

RESEARCH ARTICLE

A ketogenic diet combined with exercise alters mitochondrial function in human skeletal muscle while improving metabolic health

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Miller VJ, LaFountain RA, Barnhart E, Sapper TS, Short J, Arnold WD, Hyde PN, Crabtree CD, Kackley ML, Kraemer WJ, Villamena FA, Volek JS. A ketogenic diet combined with exercise alters mitochondrial function in human skeletal muscle while improving metabolic health. *Am J Physiol Endocrinol Metab* 319: E995–E1007, 2020. First published September 28, 2020; doi:10.1152/ajpendo.00305.2020.—Animal data indicate that ketogenic diets are associated with improved mitochondrial function, but human data are lacking. We aimed to characterize skeletal muscle mitochondrial changes in response to a ketogenic diet combined with exercise training in healthy individuals. Twenty-nine physically active adults completed a 12-wk supervised exercise program after self-selection into a ketogenic diet (KD, $n = 15$) group or maintenance of their habitual mixed diet (MD, $n = 14$). Measures of metabolic health and muscle biopsies (vastus lateralis) were obtained before and after the intervention. Mitochondria were isolated from muscle and studied after exposure to carbohydrate (pyruvate), fat (palmitoyl-L-carnitine), and ketone (β -hydroxybutyrate+acetoacetate) substrates. Compared with MD, the KD resulted in increased whole body resting fat oxidation ($P < 0.001$) and decreased fasting insulin ($P = 0.019$), insulin resistance [homeostatic model assessment of insulin resistance (HOMA-IR), $P = 0.022$], and visceral fat ($P < 0.001$). The KD altered mitochondrial function as evidenced by increases in mitochondrial respiratory control ratio (19%, $P = 0.009$), ATP production (36%, $P = 0.028$), and ATP/H₂O₂ (36%, $P = 0.033$) with the fat-based substrate. ATP production with the ketone-based substrate was four to eight times lower than with other substrates, indicating minimal oxidation. The KD resulted in a small decrease in muscle glycogen (14%, $P = 0.035$) and an increase in muscle triglyceride (81%, $P = 0.006$). These results expand our understanding of human adaptation to a ketogenic diet combined with exercise. In conjunction with weight loss, we observed altered skeletal muscle mitochondrial function and efficiency, an effect that may contribute to the therapeutic use of ketogenic diets in various clinical conditions, especially those associated with insulin resistance.

fat oxidation; insulin resistance; ketogenic diet; mitochondria; skeletal muscle

INTRODUCTION

Skeletal muscle mitochondrial function is an important determinant of overall metabolic health. Mitochondrial impairment is often associated with oxidative stress and a decline in ATP production, each of which can promote or exacerbate chronic disease (12, 62, 89). Further signifying the importance role of skeletal muscle in metabolic health, it accounts for ~90% of insulin-mediated glucose uptake in healthy humans (13). Consistent with this, mitochondrial dysfunction in skeletal muscle is associated with insulin resistance and type 2 diabetes, possibly because of impairment of fat oxidation (10, 14, 68). Although endurance exercise is a well-known stimulus to enhance fat oxidation (2) and mitochondrial function (33, 68), the impact of diet composition on the mitochondrial adaptations that occur during exercise training remains less clear.

Carbohydrate-restricted diets are a practical and sustainable lifestyle approach to manage conditions closely associated with insulin resistance, including metabolic syndrome (35, 82, 83, 86) and type 2 diabetes (5, 18). Through a combination of decreasing glucose availability and insulin levels, carbohydrate restriction facilitates mitochondrial fat oxidation in skeletal muscle. This occurs acutely because of the decreased presence of insulin (69) and also occurs chronically, at least partly through upregulated AMP-activated protein kinase (AMPK) signaling (51, 79). Ketogenic diets are particularly promising in this regard because the low carbohydrate and moderate protein intakes promote ketogenesis, which may further strengthen bioenergetic signaling that upregulates mitochondrial fat oxidation and endogenous antioxidant defense and downregulates inflammation (51, 54). Exercise is another practical approach to enhancing mitochondrial function (33, 68). Given the overlap in underlying mechanisms between adaptations to ketogenic diets and exercise training, it is possible that the combination may be synergistic (51).

Investigations of the effects of ketogenic diets on skeletal muscle and mitochondrial function have been done almost entirely in animal models with diet formulations that would not be sustainable for humans. For example, ketogenic diets used in animal experiments are comprised of processed chow and are often devoid of any carbohydrate and low in protein to ensure induction of ketosis (51). Many animal studies focused on mitochondrial function were based on diets including only 10% or

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less of energy as protein (51), which is likely adequate for maintenance of nitrogen balance but not growth or reproduction (53). Formulation of ketogenic diets used in human research has improved from the highly restrictive diets used to treat epilepsy. However, relatively few studies (5, 61, 87) have adequately addressed all the components necessary to make the diet safe, effective, and sustainable, such as the need to emphasize increased sodium requirements and provide adequate but not excessive protein intake. Many ketogenic diet studies also fail to demonstrate that nutritional ketosis was consistently maintained. The objective of the present investigation was to explore how a well-formulated ketogenic diet (51) with frequent monitoring of ketosis impacted measures of skeletal muscle metabolic and mitochondrial function in healthy adults during a 12-wk exercise training program.

MATERIALS AND METHODS

Experimental approach. The present investigation focused on mitochondrial function was part of a larger study designed to assess the effect of a ketogenic diet in military personnel during adaptation to a 12-wk exercise training program (40). All participants followed the same exercise training intervention and chose to continue their habitual mixed diet (MD) or a ketogenic diet (KD). Participants were allowed to self-select into the diet groups to address the commitment required to change dietary patterns and also enhance translation of the results, since people have freedom to choose the composition of their diet. Capillary β -hydroxybutyrate (BHB) was measured daily in KD participants with a handheld glucometer (Precision Xtra; Abbott Laboratories, Chicago, IL) to monitor dietary compliance. KD participants were instructed to measure BHB in the morning and before any consumption of food or beverage.

Participants. All participants had a military affiliation, with the majority of participants recruited from the Ohio State Reserve Officer Training Corps (ROTC). As intended, anthropometrics and demographics of the participants were similar to those of the US military. At the onset of the study, age, body mass, body composition, and sex were similar between groups. However, attrition resulted in the KD group having a greater mean body mass (85.7 vs. 79.8 kg, $P = 0.022$) and body mass index (BMI) (27.9 vs. 24.9 kg/m², $P = 0.005$). Despite this, there was no significant difference in body fat percentage. The intervention was completed by 15 participants in the KD group and 14 in the MD group, with 2 women in each group. Additional details on recruitment and participant characteristics have been published previously (40). All study procedures were approved by The Ohio State University Institutional Review Board, and written informed consent was provided by all participants before participation.

Dietary and exercise training interventions. KD participants attended an information session describing the guidelines and recommendations for the ketogenic diet, which were primarily focused on limiting carbohydrate and moderating protein intake with a goal of achieving daily capillary BHB readings at or above 1 mM. Participants were also guided toward implementing a well-formulated ketogenic diet emphasizing adequate sodium intake (4–5 g/day) to offset natriuresis of ketosis and a variety of whole foods including fresh full-fat meat, seafood, nonstarchy vegetables, and berries to support adequate intake of essential micronutrients. To help KD participants maintain the 1 mM BHB target, they received additional personal coaching as dictated by their daily BHB readings and were provided groceries and prepackaged ketogenic meals. MD participants were instructed to maintain their habitual diets consisting of 40% or more of carbohydrate. To minimize performance bias, MD participants were offered the opportunity to meet with a registered dietitian on the research team at any time throughout the intervention. Additional details on the KD and MD interventions have been published previously (40). All participants in

both groups were encouraged to eat to satiety, with no prescription for total energy intake.

Both study groups participated in the same training program, consisting of two weekly supervised sessions focused on periodized resistance and power training and high-intensity interval training. The program also included one weekly unsupervised session including 30 min or more of cardiorespiratory endurance training. Additional details on the exercise training protocol have been published previously (40).

Whole body substrate oxidation. Respiratory minute volumes for oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were measured at rest by indirect calorimetry (40). Whole body carbohydrate oxidation was calculated as $(4.585 \cdot \dot{V}CO_2 - 3.226 \cdot \dot{V}O_2)$, and whole body fat oxidation was calculated as $(1.695 \cdot \dot{V}O_2 - 1.701 \cdot \dot{V}CO_2)$ (36).

Cardiorespiratory exercise testing ($\dot{V}O_{2max}$). In a nonfasted state, participants were fitted with a face mask (Hans Rudolph, Shawnee, KS) and tested on a motorized treadmill (EXCMR, Inc., Columbus, OH) with the Bruce protocol (9). Testing was initiated at 1.7 mph at a 10% incline for 3 min, followed by incremental increases in speed and incline every 3 min until volitional fatigue was achieved. Maximal oxygen uptake ($\dot{V}O_{2max}$) was determined on the basis of measurement of respiratory gas exchange (ParvoMedics, Sandy, UT).

Blood analysis. Methodology for blood collection, processing, and analysis of serum glucose and insulin has been described previously (40). All samples were thawed only one time, which was immediately before analysis. Enzyme-linked immunosorbent assay (ELISA) kits were used to measure plasma C-reactive protein (CRP; catalog no. 10011236, range: 46.9–3,000 pg/mL, Cayman Chemical, Ann Arbor, MI) and serum myoglobin (catalog no. MG017C, Calbiotech, El Cajon, CA). The respective intra-assay coefficients of variation (CVs) were 10.8% and 4.1%. Plasma cortisol was measured with a competitive ELISA kit (catalog no. 7M21603, MP Biomedicals, Irvine, CA) and had an intra-assay CV of 2.9%. Serum nonesterified fatty acids (NEFA) were measured with an enzymatic assay kit (NEFA-HR(2), Wako Diagnostics, Mountain View, CA) and had an intra-assay CV of 2.4%. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as $[\text{fasting insulin } (\mu\text{U/mL}) \times \text{glucose (mM)}] / 22.5$ (46). Based on the diagnostic criteria for metabolic syndrome from the National Cholesterol Education Program's Adult Treatment Panel III, HOMA-IR threshold values of 2.27 for men and 2.12 for women were used to classify participants as insulin resistant (25).

Muscle biopsy. Muscle biopsies were taken from the vastus lateralis by a trained physician in a sterile environment. To minimize variation in muscle fiber type composition, pre- and postintervention biopsies were taken in close proximity to each other, each from the right leg and at similar depths. For each biopsy, ~10 mL of 1% lidocaine was injected into the biopsy site, a 1- to 2-cm incision was created, and a Bergstrom biopsy needle was inserted into the muscle through the incision. Suction was applied with a 60-mL syringe to draw muscle into the needle for cutting. The needle was rotated after each cut to allow up to three separate cuts of tissue per needle insertion. After the withdrawal of the needle from the muscle and removal of the tissue samples, the needle was reinserted into the incision to repeat the process a second time, leading to a total yield of ~300 mg of tissue. The sample was immediately placed in ice-cold phosphate-buffered saline (PBS, free of Ca and Mg). Visible blood, fat, and connective tissue were removed, and 100–140 mg of the remaining sample was used for mitochondria isolation. A remaining portion of the sample was placed in a microcentrifuge tube, frozen in liquid nitrogen, and stored at -80°C for future analysis, including determination of glycogen and triglyceride content. Stored samples were partially thawed for further aliquoting and fully thawed an additional time immediately before analysis.

Muscle glycogen. As previously described (84), muscle samples (~10 mg) were boiled for 2 h in 500 μL of 2 M HCl to hydrolyze glycogen content. After boiling, each sample was weighed, and water was added in an amount to compensate for the loss of solution during boiling. Each sample was then neutralized with 500 μL of 2 M NaOH and

50 μ L of 2 M Tris. Sample (5 μ L) was combined with 100 μ L of Glucose Hexokinase Reagent (catalog no. TR15421, ThermoFisher Scientific, Waltham, MA) in a 96-well microplate. Absorbance was read at 350 nm (Synergy H1; BioTek, Winooski, VT) and compared with a standard curve for glucose determination. The final glucose concentration was normalized to the wet weight of each sample. The intra-assay CV was 4.3%.

Muscle triglyceride. After being freeze-dried overnight, 0.5–2.5 mg of dried muscle was manually homogenized with a pestle in 0.5 mL of methanol (19). Chloroform (500 μ L) was added to the homogenate, and the solution was incubated overnight at 4°C for lipid extraction. $MgCl_2$ (1 mL, 400 μ M) was added to the solution and then centrifuged at 3,500 rpm for 30 min at 4°C. The aqueous phase of the solution was discarded, and 250 μ L of the chloroform phase was transferred to a new tube and heated at 70°C until complete evaporation occurred (20–30 min). The remaining residue was resuspended in 100 μ L of 250 mM KOH dissolved in ethanol and incubated at room temperature for 30 min. Triglyceride content was hydrolyzed with the addition of 25 μ L of 1 M HCl, and the absorbance of the resulting glycerol was enzymatically enhanced by adding 1 mL of Free Glycerol Reagent (catalog no. F6428, Sigma-Aldrich, St. Louis, MO). Absorbance was read at 540 nm (Synergy H1; BioTek, Winooski, VT) from a 96-well microplate and compared with a standard curve derived from tripalmitin for glycerol determination. The final glycerol concentration was normalized to the dry weight of each sample. The intra-assay CV was 2.1%.

Mitochondria isolation. Immediately after the biopsy, each sample of muscle tissue was minced with scissors for 90 s in 990 μ L of ice-cold isolation buffer consisting of (in mM) 100 sucrose, 100 KCl, 50 Tris, 1 KH_2PO_4 , and 0.1 EGTA with 0.2% BSA, pH 7.4 (81). Protease from *Bacillus licheniformis* (catalog no. P5380, Sigma-Aldrich, St. Louis, MO) was added to the minced tissue, resulting in a final concentration of 0.2 mg/mL, and the solution was incubated for 2 min. The minced tissue was then transferred to a 15-mL PTFE-glass tissue grinder (VWR, Radnor, PA) and homogenized, while on ice, at 610 rpm for three 15-s intervals separated by 5 s each. Immediately after homogenization, 3 mL of isolation buffer was added to the homogenization vessel, and the homogenate was divided into three 1.5-mL microcentrifuge tubes and centrifuged at 700 g for 10 min at 4°C. Supernatant from each tube was transferred to a new 1.5-mL microcentrifuge tube and centrifuged at 10,000 g for 10 min at 4°C. Supernatant from each tube was discarded, and the pellets were resuspended into a single microcentrifuge tube with 1 mL of ice-cold isolation buffer. The resuspension was then centrifuged at 7,000 g for 10 min at 4°C, the supernatant was discarded, and the final pellet was resuspended with a solution (0.6 μ L/mg muscle) consisting of (in mM) 225 mannitol, 75 sucrose, 10 Tris, and 0.1 EDTA with 0.2% BSA, pH 7.4. A 5- μ L aliquot of the final suspension was stored at –80°C for the measurement of protein content (Pierce BCA Protein Assay Kit; Thermo Scientific, Waltham, MA). The final suspension was incubated on ice for 30–40 min before further analysis.

Substrates for mitochondria testing. Three substrate combinations were used to evaluate mitochondrial function relative to carbohydrate, fat, and ketone metabolism. For each assay, the final concentrations of the three respective substrate combinations were 5 mM pyruvate (catalog no. 107360, Sigma-Aldrich, St. Louis, MO) + 2 mM malate (catalog no. M1000, Sigma-Aldrich, St. Louis, MO), 0.01 mM L-palmitoylcarnitine chloride (catalog no. P1645, Sigma-Aldrich, St. Louis, MO) + 2 mM malate, and 1 mM lithium acetoacetate (catalog no. A8509, Sigma-Aldrich, St. Louis, MO) + 1 mM sodium-R-3-hydroxybutyrate (catalog no. 54965, Sigma-Aldrich, St. Louis, MO). The concentrations for the carbohydrate- and fat-derived substrates have been reported to maximize mitochondria respiration (70). Ketone concentrations were based on available evidence, which was limited to brain mitochondria (39, 43).

Mitochondrial respiration and membrane potential. Mitochondrial O_2 consumption and membrane potential were simultaneously measured with a Clark-type electrode (Oxygraph Plus; Hansatech, King's

Lynn, UK) fitted with a tetraphenylphosphonium (TPP)-selective electrode (catalog no. KWIKTPP-2, World Precision Instruments, Sarasota, FL) and a reference electrode (catalog no. DRIFEF-2, World Precision Instruments, Sarasota, FL). Heated water was circulated through the water jacket surrounding the reaction chamber of the electrode to maintain the temperature of the mitochondria suspension at 37°C. The reaction chamber was filled with 0.5 mL of reaction buffer consisting of (in mM) 225 mannitol, 75 sucrose, 10 KCl, 10 Tris, 10 K_2HPO_4 , 0.08 $MgCl_2$, and 0.1 EDTA with 0.2% BSA, pH 7.2 (71, 81). The reaction solution was supplemented with 10 μ L of 0.1 mM tetraphenylphosphonium chloride + 1 mM KCl, which was added in three successive volumes (5, 2.5, and 2.5 μ L) to establish a calibration curve for estimation of TPP concentration (4). On the basis of the ability of TPP to diffuse across the inner mitochondria membrane, changes in TPP concentration can be used to estimate membrane potential (38, 52). Contents of the reaction chamber were stirred at 60% of max speed. State 4 mitochondria respiration was initiated with the addition of 8 μ L of mitochondria suspension to the reaction chamber. State 3 respiration was initiated with the addition of 10 μ L of 10 mM K-ADP, resulting in a final ADP concentration of ~0.2 mM. Measurements of respiration and changes in membrane potential were repeated for each of the three substrate solutions. State 3 and 4 respiration values were normalized to the protein concentrations of the final suspension of isolated mitochondria. Respiratory control ratio (RCR) was calculated as the quotient of state 3 and state 4 O_2 consumption rates. Membrane potential was calculated with the Nernst equation with the observed changes in TPP concentration (4, 38).

Mitochondrial H_2O_2 production. The rate of mitochondrial H_2O_2 production during state 4 respiration was measured with Amplex UltraRed (Invitrogen, Carlsbad, CA), which reacts with horseradish peroxidase in the presence of H_2O_2 to form a fluorescent by-product (64, 93). A 10- μ L volume of 10 $\times$ diluted mitochondria suspension was incubated at 37°C in a total well volume of 200 μ L consisting of the same reaction buffer used for respiration measurement, 15 U/mL horseradish peroxidase (catalog no. P6782, Sigma-Aldrich, St. Louis, MO), and 50 mM Amplex UltraRed. Also included was 90 U/mL superoxide dismutase (catalog S7571, Sigma-Aldrich, St. Louis, MO) to maximize conversion of O_2^- to H_2O_2 . A separate reaction was prepared for each of the three substrate combinations. Fluorescence was measured from a 96-well microplate for 10 min at 50-s intervals with excitation and emission wavelengths of 530 and 590 nm, respectively (Synergy H1; BioTek, Winooski, VT). The H_2O_2 production rate was calculated based on the average rate of change throughout all reads. The intra-assay CV was 7.2%.

Mitochondrial ATP production. The rate of mitochondrial ATP production during state 3 respiration was measured with a luciferase-based assay (ATP Bioluminescence Assay Kit CLS II; Roche, Basel, Switzerland). In the presence of ATP, luciferase converts luciferin into a detectable bioluminescent by-product (41, 91). A 5- μ L volume of 40 $\times$ diluted mitochondria suspension was incubated at 37°C in a total well volume of 100 μ L consisting of the same reaction buffer used for respiration measurement, 50 μ L of luciferase reagent, and 0.5 mM K-ADP. A separate reaction was prepared for each of the three substrate combinations. Bioluminescence was measured from a 96-well microplate for 8 min at 123-s intervals (Synergy H1; BioTek, Winooski, VT), and ATP production rate was calculated based on the average rate of change throughout the first three reads. The intra-assay CV was 5.5%.

Oxidative damage. The remaining suspension of isolated mitochondria was stored at –80°C and later thawed for measurement of protein carbonyls and nitrotyrosine. Both markers were measured with OxiSelect ELISA kits (catalog nos. STA-310 and STA-305, respectively, Cell Biolabs, San Diego, CA). Intra-assay CVs were 5.2% for protein carbonyl and 10.1% for nitrotyrosine. Plasma thiobarbituric acid reactive substances (TBARS), which is a marker of lipid peroxidation, was measured with a colorimetric assay kit (catalog no. 700870, Cayman Chemical, Ann Arbor, MI). The intra-assay CV was 4.6%.

Table 1. Tests with limited sample sizes

	Mixed Diet	Ketogenic Diet
Mitochondrial O ₂ consumption (states 3 and 4)*		
Fat and carbohydrate substrates	12	14
Ketone substrate	11	13
Mitochondrial respiratory control ratio*		
Fat and carbohydrate substrates	12	14
Ketone substrate	11	13
Mitochondrial membrane potential†	10	13
Mitochondrial ATP production‡	14	13
Mitochondrial ATP/O ₂		
Fat and carbohydrate substrates*‡	12	12
Ketone substrate	11	11
Mitochondrial ATP/H ₂ O ₂ ‡	14	13
Muscle triglyceride§¶	13	13
Plasma C-reactive protein§	14	14
Plasma cortisol§	14	14
Serum glucose§	14	14
Serum insulin§	14	14
Serum myoglobin§	14	14

*Three participants (all substrates) excluded because of equipment failure, 2 participants (ketone substrate only) excluded because of abnormally high values not consistent with ATP data; †6 participants excluded because of equipment failure; ‡2 participants excluded because of abnormally low values not consistent with O₂ consumption data; §1 participant excluded because of limited sample availability; ¶2 participants excluded because of suspected contamination with extramitochondrial lipid.

Statistical analysis. Data are reported as means and standard deviations. Independent *t* tests or Wilcoxon rank sum tests, depending on normality of the data, were used for preintervention participant characteristics and pairwise comparisons associated with repeated-measures analyses. Linear mixed-effects models were used for two- and three-factor repeated-measures analyses. Random intercepts were used to account for the nonindependence of repeated measures among participants. Body mass, HOMA-IR, CRP, and visceral fat were included as covariates in all models to account for preintervention between-group differences. Normality and variance of model residuals and random intercepts were analyzed for validation of model assumptions. Where appropriate, transformation based on Box-Cox log-likelihood or trial and error was used to improve model diagnostics. Bonferroni correction was used with each model to adjust the significance level of $\alpha=0.05$ for the following multiple comparisons: between-group comparisons at the pre- and postintervention time points, pre- vs. postintervention within-group comparisons for each group, main effect of time, and time $\times$ diet interaction. This resulted in a significance level of $\alpha=0.003$ for models of mitochondria markers including the three respiratory substrates (18 total comparisons) and $\alpha=0.008$ for all remaining models (6 total comparisons). Spearman's correlations (ρ) were used in place of Pearson's correlations (r) where appropriate based on lack of a linear relationship. Analyses were completed with R 3.4.0 (65). Because of technical complications and limited sample availability, sample sizes for statistical analyses differed across the tests (Table 1).

RESULTS

Dietary compliance, weight loss, and metabolic health. Capillary BHB and weight loss have been reported elsewhere (40). In brief, average daily BHB concentration was >1 mM in KD, indicating excellent dietary compliance. The KD group lost more body mass (-7.7 ± 3.2 vs. 0.1 ± 1.8 kg, $P < 0.001$), fat mass (-5.9 ± 2.7 vs. -0.6 ± 1.9 kg, $P < 0.001$), and visceral fat (-0.5 ± 0.4 vs. 0.0 ± 0.2 kg, $P < 0.001$) than the MD group. Although all participants were generally healthy upon entry to the study, the KD group had higher concentrations of fasting

insulin (10.5 ± 4.7 vs. 8.0 ± 4.6 μ IU/mL, $P = 0.039$) and CRP (1.2 ± 1.0 vs. 0.5 ± 0.3 μ g/mL, $P = 0.017$). There were also tendencies for greater HOMA-IR (2.1 ± 1.2 vs. 1.5 ± 0.9 , $P = 0.056$) and visceral fat (1.3 ± 0.7 vs. 0.8 ± 0.7 kg, $P = 0.063$). These differences suggest subtle early-stage metabolic impairment in some of the KD participants, which is further supported by 40% of the KD participants (6 of 15) starting the study insulin resistant (based on HOMA-R) compared with 2 of 14 MD participants. After the intervention, visceral fat, insulin, and HOMA-IR significantly decreased in the KD group but not the MD group (40). Furthermore, all six instances of insulin resistance in the KD group were reversed, indicating the resolution of metabolic impairment.

Mitochondrial protein content and function. Exercise training increased mitochondrial protein content independent of diet (Fig. 1), but the increase is not significant after correction for multiple comparisons. The RCR with the fat substrate increased in the KD group but not the MD group (Fig. 2C), suggesting increased capacity for fat oxidation. State 3 O₂ consumption (Fig. 2A) and RCR (Fig. 2C) were much lower with the ketone substrate than the carbohydrate and fat substrates, indicating minimal oxidation of ketones. Mitochondrial H₂O₂ production increased for all substrates in the MD group, leading to a trend in the time $\times$ diet interaction (Fig. 2D).

ATP production (Fig. 3A), membrane potential (Fig. 3B), and the ratios of ATP production to state 3 O₂ consumption (ATP/O₂; Fig. 3C) and H₂O₂ production (ATP/H₂O₂; Fig. 3D) each showed a clear between-group contrast in the direction of change, indicating increased capacity and efficiency for the KD group relative to the MD group. Similar to state 3 O₂ consumption, ATP production with the ketone substrate was much less compared with the fat and carbohydrate substrates (Fig. 3A). This observation is clearly independent of diet and time (Fig. 4, A and B), further indicating a lack of ketone oxidation.

Minimal change in $\dot{V}O_{2\max}$ was observed, suggesting against influence of whole body maximal oxygen uptake on mitochondrial function. Maximal oxygen consumption increased in the KD group when expressed relative to total body mass (44.9 ± 5.9 to 48.5 ± 6.2 mL $\cdot$ kg $^{-1}\cdot$ min $^{-1}$, $P = 0.050$) but did not change in the MD group (45.9 ± 8.2 to 45.8 ± 6.9 mL $\cdot$ kg $^{-1}\cdot$ min $^{-1}$, $P =$

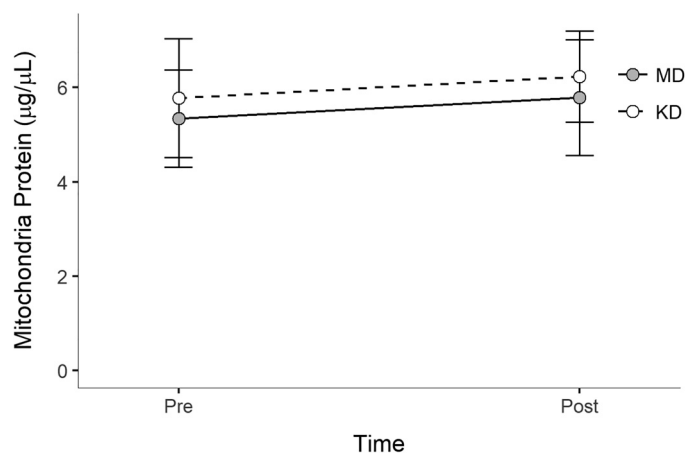


Fig. 1. Protein concentration of isolated mitochondria (means $\pm$ SD) before and after 12 wk of a ketogenic (KD) or mixed (MD) diet combined with exercise training: effect of time, $P = 0.016$; time $\times$ diet interaction, $P = 0.305$. Post, post-intervention; Pre, preintervention.

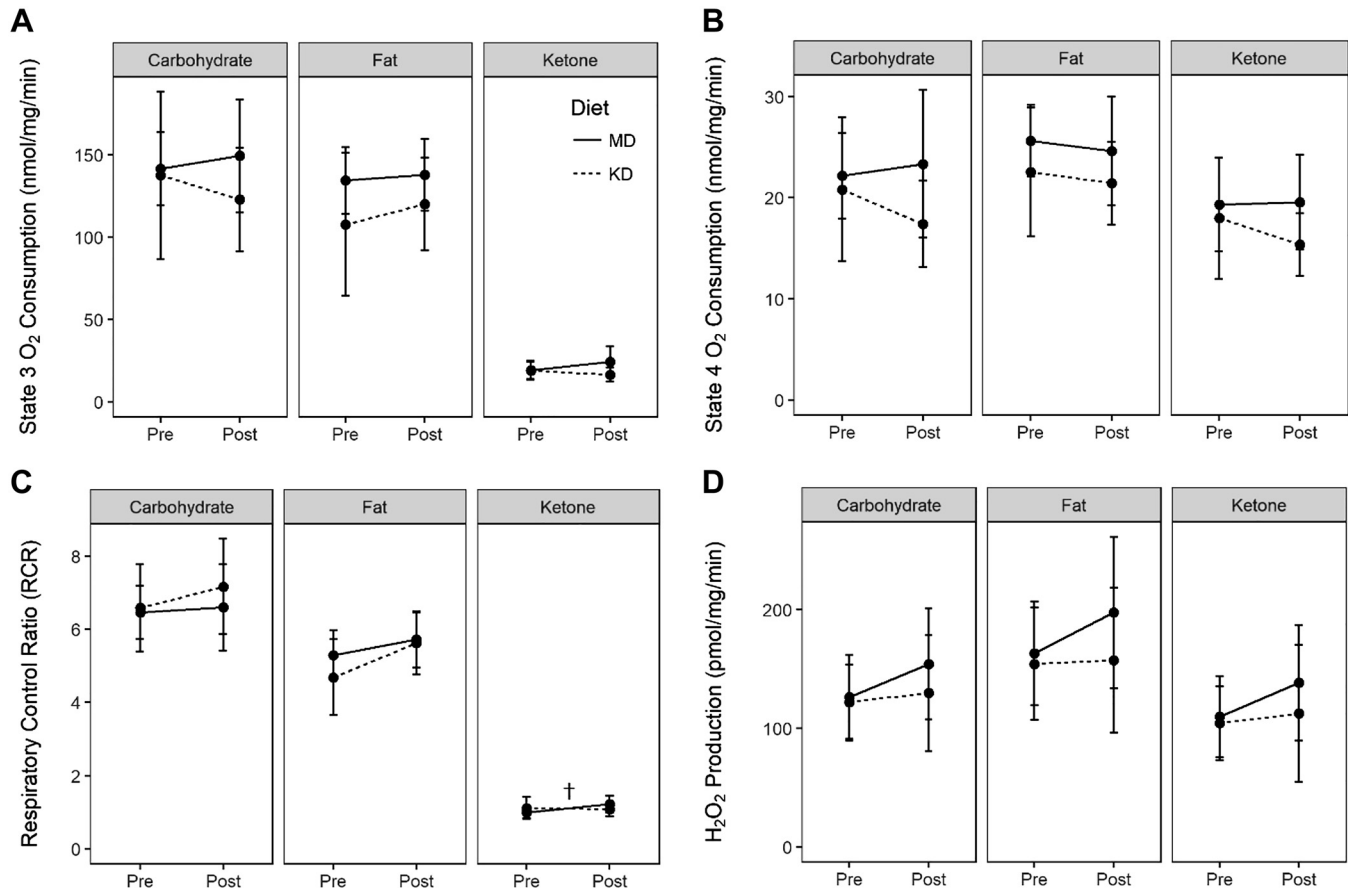


Fig. 2. General function (means $\pm$ SD, by substrate) of isolated mitochondria before and after 12 wk of a ketogenic (KD) or mixed (MD) diet combined with exercise training. A: state 3 O₂ consumption: effect of time, $P = 0.480$; time $\times$ diet interaction, $P = 0.110$. B: state 4 O₂ consumption: effect of time, $P = 0.010$; time $\times$ diet interaction, $P = 0.091$. C: respiratory control ratio: effect of time, $P = 0.003$; time $\times$ diet interaction, $P = 0.581$. D: H₂O₂ production: effect of time, $P = 0.001$; time $\times$ diet interaction, $P = 0.098$. Carbohydrate, pyruvate + malate; Fat, palmitoylcarnitine + malate; Ketone, β -hydroxybutyrate + acetoacetate; Post, postintervention; Pre, preintervention. $\dagger P < 0.002$ for within-group difference between time points for MD.

0.944). Absolute $\dot{V}O_{2\max}$ was unchanged in both groups (KD: 3.8 ± 0.6 to 3.8 ± 0.6 L/min, $P = 0.934$; MD: 3.6 ± 0.5 to 3.7 ± 0.5 L/min, $P = 0.463$), as was $\dot{V}O_{2\max}$ normalized to lean mass (KD: 62.5 ± 8.0 to 63.5 ± 7.9 mL $\cdot$ kg⁻¹ $\cdot$ min⁻¹, $P = 0.720$; MD: 61.0 ± 7.4 to 62.0 ± 6.8 mL $\cdot$ kg⁻¹ $\cdot$ min⁻¹, $P = 0.670$). Normalizing mitochondrial ATP production to absolute $\dot{V}O_{2\max}$ did not change between- or within-group differences. With or without normalization to body mass, $\dot{V}O_{2\max}$ did not correlate with any of the primary markers of mitochondrial function (O₂ consumption, membrane potential, ATP production, and H₂O₂ production). Similar to $\dot{V}O_{2\max}$, there were no postintervention correlations between body mass, fat mass, or fat percentage and any of the primary mitochondrial markers. Likewise, none of the anthropometric measures was a significant predictor in linear regression for any of the primary mitochondrial markers.

Stepwise multiple linear regression was run to identify potential explanations for preintervention differences in state 3 O₂ consumption, membrane potential, and ATP production, all with the fat substrate. Nearly 30 possible predictor variables were included to represent anthropometric, demographic, and metabolic characteristics. CRP emerged as the best predictor based on inverse correlations with preintervention ATP production ($\rho = -0.42$, $P = 0.032$) and membrane potential ($\rho = -0.45$,

$P = 0.021$) and a tendency for an inverse correlation with preintervention state 3 O₂ consumption ($\rho = -0.36$, $P = 0.069$).

Shift in muscle and whole body macronutrient metabolism. At the whole body level, the KD participants oxidized equal amounts of fat and carbohydrate at baseline compared with nearly three times as much fat after intervention (Fig. 5B). A similar shift was observed in skeletal muscle, in regard to both energy stores and mitochondrial capacity. Consistent with these results, KD participants had a nonsignificant increase in NEFA (KD: 0.43 ± 0.22 to 0.65 ± 0.29 mM, $P = 0.030$; MD: 0.30 ± 0.13 to 0.30 ± 0.13 mM, $P = 0.982$), resulting in a significant postintervention difference ($P = 0.001$). In MD participants, resting concentrations of intramuscular glycogen remained stable (Fig. 5C) and intramuscular triglycerides decreased 28% (Fig. 5D). In contrast, glycogen decreased 14% and triglycerides increased 82% in KD participants. Consistent with these changes, whereas the capacity of mitochondria to oxidize fat versus carbohydrate (MITO F/C) remained relatively stable in MD participants, it increased by 30% in KD participants (Fig. 5A). A shift in muscle ketone metabolism is also apparent despite the small magnitude of ketone oxidation. In KD participants, mitochondrial capacity to produce ATP from ketones increased relative to the capacity to produce ATP from carbohydrate or fat (Fig. 4C). In contrast, this capacity decreased in MD participants.

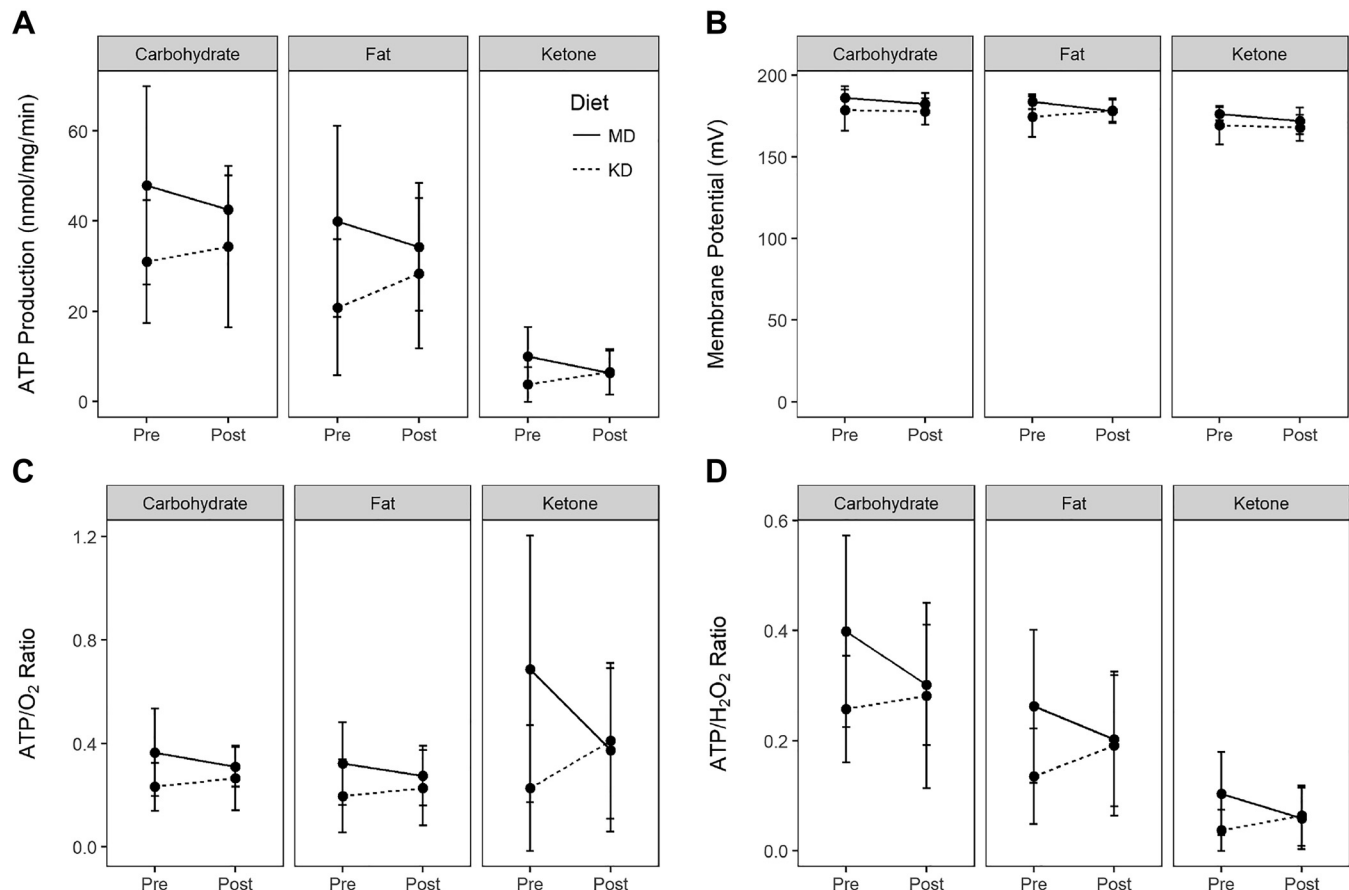


Fig. 3. Capacity and efficiency (means $\pm$ SD, by substrate) of isolated mitochondria before and after 12 wk of a ketogenic (KD) or mixed diet (MD) combined with exercise training. A: ATP production: effect of time, $P = 0.198$; time $\times$ diet interaction, $P = 0.003$. B: membrane potential: effect of time, $P = 0.019$; time $\times$ diet interaction, $P = 0.652$. C: ATP production relative to state 3 O₂ consumption: effect of time, $P = 0.177$; time $\times$ diet interaction, $P = 0.002$. D: ATP production relative to H₂O₂ production: effect of time, $P = 0.413$; time $\times$ diet interaction, $P = 0.003$. Carbohydrate, pyruvate + malate; Fat, palmitoylcarnitine + malate; Ketone, β -hydroxybutyrate + acetoacetate; Post, postintervention; Pre, preintervention.

Oxidative damage and exercise tolerance. Despite increases in mitochondrial H₂O₂ production, mitochondrial nitrotyrosine decreased in the MD group (Fig. 6B). No other significant differences were observed for mitochondrial protein carbonyls (Fig. 6A) or nitrotyrosine. Likewise, no significant effects or differences were observed for plasma TBARS (Fig. 6D). Serum myoglobin decreased in the KD group, resulting in a lower postintervention concentration compared with the MD group (Fig. 6C). No significant differences were observed for plasma cortisol.

DISCUSSION

Our primary objective was to generate novel data on skeletal muscle mitochondrial function in humans adapted to a well-formulated and precisely monitored ketogenic diet. As expected, the ketogenic diet resulted in a profound shift toward reliance on fat oxidation, as indicated by the nearly threefold greater contribution of fat versus carbohydrate to whole body resting energy expenditure. In contrast, carbohydrate oxidation remained as the primary contributor to energy expenditure in the MD group, indicating that increased fat oxidation was attributed to adaptation to the ketogenic diet rather than exercise training. The increased whole body fat oxidation and decreased reliance on

carbohydrate oxidation were accompanied by a small decrease in muscle glycogen, a large increase in muscle triglycerides, and an increased mitochondrial capacity to produce ATP from fat. Given that skeletal muscle accounts for a significant portion of resting energy metabolism (47), this observed shift in muscle metabolism may have been the primary driver of the shift in whole body metabolism.

The small decrease in resting muscle glycogen (14%) after 12 wk of a ketogenic diet was significant but less than expected relative to the traditional views that emphasize a high-carbohydrate intake to maintain glycogen from day to day. After 4 wk of a ketogenic diet in elite cyclists, resting muscle glycogen decreased 47% (61), but in elite endurance athletes who adapted to a ketogenic diet for at least 6 mo, resting muscle glycogen was similar to matched athletes who consumed a high-carbohydrate diet (84). In contrast, Webster et al. (90) reported that a group of well-trained male cyclists habituated to a low-carbohydrate, high-fat diet for at least 6 mo had $\sim 45\%$ lower resting muscle glycogen compared with a group of cyclists consuming a mixed diet. The reasons for these disparate results remain speculative but could relate to differences in the composition of ketogenic diet, training histories, or other unknown factor(s). As demonstrated in a number of mammals and reviewed by Fournier et al. (20), there can be significant depletion of

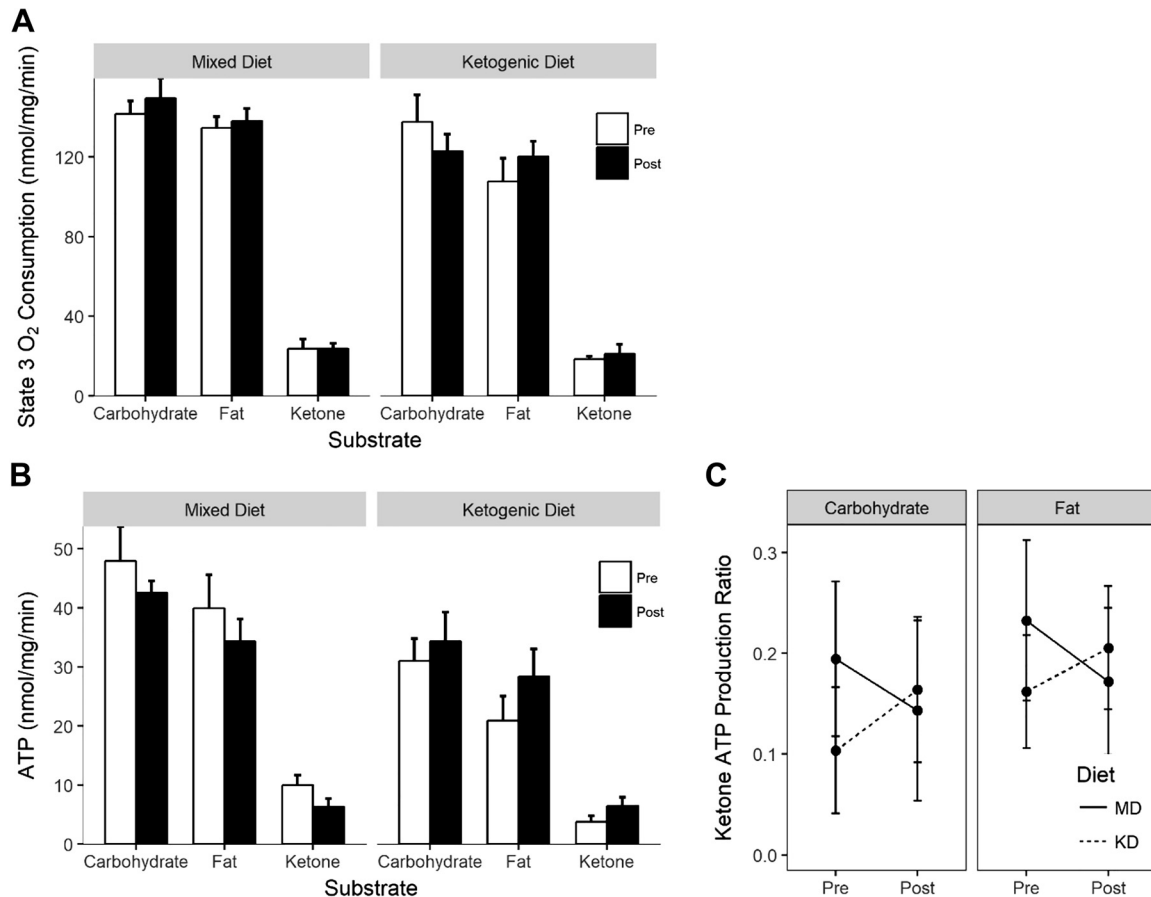


Fig. 4. Ketone metabolism (means $\pm$ SD) in isolated mitochondria before and after 12 wk of a ketogenic (KD) or mixed (MD) diet combined with exercise training. **A:** state 3 O₂ consumption by substrate and time; state 3 O₂ consumption with the ketone substrate (β -hydroxybutyrate + acetoacetate) was $\sim$ 6–8 times lower compared with the carbohydrate (pyruvate + malate) and fat (palmitoylcarnitine + malate) substrates regardless of diet or time. **B:** ATP production by substrate and time; ATP production with the ketone substrate was $\sim$ 4–8 times lower compared with the carbohydrate and fat substrates regardless of diet or time. **C:** ATP production with the ketone substrate relative to the carbohydrate and fat substrates: effect of time, $P = 0.247$; time $\times$ diet interaction, $P < 0.001$. Post, postintervention; Pre, preintervention.

glycogen in the absence of carbohydrate intake and despite prodigious amounts of exercise (49), with lactate, glycerol, and amino acids being the likely carbon sources. Although we did not assess the breakdown and synthesis of glycogen in response to a single bout of exercise in this study, we previously reported similar glycogen breakdown during exercise and synthesis rates after exercise in ketogenic and high-carbohydrate athletes (84).

Although speculative, the small decrease in intramuscular glycogen and the increases in BHB and NEFA may have been contributing factors in the changes observed in mitochondrial capacity. Given that glycogen inhibits AMPK activity (78) and that AMPK signaling regulates mitochondrial fat oxidation (30, 48), increased activity of AMPK facilitated through partial depletion of glycogen may have been a primary mechanism through which mitochondrial function changed in the KD group. This is further supported by the fact that AMPK is inhibited by insulin (34), which decreased in the KD group. In addition, both insulin and glucose can inhibit β -oxidation independently of AMPK, especially in conjunction with underlying insulin resistance (73, 88), indicating that the improvement in HOMA-IR in the KD group may have also contributed to the increase in fat oxidation. BHB is known to be a class I histone deacetylase (HDAC) inhibitor (74), and class I HDAC inhibition has been

shown to increase mitochondrial oxidative capacity in mouse skeletal muscle through increased expression of peroxisome proliferator-activated receptor (PPAR) γ coactivator 1- α (23). Likewise, free fatty acids have been shown to upregulate mitochondrial fatty acid oxidation in rat skeletal muscle, likely through activation of PPAR δ (24).

Fat and carbohydrate oxidation generally have a reciprocal relationship, which is consistent with the changes observed in mitochondrial fat metabolism not being mirrored by similar changes in carbohydrate metabolism. As metabolism shifts toward greater reliance on fat oxidation, the ratio of FADH₂ to NADH available for oxidative phosphorylation can more than double (77). In the carbohydrate-fed state, characterized by abundant glucose availability, complexes I and III of the electron transport chain tend to be associated in supercomplexes, favoring oxidation of NADH. As the ratio of FADH₂ to NADH increases during greater reliance on fat oxidation, complex I is dissociated from complex III, facilitating greater oxidation of FADH₂ (3, 26, 28, 42). Therefore, it appears that oxidative phosphorylation can be optimized according to dietary macronutrient ratios, but at the cost of decreased efficiency for the less abundant macronutrient (carbohydrate in this case). In addition, dietary carbohydrate restriction and lower insulin levels, as well

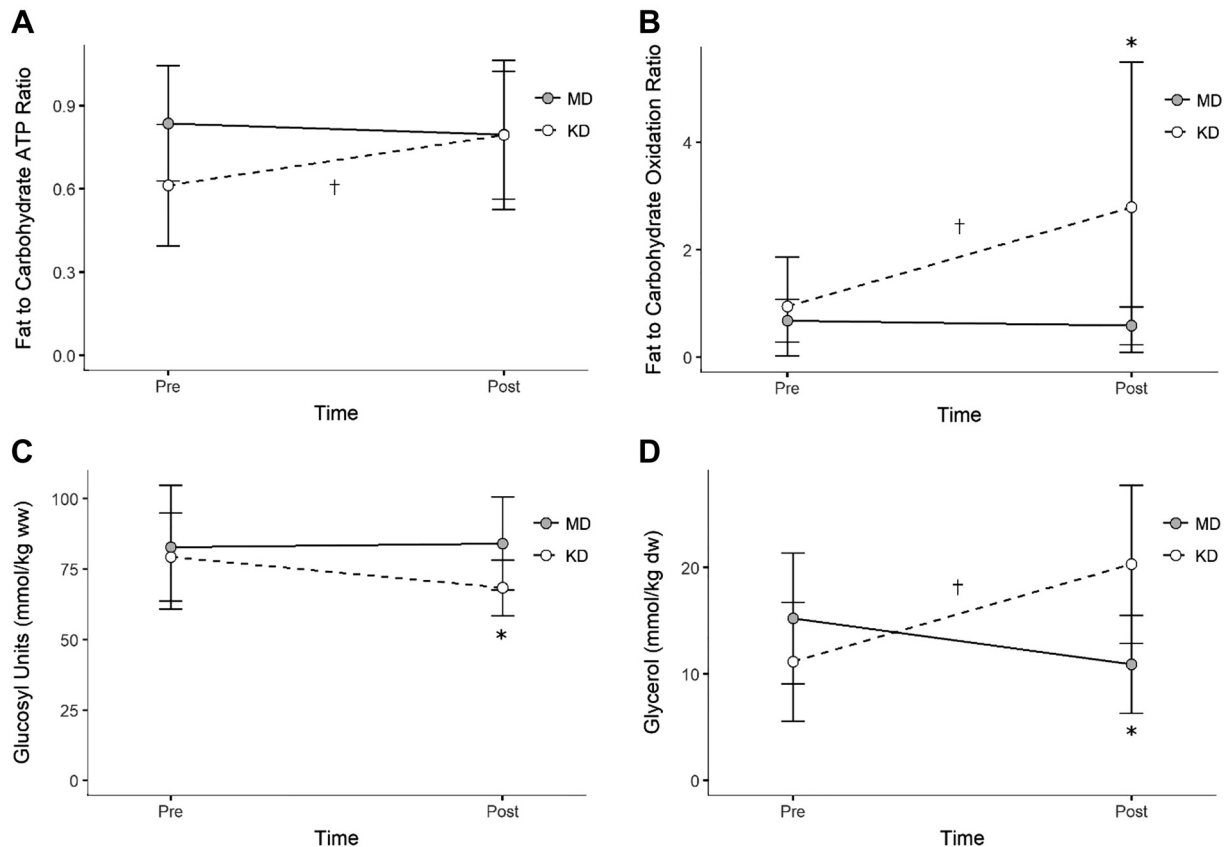


Fig. 5. Shift in macronutrient metabolism (means $\pm$ SD) in response to 12 wk of a ketogenic (KD) or mixed (MD) diet combined with exercise training. A: ratio of mitochondrial ATP production from fat substrate (palmitoylcarnitine + malate) to that with carbohydrate substrate (pyruvate + malate): effect of time, $P = 0.019$; time $\times$ diet interaction, $P = 0.001$. B: ratio of whole body fat oxidation to carbohydrate oxidation: effect of time, $P = 0.052$; time $\times$ diet interaction, $P = 0.001$. C: glycogen content (measured as glucosyl units) per wet weight (ww) of muscle tissue: effect of time, $P = 0.359$; time $\times$ diet interaction, $P = 0.099$. D: triglyceride content (measured as glycerol) per dry weight (dw) of muscle tissue: effect of time, $P = 0.060$; time $\times$ diet interaction, $P < 0.001$. Post, postintervention; Pre, preintervention. * $P < 0.008$ for between-group difference at associated time point; † $P < 0.008$ for within-group difference between time points for associated diet group.

as increased BHB and fatty acids, are all highly relevant signals of a ketogenic diet that promote inhibition of pyruvate dehydrogenase (57–59, 63), representing multiple mechanisms through which increased reliance on fat oxidation may inhibit carbohydrate metabolism.

Despite increased rates of fat oxidation and ketogenesis, oxidation of ketones in skeletal muscle was minimal. This is consistent with a recent study showing oxidation of ketones by cardiac and skeletal muscle mitochondria isolated from rats to be about 4-fold less compared with pyruvate, and this was despite the concentrations of ketones being 10 times the concentrations used in the present study (60). Similar results were observed in permeabilized muscle fibers, including human skeletal muscle. This minimal rate of ketone oxidation is likely due to the fact that succinyl CoA:3-ketoacid CoA transifuse (SCOT), an enzyme critical to ketolysis, is minimally expressed in human skeletal muscle compared with the heart, kidneys, and brain (22). Given the role of ketones as an alternative fuel source during starvation, it is logical to suspect that muscle spares ketones for more critical organs. However, consistent with the shift toward fat oxidation in the KD group, mitochondrial capacity to produce ATP from ketone oxidation increased relative to capacities from carbohydrate and fat oxidation, which warrants future investigation of potential changes in skeletal muscle SCOT activity during a ketogenic diet. Use of exogenous ketone supplementation as a

performance aid has recently increased in popularity. Prior work from our laboratory (37) indicates that ketone salts combined with caffeine and amino acids enhance physical performance. However, most studies have failed to support a performance benefit of exogenous ketones (45). Either way, our observation of minimal ketone oxidation, combined with the relatively low levels of SCOT, indicates that the mechanism of enhancement is not related to ketones acting as a direct energy substrate in skeletal muscle. This is consistent with prior observations of ketone uptake in skeletal muscle during fasting-induced ketosis, including saturation of uptake at low concentrations during rest and a decline in uptake during exercise (6).

A potential limitation of our ketone oxidation results is that there is not a clear understanding of whether or not different salts of BHB and acetoacetate uniquely influence mitochondrial function. More specifically, it is unclear whether our results were influenced by the sodium and lithium salts we used. Robust respiration has been reported with the sodium salt of pyruvate in mitochondria isolated from heart (66, 72, 92). The concentration of sodium salt used in these experiments was 5- to 16-fold greater than that used in the present study. Similarly, robust respiration has been reported in mitochondria isolated from brain (39) with the same 1 mM concentrations for lithium acetoacetate and sodium-BHB as used in the present study. In mitochondria isolated from heart, respiration rates with 1 mM of

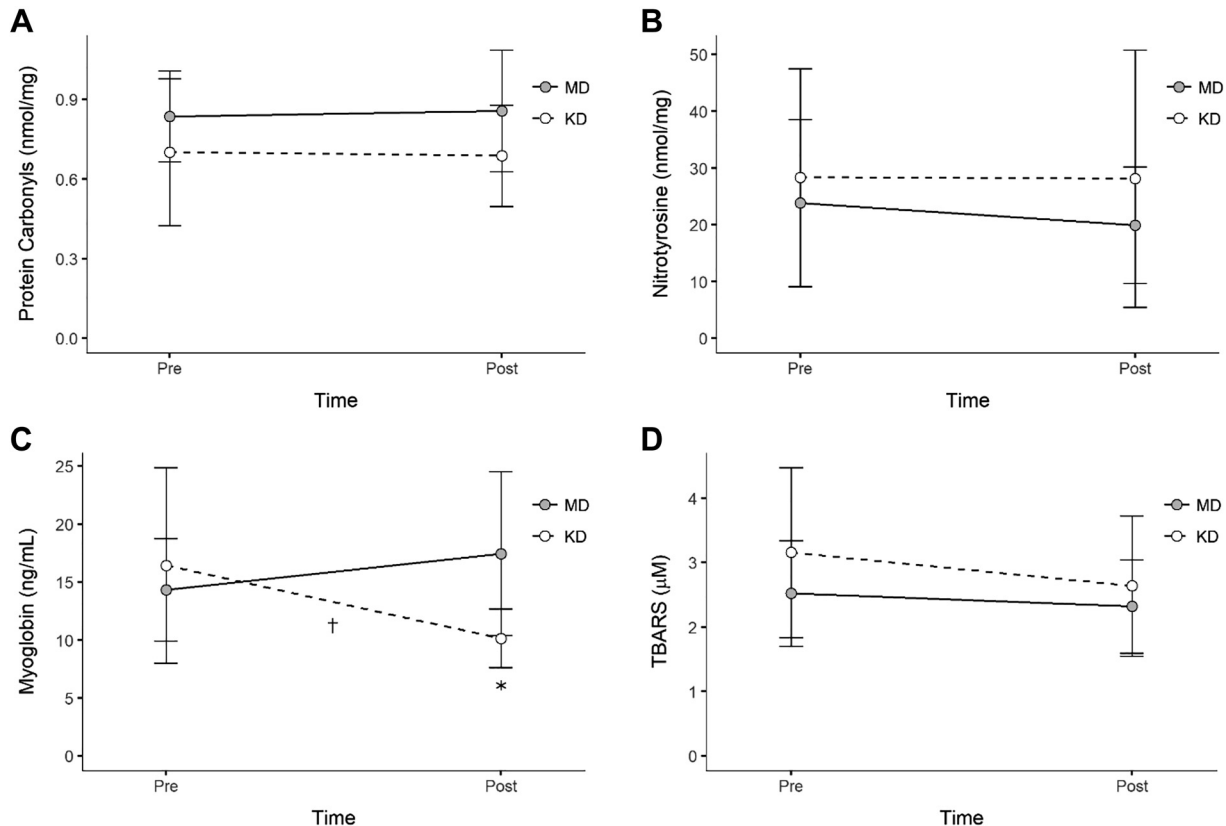


Fig. 6. Oxidative damage (means $\pm$ SD) before and after 12 wk of a ketogenic (KD) or mixed (MD) diet combined with exercise training. A: mitochondrial content of protein carbonyls: effect of time, $P = 0.286$; time $\times$ diet interaction, $P = 0.839$. B: mitochondrial content of nitrotyrosine: effect of time, $P = 0.226$; time $\times$ diet interaction, $P = 0.707$. C: serum myoglobin: effect of time, $P = 0.190$; time $\times$ diet interaction, $P = 0.005$. D: plasma thiobarbituric acid reactive substances (TBARS): effect of time, $P = 0.257$; time $\times$ diet interaction, $P = 0.589$. Post, postintervention; Pre, preintervention. * $P < 0.008$ for between-group difference at associated time point; † $P < 0.008$ for within-group difference between time points for associated diet group.

lithium acetoacetate alone have been reported to be three- to fourfold greater (55) than the rates we observed with acetoacetate and BHB combined. Although further research is warranted, we do not believe the salt forms of acetoacetate and BHB used in the present investigation explain the low rates of mitochondrial ketone oxidation.

Prompted by the low rates of ketone oxidation, we took the limited opportunities for additional testing of state 3 O_2 consumption with fresh mitochondrial isolations to assess potential limitations of the ketone-based substrate mix. Addition of 2 mM malate, the same concentration used in the carbohydrate- and fat-based substrates, increased O_2 consumption in the two samples for which usable data were obtained, but to a minimal extent that was still three- to fourfold lower compared with the carbohydrate-based substrate. In an additional two samples there was no difference in O_2 consumption with the use of an alternative sodium-BHB reagent (catalog no. H6501, Sigma-Aldrich, St. Louis, MO), and in another sample a tripling of the acetoacetate and BHB concentrations similarly had no effect. The lack of effect from increased concentration was further confirmed in several samples for which the typical volume of ketone substrate was added for a second and a third time. Collectively, this suggests that the ketone concentration, the lack of malate, and the particular BHB reagent do not explain the minimal rates of ketone oxidation.

The decreased ratio of mitochondrial ATP production to H_2O_2 production in the MD group is indicative of decreased efficiency of energy production relative to oxidative burden. The increase in H_2O_2 production with all three substrates in the MD group, but not the KD group, suggests that the ketogenic diet inhibited reactive oxygen species (ROS) production, upregulated endogenous antioxidant defense, or a combination of both. It is reasonable to suspect that upregulation of antioxidant defense was at least a contributing factor, possibly through induction of mitohormesis (51). However, given the lack of an increase in mitochondrial protein carbonyls and nitrotyrosine in the MD group, the increase in mitochondria ROS production was likely not of sufficient magnitude to alter these markers of mitochondrial oxidative damage. Collectively, these data suggest that neither intervention had a strong influence on the overall oxidative burden.

The significant decrease in serum myoglobin in the KD group (38%) and the nonsignificant increase in the MD group (22%) implies that the ketogenic diet may have minimized the stress response to exercise and/or enhanced recovery from exercise, which is consistent with anecdotal reports from elite endurance athletes (85). Increased myoglobin in the blood is indicative of damage to the sarcolemmal membrane and is therefore commonly used to assess the physiological burden of exercise training (8). However, exercise-induced elevations in myoglobin

generally resolve within 24 h (7, 17, 31). Given that participants were instructed to avoid intense activity for 48 h before testing and that the resting myoglobin levels we observed are much lower compared with exercise-induced elevations, the changes we report are more likely to reflect longer-term recovery patterns from exercise that specifically impact sarcolemmal permeability. Lipid peroxidation induced by mitochondrial ROS production can attack the fatty acids and proteins comprising cell membranes, thereby compromising membrane integrity (29). The fact that we did not detect any differences between groups in oxidative damage markers (i.e., plasma TBARS and mitochondrial protein carbonyls) suggests that lipid peroxidation or antioxidant defense mechanisms were not responsible for improved membrane integrity with the ketogenic diet. Although, it is possible that other biomarkers such as F2-isoprostanes may be more indicative of heightened oxidative stress and should be explored in future studies.

Whether the increase in resting myoglobin in the MD group contributed to their decline in mitochondrial function remains speculative. Exhaustive exercise can lead to mitochondrial impairment that is associated with elevations in myoglobin (15, 44) and appears to be a strong predictor of increased myoglobin concentration in blood (15), suggesting that muscle resilience is dependent on mitochondrial function. Examples of mitochondrial alterations resulting from exhaustive exercise include abnormal structural morphology, decreased respiration and ATP production, and increased H_2O_2 production (56). Increased mitochondrial Ca^{2+} content (16) and faster opening of the mitochondrial permeability transition pore (mPTP) (44) may also occur, which can lead to further impairment including cellular apoptosis. Given the potential of these effects to persist for several days after an exhaustive bout of activity (21, 67), frequent bouts of intense exercise may result in accumulation of mitochondrial impairment that may take longer to resolve. Although it is tempting to speculate that the ketogenic diet enhanced mitochondrial resilience to exercise-induced damage, and thus possibly contributed to the diverging responses between the KD and MD groups, more research is needed to assess the potential for some of the acute effects of strenuous exercise on mitochondrial function to linger and become chronic in the context of consistently repeated bouts.

Insulin resistance, obesity, and inflammation are strongly associated with mitochondrial impairment (1, 14). The slightly higher preintervention values for CRP, HOMA-IR, and visceral fat in the KD group indicate early-stage metabolic impairment. It is not clear whether insulin resistance is a cause or effect of mitochondrial impairment (10, 14). Since normal insulin function supports mitochondrial function, it has been proposed that insulin resistance causes mitochondrial impairment (1). However, mitochondrial dysfunction caused by oxidative stress and/or impaired fatty acid oxidation may also contribute to insulin resistance. In the present investigation, insulin sensitivity improved in the KD group in conjunction with a prominent increase in fat oxidation (both systemically and in skeletal muscle mitochondria) and minimal change in mitochondrial H_2O_2 production, suggesting that impairment of fat oxidation was a more important factor for insulin resistance than oxidative stress.

Whether the mitochondrial functional changes were solely attributed to keto-adaption cannot be definitively determined

given the concurrent exercise training, significant weight and fat loss, and improvements in HOMA-IR in the KD group. The changes in multiple mitochondrial markers despite inclusion of HOMA-IR, CRP, and visceral fat as covariates suggests that the mitochondrial and metabolic changes observed were primarily a result of the dietary intervention. This conclusion is consistent with prior evidence showing that dietary carbohydrate restriction improves many of the features of insulin resistance independent of weight loss (35), that weight loss (independent of exercise) has minimal impact on mitochondrial function in skeletal muscle (76, 80), and that ketogenic diets increase whole body fat oxidation (27, 84) and activity of mitochondrial enzymes (11, 27, 32, 75) in skeletal muscle beyond the effects of exercise. Similarly, although both groups completed the same exercise training, it is unclear to what extent this training may have contributed to mitochondrial changes. $\dot{V}\text{O}_{2\text{max}}$ was not expected to increase given that the training protocol primarily consisted of resistance exercise, and the change in $\dot{V}\text{O}_{2\text{max}}$ that we did observe can be attributed to weight loss. Although we can therefore conclude that aerobic capacity was most likely not a contributing factor, a more definitive conclusion on the influence of exercise is not possible.

Further research is needed to explore the extent to which ketogenic diets may enhance mitochondrial function in healthy individuals independent of significant changes in fat loss and insulin sensitivity. Given the involvement of both insulin resistance and mitochondrial impairment in a wide variety of degenerative diseases, if enhancement of mitochondrial function is a mechanism through which ketogenic diets improve insulin sensitivity, this would imply potential for major improvements in public health.

In conclusion, this investigation is the first to provide evidence demonstrating that a closely monitored state of nutritional ketosis achieved through a sustainable ketogenic diet alters mitochondrial function in human skeletal muscle. During 12 wk of exercise training, a ketogenic diet resulted in greater mitochondrial capacity and efficiency, particularly in the context of fat oxidation as indicated by increased total ATP production as well as ATP production relative to O_2 consumption and H_2O_2 production. The ketogenic diet was associated with greater reliance on fat relative to carbohydrate, which was accompanied by a modest decrease in resting muscle glycogen and a larger increase in muscle triglycerides. The ketogenic diet improved metabolic health markers and resolved indicators of insulin resistance in conjunction with weight loss and exercise, implying that alteration of mitochondrial function is worthy of consideration as a mechanism through which ketogenic diets exert therapeutic benefits. Such evidence could provide new insights into managing insulin resistance and associated conditions, which currently affect over half of American adults (50).

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DISCLOSURES

V.J.M. is founder of Fitfilled, LLC. J.S.V. receives royalties for low-carbohydrate nutrition books. He is founder, consultant, and stockholder of Virta Health Corp; a member of the scientific advisory boards for Atkins Nutritionals Inc, UCAN Co, and Advancing Ketone Therapeutics; and has received honoraria from Metagenics and Pruvit. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

V.J.M., R.A.L., E.B., P.N.H., W.J.K., F.A.V., and J.S.V. conceived and designed research; V.J.M., R.A.L., T.S.S., J.S., W.D.A., P.N.H., C.D.C., and M.L.K. performed experiments; V.J.M., T.S.S., and J.S.V. analyzed data; V.J.M. and J.S.V. interpreted results of experiments; V.J.M. prepared figures; V.J.M. drafted manuscript; V.J.M., R.A.L., E.B., T.S.S., J.S., W.D.A., P.N.H., C.D.C., M.L.K., W.J.K., F.A.V., and J.S.V. edited and revised manuscript; V.J.M., R.A.L., E.B., T.S.S., J.S., W.D.A., P.N.H., C.D.C., M.L.K., W.J.K., F.A.V., and J.S.V. approved final version of manuscript.

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