Hepatic steatosis, inflammation, and ER stress in mice maintained long-term on a very low-carbohydrate ketogenic diet

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ABSTRACT

Low carbohydrate diets are used to manage obesity, seizure disorders, and malignancies of the central nervous system. These diets create a distinctive, but incompletely defined, cellular, molecular, and integrated metabolic state. Here, we determine the systemic and hepatic effects of long-term administration of a very low-carbohydrate, low-protein, and high-fat ketogenic diet, serially comparing these effects to a high simple carbohydrate, high-fat Western diet and a low-fat, polysaccharide-rich control chow diet in C57BL/6J mice. Longitudinal measurement of body composition, serum metabolites, and intrahepatic fat content, using in vivo magnetic resonance spectroscopy, reveal that mice fed the ketogenic diet over 12 weeks remain lean, euglycemic, and hypoinsulinemic, but accumulate hepatic lipid in a temporal pattern very distinct from animals fed the Western diet. Ketogenic diet-fed mice ultimately develop systemic glucose intolerance, hepatic endoplasmic reticulum (ER) stress, steatosis, cellular injury, and macrophage accumulation, but surprisingly, insulin-induced hepatic Akt1 phosphorylation and whole-body insulin responsiveness are not impaired. Moreover, while hepatic Pparg mRNA abundance is augmented by both high-fat diets, each diet confers splice variant specificity. The distinctive nutrient milieu created by long-term administration of this low carbohydrate, low protein ketogenic diet in mice evokes unique signatures of nonalcoholic fatty liver disease and whole-body glucose homeostasis.
69  **Key words:** nutrient state – magnetic resonance spectroscopy (MRS) – PPARγ –
70  insulin resistance – unfolded protein response (UPR) – Xbp1 splicing – high-fat diets –
71  fatty liver disease
72
INTRODUCTION

Therapeutic use of reduced carbohydrate diets has been extensively studied for the amelioration of a multitude of clinical states, including obesity and its metabolic complications, seizure disorders, and malignancies of the central nervous system [see refs. (21, 23, 26, 36, 41, 54, 55, 59, 64) and references therein]. Nonetheless, the full scope of metabolic effects incurred by low-, and very low (ketogenic)-carbohydrate diets has only been preliminarily characterized. In wild-type mice, ketogenic diets result in weight loss and increased hepatic and myocardial fatty acid oxidation, compared to mice maintained on standard chow diets rich in polysaccharides (5, 35, 62). While leptin-deficient obese (ob/ob) animals maintained on ketogenic diets for seven weeks exhibit persistent weight gain, glucose tolerance and insulin resistance improve compared to ob/ob mice maintained on standard chow diet (3, 57). Collectively, encouraging pre-clinical and clinical studies of low carbohydrate ketogenic diets has favored their continued study for anticonvulsant therapy, adjunctive therapy for brain cancers, and weight loss for obesity.

Cardiovascular disease attributable to obesity, insulin resistance, and diabetes is increasing markedly (20, 22). Insulin resistance is highly correlated with ectopic lipid accumulation, particularly in the liver. Consequently, the pathogeneses of systemic insulin resistance and diabetes have been linked to nonalcoholic fatty liver disease (NAFLD). NAFLD is an independent predictor of cardiovascular disease – a stronger predictor than peripheral or visceral fat mass (10, 11, 18, 19). A critical – but as yet only preliminarily defined – influence over the development of NAFLD and possibly its complications, is distribution of macronutrient classes within the diet (34, 56). While low-
carbohydrate diets are effective for weight loss, comprehensive determination of their relationships with fatty liver remains ongoing. The importance of further understanding the impact of low carbohydrate diets on metabolic states in rodent and humans models is additionally underscored by case reports of humans that reveal variations in the range of metabolic response to these diets (7, 13). Therefore, to measure the long-term effects of high-fat diets of varying carbohydrate content on liver and systemic metabolism, C57BL/6J mice were serially profiled while maintained on either (i) a commonly-studied ‘Ketogenic diet’ (KD) very high in fat content, nearly devoid of carbohydrate, and also reduced in protein; (ii) a high-fat ‘Western diet’ (WD) enriched in simple carbohydrates; or (iii) a standard low-fat polysaccharide-rich chow control diet for 12 weeks.

MATERIALS AND METHODS

Animals and diets. C57BL/6J wild-type mice were maintained on Lab Diet (5053) ad libitum and autoclaved water on cedar chip bedding. Lights were off between 1800 and 0600. Beginning at the age of six weeks, male mice were maintained for 12 weeks on either (i) KD (Bio-Serv F3666); (ii) WD (Harlan-Teklad TD.96132) which induces hyperglycemia, glucose intolerance, and insulin resistance in rodents (2, 49); or (iii) standard low-fat, polysaccharide-rich chow (Lab Diet 5053). Macronutrient compositions of these three diets are indicated in Table 1. KD is a 7.5 kcal/g formulation in which 95.1% of calories are from fat (lard and butter fat), 4.5% from protein (casein), and 0.4% from carbohydrates (dextrose). Fatty acid distribution in KD is 41% saturated, 43% monounsaturated, 16% polyunsaturated, and 0% trans. WD is a 4.5 kcal/g formulation
in which 40% of calories are from fat (beef tallow and shortening), 19% from protein (casein), and 41% from carbohydrates (sucrose, corn starch, and maltodextrin). Fatty acid distribution in WD is 41% saturated, 35% mono-unsaturated, 7% polyunsaturated, and 17% trans. Both custom diets are fortified with essential micronutrients and are soy meal (phytoestrogen)-free. See Discussion for a description of the relative advantages and disadvantages of using and comparing the responses to each of these diets. To optimize experimental design, caloric consumption was measured in cohorts of animals that were not examined using the in vivo MRS protocol (see below). All experiments were performed after protocol approval by the Animal Studies Committee at Washington University.

**Metabolite and insulin measurements.** Blood, and resulting serum samples were acquired from animals that had been fasted for 5h, maintained on fresh cedar chip bedding, and were collected mid-morning. Serum metabolites and insulin were measured as previously described (62). Hepatic triglycerides (TG), using a Folch extract of liver and biochemical quantification, were quantified as previously described (14). Serum alanine aminotransferase (ALT) was measured using an assay from Teco Diagnostics, according to manufacturer’s instructions. Within each diet, no differences among serum measurements within any of the nutritional groups were observed between cohorts of animals that were examined using the in vivo MRS protocol (see below) and those for whom liver MRS was not performed.

**Measurements of body composition.** Percent body fat and lean body mass were determined in awake animals using an EchoMRI instrument (Echo Medical Systems).
Magnetic resonance spectroscopy. For a subset of mice within each nutritional treatment group, hepatic TG was quantified longitudinally using in vivo MRS. Localized MR spectra of mice were collected in an Oxford Instruments (Oxford, UK) 4.7-tesla, 40-cm horizontal-bore magnet. The magnet is equipped with Agilent/Magnex Scientific (Yarnton, UK) actively shielded gradient coils (21-cm inner diameter, ~30 G/cm, ~200 μs rise time) and International Electric Company (Helsinki, Finland) gradient power amplifiers and is interfaced with a Agilent/Varian NMR Systems (Santa Clara, CA) DirectDrive console. All data were collected using a Stark Contrast (Erlanger, Germany) 2.5-cm birdcage RF coil. Animals were anesthetized with isoflurane anesthetic and maintained on 1% isoflurane/99%oxygen (v/v) during the spectroscopy experiments.

Localized proton spectroscopy data were collected using the LASER pulse sequence (27), as described previously (25). Briefly, for each animal, a set of respiratory-gated, spin-echo transaxial images of the liver was collected, to allow placement of voxels for the localized spectroscopy. Synchronization of MR data collection with animal respiration was achieved with a home-built respiratory-gating unit (24) and all images and spectra were collected during post-expiratory periods. For the measurement of liver lipid levels, we selected voxels that were 4 mm × 4 mm (in-plane) × 3 mm. Spectroscopy data from three non-overlapping voxels, all in the same transaxial plane of the liver, were collected for each mouse and the results obtained from these three voxels were averaged together. Respiratory-gated LASER spectra were collected with echo time (TE) = 39 ms; recycle time (TR) ~ 1 s (the exact value of TR depends upon respiratory rate, and thus varies slightly from animal to animal); sweep width (SW) = 7812 Hz; acquisition time (AT) = 262 ms; number of data points
(NP) = 4096. Amplitudes of water and lipid signals were measured using Bayesian Probability Theory methods (38, 39) and were corrected for differences in T1 and T2 values for water and lipid, as described previously (25).

**Gene expression analysis.** Quantification of gene expression was performed using real-time RT-qPCR using the ΔΔCt approach as described, normalizing to Rpl32, using primer sequences listed within Table S1 (62). All RT-qPCR reactions used the ThermoFisher Abgene 2X Sybr green reagent with 900 nM each primer, with the exception of X-box binding protein 1 (XBP1) Xbp1-S primers, which also included 20 mM NH₄SO₄ and 3 μM each primer. Xbp1-S, Xbp1-U, Xbp1 (total), and Xbp1 (traditional) primer sets quantitatively amplify rat and mouse Xbp1 sequences. Migrating species within the 2% agarose gel-based RT-PCR assay of Xbp1, shown in Fig. 5A and 5D, were densitometrically quantified using QuantityOne software (Bio-Rad).

**Immunoblot.** Immunoblots measuring hepatic and gastrocnemius phospho-Akt1 [p-Akt1 (Ser473)] and total Akt were performed as previously described, using rabbit antibodies from Cell Signaling Technologies (#9271 and #9272, respectively) (14). Phospho- and total-eukaryotic initiation factor 2α (eIF2α) were measured on immunoblots using rabbit antibodies from Cell Signaling Technologies (#9721 and #9722, respectively) and donkey anti-rabbit IgG F(ab′)² fragment conjugated to horseradish peroxidase (GE Healthcare, NA9340). C/EBP-homologous protein/Growth arrest and DNA damage-inducible gene 153 (CHOP/GADD153) was measured using mouse monoclonal anti-GADD153 (Santa Cruz, sc-7351) and donkey-anti-mouse IgG conjugated to horseradish peroxidase (Jackson Immunoresearch, #715-035-151). p62 was measured using mouse monoclonal anti-p62 (Abcam, ab56416). Microtubule
associated protein 1 light chain 3 (LC3) was measured using rabbit polyclonal anti-LC3 (Novus Biologicals, #NB100-2220). Band intensities were densitometrically quantified using QuantityOne software.

**Glucose and insulin tolerance tests.** Mice were fasted for 5h prior to each test. Baseline glucose measurements were taken in duplicate using a tail snip and a glucometer (Aviva). For glucose tolerance tests, a 10% dextrose solution was administered intraperitoneally, 1 mg glucose/g body weight. For insulin tolerance tests, human insulin (Eli Lilly) was administered intraperitoneally, 0.75 U/kg body weight. Glucose measurements were performed in duplicate 30, 60, and 120 minutes after injection. Animals were then restored to their maintenance diets. All glucose and insulin tolerance tests were separated by at least 72h, and at least 72h lapsed between glucose or insulin tolerance tests and subsequent blood/tissue collections.

**Histology.** Liver specimens from freshly-killed mice were harvested and fixed in 10% neutral buffered formalin (Fisher). Tissue was embedded in paraffin, microtome-sectioned, stained with hematoxylin and eosin, and photographed using standard methods. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stains were performed using In Situ Cell Death TMR red Detection Kit from Roche (12156792910) according to manufacturer's instructions, using previously described methods (15). 4',6-diamidino-2-phenylindole (DAPI) was used as a cell nuclear counterstain (40 ng/mL in phosphate buffered saline for 5 min) for TUNEL stains. For F4/80 immunostains, rat anti-F4/80 (Abcam, ab6640) was incubated for 1h at room temperature on liver cryosections (diluted 1:50 in 1% bovine serum albumin/0.1% Triton X-100 in phosphate buffered saline), followed by Alexa fluor 594 conjugated goat anti-
rat IgG (Invitrogen), diluted 1:100 in 1% bovine serum albumin/0.1% Triton X-100 in phosphate buffered saline and coverslip application as previously described (1). All slides were examined, images acquired, and morphometric quantifications performed using a Zeiss Axioskop microscope and AxioVision software (version 4.7).

**Cell culture.** Culture of neonatal rat cardiomyocytes (NRCMs) was performed as previously described (62). DMSO or thapsigargin (Sigma, dissolved in DMSO; final \([\text{thapsigargin}] = 1.5 \mu M\) in culture medium; final [DMSO] in culture medium, 0.1%) was added to individual wells of 12-well plates for indicated durations.

**Statistical analyses.** Analyses were performed with GraphPad software (Prism), using tests described within the text and figure legends.

**RESULTS**

**Comparison of ketogenic diet to western diet on body composition and baseline metabolic parameters.** To compare the systemic effects of high-carbohydrate versus very low-carbohydrate high-fat diets, we initiated a KD and a WD in cohorts of 6 week-old C57BL/6J male mice, and compared them to strain-, age- and gender-matched mice maintained on standard low-fat, polysaccharide-rich chow (**Table 1**). This commonly-studied KD is a low-protein formulation, designed and used here because induction of ketosis in rodents is favored by low-carbohydrate and low-protein content (3-5, 8, 14, 34, 35, 57, 62). In this study, each diet was maintained for 12 weeks. Consistent with previously published results, mice maintained on KD exhibited decreased weight compared to WD and chow-fed groups after both 6 and 12 weeks (**Fig. 1A**) (34, 35). While consumption of KD is relatively reduced by mass, caloric density is higher in KD
than in WD or chow. Therefore, caloric intake was higher in KD-fed mice (Fig. 1B). The discordance between caloric intake and body weight is likely explained by increased metabolic rate in KD-fed mice (34, 35). As expected, total body fat, measured by MRS, was significantly elevated in WD-fed mice at both 6 and 12 weeks, compared to chow-fed mice, but body fat percentage in KD mice was commensurate with that of chow-fed controls (Fig. 1C). Conversely, lean mass was significantly reduced after both 6 and 12 weeks of the KD diet, and after 12 weeks of the WD, compared to chow-fed controls (Fig. 1D).

Analysis of 5h fasting serum metabolites revealed euglycemia in KD-fed mice, but reduced mean serum insulin concentrations and HOMA-IR indices at both 6 and 12 weeks (Table 2). As expected, WD-fed animals exhibited hyperglycemia, hyperinsulinemia, and therefore, significantly elevated HOMA-IR indices. Serum βOHB was not statistically elevated in WD-fed mice, but as expected, was markedly increased in KD-fed mice.

**Unique natural history and histopathology of hepatic steatosis in ketogenic diet-fed mice.** To determine the time course over which lipid accumulates in the liver of WD and KD-fed mice, we performed serial measurements of hepatic TG content in living chow-, WD-, and KD-fed mice over 12 weeks. At the start of the study (6 weeks of age), hepatic TG content in all animals was <1%, and TG content did not increase over 2% in the chow group throughout the duration of the study (Fig. 2A). By contrast, hepatic TG rose sharply in the KD group to 6.5±0.6% after three weeks, whereas the hepatic TG content in the WD group at this time point was only 1.9±0.4% (p < 0.01, KD versus WD by ANOVA). However, by six weeks duration, TG comprised ~6% of liver mass in both
WD and KD groups, and in the second six weeks, hepatic TG content increased more sharply in the WD group to 20.5±0.2%, versus 11.5±1.7% in the KD group at week 12 (p < 0.001, WD versus KD by ANOVA). The findings at 12 weeks were corroborated by biochemical quantification of hepatic TG (Fig. 2B). Taken together, these findings indicate that both very low- and high-carbohydrate, high-fat diets induce hepatic steatosis, but with markedly different kinetics and to different magnitudes.

To determine the microscopic anatomical changes that accompany hepatic lipid accumulation in these nutritional states, liver sections from mice fed chow, KD, or WD for 12 weeks were stained with hematoxylin and eosin. While sections from liver of mice fed the WD exhibited extensive peri-portal small and large droplet macrovesicular steatosis, peri-central hepatocytes were spared, and there was no evidence of inflammation, apoptosis, or regeneration (Fig. 3A-B). In contrast, sections of liver from KD-fed mice exhibited more isolated foci of hepatocytes with small and large droplet macrovesicular steatosis, often in a subcapsular distribution (Fig. 3C-D). Moreover, numerous hepatocellular mitoses, features of regeneration, and parenchymal inflammatory foci were uniquely evident in the liver sections from KD-fed mice (Fig. 3E-I). Liver sections from KD-fed mice also exhibited pyknotic hepatocytes, indicative of apoptosis, and TUNEL+ hepatocytes were more frequent in liver sections from KD-fed mice than from WD-fed or chow-fed mice (Fig. S1). No evidence of ballooning or fibrosis was observed in livers from either WD or KD. By immunostaining, F4/80+ sinusoidal cells (Kupffer cells, i.e. macrophages) were accentuated in periportal regions in all mice. However, the number of F4/80+ sinusoidal cells was significantly greater in liver sections from KD-fed mice than from WD-fed or chow-fed mice (Fig. 4A-D). Cross-
sectional area (CSA) of F4/80+ Kupffer cells was also greater in liver sections of KD-fed mice, compared to chow-fed mice or WD-fed mice (Fig. 4E). An additional marker of liver parenchymal injury, serum ALT, was selectively elevated in KD-fed mice (68.5±5.4 U/L) over chow-fed mice (41.6±4.3) and WD-fed mice (47.1±6.6, p < 0.05 by ANOVA with Tukey post-hoc testing, for comparison of KD versus chow and versus WD, n=6/condition). Taken together, our findings indicate that a chronic ketogenic diet causes an injury pattern in wild-type mice similar to a NAFLD phenotype.

Molecular phenotyping of livers from ketogenic diet-fed mice reveals splice variant-specific induction of Pparg transcript, suppressed markers of de novo lipogenesis, induced markers of fatty acid oxidation, and engagement of the unfolded protein response. To determine if the distinct temporal pattern of lipid accumulation and histopathology in livers from 12-week KD-fed mice was associated with distinct molecular responses, compared to livers from WD-fed mice, we performed gene expression analyses using real-time RT-qPCR. As expected, a central regulator of energy expenditure, glucose and fatty acid homeostasis, Fgf21, was markedly induced in livers of WD and KD-fed mice, compared to livers of chow-fed mice (Table 3) (16, 44, 65). While the genes encoding peroxisome proliferator activated receptor (PPAR)α and PPARδ were not significantly induced by WD or KD, Pparg was statistically significantly induced ≥2-fold by each diet, compared to chow. Further examination, using Pparg splice variant-specific primers, revealed a striking difference between livers of WD and KD-fed mice: Pparg2 was selectively induced by WD, and Pparg1 was selectively induced by KD (Table 3). Despite induction of Fgf21, abundance of the mRNA encoding the co-activator PGC-1α was not altered (17, 48); mRNA encoding PGC-1β was mildly
reduced in liver of KD-fed animals. As expected, due to the high-simple carbohydrate content of Western diet, mRNAs encoding key mediators of de novo lipogenesis (Srebf1, Fasn, Acaca, G6pdx, Scd1, Gck) were induced in livers of WD-fed animals, but significantly down-regulated in livers of KD-fed mice, compared to livers of chow-fed animals. Mediators of fatty acid transport and oxidation (Slc27a1, Cd36, Acsl1, Pdk4, Cpt1a, Acadm) were up-regulated over the chow-fed group in livers of both WD and KD-fed animals. The gene encoding a key ketogenic enzyme, HMGCS2, was up-regulated both by WD and KD, but to a significantly greater extent in the KD group. Interestingly, gluconeogenic enzymes encoded by Pck1 and G6pc were not altered by WD or KD.

Markers of inflammation (Tnf, Il1b, Il6) and fibrosis (Col1a2) were not significantly elevated in livers of WD or KD mice, but markers of monocyte/macrophage accumulation (Cd68; Emr1, which encodes F4/80; and Itgam, which encodes CD11b) were modestly elevated in livers from KD-fed mice, and less notably in livers from WD-fed mice. Furthermore, Nlrp3 and Pycard, which encode mediators of the inflammasome, were elevated in livers of KD-fed, but not WD-fed mice (32, 58).

While mildly increased gene expression of markers of the macrophage lineage was observed with both diets, immunohistochemical co-localization of PPARγ1 or PPARγ2 signal with F4/80 was not observed with either WD or KD (data not shown). To determine if nutritionally-induced hepatic steatosis was also associated with up-regulation of genes encoding lipid droplet-associated proteins, we measured the abundance of Cidea, Cideb, and Cidec (Fsp27) mRNAs, and observed increased abundance over chow of both PPARγ target genes Cidea and Cidec (42). Relative to chow, the extent of Cidea upregulation was markedly greater in livers of WD-fed mice,
compared to those from KD-fed mice, which is consistent with Cidea induction under
conditions in which Srebf1 is active (30).

**ER stress.** High-fat diets, hepatic steatosis, insulin resistance, and inflammation have
been linked through the induction of ER stress and mediators of the unfolded protein
response (UPR) (33, 40, 45, 47, 52, 61). A mediator of ER stress, XBP1, is a critical
transcriptional regulator of lipogenic enzymes (40, 69). To determine if ER stress is
induced in liver of mice fed these two high-fat diets, we measured classical biomarkers
of the ER stress response pathway. The induction of non-canonical splicing of the
mRNA encoding the UPR transcription factor mediator XBP1 was measured using both
traditional RT-PCR and novel RT-qPCR assays. A traditional RT-PCR assay was
performed to measure the relative abundance of the spliced and unspliced variants in
liver of mice fed chow, WD, or KD for 12 weeks. While a significant increase in the Xbp1
spliced-to-unspliced ratio was not observed at 12 weeks with either WD or KD,
compared to chow, liver of KD-fed mice exhibited a ~2-fold ($p = 0.07$) increase in this
ratio (Fig. 5A, D).

This traditional approach to quantify ER stress-induced Xbp1 splicing employs
two primers that flank the spliced exon of Xbp1, which simultaneously generates two
amplicons, in an internally-competitive PCR reaction. To perform a directly quantitative
measurement of the spliced and unspliced Xbp1 transcript abundances, we designed
primer sets that selectively amplify the rat and mouse orthologs of either the spliced,
unspliced, or total Xbp1 transcript pools, whose abundances are measured in three
separate real-time RT-qPCR reactions (see Table S1 for primer sequences, Fig. S2A-B
for a schematic of the approach, and Materials and Methods for a description). Pilot
experiments, performed using cultured primary neonatal rat cardiomyocytes treated with either DMSO vehicle or 1.5 μM thapsigargin for durations between 10-180 min, revealed marked time-dependent thapsigargin-induced increases of the spliced variant of Xbp1, mild increases in total transcript abundance, and a mild reciprocal decrease in abundance of unspliced Xbp1 variant abundance (Fig. S2C-E). Therefore, these primer sets form a novel and generalizable adjunctive tool to measure the magnitude of Xbp1 splicing as a biomarker of ER stress in rat or mouse tissues.

These primer sets were used to quantify transcript abundances in liver of 12-week chow-, WD-, and KD-fed mice. The spliced form of Xbp1 mRNA was ~2-fold elevated in livers of KD-fed mice compared with those of chow-fed mice (n=5/group; \( p < 0.05 \)), but not significantly elevated in livers of WD-fed animals (Fig. 5E). Abundances of unspliced and total Xbp1 mRNAs were not statistically different from chow. To determine if additional downstream mediators of the UPR were induced by the KD, we performed immunoblot of hepatic protein lysates, measuring the abundance of proapoptotic CHOP/GADD153, and observed statistically significant increases in livers of 12 week KD-fed, but not WD-fed animals (Fig. 5B, F). In addition, phosphorylation of the translational regulator upstream of CHOP, eIF2α, was increased within liver lysates of KD-fed mice, but not those of WD- or chow-fed mice (Fig. 5C, G). The causes of modestly slower SDS-PAGE migration of CHOP and eIF2α within lysates of cultured neonatal rat cardiomyocytes, compared to those from adult mouse liver, are unknown (51). Taken together, we concluded that UPR pathways are activated in livers of KD-fed mice.
Induction of the UPR has been recently linked to impaired autophagic flux, a possible contributor to hepatic steatosis-induced insulin resistance (66). We therefore measured biomarkers of autophagy progression – differential processing and migration of LC3 on SDS-PAGE and p62 abundance (6). Despite evidence for ER stress in livers of KD-fed mice, no evidence of altered autophagic flux was observed (Fig. S3).

**Glucose intolerance, but retained insulin responsiveness of ketogenic diet-fed mice.** As described above, when independently compared to chow and WD-fed mice, serum insulin concentrations and HOMA-IR indices were significantly reduced in KD-fed mice after 6 and 12 weeks. To determine the ability of KD-fed mice to dispose an exogenous glucose load, intraperitoneal glucose tolerance tests were performed after 6 and 12 weeks of administration of the diets. Consistent with previous results (35), glucose excursion was not different between chow-fed and KD-fed mice after 6 weeks on the diet, but as expected, WD-fed mice were glucose intolerant (Fig. 6A). On the other hand, after 12 weeks on the diet, KD-fed mice exhibited glucose intolerance (Fig. 6B). Strikingly, however, insulin tolerance tests performed after 12 weeks of the KD revealed that, despite glucose intolerance, systemic insulin responsiveness was significantly greater in KD-fed mice relative to chow-fed mice (Fig. 6C). Due to insulin-stimulated blood glucose values that fell below 30 mg/dL, two of the 12 week KD-fed animals, but none of the chow-fed animals (n=8/group) required termination of the insulin tolerance test with rescue using an intraperitoneal 50% dextrose solution.

To determine the influence of these nutrient states on insulin signaling in liver, we quantified p-Akt1 in liver of 5h fasted chow-, WD-, and KD-fed animals, in the basal and insulin-stimulated states (10 min, 0.75 U/kg total body weight intraperitoneally, n=4...
animals/group). In the basal state, KD-fed mice exhibited a significant decrease in liver
[p-Akt1]/[total Akt], consistent with low circulating insulin concentrations (Fig. 7A-B).
However, insulin-stimulated Akt1 phosphorylation remained intact in livers of KD-fed
mice. As expected, insulin-stimulated Akt1 phosphorylation was blunted in livers of WD-
fed mice (Fig. 7C-D). Similar findings were observed in skeletal muscle of basal and
insulin-stimulated mice (Fig. S4). Therefore, despite mild hepatic steatosis, induction of
ER stress, an hepatic inflammatory phenotype, and systemic glucose intolerance,
systemic insulin sensitivity remains intact, and perhaps increased, in mice fed a low-
carbohydrate high-fat ketogenic diet for 12 weeks.

DISCUSSION
To determine the fundamental metabolic responses elicited by low-carbohydrate diets,
the effects of a nearly zero carbohydrate, low-protein, high-fat ketogenic diet have been
studied using C57BL/6J mice by our group and others (3-5, 14, 34, 35, 57, 62). These
studies have indicated, in wild-type mice, that weight is reduced by maintenance on this
diet, and glucose tolerance and systemic insulin resistance are improved in ketogenic
diet-fed ob/ob mice mice (3, 35). Nonetheless, administration of this diet causes hepatic
steatosis, and was recently linked to the accumulation of diacylglycerols and
development of hepatic insulin resistance in wild-type mice (34). The results presented
here extend our insight into the effects of this low carbohydrate diet on hepatic and
systemic metabolism, and open important new avenues for exploration. These studies
are the first to serially compare and contrast the effects of a high-simple sugar, high-fat
Western diet to a very low-carbohydrate, high-fat ketogenic diet over 12 weeks.
Compared to standard chow and to WD-fed animals, we observed that the KD decreased body weight and reduced serum insulin concentrations and HOMA-IR indices. However, while glucose tolerance of 6-week KD-fed mice was commensurate with that of chow-fed animals, systemic glucose intolerance emerges between 6 and 12 weeks of the KD. For unknown reasons, both WD and KD were associated with decreases in serum triglyceride concentrations between 6 and 12 weeks of the diet. Abnormalities of very low density lipoprotein (VLDL) secretion may develop after prolonged ingestion of these diets.

Despite mild hepatic steatosis, inflammation, injury, and ER stress in 12 week KD-fed mice, the systemic response to insulin, measured using insulin tolerance tests, was preserved in KD-fed mice. The apparent discrepancy between our findings and those of another recent report, in which hyperinsulinemic-euglycemic clamp studies revealed blunted insulin-mediated suppression of hepatic glucose production in mice fed this KD for 5 weeks (34), can prospectively be explained in part by (i) relatively reduced lean body mass in KD-fed mice, resulting in a proportionately higher insulin dose in insulin tolerance tests, when corrected per gram of lean body mass, and (ii) disparate phenotypes between liver and muscle in KD-fed mice. While KD-induced hepatic insulin resistance was evident in the studies presented by Jornayvaz and colleagues, impairment of insulin-stimulated peripheral glucose disposal in KD-fed mice was much more subtle, and was not observed in skeletal muscle and white adipose tissue (34). Therefore, our observation of enhanced systemic response to insulin in KD-fed mice likely reflects augmentation of insulin-mediated peripheral glucose disposal, relative to the chow group, that overrides prospective impairment of insulin-mediated
suppression of hepatic glucose production – indicating that, in the context of the KD in mice, hepatic insulin resistance may confer a smaller contribution to overall glucose homeostasis than peripheral disposal. Furthermore, our studies revealed no evidence of impaired hepatic insulin-stimulated Akt1 phosphorylation in mice fed the KD for 12 weeks, underscoring the importance of targets downstream of Akt1 signaling. However, abundances of the mRNAs encoding key gluconeogenic mediators were not changed within liver of KD-fed mice, compared to chow-fed controls. These studies also raise the possibility that long-term KD maintenance also alters physiological glucose-stimulated insulin secretion by pancreatic β cells.

The use of MRS to longitudinally quantify hepatic triglyceride revealed remarkably diet-specific signatures of lipid accumulation. Unlike WD-fed mice, livers of mice fed the KD exhibited early lipid accretion over the first three weeks. After week 6, lipid content increased rapidly within the liver of WD-fed mice, while TG content within KD-fed mice increased at a much slower rate. A key molecular difference between the livers of WD and KD-fed mice, induction of the \textit{de novo} lipogenesis program in liver of WD-fed mice – and its suppression in KD-fed mice – prospectively contributes to differences in hepatic lipid content kinetics, and the histopathological differences between livers of WD- and KD-fed mice. Another potential explanation for differences observed between our study, and those of others that have evaluated the effects of this KD on hepatic and systemic metabolism, is duration – 12 weeks is the longest reported administration of this diet to mice. Changes in the rate of increase in triacylglycerol accumulation also likely reflect altered flux of toxic lipid intermediates, including ceramides and diacylglycerols (46).
How does an extreme ketogenic diet provoke a robust parenchymal inflammatory response, hepatocellular damage, and ER stress, which were not observed in livers of mice fed Western diet? Low protein content (see below) may contribute to the KD-induced injury pattern. However, sustained delivery of high concentrations of fat may trigger inflammation and ER stress-inducing membrane remodeling in periportal hepatocytes that receive more fat than can be oxidized or exported via VLDL secretion (9). Ceramide production within macrophages or hepatocytes, favored by high intracellular concentrations of saturated fatty acids, may also trigger the inflammasome, whose biomarkers were selectively elevated in livers of KD-fed mice, ultimately favoring macrophage recruitment and activation (10, 58). Splice variants of the insulin sensitizing nuclear receptor transcription factor PPARγ exhibit distinct activities in lipogenic contexts (60, 63). The ‘adipocyte’ form, PPARγ2, is induced in liver in the setting of traditional ‘Western’ diet-induced hepatic steatosis, and while selective elimination of PPARγ2 by gene targeting reduces hepatic steatosis, its loss also worsens lipotoxicity and insulin resistance (28, 43, 50, 53, 68). Ligand-induced activation of PPARγ may also ameliorate hepatic ER stress (31, 67). Our results suggest a nutritional switch that regulates hepatic PPARγ splice isoforms. Future studies will determine the mechanisms that coordinate this switch, and the relationship between PPARγ splice variants in livers of WD versus KD-fed mice and the progression of the UPR, injury, inflammation, steatosis, and insulin resistance.

It should be noted that the ketogenic diet studied here would not be studied in or prescribed for humans. Moreover, the minimum protein requirement for normal growth, reproduction, and lactation in mice is ~14% (29). With regard to the development of
NAFLD, low protein content – particularly the amino acid methionine – and prospectively low choline, are recognized as confounding variables of the KD. In humans, diets with caloric contents of up to 75-80% fat and ≥15% protein are commonly used for clinical control of seizure disorders, and Atkins diets typically consist of 60-70% fat and up to 30% protein. Nonetheless, this particular KD is a commonly-studied low-protein formulation for mature mice, and was used here because induction of ketosis, an initial goal of the study, is favored in rodents by low carbohydrate and low protein content (3-5, 8, 12, 14, 34, 35, 57, 62). Future experiments will use high-fat, very low-carbohydrate diets that are higher in protein content, directly comparing their effects to those of the KD used in this study, to determine the influence of low protein content on the hepatic phenotypes we observed. The ability to use animals with genetic perturbations of ketone metabolism will also be helpful to measure the specific role of this metabolic pathway in the systemic response to low-carbohydrate, high-fat diets.

An alternative to carbohydrate/protein content as the basis for differences observed between WD- and KD-fed mice is the selective presence of trans fatty acids in the WD. Indeed, as trans fats independently promote hepatic injury and insulin resistance, it is possible that insulin resistance in livers of KD-fed mice would be evident if the KD also contained trans fat (37). Nonetheless, it is intriguing that, despite the absence of trans fat ingestion, KD-fed mice exhibited features of liver injury that were not observed in livers of WD (trans fat-containing)-fed mice: ER stress, inflammatory cell infiltrates, and hepatocyte loss and regeneration. In future studies, it will also be important to compare low-carbohydrate, high-fat diets that contain trans fatty acids to the effects of similar diets that lack trans fat.
Despite these limitations, study of the ketogenic nutrient milieu in animal models provides key informative insight. With increasing utilization of low- and very low-carbohydrate diets in clinical trials, it is important to explore the prospective scope of unique molecular responses to low-carbohydrate diets, particularly because case reports citing the use of ketogenic diets have illustrated unexpected and dramatic variations of metabolic response in select individuals (7, 13). Moreover, the unusual convergence of phenotypes observed in this study, i.e., mild liver fat accumulation, markers of injury and inflammation, ER stress activation and glucose intolerance, but preserved systemic insulin sensitivity, confirm that this nutritional state will continue to be a useful tool to explore the molecular mechanisms that relate hepatic response to nutrient state, insulin resistance, and adverse cardiovascular events.
ACKNOWLEDGMENTS

The authors thank Kenneth Polonsky, Brian Finck, and Joseph J.H. Ackerman for helpful advice, and Laura Kyro for assistance with graphics. This work was supported in part by NIH grants DK020579, DK073282, and U24 CA83060, and a Pilot and Feasibility Grant from the Diabetic Cardiovascular Disease Center at Washington University.
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31. **Han KL, Choi JS, Lee JY, Song J, Joe MK, Jung MH, and Hwang JK.** Therapeutic potential of peroxisome proliferators--activated receptor-alpha/gamma dual
agonist with alleviation of endoplasmic reticulum stress for the treatment of diabetes.


FIGURE LEGENDS

**Fig. 1.** Reduced body weight and percent body fat in mice fed a very low-carbohydrate, high-fat ketogenic diet for 12 weeks. (A) Body weights at time zero (six weeks of age), after 6 weeks of diet (12 weeks of age), and after 12 weeks of diet (18 weeks of age) in chow-, WD-, and KD-fed mice. (B) Caloric consumption, in kcal/mouse/day, for each diet. Mean consumption was measured on twenty consecutive days. (C) Total body fat percentage and (D) lean mass, measured by MRS in living, awake mice. Data are presented mean±SEM; n=8 mice/group; *, p < 0.05; **, p < 0.01; ***, p < 0.001 by one-way ANOVA with Tukey post-hoc testing, for (A-B), and by two-way ANOVA with Bonferroni post-hoc testing (C-D). For (C-D), open bars: 6 weeks duration on diet; closed bars: 12 weeks duration on diet.

**Fig. 2.** Distinct temporal patterns of hepatic lipid accumulation in ketogenic diet-fed, versus western diet-fed mice. (A) Hepatic lipid content (%), determined by MRS, in anesthetized mice after the indicated number of weeks maintained on the three diets. a, p < 0.001 vs. chow at same time point; b, p < 0.01 (KD vs. WD) at same time point; bb, p < 0.001 (KD vs. WD) at same time point, by two-way ANOVA with Bonferroni post-hoc testing. n=7, 7, and 9 animals/group, in the chow, WD, and KD groups, respectively. (B) Biochemical measurement of hepatic lipid content after 12 weeks of each diet. n=8 mice/group; *, p < 0.05; **, p < 0.01 by one-way ANOVA with Tukey post-hoc testing. Data are presented mean±SEM.

**Fig. 3.** NAFLD-like histopathology in ketogenic diet-fed mice. All micrographs are taken from liver sections of animals fed each diet for 12 weeks. (A) Chow-fed animal, showing normal architecture. Original magnification, 10X. (B) Western diet-fed animal;
note peri-portal steatosis with sparing of zone 3, surrounding central veins. No evidence of inflammation, injury, or regeneration was observed. Original magnification, 10X. (C-I)

Sections from ketogenic diet-fed animals. In (C), note sparse subcapsular steatosis. Original magnification, 10X. (D) Higher-power field, revealing macrovesicular steatosis and inflammation (arrowheads). Original magnification, 20X. (E) Pyknotic (apoptotic) hepatocytes (arrowhead). Original magnification, 60X. (F) Mitotic figure (arrowhead). Original magnification, 60X. Inflammatory foci (arrows) are observed in (G-I) original magnification, 10X, 20X, and 40X, respectively.

**Fig. 4.** Increased accumulation and size of macrophages in livers of ketogenic diet-fed mice. Liver sections from (A) chow-fed, (B) Western diet-fed, and (C) ketogenic diet-fed mice. Fluorescent staining for macrophages (F4/80) is bright red. Original magnification of each panel is 20X. (D) Quantification of number of F4/80+ cells/20X field in each condition; ***, p < 0.001 by ANOVA with Tukey post-hoc testing, for comparison of KD versus chow and versus WD, n=5 20X fields from three independent animals/condition. (E) Quantification of cross-sectional area (CSA, in μm²) of F4/80+ cells; ***, p < 0.001 by ANOVA with Tukey post-hoc testing, for comparison of KD versus chow and versus WD, n=25 cells/section from three replicate animals/condition.

**Fig. 5.** Induction of ER stress in liver of ketogenic diet-fed mice. (A) Ethidium bromide-stained agarose gel separates PCR amplicons derived from spliced and unspliced transcripts of Xbp1 in individual liver samples of chow-, WD-, and KD-fed mice, generated using a traditional primer pair that spans the spliced exon (see Fig. S1A for a schematic overview and Table S1 for primer sequences). NRCM, cultured
primary neonatal rat cardiomyocytes treated with vehicle (0.1% DMSO) or 1.5 μM thapsigargin (Tg) in DMSO for 180 min. (B) Immunoblot for CHOP protein (and Actin loading control) in NRCM controls (V, DMSO vehicle) and individual liver samples from each diet group. (C) Immunoblot for p-eIF2α and total eIF2α in NRCM controls (V, DMSO vehicle) and individual liver samples from each diet group. (D) Quantification of data presented in panel A, plotting ratio of spliced-to-unspliced PCR product intensity. (E) RT-qPCR measurements of the selectively-amplified spliced (Xbp1-S), unspliced (Xbp1-U), and total Xbp1 (Xbp1) cDNAs using selective primer sets (see Fig. S1B for a schematic overview, Table S1 for primer sequences, and Materials and Methods for a description). n=4-5/group. (F) Quantification of data presented in panel B, plotting ratio of CHOP/Actin. (G) Quantification of data presented in panel C, plotting ratio of p-eIF2α/total eIF-2α. *, p < 0.05; **, p < 0.01; ***, p < 0.001 by one-way ANOVA with Tukey post-hoc testing. Data are presented mean±SEM.

Fig. 6. Development of glucose intolerance, but preserved systemic insulin sensitivity, in ketogenic diet-fed mice. (A-B) Intraperitoneal glucose tolerance tests in mice maintained on each diet for (A) 6 weeks and (B) 12 weeks. AUC, mean area under the curve. (C) Intraperitoneal insulin tolerance tests after 12 weeks of chow or the ketogenic diet. *, p < 0.05; **, p < 0.01; ***, p < 0.001 by one-way ANOVA with Tukey post-hoc testing, or Student’s t-test, as appropriate. Data are presented mean±SEM. n=8 mice/group.

Fig. 7. Preserved hepatic Akt response to insulin in ketogenic diet-fed mice. Immunoblot comparisons of p-Akt1 response to insulin in livers of animals fed (A) control chow, versus KD or (C) chow versus WD, for 12 weeks. (B, D) Quantification of
p-Akt/total Akt ratios from panels A and C, respectively. *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA with Bonferroni post-hoc testing. Data are presented mean±SEM. Protein extracts from livers of chow-fed mice are presented in both panels A and C, to independently compare chow vs. KD and chow vs. WD.
### Table 1. Macronutrient composition of mouse diets used in this study

<table>
<thead>
<tr>
<th>Diet†</th>
<th>Vendor (Cat. #)</th>
<th>Carb*</th>
<th>Fat*</th>
<th>Protein*</th>
<th>kcal/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard chow</td>
<td>Lab Diet (5053)</td>
<td>62.1</td>
<td>13.2</td>
<td>24.6</td>
<td>3.4</td>
</tr>
<tr>
<td>Ketogenic diet (KD)</td>
<td>Bio-Serv (F3666)</td>
<td>0.4</td>
<td>95.1</td>
<td>4.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Western diet (WD)</td>
<td>Harlan-Teklad (TD.96132)</td>
<td>40.7</td>
<td>40.6</td>
<td>18.7</td>
<td>4.5</td>
</tr>
</tbody>
</table>

†all diets are irradiated (to sterilize) before feeding

*values represent percentage of total kcal

The ketogenic and Western diets are vitamin and mineral-fortified to match the standard chow diet
Table 2. Serum parameters in chow, WD, and KD-fed mice.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Glucose, mg/dL</th>
<th>Insulin, ng/mL</th>
<th>HOMA-IR</th>
<th>βOHB, mM</th>
<th>FFA, mM</th>
<th>TG, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow, 6 weeks</td>
<td>122.4±6.4</td>
<td>0.97±0.18</td>
<td>7.3±1.6</td>
<td>0.10±0.03</td>
<td>1.37±0.12</td>
<td>61.5±4.4</td>
</tr>
<tr>
<td>WD, 6 weeks</td>
<td>247.1±32.9***</td>
<td>1.97±0.45*</td>
<td>29.6±8.1*</td>
<td>0.27±0.02</td>
<td>0.80±0.15*</td>
<td>110.5±33.1</td>
</tr>
<tr>
<td>KD, 6 weeks</td>
<td>140.9±20.0‡</td>
<td>0.35±0.13*,†</td>
<td>1.5±0.4*,†</td>
<td>1.06±0.24***,‡</td>
<td>1.23±0.29</td>
<td>130.6±16.2**</td>
</tr>
<tr>
<td>Chow, 12 weeks</td>
<td>130.5±5.6</td>
<td>1.14±0.26</td>
<td>9.6±2.0</td>
<td>0.10±0.01</td>
<td>1.03±0.07</td>
<td>102.9±12.0</td>
</tr>
<tr>
<td>WD, 12 weeks</td>
<td>206.1±16.9**</td>
<td>2.33±0.40*</td>
<td>34.6±8.3*</td>
<td>0.10±0.02</td>
<td>0.74±0.05</td>
<td>49.4±4.9*</td>
</tr>
<tr>
<td>KD, 12 weeks</td>
<td>129.2±6.6‡</td>
<td>0.30±0.04*,‡</td>
<td>2.7±0.4*,†</td>
<td>0.61±0.07**,‡</td>
<td>0.68±0.05</td>
<td>71.2±7.3</td>
</tr>
</tbody>
</table>

n = 7-10/group; *, p < 0.05 vs. Chow; **, p < 0.01 vs. Chow; ‼, p < 0.001 vs. Chow; †, p < 0.01 vs. WD; ‡, p < 0.001 vs. WD by two-way ANOVA with Bonferroni post-hoc analysis.

HOMA-IR, homeostatic model of insulin resistance; βOHB, β-hydroxybutyrate; FFA, non-esterified (free) fatty acids; TG, triglycerides.
Table 3. Transcriptional responses of liver from Western versus Ketogenic diet-fed mice

Relative abundance (±SEM), compared to chow, n=5/group

<table>
<thead>
<tr>
<th>Gene</th>
<th>Western diet</th>
<th>Ketogenic diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Central metabolic regulators</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fgf21</td>
<td>17.2±7.9</td>
<td>25.0±4.7</td>
</tr>
<tr>
<td>Ppara</td>
<td>1.2±0.4</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>Ppard</td>
<td>4.8±3.6</td>
<td>1.6±0.4</td>
</tr>
<tr>
<td>Pparg</td>
<td>1.9±0.2</td>
<td>2.5±0.2</td>
</tr>
<tr>
<td>Pparg1</td>
<td>1.2±0.0</td>
<td>3.3±0.3</td>
</tr>
<tr>
<td>Pparg2</td>
<td>4.4±0.5</td>
<td>1.3±0.4</td>
</tr>
<tr>
<td>Ppargc1a</td>
<td>1.5±0.7</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td><strong>De novo lipogenesis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Srebf1</td>
<td>2.9±0.5</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>Fasn</td>
<td>4.7±1.3</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>Acaca</td>
<td>3.0±0.6</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>G6pdx</td>
<td>6.3±1.0</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>Scd1</td>
<td>7.3±0.3</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>Gck</td>
<td>2.9±0.3</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td><strong>Fatty acid oxidation/ketogenesis</strong></td>
<td></td>
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<tr>
<td>Slc27a1</td>
<td>0.9±0.1</td>
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<tr>
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<tr>
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<tr>
<td>Pdk4</td>
<td>6.4±1.1</td>
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<tr>
<td>Cpt1a</td>
<td>1.8±0.2</td>
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<tr>
<td>Acadm</td>
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<td>Hmgcs2</td>
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<tr>
<td><strong>Gluconeogenesis</strong></td>
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<tr>
<td>G6pc</td>
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<td>Pck1</td>
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<td><strong>Inflammation/Fibrosis</strong></td>
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<td>Col1a2</td>
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<td>Il6</td>
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<td><strong>Lipid droplet proteins</strong></td>
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<td>Cidea</td>
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<tr>
<td>Cidec</td>
<td>7.3±2.0</td>
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*p < 0.05, 0.01, and 0.001, respectively, compared to chow Western diet (by ANOVA with Tukey post-hoc testing)
Garbow et al., Fig. 2
Garbow et al., Fig. 4

A. Chow

B. WD

C. KD

D. Mean CSA, μm², F4/80+ cells

E. Mean CSA, μm², F4/80+ cells
Garbow et al., Fig. 5

**A**

*Xbp1 spliced exon-spanning primers*

<table>
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<tr>
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**B**

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**C**

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**D**

![Graph showing the ratio of spliced/unspliced Xbp1 isoforms](image)

**E**

*Xbp1 splice variant-specific primers*

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**G**

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</table>
Garbow et al., Fig. 6

**A.** Serum glucose levels (mg/dL) over time for Chow, WD, and KD diets. AUC values are: Chow 26,439 ± 1205, WD 31,095 ± 1395, KD 33,412 ± 1421.

**B.** Serum glucose levels (mg/dL) over time for Chow, WD, and KD diets. AUC values are: Chow 22,641 ± 1404, WD 28,445 ± 1354, KD 22,525 ± 988.

**C.** Serum glucose levels (% initial value) over time for Chow, WD, and KD diets. AUC values are: Chow, WD, KD.

***Note:*** Graphs illustrate the changes in serum glucose levels across different diets and time points.
Garbow et al., Fig. 7

A

<table>
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<tr>
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<td>Basal</td>
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<tr>
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B

**p-Akt/Akt ratio, arbitrary units**

Chow KD

C

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<td>Basal</td>
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<td>Insulin</td>
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D

**p-Akt/Akt ratio, arbitrary units**

Chow WD