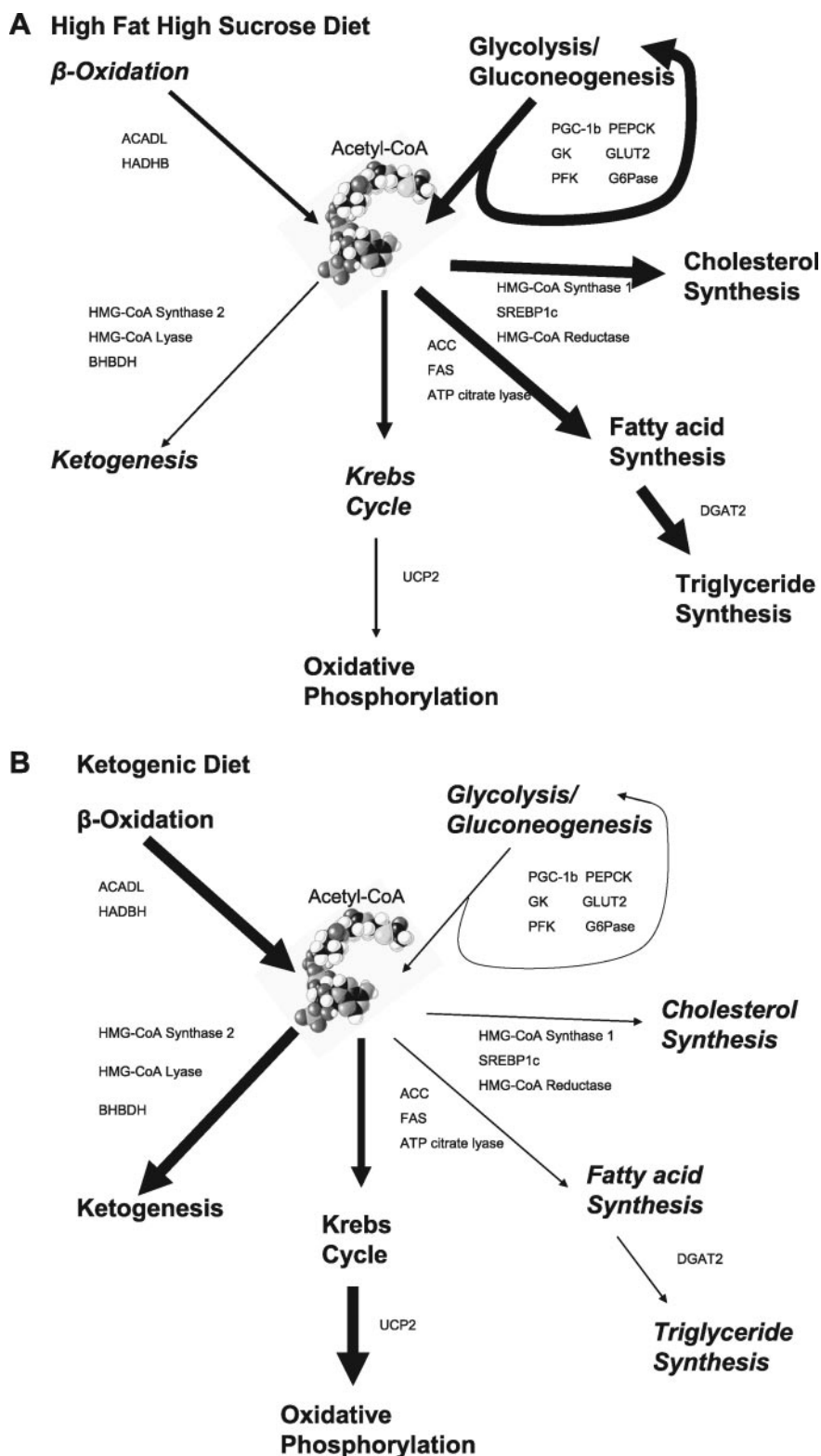


Fig. 10. Diagram showing pathways associated with fate of acetyl-CoA in both HF, where expression of lipogenic and cholesterol synthesis pathways are increased (A), and KD, where expression of ketogenic and uncoupling pathways are increased (B). Arrow thickness correlates to pathway enzyme expression and supposed flow of acetyl-CoA. ACADL, long-chain acyl-CoA dehydrogenase; HADHB, hydroxyacyl dehydrogenase-β; BHBDH, β-hydroxybutyrate dehydrogenase.

loss. Transition to ketogenic diet was associated with rapid loss of excess body weight within 14 days of starting ketogenic diet. Whereas ketogenic diet animals maintained a lower weight through the end of the study, high-fat-fed animals continued to gain weight. Weight loss was secondary to a large reduction in fat mass that returned to the level of chow-fed animals. Compared with obese high-fat-fed animals, leptin levels were markedly decreased in ketogenic diet animals and were somewhat lower than chow-fed animals. Insulin levels were also dramatically reduced and accompanied by improved glucose tolerance in the ketogenic diet-fed group. Evaluation of hypothalamic neuropeptides revealed a pattern that is expected of fasted animals and similar to that seen in animals fed ketogenic diet from the onset. POMC mRNA expression was decreased. AgRP was substantially increased, and an increase in NPY was also seen (4, 36). The more robust increases in orexigenic gene expression seen in this group of animals may be explained by the fact that the animals were obese to start with and are compensating for lost body weight and the associated rapid drop in circulating leptin (4).

Analysis of hepatic gene expression revealed changes that were similar to those seen in animals fed ketogenic diet from the onset. Overall, the findings in diet-induced obesity animals transitioned to ketogenic diet indicate that established diet-induced obesity does not preclude the ability of ketogenic diet to alter the metabolic state of the animal and cause weight loss. This state involves increased energy expenditure in the context of reduced weight and a hormonal and hypothalamic profile more consistent with reduced calorie availability (rather than ad libitum feeding), increased fatty oxidation in the liver, decreased fatty acid synthesis, and decreased gluconeogenesis and increased expression of UCP2.

To identify peripheral potential upstream regulators that would explain these findings, we also evaluated the activity of the nutrient sensor AMPK. AMPK responds to ATP depletion and is activated by low glucose (27); AMPK may also be inhibited by insulin in some cases (50). Activation of AMPK leads to decreased fatty acid synthesis and increased fatty acid oxidation (20, 27). In the liver, ketogenic diet feeding is associated with a twofold increase in AMPK in liver and more than threefold increased in soleus muscle. Concomitantly, activity of ACC declines by 25% in liver and 56% in soleus. ACC plays a pivotal role in lipogenesis, as it is required for the carboxylation of acetyl-CoA to produce malonyl-CoA; thus, the enzyme serves as a metabolic switch, and decreased activity is associated with a decrease in fatty acid synthesis and increased fatty acid oxidation.

The activation of AMPK, which may be facilitated by both low glucose and extremely low levels of insulin and the concomitant decreases in ACC, FAS, and SCD-1 as well as the activation of pathways involved in hepatic lipid oxidation, is consistent with the decrease in body weight and increase in energy expenditure seen with ketogenic diet feeding. This profile is similar to that seen in other mice, where genetic or pharmacological interventions targeting lipogenesis result in a lean phenotype. For example, SCD-1 deletion leads to a lean phenotype, even in the presence of a high-fat diet (35). This appears to be a result of impaired triglyceride synthesis leading to increased fatty acid oxidation. Similarly, inhibition of the enzyme fatty acid synthase reverses diet-induced obesity and is also associated with an increase in fatty acid oxidation (46).

Acetyl-CoA can be utilized in a number of ways, depending on the metabolic state of the animal, as can be seen in Fig. 10. Acetyl-CoA may enter cholesterol synthesis, fatty acid synthesis, oxidative phosphorylation, or ketogenesis. In the high-fat-fed animals, pathways associated with fatty acid and cholesterol synthesis are increased, suggesting an increased flow of excess acetyl-CoA into storage, as can be seen in Fig. 10A. In the case of animals fed ketogenic diet (Fig. 10B), lipid and cholesterol synthesis pathways are reduced, and instead ketogenic pathways are increased, leading to enhanced flow of excess acetyl-CoA into ketone production. The increase in UCP2 also suggests increased uncoupling and associated increased flow through oxidative phosphorylation. Increased uncoupling in the liver via overexpression of UCP1 has been shown to increase metabolic rate in mice and may, therefore, contribute to increased O<sub>2</sub> consumption in ketogenic diet (15).

A previous study, based on a 72-h feeding protocol, showed that a high-saturated-fat diet resulted in increased expression of fatty acid synthesis and lipogenesis pathways in the liver (32). These results are very different from those seen in our study; however, the composition of the diet used in the study was much more closely related to our HF diet, containing large amounts of simple sugars that were not present in the control diets. Hence, the changes seen in the study may be related to the change in carbohydrate type, and as such, the gene changes seen match our high-fat, high-carbohydrate group very closely. In fact, the changes seen in the study are largely associated with insulin responsive genes (e.g., SREBP-1c), suggesting that a rise in insulin response to the diet is responsible more than the diet saturated fat content.

In conclusion, feeding of a ketogenic diet with a high content of fat and very low carbohydrate leads to distinct changes in metabolism and gene expression that appear consistent with the increased metabolism and lean phenotype seen. Through a specific dietary manipulation, weight loss can occur secondary to distinct metabolic changes and without caloric restriction. Further investigation into the mechanisms of this model may lead to new targets for drug therapy as well as new insights into the role of diet composition in energy balance.

## GRANTS

This study was supported by National Institute of Diabetes and Kidney Diseases Grant DK-069983-01 to E. Maratos-Flier and by a pilot and feasibility award from a pilot and feasibility grant (BONRC-DK-46200) to A. R. Kennedy. Metabolic analysis of mice was done by the animal physiology core funded by PPG-DK-56116-07. Affymetrix analysis was supported by an unrestricted sponsored research grant from Takeda pharmaceuticals.

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