Effects of a ketogenic diet on adipose tissue, liver, and serum biomarkers in sedentary rats and rats that exercised via resited voluntary wheel running

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Low-carbohydrate ketogenic diets (KDs) comprise high amounts of fat with minimal carbohydrates (typically <50 g/day in humans), which sway metabolism to oxidize dietary lipids and adipose lipid stores for energy (4). KDs were developed in the 1920s to combat epileptic seizures (4). However, many current-day research efforts have examined the effects of KDs on whole body adiposity and weight management. For instance, short-term (i.e., 4–6 wk) and long-term (i.e., up to 12 mo) studies (10, 11, 17, 33, 47–49) have demonstrated that KDs induce greater fat loss compared with other diets in overweight individuals. Researchers are also beginning to delve into the mechanistic effects of a KD on adiposity and inflammation in rodent models, and one study reported that mice fed a KD presented lower mRNA expression patterns of inflammatory markers (i.e., TNF-α, IL-6, Emr1, CD68, Itgam, and Nlrp3) in white adipose tissue, while increasing energy expenditure compared with mice fed a standard chow diet (2). However, equivocal data exist (6) that demonstrate that 4 wk of KD feeding in rats increased fat mass, reduced lean mass, reduced HDL (“good”) cholesterol levels, and increased insulin resistance compared with rats fed a low-fat diet. Interestingly, a more recent rodent study (18) examining the effects of three different KDs (fat-protein percentages of diet: 75/10 vs. 65/20 vs. 55/30) reported: 1) the 55/30 group presented a greater retention in whole body nitrogen, as well as an increased carcass protein composition compared with the 75/10 group, and 2) the 55/30 group presented significantly less total carcass mass, as well as liver fat composition compared with the 75/10 group. These data imply that KDs with higher percentages of protein (~25–30%) and lower percentages of fat (~55–60%) may likely result in more favorable alterations in body composition and reduce liver fat accumulation compared with lower-protein/higher-fat KDs. Moreover, these data suggest that there is inconsistency in the scientific literature regarding how ketogenic dieting affects whole body adiposity, as well as general health markers in rodents.

Beyond dieting, physical activity is also known to decrease whole body adiposity, as this was demonstrated in both physically active humans and rodents compared with sedentary counterparts (9, 31). Although there are likely numerous mechanisms at play, increased physical activity levels are shown to affect adipose tissue in the following manners: 1) an increased fat mobilization from adipose tissue (9), and 2) a decreased activation of adipogenesis-related mechanisms (13). Although largely understudied, it stands to reason that the synergistic effects of combining an increase in physical activity, while implementing a KD is an attractive strategy to promote weight loss. Notwithstanding, studies demonstrating the synergistic

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effects of KDs and exercise on body composition are very limited. In fact, only one study to our knowledge (24) examined untrained overweight women that consumed either a KD or a mixed-macronutrient diet for 10 wk while engaging in exercise training (i.e., 60–100 min of various resistance exercise twice a week). The KD group lost fat mass without experiencing alterations in lean mass, whereas their counterparts gained lean body mass but did not lose fat mass. However, it should be noted that the effects of the KD on adipose tissue and/or liver biomarkers were not reported in the aforementioned human study and, thus, remain unexplored with regard to the potential synergistic effects that the KD and exercise may exert on these biomarkers. Therefore, the purpose of this study was to examine the effects of a KD, vs. other diets (Western diet and standard chow), on adipose tissue, liver, and serum parameters in sedentary rats, as well as rats that performed progressively loaded voluntary wheel running. We hypothesized that body mass, feed efficiency, adipose tissue characteristics (i.e., mass, adipocyte size, and protein expression patterns suggestive of decreased lipogenesis-related mechanisms and reduced inflammation), liver characteristics (i.e., triglyceride storage and protein expression patterns suggestive of decreased lipogenesis-related mechanisms and reduced inflammation), and serum biomarkers (i.e., insulin, glucose, and blood lipids) would be the lowest in KD-fed rats due to the potent effects that this diet has on reducing serum insulin levels (38) and that exercise would synergistically improve these parameters.

METHODS

Animal husbandry. Prior to initiating this study, all experimental procedures were approved by Auburn University’s Institutional Animal Care and Use Committee (protocol no. 2015–2612). Male Sprague-Dawley rats (~9–10 wk of age, ~300–325 g) were purchased (Harlan Laboratories, Indianapolis, IN) and allowed to acclimate in the animal housing facility for 1 wk prior to experimentation. During acclimation, animals were provided water and fed a standard rodent chow (24% protein, 58% carbohydrate, 18% fat; Harlan Laboratories) ad libitum in a maintained ambient temperature and constant 12:12-h light-dark cycle.

Dietary and physical activity experimental protocol. For a 6-wk period after acclimation, rats were provided isocaloric amounts with one of three diets: 1) 18 animals were provided 17 g/day of a ketogenic diet (KD) (5.2 kcal/g, 20.2% protein, 10.3% carbohydrate, 69.5% fat; Harlan Laboratories, Teklad TD.96355); 2) 18 animals were provided 20 g/day of a Western diet (WD) (4.5 kcal/g, 15.2% protein, 42.7% carbohydrate, 42.0% fat; Harlan Laboratories, Teklad TD.88137); and 3) 18 animals were fed 30 g/day of the control standard chow diet (SC) (3.1 kcal/g, 24.0% protein, 58.0% carbohydrate, 18% fat; Harlan Laboratories, Teklad no. 2018). A more in-depth description of the diets can be found in Table 1 below.

Ten rats from each dietary group above were double housed without a running wheel and considered sedentary (SED). Eight from each dietary group above were individually housed with a resistance loaded voluntary running wheel (Lafayette Instrument, Lafayette, IN, USA) (EX).

Skeletal muscle adaptations (but not adipose tissue, liver, or serum adaptations) between SED and EX animals in the KD and WD groups have been reported previously in detail (38), as has the exercise protocol for EX rats. Briefly, the EX group performed free-wheel voluntary running for 7 days during an acclimation phase to the activity cages (noted as days –7 to –1). Thereafter, the prescribed diets were assigned and progressive wheel resistance (as percent body mass) was applied from days 0 to 42 of the study (Fig. 2 in the RESULTS). Our previous study demonstrated that this form of progressively loaded voluntary wheel running elicited muscle hypertrophy and, thus, serves as a viable model for voluntary resistance exercise in rats.

Running distance for the EX group as well as the amount of food eaten for all rats were recorded daily. Finally, running wheels were locked 24 h before the EX animals were killed to prevent exercise-induced signaling phenomena from being detected in adipose tissue and/or liver samples.

Necropsies. On the morning of necropsies, rats were food-deprived for 6–10 h but provided water ad libitum. Animals were then transported from the campus vivarium to the School of Kinesiology and allowed to acclimate for 2 h. Thereafter, rats were euthanized under CO2 gas in a 2-liter induction chamber (VetEquip, Pleasanton, CA). Following euthanasia, a final body mass was recorded, and blood was collected from the heart using a 22-gauge syringe. Collected blood was placed in a 6-ml serum separator tube (SST). SST blood was centrifuged at 3,500 g for 10 min, and resultant serum was then aliquoted into 1.7-ml microcentrifuge tubes and stored at –80°C until analyte analysis. The right-side inguinal/subcutaneous adipose tissue (SQ), visceral/omental adipose tissue (OMAT), and scapular brown adipose tissue (BAT) depots were dissected out, and weights were recorded using a calibrated scale with a sensitivity of 0.0001 g (Mettler-Toledo; Columbus, OH). The liver was also removed for analysis but was not weighed.

Histological analysis. Approximately 1–2-cm sections were obtained from each of the SQ, OMAT, and BAT depots and placed in 10% formalin and preserved for histological analyses. Briefly, samples were removed from formalin and then washed in cold running tap water, embedded, and stored in 70% alcohol. Dehydration was accomplished by gradually increasing percentages of ethyl alcohol to replace the water content in the tissue. Hematoxylin and eosin, a final body mass was recorded, and blood was collected from the heart using a 22-gauge syringe. Collected blood was placed in a 6-ml serum separator tube (SST). SST blood was centrifuged at 3,500 g for 10 min, and resultant serum was then aliquoted into 1.7-ml microcentrifuge tubes and stored at –80°C until analyte analysis. The right-side inguinal/subcutaneous adipose tissue (SQ), visceral/omental adipose tissue (OMAT), and scapular brown adipose tissue (BAT) depots were dissected out, and weights were recorded using a calibrated scale with a sensitivity of 0.0001 g (Mettler-Toledo; Columbus, OH). The liver was also removed for analysis but was not weighed.

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Physiological Effects of a Ketogenic Diet and Exercise

Immunochemistry for OMA, SQ, and BAT uncoupling protein-1 (UCP-1) protein expression was performed as previously described elsewhere (34). Briefly, additional adipose tissue sections were prepared and placed on microscope glass slides as described above and then deparaffinized and rehydrated by boiling in a 6.0 pH solution of 10 mM sodium citrate with 0.05% Tween-20 for 30 min. Tissues were then incubated for 10 min in 3% hydrogen peroxide to inhibit endogenous horseradish peroxidase (HRP) activity. Tissue sections were blocked in normal serum and then incubated for 1 h with a primary antibody (rabbit anti-UCP-1, 1:100; Abcam, Cambridge, MA). A commercial kit (LSAB+ kit; Dako Cytomation, Carpinteria, CA) with rabbit polyclonal IgG (Abcam), and 3,3'-diaminobenzidine was used for secondary antibody staining. Tissue sections were then restained with hematoxylin, dehydrated, and enclosed with a coverslip and mounting media. Two 10× digital images per sample were obtained, as described above, and UCP-1 staining intensities (% image stained) were quantified using ImageJ (National Institutes of Health).

Western blot analysis. Another ~100 mg of tissue from each adipose depot and the liver was placed in 500 μl of 1 × cell lysis buffer (Cell Signaling, Danvers, MA) with added protease inhibitors (1 μg/ml leupeptin) and phosphatase inhibitors (2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate) and was homogenized in microcentrifuge tubes using tight-fitting pestles. Samples were centrifuged at 500 g for 5 min at 4°C. Supernatants were then subjected to a protein assay using a commercial bicinchoninic acid assay (ThermoFisher Scientific, Waltham, MA) and were prepared for Western blotting using 4X Laemmli reducing buffer (C.B.S. Scientific, San Diego, CA). Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad), and membranes were stained with Ponceau S following transfers to ensure even loading and transfer between samples. Membranes were then blocked with 5% nonfat milk powder diluted in TBS with 0.1% Tween-20 (TBST) for 1 h at room temperature. Primary antibodies directed against the proteins of interest were incubated with membranes overnight at 4°C in TBST with 5% BSA added. The primary antibodies used included anti-CC20 (1:1000; CEBPa, no. D56F10, Cell Signaling), anti-α-fatty acid synthase (FASN, no. C20G5; Cell Signaling), anti-acetyl CoA carboxylase (ACC, no. C83B10; Cell Signaling), anti-phospho-nuclear factor kappa beta p65 [phospho-p65 (Ser-536), no. 931H; Cell Signaling], anti-nuclear factor kappa beta p65 (p65, no. D14E12; Cell Signaling), anti-CD36 (no. ab78054, Abcam), anti-phospho-hormone-sensitive lipase [p-HSL (Ser-563), no. 4139, Cell Signaling], anti-hormone-sensitive lipase (HSL, no. 4107, Cell Signaling), anti-phospho-AMP-activated protein kinase alpha [p-AMPKα (Thr-172), no. 40H9, Cell Signaling], and anti-AMP-activated protein kinase alpha (AMPKα, no. DS5A2, Cell Signaling). On the following day, membranes were incubated with anti-rabbit or anti-mouse IgG secondary antibodies diluted in TBST with 5% BSA added (1:2,000; Cell Signaling) at room temperature for 1 h prior to membrane development. Membrane development was accomplished by using an enhanced chemiluminescent reagent (Amersham, Pittsburgh, PA), and band densitometry was achieved with the use of a digitized gel documentation system and associated densitometry software (UVP, Upland CA). CEBPa, FASN, ACC, and CD36 band densities were normalized to Ponceau stain densities, whereas phosphorylated protein densities were normalized to pan (or total) protein densities. All Western blot analysis data are presented as arbitrary density units.

Liver triglyceride assessment. Liver tissue (~50 mg) was pulverized on a liquid nitrogen-cooled stage, weighed, and homogenized with chloroform/methanol (2/1) to a final volume of 20× the volume of the tissue sample. After homogenization, the mixture was vortexed for 5 s every 5 min for 20 min and then centrifuged at 10,000 g for 10 min. The supernatant transferred to a new tube containing 200 μl of 0.9% NaCl solution. This mixture was vortexed and then centrifuged at a low speed (2,000 rpm) for 5 min to separate the two phases. The upper phase was removed via siphoning, and the lower chloroform phase containing the lipids was evaporated under a vacuum. The resulting dried lipids were reconstituted in 500 μl of tert-butanol: Triton X114 mix stock (6 ml tert-butanol: 4 ml Triton X114) and were vortexed. The resulting lipid mixture for each sample was used for the triglyceride analysis from a commercially available triglyceride colorimetric kit (Cayman Chemical, Ann Arbor, MI), according to manufacturer’s instructions. Liver triglyceride content was expressed as milligrams triglycerides per milligram wet liver mass.

Histology and UCP-1 immunochemistry. Immunohistochemistry for OMA, SQ, and BAT uncoupling protein-1 (UCP-1) protein expression was performed as previously described elsewhere (34). Briefly, additional adipose tissue sections were prepared and placed on microscope glass slides, as described above and then deparaffinized and rehydrated by boiling in a 6.0 pH solution of 10 mM sodium citrate with 0.05% Tween-20 for 30 min. Tissues were then incubated for 10 min in 3% hydrogen peroxide to inhibit endogenous HRP activity. Tissue sections were blocked in normal serum and then incubated for 1 h with a primary antibody (rabbit anti-UCP-1, 1:100; rabbit anti-rat UCP-1, no. ab10983; Abcam). A commercial kit (LSAB+ kit; Dako Cytomation) with rabbit polyclonal IgG (no. ab171870; Abcam), and 3,3'-diaminobenzidine was used for secondary antibody staining. Tissue sections were then restained with hematoxylin, dehydrated, and enclosed with a coverslip and mounting media. Two 10× digital images per sample were obtained, as described above, and UCP-1 staining intensities (% image stained) were quantified using ImageJ (National Institutes of Health).

Serum analyses. Commercial beta-hydroxybutyrate (BHB) colorimetric assay kits (Cayman Chemical) were used to determine blood ketone levels, and ELISAs (EMD Millipore, Billerica, MA) were used to analyze serum insulin levels. A serum chemistry profile (i.e., glucose, triglycerides, total cholesterol, ALT) was provided by Auburn University’s Veterinarian School using an automated chemistry analyzer (Roche Cobas C311; Roche Diagnostics, Indianapolis, IN).

Statistics. All dependent variables were presented as means ± SE. Unless otherwise stated in the results, statistical comparisons of all dependent variables were run using two-way ANOVAs [diet (WD vs. KD) × activity (SED vs. EX)] using SPSS v 22.0 (IBM, Armonk, NY). If a main effect for diet was observed, pairwise comparisons using Tukey post hoc tests were performed to determine which diets were statistically different. If a main effect for activity was observed, pairwise comparisons between SED vs. EX were performed. If a significant diet × activity interaction was observed: 1) one-way ANOVAs in the SED and EX treatments were performed, and a Tukey post hoc test was performed to determine which diets were statistically different. If a main effect for activity was observed, pairwise comparisons between SED vs. EX were performed. Because ketogenic diet putatively increases serum ketones and reduces serum insulin, and both of these phenomena have been posited to facilitate adipose tissue adaptations, select bivariate correlations were performed throughout to examine whether associations existed between serum insulin or BHB levels and various phenotypes. For all statistics, significance was set at P ≤ 0.05.

RESULTS

Body mass and feed efficiency. For visual simplicity, body masses throughout the 6-wk study in 3-day intervals are presented in Fig. 1A (SED rats only) and 1B (EX rats only). KD SED rats weighed less than SC SED and WD SED rats from days 4–40 (P < 0.05). KD EX rats weighed less than SC EX and WD EX rats from days 15–40 (P < 0.05). There was also
a diet effect ($P < 0.05$) for body mass at the time of death (Fig. 1C) and feed efficiency (total grams body mass gain/total kcal consumed over 6 wk) (Fig. 1D). Regarding notable significant diet effects, the following observations were noted: 1) WD- and SC-fed rats weighed 22% and 15% more, respectively, at the end of the intervention compared with KD-fed rats, 2) WD- and SC-fed rats had ~130% greater feed efficiencies at the end of the intervention compared with KD-fed rats. There were no activity effects or diet $\times$ activity interactions for body mass and feed efficiency.

Wheel-running distances in EX rats over the 6-wk intervention. Of note, the running distances of the WD EX and KD EX groups have been reported elsewhere, whereby we analyzed the muscle characteristics from these two groups of rats (38). In the current study, a one-way ANOVA indicated that total running distance over the 6-wk intervention was not different between the KD EX, WD EX, or SC EX groups ($P = 0.70$; Fig. 2B).

Energy and macronutrient intake. There was a diet effect ($P < 0.05$) for total calories (Fig. 3A), total protein (Fig. 3B), total carbohydrates (Fig. 3C) and total fat (Fig. 3D) consumed during the 6-wk intervention. Regarding notable significant diet effects, the following observations were noted: 1) WD- and KD-fed rats consumed 10% and 13% more calories, respectively, over the intervention compared with SC-fed rats; 2) SC-fed rats consumed 28% and 39% more protein, respectively, over the intervention compared with KD- and WD-fed rats, and KD-fed rats consumed 28% more protein compared with WD-fed rats; 3) SC-fed rats consumed 416% and 20% more carbohydrates, respectively, over the intervention compared with KD- and WD-fed rats, and WD-fed rats consumed 328% more carbohydrates compared with KD-fed rats; and 4) KD-fed rats consumed 60% and 321% more fat, respectively, over the intervention compared with WD- and SC-fed rats, and WD-fed rats consumed 163% more fat compared with SC-fed rats. There was an activity effect ($P < 0.05$) for total fat consumed during the 6-wk intervention (Fig. 3D). Specifically, SED animals consumed 4% more fat over the intervention. There was a diet $\times$ activity interaction ($P < 0.05$) for total calories (Fig. 3A), total protein (Fig. 3B), total carbohydrates (Fig. 3C) and total fat (Fig. 3D) consumed during the 6-wk intervention, and between- and within-diet effects are noted in Fig. 3 by superscript letters.

OMAT masses and adipocyte diameters. There were diet effects ($P < 0.05$) for absolute OMAT mass (Fig. 4A), relative (body mass-corrected) OMAT mass (Fig. 4B), average OMAT adipocyte diameter (Fig. 4C), and OMAT adipocyte diameters between 40 and 59.99 $\mu$m, 80 and 99.99 $\mu$m, and greater than 100 $\mu$m (Fig. 4, D and E). Regarding notable significant diet effects, the following observations were noted: 1) WD-fed rats presented a 93% greater absolute OMAT mass compared with KD-fed rats and a 37% greater absolute OMAT mass compared

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**Fig. 1.** Body masses and feed efficiency over the 6-wk dietary intervention. Values for a 3-day body mass for sedentary (SED) and exercised (EX) rats is presented in A and B, respectively. C: terminal body mass is presented. D: feed efficiency over the duration of the study is presented. For A and B, asterisks denote WD and SC > KD ($P < 0.05$). WD, Western diet; KD, ketogenic diet; SC, standard chow.
with SC-fed rats, and 2) WD- and SC-fed rats presented ~30% greater OMAT diameters compared with KD-fed rats. There was an activity effect ($P < 0.05$) for absolute OMAT mass (Fig. 4A), relative (body mass corrected) OMAT mass (Fig. 4B), and OMAT adipocyte diameters greater than 100 $\mu$m (Fig. 4, D and E). Specifically, SED animals presented ~50% greater absolute and relative OMAT masses compared with EX rats. There were no diet $\times$ activity interactions for OMAT mass or OMAT adipocyte variables.

**SQ masses and adipocyte diameters.** There was a diet effect ($P < 0.05$) for absolute SQ mass (Fig. 5A) and SQ adipocyte diameters between 0–19.99 $\mu$m and 20–39.99 $\mu$m (Fig. 5, D and E). Regarding notable significant diet effects, WD-fed rats presented a 54% greater absolute SQ mass compared with

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**Fig. 2.** Study design and running distances for EX rats. For exercised (EX) rats, the voluntary running wheel resistance load is presented in A, and actual running distances are presented in B. Of note, no differences were observed for total running distances over the course of the intervention between diet groups (ANOVA; $P = 0.70$).

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**Fig. 3.** Energy and macronutrient intakes over the 6-wk dietary intervention. A: total energy consumed over the 6-wk intervention is presented. B: total protein consumed over the 6-wk intervention is presented. C: total carbohydrate (CHO) consumed over the 6-wk intervention is presented. D: total fat consumed over the 6-wk intervention is presented. For all panels, $P$ values from the two-way ANOVA (i.e., main diet and activity effects and diet $\times$ activity interactions) are presented in the top left corner, main diet effects are noted with brackets above bars, and bars that do not share the same superscript letters are significantly different ($P < 0.05$).
KD-fed rats. There was an activity effect (P < 0.05) for SQ adipocyte diameters greater than 100 μm (Fig. 5, D and E). There was a diet×activity interaction (P < 0.05) for SQ adipocyte diameters between 80 and 99.99 μm, and within-group analyses indicated that EX decreased diameter size within WD-fed rats (P < 0.05), whereas this did not occur in other dietary groups (Fig. 5, D and E).

BAT masses and adipocyte diameters. There was a diet effect (P < 0.05) for absolute BAT mass (Fig. 6A), average BAT diameter (Fig. 6C), and BAT adipocyte diameters be-

Fig. 4. Omental (OMAT) mass and adipocyte diameter. A: raw OMAT mass is presented. B: Relative OMAT mass (corrected for body mass) is presented. C: average OMAT adipocyte diameter is presented. D: OMAT adipocyte diameter range for SED rats is presented. E: OMAT adipocyte diameter range for EX rats is presented. F: Representative 10× objective images of OMAT adipocyte diameters from SED and EX rats are presented (white bar = 100 μm). For A–C, P values from the two-way ANOVA (i.e., main diet and activity effects and diet×activity interactions) are presented in the top left corner, and main diet effects are noted with brackets above bars. For panels D and E, superscript deltas (Δ) indicate a diet effect, whereby KD is statistically different from WD and SC (P < 0.05).

Fig. 5. Inguinal/subcutaneous adipose tissue (SQ) masses and adipocyte diameters. A: raw SQ mass is presented. B: relative SQ mass (corrected for body mass) is presented. C: average SQ adipocyte diameter is presented. D: SQ adipocyte diameter range for SED rats is presented. E: SQ adipocyte diameter range for EX rats is presented. F: representative 10× objective images of SQ adipocyte diameters from SED and EX rats are presented (white bar = 100 μm). For A–C, P values from the two-way ANOVA (i.e., main diet and activity effects and diet×activity interactions) are presented in the top left corner, and main diet effects are noted with brackets above bars. For panels D and E, superscript deltas (Δ) indicate a diet effect, whereby KD is statistically different from WD and SC (P < 0.05), and the asterisk (*) indicates that SC EX is statistically different from SC SED (P < 0.05).
between 0–19.99 μm and 20–39.99 μm (Fig. 6, D and E). Regarding notable significant diet effects, the following was observed: 1) WD-fed rats presented a 22% greater absolute BAT mass compared with KD-fed rats, and 2) WD-fed rats presented a 21% greater average BAT adipocyte diameter compared with SC-fed rats. There was an activity effect (P < 0.05) for absolute BAT mass (Fig. 6A), relative BAT mass (Fig. 6B) and BAT adipocyte diameters between 0 and 19.99 μm (Fig. 6, D and E). Specifically, SED rats presented a 20% greater absolute BAT mass and 15% greater relative BAT mass compared with EX rats. There were no diet x activity interactions for BAT mass or BAT adipocyte variables.

OMAT protein expression patterns. There was an activity effect (P < 0.05) for OMAT ACC (Fig. 7A), CEBPα (Fig. 7C), phospho/total p65 (Fig. 7D) phospho/total AMPKα (Fig. 7E), and phospho/total HSL (Fig. 7F) protein expression patterns. Specifically, SED rats presented 61% lower ACC levels, 107% greater CEBPα levels, 45% lower phospho/total p65 levels, 173% greater phospho/total AMPKα levels, and 69% greater phospho/total AMPKα levels compared with EX rats. There were no main effects for OMAT CD36 protein levels (Fig. 7B), nor were there diet x activity interactions for any of the measured OMAT proteins.

SQ protein expression patterns. There were diet effects (P < 0.05) for SQ CD36 (Fig. 8B) and phospho/total HSL (Fig. 8F) protein expression levels. Regarding notable significant diet effects, the following was observed: 1) WD-fed rats presented 58% greater CD36 protein levels compared with KD-fed rats, and 2) SC-fed rats presented 284% and 109% greater phospho/total HSL protein levels, respectively, compared with WD- and KD-fed rats. There were activity effects (P < 0.05) for SQ CD36 (Fig. 8B), CEBPα (Fig. 8C), and phospho/total AMPKα (Fig. 8E) protein expression patterns. Specifically, SED rats presented 25% lower CD36 levels, 654% greater CEBPα levels, and 73% greater phospho/total AMPKα levels. There were no main effects for SQ ACC or phospho/total p65 protein levels (Fig. 8, A and D), nor were there diet x activity interactions for any of the measured SQ proteins.

OMAT, SQ, and BAT UCP-1 immunohistochemistry. There was an activity effect (P < 0.05) for OMAT and BAT UCP-1 histological protein expression (Fig. 9, A and C). Specifically, SED rats had 12% less OMAT UCP-1 staining and 25% less BAT UCP-1 staining compared with EX rats. There were no main effects for SQ UCP-1 histological protein expression (Fig. 9B), nor were there diet x activity interactions for UCP-1 histological protein expression in any depots measured.

Liver triglycerides and protein expression patterns. There was a diet effect (P < 0.05) for liver triglycerides (Fig. 10A) and phospho/total p65 protein expression (Fig. 10D). Regarding notable significant diet effects, the following was observed: 1) WD-fed rats presented 165% and 107% greater liver triglycerides, respectively, compared with KD- and SC-fed rats, and 2) WD- and SC-fed rats presented 175% and 326% greater phospho/total p65 protein levels, respectively, compared with KD-fed rats. There was an activity effect (P < 0.05) for liver phospho/total p65 protein expression (Fig. 10D). Specifically, SED rats presented 105% greater phospho/total p65 protein levels compared with EX rats. There were no main effects for liver FASN (Fig. 10B), liver ACC (Fig. 10C), or liver phospho/total AMPKα (Fig. 10E) protein expression, nor were there diet x activity interactions for any of the measured liver proteins.

Serum biomarkers. Serum biomarkers are presented in Table 2. There were diet effects (P < 0.05) for insulin, glucose, BHB, triglycerides, total cholesterol, and ALT. Regarding notable significant diet effects, the following was observed: 1) WD- and SC-fed rats presented 203% and 218% greater insulin, respectively, compared with KD-fed rats; 2) WD- and SC-fed rats presented 35% and 43% greater glucose, respec-
tively, compared with KD-fed rats; 3) WD- and KD-fed rats presented 73% and 145% greater BHB, respectively, compared with SC-fed rats; 4) WD-fed rats presented 253% and 73% greater triglyceride levels compared with KD- and SC-fed rats, respectively, and SC-fed rats presented 104% greater triglyceride levels compared with KD-fed rats; 5) WD- and SC-fed rats presented 20% and 31% greater total cholesterol levels, respectively, compared with KD-fed rats; and 6) SC-fed rats presented 43% and 49% greater ALT levels compared with KD- and WD-fed rats, respectively. There were activity

Fig. 7. OMAT protein expression patterns. A: OMAT acetyl-CoA carboxylase (ACC) protein expression patterns are presented. B: OMAT cluster of differentiation/fatty acid translocase (CD36) protein expression patterns are presented. C: OMAT CCAAT/enhancer-binding protein alpha (CEBPα) protein expression patterns are presented. D: OMAT phosphorylated/pan-p65 protein expression patterns are presented. E: OMAT phosphorylated/pan 5′ AMP-activated protein kinase alpha 1 subunit (AMPKα) protein expression patterns are presented. F: OMAT phosphorylated/pan hormone-sensitive lipase (HSL) protein expression patterns are presented. G: representative Western blot analysis images are presented. For panels A–F, P values from the two-way ANOVA (i.e., main diet and activity effects and diet × activity interactions) are presented in the top left corner.
effects ($P < 0.05$) for insulin, BHB, triglycerides, and total cholesterol. Specifically, SED rats presented 49% greater insulin levels, 59% greater BHB levels, 112% triglyceride levels, and 11% greater total cholesterol levels compared with EX rats. There were diet $\times$ activity interactions for triglycerides and total cholesterol, and between- and within-diet effects are noted in Table 2 by superscript letters and symbols.

Fig. 8. SQ protein expression patterns. A: SQ ACC protein expression patterns are presented. B: SQ CD36 protein expression patterns are presented. C: SQ CEBPα protein expression patterns are presented. D: SQ phosphorylated/pan-p65 protein expression patterns are presented. E: SQ phosphorylated/AMPKα protein expression patterns are presented. F: SQ phosphorylated/pan HSL protein expression patterns are presented. G: representative Western blotting images are presented. For panels A–F, $P$ values from the two-way ANOVA (i.e., main diet and activity effects and diet $\times$ activity interactions) are presented in the top left corner, and main diet effects are noted with brackets above bars.
Select bivariate correlations of serum insulin or BHB with body and adipose tissue masses. Correlations between serum insulin vs. select whole body or adipose tissue parameters are presented in Fig. 11. Serum insulin was positively associated with body mass (\( r = 0.54, P = 0.05 \)), feed efficiency (\( r = 0.57, P = 0.05 \)), relative OMAT mass (\( r = 0.57, P = 0.05 \)), and relative SQ mass (\( r = 0.31, P = 0.05 \)). Serum BHB was not associated with body mass (\( r = 0.14, P = 0.35 \)), feed efficiency (\( r = -0.02, P = 0.89 \)), relative OMAT mass (\( r = -0.03, P = 0.83 \)), or relative SQ mass (\( r = 0.06, P = 0.67 \)) (data not shown).

**DISCUSSION**

The purpose of this study was to examine the effects of a KD vs. other diets, on adipose tissue, liver, and blood parameters in sedentary rats, as well as rats that performed progressively loaded voluntary wheel running. Irrespective of exercise intervention, KD-fed rats exhibited significantly lower: 1) body mass and feed efficiencies, 2) average OMAT adipocyte size, and 3) serum insulin, triglyceride, and total cholesterol levels. Regarding notable diet×activity interactions, the following was observed: 1) EX decreased serum triglycerides within the WD and SC groups, and 2) EX decreased serum total cholesterol within the WD group. It should be finally noted that, in all animals, there were significant positive associations with serum insulin vs. body mass, feed efficiency, relative OMAT mass, and relative SQ mass; this underscores the potential role that insulin has in accruing body mass and fat mass and why ketogenic dieting may be effective in weight maintenance or decreases in adiposity. However, contrary to our hypothesis, KD-fed EX rats did not experience an attenuated gain in body mass and/or the measured adipose tissue depot masses and/or improvements in adipose tissue, liver and/or serum biomarkers. These findings are discussed in greater detail below.
gain. Moreover, while we did not utilize whole body scanning to assess how the dietary and/or exercise interventions affected whole body fat and lean tissue mass, there are rodent data suggesting that mice fed a ketogenic diet over a 12-wk period contained less whole body fat compared with mice fed a Western diet, whereas body fat was not different in ketogenic diet-fed vs. standard chow-fed mice (27). While the mechanism of lowered feed efficiency was not ascertained in the current study, past research has elucidated potential mechanisms responsible for KD-induced alterations in metabolic characteristics which, in turn, could lower feed efficiency. For instance, Jornayvaz et al. (26) measured 6-h energy expenditures in KD-fed vs. SC-fed mice after a 5-wk feeding period and reported that KD-fed animals presented a 17% increase in energy expenditure compared with SC-fed mice despite similar activity counts. Hence, these data suggest a potential KD-induced increase in thermogenesis that is independent from physical activity. Frommelt et al. (18), while not measuring energy expenditure, reported that rats fed a lower-protein ketogenic diet over a 4-wk period presented urine that contained 70% more gross energy compared with rats fed standard chow. Hence, relating the aforementioned findings to our

Table 2. Serum biomarkers

<table>
<thead>
<tr>
<th>Serum Biomarker</th>
<th>SED</th>
<th>EX</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin, ng/ml</td>
<td>WD: 7.3 ± 1.3</td>
<td>WD: 3.4 ± 0.9</td>
<td>Diet P &lt; 0.05 (WD = SC &gt; KD)</td>
</tr>
<tr>
<td></td>
<td>KD: 0.9 ± 0.2</td>
<td>KD: 3.0 ± 0.4</td>
<td>Activity P &lt; 0.05 (SED &gt; EX)</td>
</tr>
<tr>
<td></td>
<td>SC: 7.3 ± 1.0</td>
<td>SC: 4.0 ± 0.9</td>
<td>D•A P = 0.58</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>WD: 12.3 ± 0.9</td>
<td>WD: 12.9 ± 1.3</td>
<td>Diet P &lt; 0.05 (WD = SC &gt; KD)</td>
</tr>
<tr>
<td></td>
<td>KD: 7.6 ± 0.4</td>
<td>KD: 11.3 ± 1.0</td>
<td>Activity P = 0.48</td>
</tr>
<tr>
<td></td>
<td>SC: 14.2 ± 1.5</td>
<td>SC: 12.1 ± 2.4</td>
<td>D•A P = 0.06</td>
</tr>
<tr>
<td>BHB, mM</td>
<td>WD: 0.45 ± 0.07</td>
<td>WD: 0.30 ± 0.03</td>
<td>Diet P &lt; 0.05 (WD = KD &gt; SC)</td>
</tr>
<tr>
<td></td>
<td>KD: 0.65 ± 0.20</td>
<td>KD: 0.40 ± 0.04</td>
<td>Activity P &gt; 0.05 (SED &gt; EX)</td>
</tr>
<tr>
<td></td>
<td>SC: 0.27 ± 0.02</td>
<td>SC: 0.16 ± 0.03</td>
<td>D•A P = 0.34</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>WD: 320 ± 35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>WD: 111 ± 16&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>Diet P &lt; 0.05 (WD &lt; SC &gt; KD)</td>
</tr>
<tr>
<td></td>
<td>KD: 70 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>KD: 57 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Activity P &lt; 0.05 (SED &lt; EX)</td>
</tr>
<tr>
<td></td>
<td>SC: 163 ± 21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>SC: 92 ± 17&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>D•A P &lt; 0.05 (symbols denoted)</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>WD: 89.9 ± 3.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>WD: 67.6 ± 2.3&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>Diet P &lt; 0.05 (WD = SC &gt; KD)</td>
</tr>
<tr>
<td></td>
<td>KD: 67.7 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>KD: 65.8 ± 3.3&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>Activity P &lt; 0.05 (SED &lt; EX)</td>
</tr>
<tr>
<td></td>
<td>SC: 87.0 ± 5.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>SC: 87.7 ± 5.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>D•A P &lt; 0.05 (symbols denoted)</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>WD: 49.3 ± 5.8</td>
<td>WD: 42.5 ± 1.9</td>
<td>Diet P &lt; 0.05 (SC &lt; KD = WD)</td>
</tr>
<tr>
<td></td>
<td>KD: 46.0 ± 4.2</td>
<td>KD: 50.6 ± 3.1</td>
<td>Activity P = 0.36</td>
</tr>
<tr>
<td></td>
<td>SC: 65.0 ± 3.5</td>
<td>SC: 73.4 ± 5.7</td>
<td>D•A P = 0.11</td>
</tr>
</tbody>
</table>

Values for SED and EX are given as means ± SE. SED, sedentary rats; EX, exercised rats; BHB, β-hydroxybutyrate; ALT, alanine transaminase; D•A, diet•activity interaction. Superscript letters that differ within SED or EX groups indicate significant between-diet differences (P < 0.05). Asterisks (*) denote a within-diet alteration between the SED and EX condition. Bolded values indicate biomarkers that presented a significant D•A interaction.
findings suggests that KD-fed rats could have presented increases in either thermogenesis and/or an increase in urine energy excretion to lower feed efficiency.

Regarding adipose tissue masses, KD-fed rats presented lower raw OMAT and SQ masses and lower relative OMAT masses compared with the WD-fed rats. Adipose tissue expands via hypertrophy or hyperplasia, with visceral adipocyte hypertrophy being the most deleterious regarding health consequences (13). The KD-fed rats in our study had significantly smaller OMAT average adipocyte sizes compared with the WD- and SC-fed rodents, but SQ adipocyte sizes were similar between diets. Indeed, smaller adipocytes are more insulin-sensitive than larger adipocytes based upon previous research in visceral adipocytes (40). Enlarged visceral adipocytes are shown to contribute to increased blood lipid concentrations (41) and, in obese states, adipocytes reach maximal expanding capacity (42), thereby inhibiting storage of new lipids and causing a dysfunctional increase in lipolysis rates (15). These events lead to an accumulation of free fatty acids in the plasma (15), which may result in an increased risk for Type 2 diabetes and cardiovascular disease (14, 39). Although we did not assess adipocyte insulin sensitivity or whether larger adipocyte size was associated with increases in serum-free fatty acid levels, it is noteworthy to mention that KD rats, which had lower OMAT-average adipocyte sizes, presented lower levels of serum glucose, serum insulin, and serum lipid (i.e., triglyceride and cholesterol) levels compared with the other dietary treatments. The ability of KD feeding to reduce glucose and insulin levels has been well established (16, 36). However, it is paradoxical that KD-fed rats, which consumed substantially more dietary fat, presented significant decrements in serum triglyceride and total cholesterol levels. Indeed, this too has been reported in rodent literature (27), and it could be related to KD-induced reductions in insulin which, in turn, decreases liver fatty acid and cholesterol biosynthesis pathways. Notwithstanding, these data continue to suggest that, at least in an animal model, KD feeding may confer positive adaptations related to improving metabolic outcomes.

We also sought to examine how the different diets with or without exercise affected protein expression levels related to lipogenic (CEBPα, ACC, CD36), lipolytic (phospho/pan-AMPKα and phospho/pan-HSL), inflammatory (phospho/pan-p65), and thermogenic (UCP-1) processes. Remarkably, while KD-fed rats presented lower adipose tissue masses compared with WD-fed rats and lower OMAT adipocyte diameters compared with both diets, most of the assayed proteins did not reveal a diet effect (with the exception of SQ CD36 being lower in KD vs. WD rats). While this finding is difficult to reconcile, this may be due to the sampling time point of our study, whereby changes in the aforementioned proteins (or associated genes) occurred earlier in the intervention. Moreover, there is evidence to suggest that KD-induced metabolic alterations are largely due to increases in the liver production of and subsequent increases in serum fibroblast growth factor 21 (FGF21) levels. In this regard, Badman et al. (3) reported that 1) mice fed a KD for 30 days presented robust liver FGF21 mRNA expression levels compared with mice fed a standard chow diet, and 2) adenoviral knockdown of hepatic FGF21 mRNA levels in mice fed a KD reduced circulating FGF21 levels, decreased liver mRNAs for hydroxyacyl-CoA dehydrogenase, and long- or medium-chain acyl-CoA dehydrogenase, and robustly increased serum and liver triglyceride levels. Moreover, FGF21 treatments over a 7-day period in mice has been shown to elicit dose-dependent effects on weight loss, and this effect is abrogated in adipose tissue-specific FGF receptor-knockout mice (1). Thus, potential KD-induced increases in liver FGF21 production and secretion, as well as potential increases adipose tissue FGF signaling could be responsible for the KD-induced metabolic phenomena observed in the current study. However, these phenomena were not assessed in the current study and, thus, we cannot firmly assert these hypotheses. Hence, it will be fruitful to examine time course studies related to how ketogenic dieting affects adipose tissue signaling markers assessed in the current study, as well as serum FGF21 levels and adipose tissue signaling.
events that occur as an effect of KD-induced increases in this ligand. 

Effects of progressive voluntary wheel running on adipose tissue characteristics. Raw and relative OMAT masses, as well as very large SQ and OMAT adipocytes (>100 μm in diameter), were reduced in the EX rodents compared with the SED rodents, independent of diet. This is noteworthy given the aforementioned associations between large adipocytes and potential metabolic consequences. Notwithstanding, a physical activity-induced decrease in visceral adiposity with up to 4 wk of unloaded voluntary wheel running in rats has been previously reported by our group (13, 35), so our current findings are not unfounded. However, despite lower OMAT masses, the EX rats presented paradoxical changes in select protein expression patterns, indicative of increased lipogenesis (i.e., higher ACC protein, lower phospho/pan-AMPKα protein, and lower phospho/pan-HSL protein expression patterns) and inflammation (i.e., increased OMAT phospho/pan-p65 protein). Although these findings are difficult to reconcile, this increase in OMAT lipogenic and inflammatory related protein expression with exercise may be involved with the exercised-induced “catch-up fat” phenomenon that was previously reported (28). Alternatively stated, exercise-induced decreases in visceral fat may trigger a negative feedback mechanism, whereby lipogenic gene upregulation occurs to “prime” the tissue to store lipid. Therefore, aside from the dietary related themes of this study, these data continue to suggest that it is beneficial to maintain physical activity levels to reduce adipose tissue mass accretion that occurs with the cessation of physical activity.

Interestingly, our histology data suggest that, regardless of diet, progressive voluntary wheel running increases UCP-1 protein expression in the OMAT and BAT depots. Mechanisms involving the induction of UCP-1 expression are still not fully elucidated, but are thought to stem from elevated norepinephrine levels (32). Given that exercise increases circulating catecholamines in a pulsatile fashion, this may be the mechanism of action related to EX-induced increases in UCP-1 expression.

What should be finally noted regarding our exercise model is its unique ability to increase skeletal muscle hypertrophy as reported by our group (38) as well as others who have used similar resistance-loaded voluntary wheel running models in rats (23, 30). We contend that this exercise model is a mode of voluntary resistance exercise, given the hypertrophic response observed in the aforementioned studies, albeit what also cannot be ignored is the fact that this model also possesses endurance-like features (i.e., rats can run for long sustained distances rather than performing conventional resistance exercise bouts with predetermined numbers of sets and repetitions of resistance-loaded exercise). In this regard, the data observed herein may have differed if rats had been solely endurance-trained via treadmill running or unloaded voluntary wheel running, whereby rats are able to run higher nightly volumes. Specifically, endurance training facilitates skeletal muscle adaptation toward a more oxidative phenotype, which relies more upon the usage of fatty acids for fuel (21), and recent human data by Volek et al. (46) suggest that elite long-distance runners engaged in long-term ketogenic dieting possessed 1.0 kg less total body fat and presented a 2-fold increase in fat oxidation during a 3-h exercise bout compared with counterparts who consumed high-carbohydrate diets. Thus, given the data above, we posit that endurance-trained rodents on a ketogenic diet may present more robust decreases in fat mass and/or more robust changes in the assayed adipose tissue targets compared with the rats in our current study.

Effects of KD on liver physiology. Interestingly, select markers of liver damage (i.e., serum ALT and hepatic triglyceride accumulation) and inflammation (i.e., phosphorylated/pan-p65) were reduced in KD rodents compared with SC-fed and/or WD-fed rats. These findings contradict previous research, which has reported that KD-fed rodents exhibited increases in liver inflammation (19) or alterations in liver-related biomarkers suggestive of de novo lipogenesis (26, 37). A possible explanation for the contradicting KD-associated liver damage reported in the previous studies may be attributed to the protein content of the respective diets used and/or the fact that the aforementioned studies used murine models compared with our rat model. More specifically, we utilized a KD formula comprising 20% protein, whereas the aforementioned studies fed mice a ketogenic diet composed of <10% protein. Impairment of normal growth, organ development and function have been shown to occur when diets consist of <14% protein (20). Therefore, our data, as well as other similar reports, underscore the importance of examining the dietary composition of different ketogenic diets when making conclusions or extrapolating results related to changes in physiology.

Associations between serum insulin and BHB with body masses, feed efficiencies and fat pad masses. As stated above, KD-fed, as well as EX rats, presented lower insulin levels, and there were positive associations between serum insulin and indices of adiposity (Fig. 11). This is noteworthy given that 1) previous literature is in agreement with these findings suggesting that reducing insulin also reduces adiposity (8, 27, 48), and 2) insulin is an anabolic hormone [i.e., it inhibits tissue breakdown and promotes tissue storage of nutrients (12)]. Moreover, Jensen et al. (25) demonstrated that moderate reductions in circulating insulin levels result in large increases in lipolysis, as metabolism is shifted toward fat oxidation. Reducing insulin levels through carbohydrate restriction also decreases lipogenesis (12, 22, 43), this being a phenomenon that allows ingested fats to be metabolized for fuel rather than stored.

Some literature suggests that KD-induced increases in serum ketones may confer positive physiological adaptations in adipose tissue leading to fat loss (i.e., increased thermogenesis-related mechanisms) (44, 45), although opposing literature reported that ketone bodies inhibit lipolysis in vitro (7, 29). Notwithstanding, there were no associations when correlating serum BHB to body masses, feed efficiencies, or fat pad masses. Moreover, EX rats presented lower serum BHB levels, while also presenting more favorable adipose tissue characteristics (i.e., reduced OMAT mass, a reduced percentage of OMAT and SQ adipocyte diameters <100 μm, reduced OMAT, and SQ CEBPα protein expression, increased OMAT, and BAT UCP-1 expression) which, again, questions the role of circulating ketones in promoting favorable adaptations in adipose tissue phenotypes. Therefore, our findings collectively suggest that the lower insulin levels associated with ketogenic dieting or exercise may play a key role in attenuating the accumulation of adipose tissue, whereas KD-induced increases in serum ketones may play a lesser role in body fat regulation.
Perspectives and Significance

This study is not without limitations. First, this study was performed in younger rodents and, thus, the physiological changes may be different in magnitude or time-course (or different altogether) when related to human adults. Second, while isocaloric amounts of chow were provided to each group, we were unable to maintain an isocaloric status between diets; specifically, the SC group consumed fewer calories than the WD and KD groups. In spite of this limitation, body masses in the SC group were greater than the KD group, and KD-fed rats demonstrated the lowest feed efficiency compared with both diet groups. While others have postulated potential mechanisms whereby KD-feeding reduces feed efficiency in rodents [i.e., through urinary ketone loss rather than increases in daily energy expenditure or locomotor activity (5, 18)], this mechanism continues to remain unsolved and requires further investigation.

In spite of the current limitations, this study provides a comprehensive examination of adipose tissue, liver, and serum alterations with ketogenic dieting compared with other rodent diets in exercise-trained vs. sedentary rats. Overall, the tested KD reduced body mass, reduced feed efficiency, reduced OMAT average adipocyte size, and improved serum glucose, insulin, and lipid markers compared with the WD and SC. However, KD-fed EX rats did not experience synergistic improvements in body mass and/or the measured adipose tissue depot masses and characteristics. The cellular mechanisms leading to reduced adipose tissue mass and cell size from a KD or EX may be driven by lower levels of insulin, which dampens the signal to store fat and inhibit lipolysis; thus, more time-course mechanistic studies are needed to examine these processes. Finally, the tested KD did not cause adverse liver responses, but on the contrary, it lowered hepatic phospho-pan-p65 protein expression, hepatic triglyceride content, and serum ALT levels, which are all suggestive of improved liver health over time.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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Physiological effects of a ketogenic diet and exercise


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