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Article

Decreased Hepatic Triglyceride Content in Obese Male Mice Fed a Ketogenic Diet is Due to a Reduction in Lipid Anabolism Associated with Lower Insulin Signaling

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Abstract

The efficacy of ketogenic diets (KD) in inducing weight loss is well documented, but the changes in peripheral and hepatic insulin sensitivity and their associated effects on fatty acid anabolism remain to be elucidated. The present study examined the impact of a chow diet (CD) versus a one-month KD on peripheral and hepatic insulin sensitivity in male mice with obesity due to two months on a high-fat diet. The subsequent effect of instauration of one-month CD in both subgroups was also investigated. KD mice presented higher weight, serum leptin and high-molecular weight adiponectin levels along with reduced HOMA-IR index. Phosphorylation of STAT3 was increased, while the phosphorylation of insulin receptor, Akt and cAMP response element-binding protein, the activation of ATP citrate lyase and levels of fatty acid synthase, were reduced in KD mice. After instauration of CD in both subgroups, those mice previously fed KD exhibited lower HOMA-IR index and serum and hepatic triglycerides. These findings suggest that KD intervention improves peripheral insulin sensitivity through the reduction in hepatic insulin signaling that inhibits fatty acid anabolism, thereby reducing hepatic triglyceride content.

Keywords: fatty acid anabolism; high-fat diet; insulin signaling; ketogenic diet; liver; leptin; triglyceride

1. Introduction

Obesity, resulting from an imbalance in energy homeostasis often associated with increased high fat intake, is frequently associated with resistance to the actions of insulin [1] and is one of the factors that most contribute to the appearance of metabolic syndrome [2]. The obesity epidemic has become a major public health problem in recent decades and the pathophysiology of obesity, which underlies its progressive and self-sustaining progression, hampers the development of effective and safe therapies for weight loss. Although understanding the molecular mechanisms that regulate energy intake and homeostasis has allowed the development of effective drugs for weight reduction [3], the use of different nutritional interventions remain the most common therapy for weight loss and obesity-associated comorbidities [4].

Hyperglycemia and hyperlipidemia are risk factors in patients with obesity, as well as a low-grade systemic inflammatory profile [5]. Among the dietary strategies used to control some of these obesity-associated comorbidities is the use of ketogenic diets (KD), mainly composed of fat and protein, with small amounts of carbohydrate. KD decrease serum insulin concentrations, independent of weight loss [6]. This decline in circulating insulin stimulates hepatic lipolysis resulting in the generation of ketone bodies from β -oxidation of fatty acids that are secreted as additional fuel [7]. This diet also affects lipid anabolism, downregulating lipogenic enzyme activity in the liver [8]. However, data on systemic and hepatic inflammation in response to KD are contradictory [9,10] and the effects of transition from a KD to a normal diet have been scarcely studied [11].

Leptin, which is mainly produced in white adipose tissue, is a pro-inflammatory cytokine and can affect insulin actions. In fact, in the liver leptin has been shown to interfere with insulin signaling, affecting its important actions in this tissue [12]. Hyperleptinemia, which is frequent in obesity, can inhibit Akt activation through increased signal transducer and activator of transcription 3 (STAT3) and suppressor of cytokine signaling 3 (SOCS3) levels [13]. In addition, leptin favors the release of macrophage chemoattractant factors, increasing insulin resistance, augmenting proinflammatory cytokines, and promoting resistance to insulin action [14]. In contrast, deletion of liver-SOCS3 improves hepatic insulin sensitivity and lipogenesis [15]. In fact, insulin plays a crucial role in fatty acid synthesis, modulating activity and levels of key enzymes involved in this process, contributing not only to changes in the serum lipid profile but also in hepatic lipid accumulation, factors that are closely related to inflammation [16].

We hypothesized that intake of a KD can reduce serum and hepatic lipid levels through an inhibition of lipid anabolism in the liver related to a decrease in insulin action, related to cytokine environment. Thus, our aims were to evaluate the effects of one-month of a KD compared to intake of a normal diet in HFD-induced obese mice on peripheral and hepatic insulin sensitivity and its relationship with fatty acid anabolism and inflammatory environment, as well as the effect of an additional period of normal diet intake in both groups of mice on the same parameters.

2. Materials and Methods

2.1. Materials

All reagents were from Merck (Darmstadt, Germany) unless otherwise stated. Antibodies against vinculin were from Thermo Fisher Scientific (Waltham, MA, USA), and long-chain acyl-CoA synthetase (ACSL)1, phosphorylated (p) ATP citrate lyase (ACL) and fatty acid synthase (FASN) from Cell Signaling Technology (Danvers, MA, USA). The Immun-Star Western C kit was from Bio-Rad Laboratories (Hercules, CA, USA). The secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from Thermo Fisher Scientific.

2.2. Ethical Statement

This study was designed and performed according to the European Communities Council Directive (2010/63/UE) and the Spanish Royal Decree 53/2013 concerning the protection of experimental animals. It was approved by the Ethical Committee of Animal Experimentation of the Hospital Puerta de Hierro of Madrid and the Animal Welfare Organ of the Community of Madrid (PROEX 009.4/24, 12 February 2024). The number of animals used in this study was reduced to the minimum required. Mice were maintained at 22 ± 2 °C with free access to tap water in a stable 12-h light–dark cycle. The study design and reporting of the results adheres to the ARRIVE guidelines ([https:// arriveguidelines.org](https://arriveguidelines.org)).

2.3. Animals and Diets

Fifty-six six-week-old C57BL/6J male mice were purchased from Envigo RMS Spain (Sant Feliu de Codines, Barcelona, Spain). Upon their arrival, animals were weighed, and 2 mice were randomly placed in each cage. They had free access to standard rodent chow and tap water while being allowed to acclimate to their new environment for one week.

For the study, one group of 21 mice was fed a standard rodent chow diet (CD, 6% Kcal from fat, 17% Kcal from proteins, 77% from carbohydrates, 3.41 kcal/g, Panlab, Barcelona, Spain) during the 2, 3 or 4 months and served as controls (n = 7 per group). Another group of 35 mice received a HFD (62% Kcal from fat, 18% Kcal from proteins, 20% Kcal from carbohydrates, 5.1 kcal/g, LabDiet, Sodispan Research SL, Madrid, Spain) for 2 months. After this period, 7 animals were euthanized (HH) and the rest of animals were subdivided into 2 groups; one received the CD and the other a KD (84% Kcal from fat, 11% Kcal from proteins, 5% Kcal from carbohydrates, 6.4 kcal/g, Altromin, Lage, Germany; n=14 per group). After one month, 7 animals of both groups were euthanized (HHC and HHK, respectively) and the rest of mice were fed with CD, being euthanized one month later (HHCC and HHKC, respectively). Thus, this resulted in eight experimental groups (n = 7 per group): Chow and HH at 2 months; chow, HHC and HHK at 3 months; and chow, HHCC and HHKC at 4 months.

Body weight and food intake were monitored weekly in all groups until the termination of the study. Food intake was monitored by weighing the amount of food remaining of a previously determined quantity, with the pieces that fell into the cages being taken into account. Mean energy efficiency during the entire study was calculated as the weight gained in grams per the total number of calories consumed. Animals were euthanized by decapitation between 8.00 and 9:00 h after a fasting period of 12 h and the whole liver, without removal lipids, was dissected, weighed and prepared for biochemical determinations. Inguinal fat was also dissected and weighed. Peripheral blood was collected and centrifuged at $1800 \times g$ for 10 min at 4°C and serum was aliquoted and frozen at -80°C until processed.

2.4. Glycemia and Serum and Tissue Free Fatty Acid (FFA) and Triglyceride (TG) Levels

Freestyle Optimum Neo glucometer (Abbott, Whitney, UK) was employed for determining serum glucose levels.

Circulating concentrations of FFA and TG were determined by using colorimetric kits (AkrivisBio, Fremont, CA, USA), following the manufacturer's instructions. To determine the levels in liver, extraction of total lipids was performed following the method of Folch et al. [17], and then measured by using the same kits. The average coefficients of variation were lower than 10%.

2.5. ELISAs

2.5.1. Adiponectin, Insulin and Leptin

The levels of adiponectin in serum were determined by using an ELISA kit from Bio Vendor (Brno, Czech Republic) and insulin and leptin were measured with kits from Merck, following the manufacturer's recommendations. Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated according to the equation:

$$\text{HOMA-IR} = [\text{glycemia (mmol/L)} \times \text{insulin (mU/L)}] / 22.5$$

2.5.2. High Molecular Weight (HMW)-Adiponectin

Serum HMW-adiponectin concentrations were measured using an ELISA kit from Fujirebio (Tokyo, Japan) in diluted samples (from 1/50 to 1/500) with assay buffer and determined as indicated by the manufacturer.

2.5.3. Phosphorylation of Insulin Receptor

The solid phase sandwich ELISA was from Assay Solution (Woburn, MA, USA) and detects phosphorylated insulin receptor β . After incubation with lysates of liver, the ligand was bound by

the monoclonal antibody in the microplate. The absorbance is proportional to the quantity of insulin receptor phosphorylated. Intra- and inter-assay variation coefficients were lower than 10% in all assays.

2.6. Tissue Homogenization and Protein Quantification

For detection of ACSL1, phosphorylated (p) activating transcription factor 2 (pATF-2), pSer473-Akt, Akt, pATP-CL, pSer133-cAMP response element binding protein (CREB), CREB, FASN, interleukin (IL)-1 β , IL-6, p-insulin receptor (p-IR), pThr389-p70 S6 kinase (pThr389p70 S6K), p70 S6K, pSer2448-mammalian target of rapamycin (pSer2448mTOR), mTOR, pSer380-phosphatase and tensin homolog (pSer380PTEN), PTEN, pSer727STAT3, STAT3, tumor necrosis factor (TNF)- α , β -actin, and vinculin, 30 mg of liver of the same lobe in all animals were homogenized on ice in 350 μ L of lysis buffer (Merck). The lysates were frozen during 16 h at -80°C . Later, samples were centrifuged at $12,000 \times g$ for 5 min at 4°C and the supernatants kept at -80°C until assayed [18]. Protein levels were measured by the Bradford procedure (Bio-Rad Laboratories).

2.7. Western Blotting

Western blotting was performed as previously described [19]. Twenty μ g of protein was resolved on 10% sodium-dodecyl-sulphate-denaturing polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and then incubated with antibodies. Peroxidase activity was detected by using an ECL system (Bio-Rad Laboratories), and the chemiluminescent signal was calculated with ImageQuant Las 4000 Software (GE Healthcare Life Sciences, Barcelona, Spain). Gel-loading variabilities were normalized with vinculin.

2.8. Multiplexed Bead Immunoassays

Phosphorylated levels of ATF-2 and phosphorylated and total levels of Akt, CREB, PTEN, mTOR, p70 S6K and STAT3 in liver, and serum and tissue levels of IL-1 β , IL-6 and TNF- α , were measured by multiplexed bead immunoassays (Bio-Rad Laboratories and Merck), as stated [20]. Phosphorylated ATF-2 was normalized with β -actin. Concisely, antibodies coupled to magnetic beads and lysates were incubated overnight at room temperature or 4°C . Subsequently, after washing with a magnetic separation block (Merck), the corresponding antibodies coupled to biotin were added. After incubation for 30 min at room temperature, a streptavidin-phycoerythrin complex was added and incubated during the same-time period. At least 50 beads per analyte were examined in the Bio-Plex suspension array system 200 (Bio-Rad Laboratories). Raw data (median fluorescence intensity, MFI) were evaluated with the Bio-Plex Manager Software 6.2 (Bio-Rad Laboratories).

2.9. Statistical Analysis

Data are summarized as mean $\pm$ standard error of the mean (SEM). The analysis of all data was carried out using Student's t-test for unpaired samples or one-way ANOVAs followed by Bonferroni's post hoc tests. Linear regression analysis was used to determine the possible relationships between specific parameters. In all analyses $p < 0.05$ was considered significant. Analyses were performed using Statview software (Statview 5.01, SAS Institute, Cary, NC, USA),

3. Results

3.1. Weight and Caloric Intake Differ Between KD and CD

After two months of HFD there was a significant increase in weight gain and caloric intake (Figure 1A and 1D, respectively). After one month on a CD, obese mice had a decrease in body weight, although there was no difference in caloric intake compared to controls. KD caused an increase in weight with respect to the obese animals fed CD, as well as an increase in caloric intake with respect to the other two experimental groups (Figure 1B and 1E).

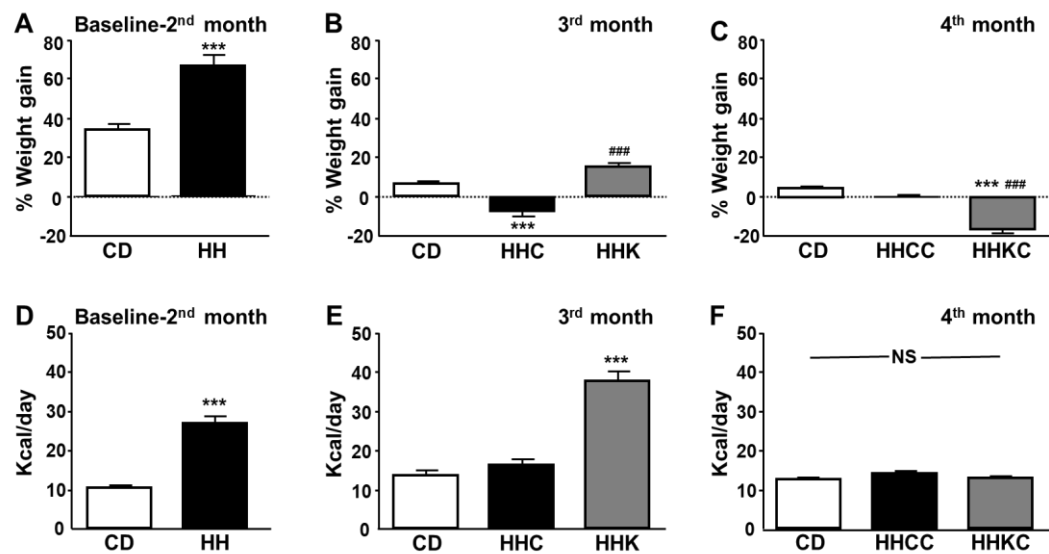


Figure 1. Percent weight gain and mean caloric intake per day from baseline to the end of month 2 (**A and D, respectively**), from the end of month 2 to the end of month 3 (**B and E, respectively**), and from the end of month 3 to the end of month 4 (**C and F, respectively**). Data are presented as mean $\pm$ SEM. CD, chow diet; HH, high-fat diet (HFD) for 2 months; HHC, HH followed by CD for 1 month; HHK, HH followed by ketogenic diet (KD) for 1 month; HHCC, HHC plus CD for 1 month; HHKC, HHK followed by CD for 1 month. NS, non-significant. *** $p < 0.001$ vs. CD; ### $p < 0.001$ vs. HHC or HHCC.

In the fourth month, when CD was administered to both groups of obese mice, we detected a decrease in weight in mice previously treated with KD, with no change in caloric intake in any of the three groups (Figure 1C and 1F).

3.2. Differences in Weight Gain, Caloric Intake and Energy Efficiency During the Entire Study Period

Total weight gain at the end of the study was increased in mice that received the HFD regardless of subsequently receiving a KD and/or CD or KD, with no differences between these latter two groups (Figure 2A). Caloric intake was augmented in both experimental groups, being higher in those supplemented with KD (Figure 2B), and energy efficiency was reduced with this reaching statistical significance in obese mice receiving a KD (Figure 2C).

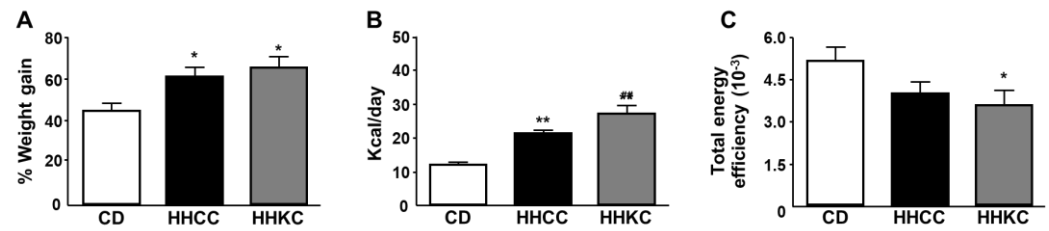


Figure 2. Percent weight gain, mean caloric intake per day and energy efficiency from baseline to the end of study (**A, B and C, respectively**). Data are presented as mean $\pm$ SEM. CD, chow diet during 4 months; HHCC, high fat diet (HFD) for 2 months followed by CD for 2 months; HHKC, HFD for 2 months, followed by a ketogenic diet (KD) for 1 month and then CD for 1 month. * $p < 0.05$, ** $p < 0.01$ vs. CD; ## $p < 0.01$ vs. HHCC.

3.3. General Characteristics of the Experimental Groups

3.3.1. KD or CD Administration in Obese Mice (3 Months)

We found that the percentage of liver weight was reduced in HHK with respect to CD mice. Percentage of inguinal fat was increased in HHK compared to the other experimental groups. Serum glucose and insulin levels and HOMA-IR were lower in HHK than HHC mice. Circulating leptin levels were increased in HHK with respect to CD and HHC mice. No changes in total adiponectin were detected among the experimental groups, however, high molecular weight (HMW)-adiponectin levels were increased in HHK with respect to the CD and HHC groups (Table 1).

Table 1. Weight of liver, inguinal fat, glucose and circulating levels of hormones and adipokines at 3 and 4 months.

Parameter (3 months)	CD	HHC	HHK
Liver weight (% BW)	4.50 ± 0.12	4.44 ± 0.24	3.91 ± 0.20 *
Inguinal fat (% BW)	1.15 ± 0.18	1.07 ± 0.19	2.94 ± 0.81 * #
Serum glucose (mg/dL)	130.3 ± 5.9	105.3 ± 5.2	89.6 ± 7.8 **
Serum insulin (ng/mL)	2.07 ± 0.26	2.91 ± 0.27	1.92 ± 0.21 #
HOMA-IR	16.03 ± 2.42	19.67 ± 2.63	10.93 ± 1.57 #
Serum leptin (ng/ml)	3.77 ± 0.36	2.84 ± 0.45	15.91 ± 4.18 * #
Serum ADP (µg/mL)	3.88 ± 0.17	2.92 ± 0.43	3.58 ± 0.19
Serum HMW-ADP (µg/mL)	1.87 ± 0.11	1.57 ± 0.32	2.94 ± 0.05 *** ###
Parameter (4 months)	CD	HHCC	HHKC
Liver weight (% BW)	4.53 ± 0.12	5.08 ± 0.25	4.34 ± 0.07 #
Inguinal fat (% BW)	1.03 ± 0.27	1.04 ± 0.12	2.24 ± 0.41 ** ##
Serum glucose (mg/dL)	113.1 ± 3.8	102.6 ± 8.00	117.8 ± 7.3
Serum insulin (ng/mL)	1.76 ± 0.08	3.05 ± 0.33 **	1.68 ± 0.23 ##
HOMA-IR	12.59 ± 0.87	21.21 ± 3.44 *	12.54 ± 1.73 #
Serum leptin (ng/ml)	3.40 ± 0.79	4.82 ± 0.72	6.26 ± 1.22
Serum ADP (µg/mL)	3.02 ± 0.13	2.75 ± 0.17	2.89 ± 0.12
Serum HMW-ADP (µg/mL)	1.60 ± 0.28	1.51 ± 0.25	1.93 ± 0.42

Values are means ± SEM of seven animals. ADP, adiponectin; BW, body weight; CD, chow diet; HHC, high fat diet (HFD) during 2 months plus CD during 1 month; HHCC, HHC plus CD during another month; HHK, HFD plus a ketogenic diet (KD) during 1 month; HHKC, HHK plus CD during one month; HOMA-IR, homeostatic model assessment for insulin resistance; IL, interleukin; HMW; high molecular weight. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. CD; # p < 0.05, ## p < 0.01, ### p < 0.001 vs. HHCC.

3.3.1. CD Administration to HHC and HHK (4 Months)

The percentage of liver weight was lower in HHKC with respect to HHCC mice. Percentage of inguinal fat depot was increased in HHKC. There were no differences in serum glucose levels, but serum insulin and HOMA-IR index were increased in HHCC compared to CD and HHKC mice. No changes in leptin, adiponectin and HMW-adiponectin were detected (Table 1).

3.4. KD Generates a Better Cytokine Profile than CD in the Circulation, but Not in Liver

At 3 months, no changes in hepatic concentrations of IL-1β or IL-6 were found after obese mice received a CD or KD for one month; however, TNF-α was increased in the liver of KD fed mice (Table 2). After an additional month of the CD or reintroduction of the CD to the KD group for one month, an increase in liver IL-1β was seen in both groups and IL-6 was augmented in mice previously fed with the KD, with TNF-α values no longer being different between groups (Table 2).

Table 2. Hepatic and circulating levels of cytokines at 3 and 4 months.

Parameter (3 months)	CD	HHC	HHK
Liver IL-1β (ng/mg protein)	21.76 ± 2.67	21.14 ± 3.54	19.61 ± 2.50
Liver IL-6 (ng/mg protein)	22.52 ± 4.83	22.94 ± 1.68	17.83 ± 4.01

Liver TNF-α (ng/mg protein)	6.41 ± 0.54	7.92 ± 1.01	16.33 ± 2.28 ** #
Serum IL-1β (pg/mL)	108.1 ± 19.0	152.4 ± 25.5	69.6 ± 18.2 #
Serum IL-6 (pg/mL)	16.07 ± 2.54	23.76 ± 8.95	20.0 ± 4.16
Serum TNF-α (pg/mL)	9.87 ± 1.10	20.77 ± 1.91 ***	11.38 ± 1.24 ###
Parameter (4 months)	CD	HHCC	HHKC
Liver IL-1β (ng/mg protein)	8.58 ± 2.27	17.31 ± 1.23 **	16.26 ± 1.02 **
Liver IL-6 (ng/mg protein)	12.85 ± 1.50	16.37 ± 0.63	19.34 ± 1.18 **
Liver TNF-α (ng/mg protein)	5.57 ± 1.97	5.12 ± 1.74	3.83 ± 1.35
Serum IL-1β (pg/mL)	145.8 ± 21.7	152.3 ± 25.7	62.3 ± 7.9 ** #
Serum IL-6 (pg/mL)	18.20 ± 3.24	21.13 ± 5.06	12.84 ± 3.53
Serum TNF-α (pg/mL)	11.29 ± 0.85	20.63 ± 3.29 **	9.54 ± 2.02 #

Values are means ± SEM of seven animals. CD, chow diet; HHC, high fat diet (HFD) during 2 months plus CD during 1 month; HHCC, HHC plus CD during another month; HHK, HFD plus KD during 1 month; HHKC, HHK plus CD during one month; IL, interleukin; TNF, tumor necrosis factor. ** p < 0.01, *** p < 0.001 vs. CD; # p < 0.05, ## p < 0.01, ### p < 0.001 vs. HHCC.

Serum IL-1β levels were decreased in HHK compared to HHC with no changes in IL-6. Circulating TNF-α was augmented in HHC compared to CD and HHK. These changes remained after reintroduction of a chow diet in both groups (Table 2).

3.5. KD Reduces Activation and Levels of Enzymes of Fatty Acid Anabolism and Akt-Related Signaling in Obese Mice

When obese mice were put on a CD or KD for one month there was a decrease in the phosphorylation of ATP-CL in the liver, with this decrease being more pronounced with the KD (Figure 3A). No change in ACSL1 was found (Figure 3B), while the KD induced a reduction in FASN levels (Figure 3C).

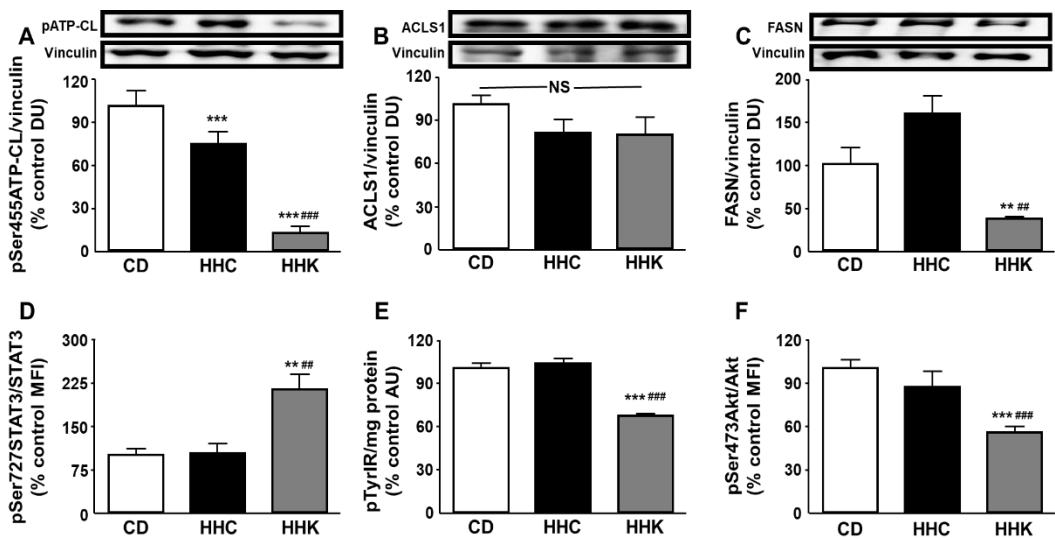


Figure 3. Effect of CD or KD on enzymes of fatty acid anabolism and leptin- and insulin-related signaling in the liver. Relative protein levels of (A) ATP citrate lyase (ATP-CL) phosphorylated at Ser455 (pSer455ATP-CL), (B) long-chain acyl-CoA synthetase (ACSL)1, (C) fatty acid synthase (FASN), (D) signal transducer and activator of transcription 3 (STAT3) phosphorylated at Ser727 (pSer727STAT3), (E) insulin receptor (IR) phosphorylated at Tyr residues (pTyrIR) and (F) Akt phosphorylated at Ser473 (pSer473Akt). Data are presented as mean ± SEM. CD, chow diet; HHC, high-fat diet (HFD) for 2 months followed by CD for 1 month; HHK, HFD for 2 months followed by ketogenic diet (KD) for 1 month. AU, absorbance units; DU, densitometry units; MFI, median fluorescence intensity; NS, non-significant. ** p < 0.01, *** p < 0.001 vs. CD; # p < 0.01, ## p < 0.001 vs. HHC.

Phosphorylation of STAT3 was increased in the liver of mice fed a KD (Figure 3D) and phosphorylation of IR and Akt was diminished in the same group of mice (Figure 3E and 3F, respectively). No changes in phosphorylation of any of these parameters were detected in CD-fed obese mice (Figure 3D, 3E and 3F).

3.6. Decreased Hepatic FASN Levels in KD-Fed Mice Are Associated with Changes in Activation of Downstream Akt Signaling

Obese mice fed a KD had increased phosphorylation of PTEN and decreased phosphorylation of mTOR (Figure 4A and 4B, respectively), with no changes in these factors when fed a CD. Both diets decreased p70 S6K phosphorylation (Figure 4C) and only the KD diminished CREB and ATF-2 activation (Figure 4D and 4E, respectively).

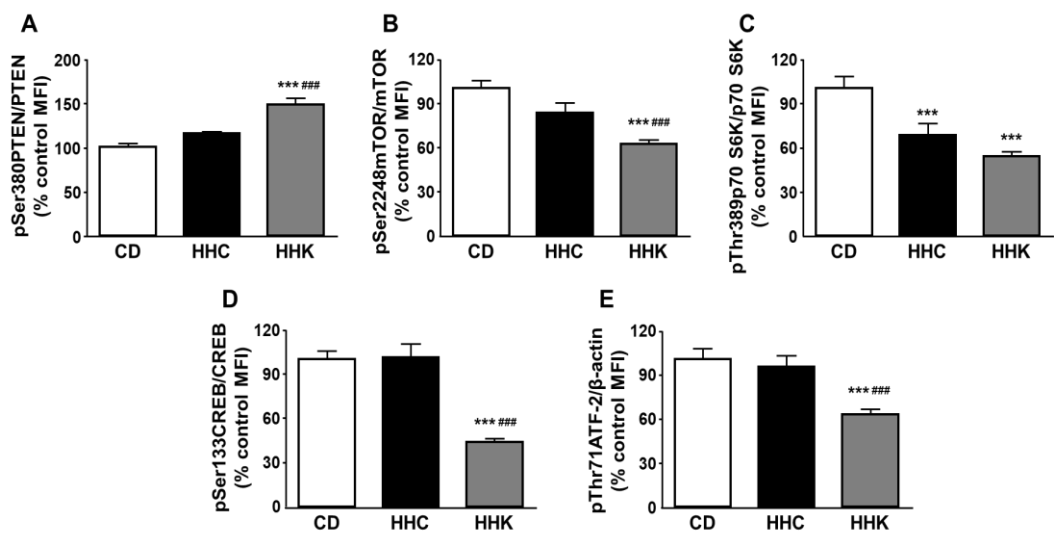


Figure 4. Effect of CD or KD in obese mice on downstream Akt-related signaling. Relative protein levels of (A) phosphatase and tensin homolog (PTEN) phosphorylated at Ser380 (pSer380PTEN), (B) mammalian target of rapamycin (mTOR) phosphorylated at Ser2248 (pSer2248mTOR), (C) p70 S6 kinase (p70 S6K) phosphorylated at Thr389 (pThr389p70 S6K), (D) cAMP response element binding protein (CREB) phosphorylated at Ser133 (pSer133CREB) and (E) activating transcription factor 2 (ATF-2) phosphorylated at Thr71 (pThr71ATF-2). Data are presented as mean $\pm$ SEM. CD, chow diet; HHC, high-fat diet (HFD) for 2 months followed by CD for 1 month; HHK, HFD for 2 months followed by ketogenic diet (KD) for 1 month. MFI, median fluorescence intensity. *** $p < 0.001$ vs. CD; ### $p < 0.001$ vs. HHC.

3.7. Effect of Reintroduction of a CD to Obese Mice previously Fed a KD on Lipid Anabolism Enzymes and Insulin-Related Signaling

Chow diet administered to the HHK group, as well as continued intake of the CD in the obese group, did not change phosphorylation levels of ATP-CL in the liver (Figure 5A), but reduced ACSL1 in the HHK mice (Figure 5B). Hepatic levels of FASN were reduced in both groups (Figure 5C). Phosphorylation of IR and CREB were decreased in HHCK mice (Figure 5D and 5E, respectively) and ATF-2 activation was diminished in HHCC mice (Figure 5F).

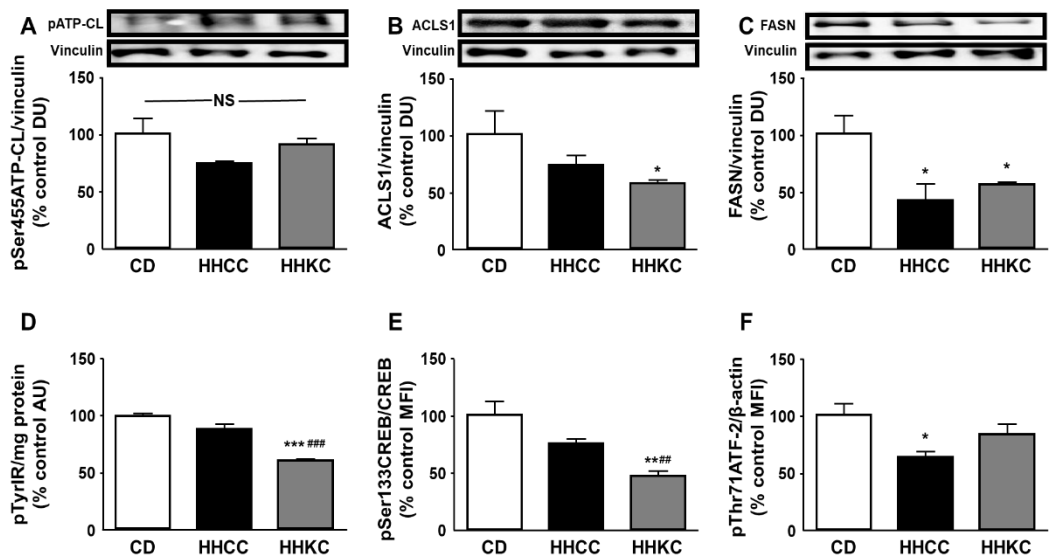


Figure 5. Effect of CD on enzymes of fatty acid anabolism and insulin-related signaling in obese mice fed previously with CD or KD. Relative protein levels of (A) ATP citrate lyase (ATP-CL) phosphorylated at Ser455 (pSer455ATP-CL), (B) long-chain acyl-CoA synthetase (ACSL)1, (C) fatty acid synthase (FASN), (D) insulin receptor (IR) phosphorylated at Tyr residues (pTyrIR), (E) cAMP response element binding protein (CREB) phosphorylated at Ser133 (pSer133CREB) and (F) activating transcription factor 2 (ATF-2) phosphorylated at Thr71 (pThr71ATF-2). Data are presented as mean ± SEM. CD, chow diet, HHCC, high-fat diet (HFD) plus CD for 2 months; HHKC, HFD followed by ketogenic diet (KD) for 1 month and CD for another month. AU, absorbance units; DU, densitometry units; MFI, median fluorescence intensity; NS, non-significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. CD; # $p < 0.01$, ## $p < 0.001$ vs. HHCC.

3.8. Changes in Hepatic Insulin Content After CD or KD Intake in Obese Mice and Reintroduction of CD to Both Groups of Mice

Given the existence of changes in factors involved in hepatic insulin signaling, we also analyzed the concentration of this hormone in the liver, as well as the possible correlation with serum concentrations. Hepatic levels were decreased in HHC, with no variation between CD and HHK (Figure 6A). When we studied the effect of reintroduction of CD, we found an increase in HHKC with respect to the other experimental groups (Figure 6C).

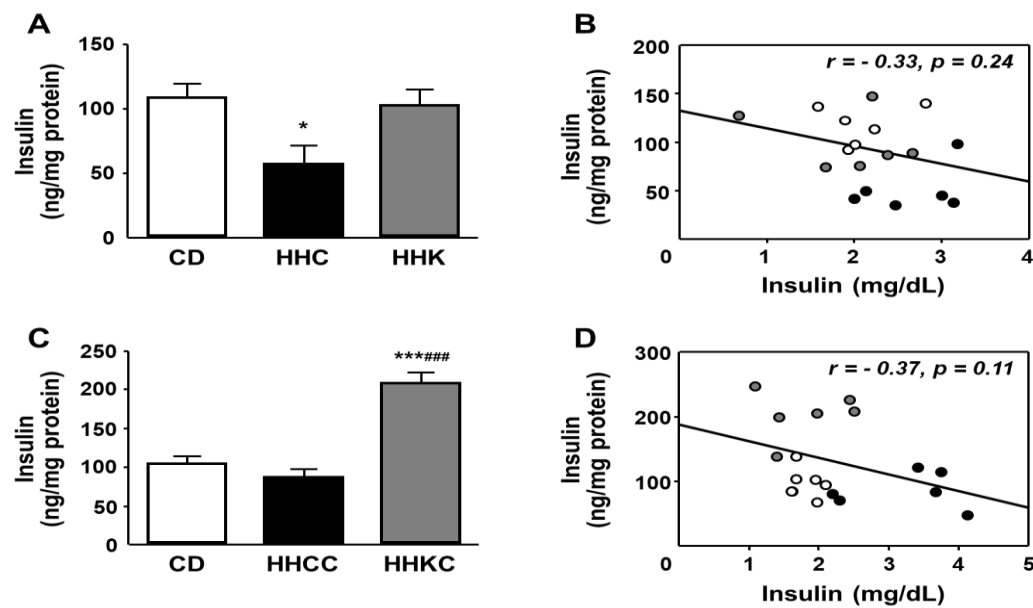


Figure 6. Effect of CD or KD in obese mice (**A**) and reintroduction of CD to both groups (**C**) on hepatic insulin levels and linear correlations between serum insulin and hepatic insulin levels in these groups (**B and D, respectively**). Data are presented as mean ± SEM. CD, chow diet, HHC, high-fat diet (HFD) for 2 months followed by C for 1 month; HHK, HFD for 2 months followed by ketogenic diet (KD) for 1 month, HHCC, HHC plus CD for 1 month; HHKC, HHK plus CD for 1 month. * $p < 0.05$, *** $p < 0.001$ vs. CD; ### $p < 0.001$ vs. HHCC. Correlation coefficients (r) and p values are represented for each analysis. Dots represent the individual data.

We did not find significant correlations between hepatic and serum insulin levels at either of the two time points studied (Figure 6B and 6D, respectively).

3.9. KD Causes a Reduction in Hepatic TG Content

There were no changes in hepatic FFA concentrations in the HHC, HHK, HHCC or HHKC groups (Figure 7A and 7C). Triglyceride content was diminished in HHK mice compared to the CD and HHC (Figure 7B) groups and augmented in HHCC with respect to CD and HHKC (Figure 7D).

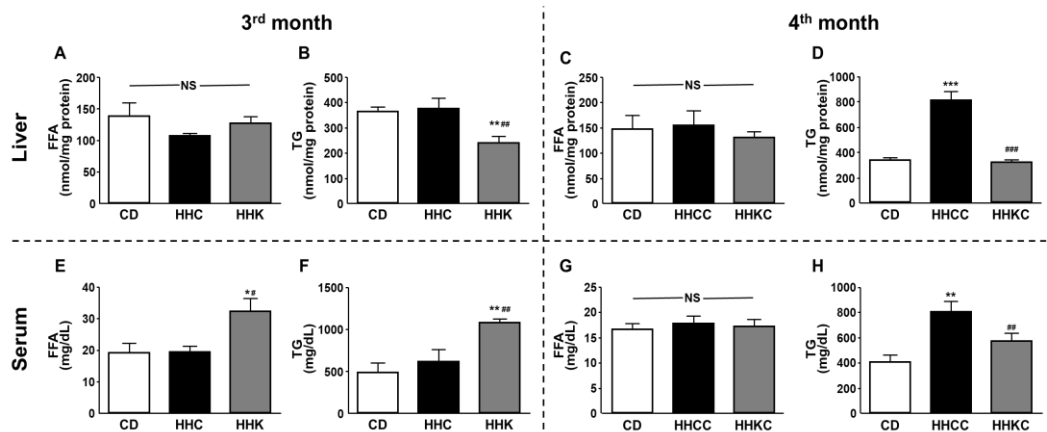


Figure 7. Effects of CD or KD in obese mice and reintroduction of CD in both groups on hepatic and circulating levels of free fatty acids (FFA) and triglycerides (TG). Hepatic levels of FFA and TG in obese mice fed with CD or KD (**A and B, respectively**) and after reintroduction of CD (**C and D, respectively**). Serum levels of FFA and TG in obese mice fed with CD or KD (**E and F, respectively**) and after reintroduction of CD (**G and H, respectively**). Data are presented as mean ± SEM. CD, chow diet, HH, high-fat diet (HFD) for 2 months; HHC, HH followed by CD for 1 month; HHK, HH followed by ketogenic diet (KD) for 1 month; HHCC, HHC plus CD for 1 month; HHKC, HHK followed by CD for 1 month. NS, non-significant. ** $p < 0.01$, *** $p < 0.001$ vs. CD; ## $p < 0.01$, ### $p < 0.001$ vs. HHC or HHCC.

Serum levels of FFA and TG were increased in HHK mice with respect to the CD and HHC groups (Figure 7E and 7F, respectively). At the end of the study, the content of FFA was not different between the three experimental groups (Figure 7G) and the hepatic content of TG was increased in HHCC mice, whereas HHKC showed no differences with respect to the CD group (Figure 7H).

4. Discussion

This study was conceived to analyze the possible differential effects of a ketogenic diet versus a normal chow diet, as well as the resumption of a chow diet after exposure to a ketogenic diet, on hepatic and serum triglyceride content in obese male mice, as well as its probable relationship with insulin-related signaling and fatty acid anabolism in the liver. We found that ingestion of the ketogenic diet for one month reduced the hepatic content of triglycerides and that even after the reintroduction of a chow diet for one month they maintained lower serum and hepatic concentrations of triglycerides compared to obese mice that had received the chow diet for two months. Our results

suggest that these changes are associated with attenuation of hepatic insulin signaling and a consequent reduction in the activation and levels of enzymes involved in lipid anabolism in the liver.

Obese animals fed the KD consumed more kilocalories per gram of body weight than those on the CD, but weight gain was similar in both groups at the end of the study, showing a lower energy efficiency in mice fed a KD. Although we have used an unrestricted KD, it is known that restricted KDs can be more effective for weight loss, providing metabolic benefits such as muscle mass preservation, lower metabolic efficiency, causing metabolic activation of thermogenesis, and enhancing fat loss, even when restricted KD contain more calories than other controlled diets [21,22]. In addition, KDs have been reported to have lower energy digestibility than diets with a balanced nutrient composition, suggesting a lower efficiency of energy utilization [23].

Ketogenic diets have a high percentage of lipids, and this fat is usually used for energy rather than stored [24]. In our study, an unrestricted KD was used, and we found an increase in both circulating FFA and the percentage of weight inguinal fat, which could explain the hyperleptinemia detected after administration of this diet. Higher circulating leptin levels are associated with increases in activation of its intracellular signaling, such as augmented STAT3 phosphorylation, in mice fed the KD and its activation may augment SOCS3 levels [25]. On the opposite hand, reduction in STAT3 phosphorylation decreases SOCS3 levels and insulin resistance [26], while SOCS3 is a negative regulator of insulin signaling as it is capable of binding to the IR and suppressing insulin-mediated autophosphorylation of the receptor [27].

We found that KD intake reduced circulating insulin levels, as previously reported [28,29]. We found a reduction in hepatic insulin signaling that might be consistent with reduced circulating levels of this hormone. However, hepatic insulin levels in the KD group were not different from those found in the controls, and then increased after being switched to the CD. It is important to denote that hepatic insulin clearance is involved in regulating the homeostatic level of insulin that reaches insulin target tissues, such as the liver itself, to produce appropriate insulin action. Receptor-mediated insulin uptake followed by its degradation is an important mechanism of insulin clearance. Upon its phosphorylation by the IR tyrosine kinase, carcino-embryonic antigen-related cell adhesion molecule 1 (CEACAM1) increases the rate of endocytosis and the targeting to degradation pathways [30]. Thus, the lower phosphorylation of IR found in our study may be related, at least in part, to the increase of hepatic insulin content. In addition, the low-grade hepatic inflammation found in our study, could reduce insulin-degrading enzyme activity [31], maintaining similar hepatic insulin concentrations than controls. although their circulating levels were lower.

How the intake of a KD affects blood glucose, lipid levels, cytokines and insulin sensitivity is still controversial. Some clinical studies have shown a favorable effect on circulating glucose, fatty acid levels and insulin sensitivity [32,33], while others have reported an increase in lipids and proinflammatory markers in subjects with obesity [34]. Here we found a favorable circulating profile of glucose, insulin, and cytokines with elevated levels of HMW-adiponectin and a lower HOMA-IR index in obese mice fed a KD compared to those fed a CD, with this index remaining stable even the mice ingesting the KD were changed to a CD. This is in agreement with studies showing that the administration of ketone bodies reduces circulating glucose levels [35] and that KD improves various metabolic parameters, such as HMW-adiponectin [21], and can have anti-inflammatory properties [36].

However, we found a reduction in the activation of hepatic insulin signaling both in obese mice fed KD and after reintroducing a CD to these animals. This decrease in insulin signaling was accompanied by a proinflammatory environment in the liver. The lower IR phosphorylation may be due to several factors, including a possible increase in SOCS3 levels and an inflammatory profile in the liver of these mice. As mentioned above, SOCS3, dependent on STAT3 activation, can interfere with insulin-dependent IR phosphorylation [27] and pro-inflammatory cytokines restrain Akt phosphorylation [37]. An increase in PTEN phosphorylation as seen here is reported to reduce Akt activation [38] and activation of this phosphatase has been related to an increase in IL-1 β , IL-6 or TNF- α [39], as found in our study. In summary, an adverse inflammatory profile can interfere with

several targets of intracellular insulin signaling [40,41] and KD has been reported to induce hepatic insulin resistance in mice [42,43].

Mammalian TOR and S6K are two downstream targets of Akt that promote protein synthesis and are closely related to the regulation of key enzymes in fatty acid metabolism, mainly FASN [44] through modulation of sterol regulatory element binding protein-1c (SREBP-1c), a key transcription for lipogenesis [45,46]. In mice fed KD we found a reduction in mTOR and S6K phosphorylation with a decrease in hepatic FASN levels, as well as the activation of ATP citrate lyase, which is also regulated by Akt signaling [47].

The decrease in FASN persists after reintroducing a CD to obese mice previously fed a KD diet and also appears in those who were not fed a KD. The CREB/ATF family of transcription factors integrates nutritional signals to regulate metabolism, principally in the liver [48]. Akt signaling, in addition to regulating mTOR, modulates CREB phosphorylation [49], that induces expression of FASN [50,51], which would explain, at least in part, the lower levels of this anabolic enzyme. The reduction in phosphorylation of ATF-2 could explain the reduction in FASN levels in mice following the reintroduction of a CD to obese mice that had not received a KD, as this transcription factor binds to CREB to promote their actions [52]. ACSL1 is another crucial enzyme in fatty acid metabolism, which we found to be reduced in mice fed KD plus CD. This enzyme is also regulated by insulin signaling [53] and inflammatory signals [54], factors reduced in this experimental group.

Our results showed a decrease in hepatic TG content after KD intake, with hepatic and circulating TG levels not being different from controls and actually lower than in mice that only received the CD to induce weight loss. These data are consistent with the decrease in the levels or activation of some key enzymes in lipid anabolism found here. In fact, it has been reported that under some conditions KD can reduce circulating levels of TG [55], as well as intrahepatic lipid content by reducing lipogenesis [56].

A couple of caveats should be taken into consideration when evaluating our results. A weakness of this study is the lack of data on hepatic SOCS3 levels, as well as its interaction with the insulin receptor, since it decreases its autophosphorylation [57]. A limitation related to the unrestricted KD is the increase in inguinal fat, which could modify circulating levels of FFA and TG. However, the use of an unrestricted KD has allowed us to better study the mechanisms that regulate hepatic lipid biosynthesis, because of the hyperleptinemia linked to this diet. In this regard, hyperleptinemia allowed us to better understand some of the mechanisms that modulate these biosynthetic processes, since activation of leptin signaling may promote an attenuation of insulin signaling [12,58,59].

5. Conclusions

Our results indicate that an unrestricted KD in obese male mice initially increases serum triglyceride levels, while decreasing intrahepatic fat content. Reintroducing a CD to mice previously fed a KD normalized serum triglyceride content, while those receiving only a CD to induce weight loss had elevated levels. These beneficial effects are associated with a decrease in hepatic levels of key lipid anabolism enzymes and closely related to an attenuation of hepatic insulin signaling. In summary, reduction in hepatic insulin signaling translates into an improvement in the hepatic triglyceride content of obese mice fed KD that persists after the reintroduction of CD, accompanied by a higher peripheral insulin sensitivity.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the declaration of Helsinki and approved by the Ethical Committee of Animal Experimentation of the Hospital Puerta de Hierro of Madrid and the Animal Welfare Organ of the Community of Madrid (PROEX 009.4/24, 12 February 2024).

Informed Consent Statement: Not applicable.

Data Availability Statement: All relevant data are included within the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

ACSL1	long-chain acyl-CoA synthetase 1
Akt	Protein kinase B
ATF-2	Activating transcription factor 2
ATP-CL	ATP citrate lyase
AU	Absorbance units
CD	Chow diet
CEACAM1	Carcino-embryonic antigen-related cell adhesion molecule 1
CREB	cAMP response element-binding protein
DU	Densitometry units
ELISA	Enzyme-linked immunosorbent assay
FFA	Free fatty acid
HFD	High-fat diet
HHC	HFD plus CD
HHCC	HHC plus CD
HHK	HFD plus KD
HHKC	HHK plus CD
HMW	High molecular weight
HOMA-IR	Homeostatic model assessment for insulin resistance
HRP	Horseradish peroxidase
IL	Interleukin
IR	Insulin receptor
KD	Ketogenic diet
MFI	Median fluorescence intensity
mTOR	Mammalian target of rapamycin
NS	Non-significant
PTEN	Phosphatase and tensin homolog
p70 S6K	p70 S6 kinase
SEM	Standard error of the mean
SOCS3	Suppressor of cytokine signaling 3
SREBP-1c	Sterol regulatory element binding protein-1c
STAT3	Signal transducer and activator of transcription 3
TG	Triglyceride
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor-alpha

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