Title: Calorie restriction-induced weight loss and exercise have differential effects on skeletal muscle mitochondria despite similar effects on insulin sensitivity

Authors: Menshikova E.V., Ritov V.B., Dube J.J., Amati F., Stefanovic-Racic M., Toledo F.G., Coen P.M., Goodpaster B.H.

Journal: The journals of gerontology. Series A, Biological sciences and medical sciences
Year: 2017 February
DOI: 10.1093/gerona/glw328

Calorie restriction-induced weight loss and exercise have differential effects on skeletal muscle mitochondria despite similar effects on insulin sensitivity

[*]Elizaveta V. Menshikova[b], [*]Vladimir B. Ritov[b], John J. Dube[b], Francesca Amati[b], Maja Stefanovic-Racic[b], Frederico G.S. Toledo[b], Paul M. Coen[a#,b], Bret H. Goodpaster[a#,b]

* These two authors contributed equally to this work.

Author Institutional Affiliations:

[a]Translational Research Institute for Metabolism and Diabetes, Florida Hospital, Orlando, FL 32804, USA.

[b]Division of Endocrinology and Metabolism, Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA.

[#] = new affiliation for this author

Address correspondence to:

Bret H. Goodpaster, Ph.D.
Translational Research Institute for Metabolism and Diabetes
Florida Hospital • Sanford | Burnham Medical Research Institute
301 East Princeton Street, Orlando, FL 32804, U.S.A.
bret.goodpaster@flhosp.org
Office: 407.303.1305
Fax: 407.303.7199

Running title: Insulin Resistance and Mitochondria in Aging

ABSTRACT

Background: Skeletal muscle insulin resistance (IR) and reduced mitochondrial capacity have both been reported to be affected by aging. The purpose of this study was to compare the effects of calorie restriction-induced weight loss and exercise on IR, skeletal muscle mitochondrial content and mitochondrial enzyme activities in older overweight to obese individuals. **Methods:** Insulin-stimulated rates of glucose disposal (Rd) were determined using the hyperinsulinemic euglycemic clamp before and after completing 16 weeks of either calorie restriction to induce weight loss (N=7) or moderate exercise (N=10). Mitochondrial volume density (Vd), mitochondria membrane content (cardiolipin), and activities of electron transport chain (rotenone-sensitive NADH-oxidase), TCA cycle (citrate synthase) and ß-oxidation pathway (ß-hydroxyacyl CoA dehydrogenase; ß-HAD) were measured in percutaneous biopsies of the vastus lateralis before and after the interventions. **Results:** Rd improved similarly (18.2±9.0%, $p<0.04$) with both weight loss and exercise. Moderate exercise significantly increased Vd (14.5±2.0% $p<0.05$), cardiolipin content (22.5±13.4% $p<0.05$), rotenone-sensitive NADH-oxidase (65.7±13.2% $p=0.02$) and ß-HAD (30.7±6.8% $p=<0.03$) activity, but not citrate synthase activity (10.1±4.0%). In contrast, calorie restriction-induced weight loss did not affect mitochondrial content, NADH-oxidase or ß-HAD, yet increased citrate synthase activity (44.1 ± 21.1% $p=<0.04$). Exercise (increase) or weight loss (decrease) induced a remodeling of cardiolipin with a small (2-3%), but significant change in the relative content of tetralinoleoyl cardiolipin. **Conclusion:** Exercise increases both mitochondria content and mitochondrial electron transport chain and fatty acid oxidation enzyme activities within skeletal muscle, while calorie restriction-induced weight loss did not, despite similar improvements in insulin sensitivity in overweight older adults. **Key words:** Glucose uptake, muscle metabolism, human aging, calorie ~~dietary~~ restriction, obesity.

INTRODUCTION

Skeletal muscle insulin resistance (IR) has been linked to the etiology of type 2 diabetes (T2D) in both aging (1) and obesity (2). Although the exact causes of IR remain unexplained, chronic nutritional oversupply, i.e., overweight and obesity, is considered to be a major contributor in the development of IR (3). In addition, insufficient physical activity (4) or specific age-related alterations in glucose metabolism and mitochondria function (5, 6) are associated with IR. Weight loss induced by moderate calorie restriction (CR) or chronic exercise leads to a significant drop in plasma insulin and improves insulin sensitivity in middle age obese or elderly overweight individuals (4, 7).

Insulin-dependent glucose transport has been reported to be closely associated with skeletal muscle oxidative metabolism. Low oxidative type skeletal muscle fibers have reduced content of glucose transporter Glut 4 and reduced insulin sensitivity in comparison to high oxidative fibers (8). Exercise significantly increases both Glut 4 and mitochondrial content in human skeletal muscle (9, 10). Over-expression of PGC-1β in skeletal muscle increases tissue oxidative capacity and ameliorates suppressing effect of high –fat diet on insulin dependent glucose transport (10). Obesity and T2D are associated with mitochondria remodeling as indicated by the multiple changes in mitochondrial proteome and mitochondrial enzyme activities (11-13).

Little is known about the effects of CR ~~calorie restriction~~ on mitochondria in older humans. The CALERIE study reported that while CR ~~caloric restriction~~-induced weight loss increases mitochondrial DNA content in skeletal muscle, the activities of mitochondrial enzymes were not increased (14). This study was limited, however, to middle age subjects. Bariatric surgery-induced weight loss did not increase markers of mitochondrial content in middle aged

adults, but did improve qualitative aspects of mitochondrial respiration (15). We are not aware of any study that has specifically examined the effects of CR-induced weight loss on skeletal muscle mitochondria in older subjects. Moreover, nearly all studies of mitochondria and aging have been limited to one or two measures of mitochondrial content or function, and therefore have not accounted for potential differential effects on various components of mitochondrial energetics that include ß-oxidation, the tricarboxylic acid (TCA) cycle, and electron transport chain.

In addition to the loss of muscle mass and quality associated with aging (16), there are also increases in adiposity. Given that over 70% of men and women in the U.S. over the age of 60 are either overweight or obese (17), it is critical to evaluate the specific effects of exercise and CR-induced weight loss on mitochondria in older overweight to obese humans. Therefore, this study examined the separate effects of CR-induced weight loss and exercise on several aspects of mitochondria content and performance assessed by mitochondria enzyme profiling in human skeletal muscle. ~~A second purpose~~ An exploratory aim was to evaluate whether or not alterations in the various parameters of mitochondrial content and performance, including cardiolipin molecular species, the tricarboxylic acid (TCA) cycle, electron transport chain or ß-oxidation activities, are associated with improved insulin sensitivity with CR-induced weight loss or exercise.

STUDY METHODS AND DESIGN

Participants. Seventeen overweight to moderately obese (body mass index [BMI] = 28.0 – 32.0 kg/m^2) men and women aged 60 – 75 years were recruited from the Pittsburgh metropolitan and surrounding areas. The study was approved by the University of Pittsburgh Institutional Review Board and a written informed consent was obtained from each volunteer. Participants were considered for this study if they (a) had no history of clinically significant cardiovascular disease; (b) had a resting systolic blood pressure of 150 mmHg or less and diastolic blood pressure of 95 mmHg or less; (c) were a nonsmoker; (d) was a stable weight (no gain or loss of >6 kg in 6 months); and (e) were sedentary (currently participating in aerobic exercise <2 d/wk). Participants who had T2D were excluded based on self-reported diabetic medication use, fasting glucose >120mg/dL or 2-hr glucose >200mg/dL obtained during a standard oral glucose tolerance test (75g, OGTT). Clinical baseline characteristics of the study participants are presented in Table 1. The interventions lasted 16 weeks after initial baseline assessments.

~~Dietary~~ Calorie restriction intervention. The intervention was aimed at a target weight loss of 8% -10 % of baseline weight, by a reduction in calorie intake (18). A caloric restriction of 500 – 1,000 kcal/d (<30% of calories from fat) was implemented based on the participant's baseline weight. Total caloric needs were determined by multiplying the participant's baseline weight (kg) by 25. From this, the dietitian made the required caloric adjustments to produce a negative energy intake resulting in a loss of 0.5 – 1.0 kg of body weight per week.

Exercise intervention. Subjects performed exercise nearly 5 days per week at moderate-intensity exercise (60–70% of maximal heart rate as determined during a maximal capacity aerobic test). Three sessions were supervised in our facility and two were unsupervised. Mostly, the exercise included using of a stationary cycle, treadmill, or walking as previously described (18). The

exercise program was progressive such that by the last 8 weeks, exercise sessions were performed at about 40 minutes and the intensity of the exercise was raised to 75% of their maximal heart rate. Participants wore a polar for each exercise session, and at the end of each session, an average heart rate, exercise duration, and rating of perceived exertion were recorded. Exercise sessions consisted of treadmill or outside brisk walking as the primary mode of exercise and cycling as a secondary mode. Each participant was issued a personal binder with exercise logs to record exercise session data (ie, heart rate, duration, and intensity), and the exercise logs were reviewed weekly for exercise adherence.

Body weight and lean mass. Body weight was measured weekly using a Scale Tronix electronic scale (Tronix Inc., Wheaton, IL). Dual-energy x-ray absorptiometry (DXA; GE Lunar Prodigy scanner, Encore software 2005; General Electric, Milwaukee, WI) was used to measure changes in total fat mass (FM) and fat-free mass (FFM).

Metabolic assessments. Hyperinsulinemic euglycemic glucose clamps were used to measure insulin sensitivity (4). Maximal aerobic capacity (VO_{2max}) was measured on a stationary cycle as described previously (19).

Analysis of mitochondria content and enzyme activities. Percutaneous biopsy samples of the *vastus lateralis* muscle (15–25 mg wet weight) were obtained by Bergstrom needle and flash frozen in liquid nitrogen and stored at -80C. A portion of the muscle sample was fixed for determination of Volume density by TEM. Frozen biopsy specimen was analyzed for cardiolipin content, citrate synthase, ß-HAD and electron transport chain NADH-oxidase as ~~described in the supplemental section~~ follows. Total particulate fraction that contains more than 95% of tissue mitochondria was isolated by centrifuging from skeletal muscle biopsy homogenate (20). Particulate fraction was used to assess cardiolipin content (21), activity of whole mitochondrial

electron transport chain (rotenone-sensitive NADH:O_2 oxidoreductase), citrate synthase (20), and ß-HAD (13). The soluble fraction (supernatant) was used to estimate activity of creatine kinase and remaining activities of citrate synthase and ß-HAD that were released from mitochondria due to tissue freezing. Activity of ß-HAD have been measured in the reverse reaction by standard method adapted for higher sensitivity to our HPLC technique (13). Activity of rotenone-sensitive NADH:O_2 oxidoreductase in total particulate fraction, prepared from muscle homogenate, was measured in the presence of alamethicin as described previously (20). Creatine kinase (CK) activity was measured as a marker of muscle fiber content in biopsy (22). Mitochondrial volume density (Vd), i.e., the percentage of the cell volume occupied by total mitochondrial volume, reflects mitochondrial content and was measured in biopsy specimens by stereological methods and transmission electron microscopy as described previously (23). Tetraoleoyl cardiolipin (internal standard) was purchased from Avanti Polar lipids Inc. (Alabaster, AL). 1-Pyrenyldiazomethane (PDAM) was obtained from Molecular Probes (Eugene, Oregon). Citrate Synthase, ß-HAD, and NADH:O_2 oxidoreductase activities and cardiolipin content were normalized to CK activity to control for muscle fiber content. Ratios between activity of mitochondrial electron transport chain (NADH-oxidase), ß-oxidation (β-HAD), and TCA cycle (Citrate Synthase) were also calculated to examine changes in the relative capacity for mitochondrial electron transport chain/TCA or ß-oxidation/TCA.

Statistical analysis. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS for Mac, v18). Baseline group differences were assessed using a one-way analysis of variance (ANOVA). Differences in baseline ratio variables (Metabolic Status and Gender) were determined with a Pearson's chi-squared test. A two-way repeated-measures ANCOVA was used to determine main (group, treatment) and interaction effects for all outcome

variables. Baseline values for each variable were used as covariates. Log transformations were used when ANOVA assumptions of normality were not met. Pearson's correlation coefficient was used to relate alterations in mitochondrial parameters to changes in insulin sensitivity. Statistical significance was assumed *a priori* at $P<0.05$.

RESULTS

Baseline comparison between groups. Age, Gender ratio, Weight, BMI, Fat mass and FFM were not different between groups at baseline (Table 1, P>0.05). The ratio of IGT/NGT was significantly different, with more NGT and less IGT participants in the exercise group (Table 1, P<0.05). The exercise group also had lower fasting glucose and HbA1C (Table 1, P<0.05). VO_2max (ml/min/kgFFM) was also higher in the exercise group (Table 1, P<0.05). Insulin sensitivity and the majority of mitochondrial measurements (Vd, cardiolipin content, NADH-oxidase and B-HAD activity) were not different between groups at baseline (Table 1, P>0.05).

Effects of intervention on insulin sensitivity, physical fitness and body composition. Insulin sensitivity (Rd) improved by 1.25 mg/kgFFM/min (18.9±9.8%) with calorie restriction ~~weight loss~~ and by 0.86 mg/kgFFM/min (18.3 ± 8.2%) with exercise interventions (Figure 1, both P<0.05). Neither fasting glucose nor HbA1C was significantly altered with either intervention (Table 1, P>0.05). The exercise intervention increased cardiovascular fitness as evidenced by a modest, but significant increase in VO_2max (P<0.05), which did not occur in weight loss (Table 1, P>0.05). Weight loss reduced body weight and body fat to a greater extent compared to exercise (P<0.05). Weight loss also resulted in a significant reduction in fat-free mass, which was not observed with Exercise (P<0.05).

Effects of intervention on mitochondrial content. Mitochondrial Vd was increased by exercise (P<0.05), but not CR-induced weight loss (Figure 2A, P>0.05). Similarly, exercise significantly increased cardiolipin content (22.5±13.4% P<0.05), but CR-induced weight loss did not change cardiolipin content significantly (Figure 2B, P>0.05). The predominant molecular form of cardiolipin in human skeletal muscle is tetralinoleoyl cardiolipin (TL-CL) (21) that represented ~75% of total cardiolipin at baseline (Table 2). Both interventions induced small, but statistically

significant changes in the composition of cardiolipin molecular forms (Table 2, $P<0.05$). Exercise increased by ~2% the proportion of TL-CL, and decreased the relative content of the more saturated species of cardiolipin (Table 2, $P<0.05$). In contrast, CR-induced weight loss induced ~3% reduction in the proportion of TL-CL while increasing the proportion of saturated species of cardiolipin (Table 2, $P<0.05$).

Effects of intervention on mitochondrial enzymatic activities. Exercise and CR-induced weight loss both increased citrate synthase activity normalized to biopsy creatine kinase (Time Effect, Figure 3B, $P<0.05$). Baseline level of citrate synthase activity was lower in the CR group and increased only in response to the CR intervention (both $P<0.05$). This was in contrast to the effects on ß–HAD and rotenone-sensitive NADH-oxidase; exercise increased ß-HAD and rotenone–sensitive NADH-oxidase activities by 30.7±6.8% and 65±13% (both $P<0.05$), respectively, while CR-induced weight loss did not significantly alter these mitochondrial enzyme activities (Figure 3A & C, $P>0.05$). In order to further examine whether or not the respective interventions had specific effects on these selected enzyme activities of mitochondrial TCA cycle, ß-oxidation or electron transport chain, simple ratios were calculated. Exercise significantly increased the ratios of NADH-oxidase/citrate synthase and ß-HAD/citrate synthase (Figure 4A & B, both $P<0.05$). These ratios were not affected by CR-induced weight loss ($P>0.05$), indicating a proportionately greater exercise-induced increase in both the total activity of electron transport chain and ß-HAD relative to citrate synthase. A correlation analysis was conducted within each group individually and with all participants combined to examine whether change in mitochondrial measurements (content and activity) related to improvements in insulin sensitivity. We found, however, that none of the mitochondrial content or function changes, were associated with the improved insulin sensitivity induced by these interventions (all $P>0.05$).

DISCUSSION

Effects of exercise or caloric restriction on skeletal muscle mitochondria.

The primary findings of our study were that exercise training improved skeletal muscle (*vastus lateralis*) mitochondria content, mitochondrial electron transport chain activity or β-HAD activity in overweight to obese older adults, which was in stark contrast to little effect of CR-induced weight loss on mitochondria, despite similar improvements in insulin sensitivity. Our findings are in accord with several previous reports in middle-aged subjects. We have previously demonstrated that exercise training superimposed on CR-induced weight loss increases both mitochondrial electron transport chain capacity and mitochondrial content (assessed by cardiolipin and EM) in obese insulin resistant subjects (7, 24) and in those with type 2 diabetes (23), while CR-induced weight loss alone increased neither mitochondria content, nor ETC or β-HAD (13, 24). Our data are in agreement with those from the CALERIE study, which demonstrated that CR-induced weight loss had no effect on mitochondrial cytochrome c oxidase or β-HAD activities in *vastus lateralis* biopsy in overweight middle-age volunteers (14). In contrast to CR-induced weight loss, exercise significantly increased the NADH oxidase/citrate synthase ratio**.** We hypothesize that this is related to the specific increased capacity of mitochondria to oxidize NADH (increase in ETC activity) with the increased energy demands by exercise.

One goal of our study was to quantify intervention responses in mitochondria content as an integrated indicator of changes in mitochondrial biogenesis and mitophagy, the cellular processes that regulate mitochondrial content. Exercise increased cardiolipin content, contrary to CR-induced weight loss. While this study was not statistically powered to examine correlations between changes in variables, the amount of exercise (energy expenditure per week) performed

was strongly associated (r^2=0.56, P<0.01) with the increase in cardiolipin, suggesting a dose-response effect of exercise on cardiolipin content. This suggests that the increase of insulin sensitivity after CR does not provide a sufficiently strong signal to induce robust mitochondria biogenesis in skeletal muscle of overweight elderly, or that there is a "resistance" in aged skeletal muscle to the anabolic effects of insulin (25). This hypothesis is supported by our demonstration that exercise training, but not CR-induced weight loss, increased mitochondria volume density. In the CALERIE study it was reported that CR increases the content of mitochondrial DNA (mDNA) and mitochondrial transcription factor A (TFAM) (14). The mitochondrial network contains variable number of DNA copies (26) and it is quite possible that the increase in mDNA and TFAM may be disconnected from changes in mitochondrial volume density. An alternative interpretation is that CR stimulated mitochondrial biogenesis and mitophagy (clearance) to a similar degree resulting in no change in markers of content.

A significant proportion of mitochondrial proteome can be acetylated and could be a potential target for deacetylase activity of Sirt 3 (27). High-fat diets reduce the content of Sirt 3 and leads to hyperacetylation of mitochondrial proteome in skeletal muscle and liver (28). Recent studies in rodents indicate that CR ~~calorie restriction~~ sharply increases expression of Sirt 3 in skeletal muscle mitochondria (29). Although we did not directly measure Sirt 3 expression or content, our study is the first to examine the separate effects of CR-induced weight loss and exercise on ß-oxidation, TCA cycle and electron transport chain activities in aging human muscle, thus providing a more comprehensive analysis of mitochondrial performance in response to these interventions. These data indicate that increases in mitochondria content, ß-oxidation or electron transport chain activities are not required for improved insulin sensitivity and are in agreement with our recent observations in bariatric surgery patients (15). These data suggest that

either a decreased energy supply through CR-induced weight loss or an increase in energy demand by exercise can promote beneficial effects on insulin sensitivity, providing further evidence that obesity-induced insulin resistance in skeletal muscle can be caused by either excessive influx of nutrients or reduced energy expenditure, physical inactivity or lack of exercise. It is possible that a sustained imbalance between nutritional supply and energy expenditure generates a metabolic signal that restricts insulin-dependent glucose uptake in skeletal muscle. Due to intricate nature of metabolic controls and lack of data on human skeletal muscle metabolome, it is impossible currently to specify a metabolite(s) or a signaling pathway that attenuates glucose transport in response to nutritional oversupply. However, products from incomplete ß-oxidation or reactive oxygen species emission may play a role (30, 31).

In addition to specific intervention effects on mitochondria, we found that overweight older subjects respond differently to both interventions in comparison to younger subjects. Exercise induced robust responses in both ß-oxidation and electron transport chain that were absent with CR-induced weight loss, though the increase in citrate synthase activity was significantly increased only in the CR-induced weight loss group. Although the increase in citrate synthase activity after CR may be a partial anabolic response, we cannot exclude the possibility that the increase in citrate synthase activity may be mediated by post translational modification and reflects activation of TCA cycle flux in demand for increased processing of amino acids into oxaloacetate to support oxidation of fatty acid released from adipose tissue during caloric restriction. Exercise, due to increased number and recruitment of Glut4 (32), could provide an additional flux of glucose into muscle that supports anaplerosis and spares amino acids from oxidation that is manifested as the preservation of fat-free mass in exercise contrary to weight loss. This remains to be proven, however, and should be explored further.

Effects of exercise or caloric restriction on the cardiolipin – mitochondrial remodeling?

The differential effects of exercise and CR-induced weight loss on cardiolipin composition in our study suggests differences in mitochondrial remodeling with CR-induced weight loss and exercise, which has recently been implicated in insulin resistance of obesity (33). The increases in tetralinoleoyl cardiolipin with exercise is also consistent with our prior observations that this unsaturated species can represent up to 83% of total tissue cardiolipin in muscle from highly insulin sensitive, exercise-trained young individuals (25) and can be increased with exercise (15). Cardiolipin is a mitochondria-specific phospholipid and is important for numerous mitochondria functions (34-36). However, its exact role in mitochondria is still not completely understood (34, 35). The presence of four fatty acids and molecular symmetry of many cardiolipin species (37) suggest that this phospholipid can be a "fastening tool" that maintains structural integrity of mitochondrial respiratory complexes or correct crista folding. Additionally, it was suggested that two phosphates in cardiolipin molecule can form an intermolecular proton trap that can help maintain proton gradient across mitochondrial membrane (38).

Once nascent cardiolipin is synthesized it is remodeled into a tissue specific pattern of cardiolipin molecular species (34, 37). In human skeletal muscle "mature" cardiolipin is represented primarily by tetralinoleoyl cardiolipin. Tetralinoleoyl cardiolipin is perhaps linked to fatty acid oxidation in mitochondria, since it is present in very low amounts in tissues and cells that mostly rely on the oxidation of glucose for production of ATP (brain, transformed cells). Mechanisms for cardiolipin remodeling still have not completely understood. The content of tetralinoleoyl cardiolipin in mitochondria is also sensitive to tissue metabolic state. High-fat diet leads to dramatic decrease in tetralinoleoyl cardiolipin content or total cardiolipin linoleate in rat

heart (39). Additional studies need to be performed to determine whether these small changes in cardiolipin composition with CR-induced weight loss or exercise are related to mitochondrial function or improvements in fuel metabolism, including insulin action.

The prevailing paradigm is that aging is associated with insulin resistance and declining mitochondria function (6). CR is often touted as being the best means to improve or slow age-related loss of function. Within the context of human aging, the effects of life-long CR are difficult, if not impossible, to objectively ascertain. Moreover, investigating the impact of CR-induced weight loss in older humans who are already overweight or obese – comprising more than 70% of the older U.S. population – is more likely to have a greater translation. There are very little data examining the effects of CR-induced weight loss on skeletal muscle insulin resistance and mitochondria specifically in older humans. CR-induced weight loss alone is sufficient to improve insulin sensitivity in overweight to obese older adults, which may be informative regarding the potential benefits of weight loss alone on cardiometabolic risk. It is also possible that the elderly do not improve their insulin sensitivity in response to exercise as well as younger adults (40). From these considerations, CR could be an intervention of choice to treat obesity in elderly. It was clear from our results that these benefits on insulin sensitivity could be derived with or without concomitant effects on markers of the capacity for fatty acid oxidation or oxidative phosphorylation. An additional potential pitfall of CR in the elderly is a loss of lean body mass (18). Specific molecular mechanisms underlying the loss of muscle mass during CR need to be elucidated. For example, it has recently been demonstrated that induction of PGC1 alpha, which is known to promote mitochondria biogenesis (41), can also play a role in maintaining muscle mass in models of muscle wasting (42). Therefore, it is possible that

increases in mitochondria or the transcriptional factors involved in mitochondrial biogenesis also play a role in attenuating the loss of muscle mass induced by CR ~~caloric restriction~~.

A potential limitation of the study design is that it is not a randomized controlled trial and this likely resulted in baseline differences in fasting glucose, HbA1C and VO_2max. Citrate synthase activity was also lower in the calorie restriction group at baseline and may have influenced the response to the intervention. However, we do not believe these baseline differences greatly influenced the muscle focused endpoints (IS and mitochondria) and concern is mitigated by the fact that we statistically controlled for baseline values of each variable in the ANCOVA analysis. A second limitation is that the study was not statistically powered to adequately examine change associations. We found that none of the mitochondrial content or function changes, were associated with the improved insulin sensitivity induced by these interventions. The multi-factorial etiology of insulin resistance (lipitoxicity, inflammation, autophagy) may have also confounded potential relationships between mitochondria and Rd.

In summary, CR-induced weight loss and exercise are two distinct clinical interventions that can improve insulin sensitivity despite very different effects on skeletal muscle mitochondria biogenesis and performance. Our study neither confirms nor denies a mechanistic or causal link between mitochondria and insulin resistance. The etiology of insulin resistance is multi-factorial. It is therefore likely that there are many means to improve or ameliorate insulin resistance. Mitochondria function is far more complex than simply mitochondria biogenesis or content, or even measures of enzyme activity. Further investigations should examine additional aspects of mitochondrial function in response to insulin-sensitizing exercise and CR interventions to gain a better understanding of the potential links between mitochondria and insulin resistance in humans.

Conflict of Interest

The authors have no conflicts of interest to declare.

Funding

This work was supported by the University of Pittsburgh CTRC (M01RR00056), the Obesity and Nutrition Research Center (1P30DK46204), the National Institute on Aging (R01-AG20128), and the American Diabetes Association (to BHG). PMC is supported with a career development award from the National Institute on Aging (K01AG044437).

Acknowledgments

The authors would like to acknowledge the excellent technical assistance of Krista Clark, Steve Anthony, and the nursing staff at the University of Pittsburgh CTRC. We appreciate the assistance of Jim DeLany and Kelly McCoy with plasma glucose enrichment measures. We would like to thank Peter Chomentowski for assistance in mitochondrial morphometry analysis.

FIGURE LEGENDS

Figure 1. Effect of Exercise or Calorie Restriction interventions on skeletal muscle insulin sensitivity in older sedentary individuals. Glucose Clamps were performed before (Pre) and after the interventions (Post). Data represent Mean ± SEM. * = P<0.05, Significant within-group differences between Pre- and Post-intervention. Abbreviations: Rd; Rate of disappearance of glucose, FFM; Fat free mass.

Figure 2. Effect of Exercise or Calorie Restriction interventions on mitochondrial volume density (A) or cardiolipin content (marker of mitochondrial mass) (B) in skeletal muscle biopsy from older sedentary individuals. Biopsies were analyzed before (Pre) and after the interventions (Post). Cardiolipin content was normalized to CK activity to control for muscle fiber content. Data represent Mean ± SEM. * = P<0.05, Significant within-group differences between Pre- and Post-intervention. Abbreviations: CK; Creatine Kinase activity.

Figure 3. Effect of Exercise or Calorie Restriction interventions on markers of mitochondrial mass and electron transport chain activity in skeletal muscle biopsy from older sedentary individuals. Biopsies were analyzed before (Pre) and after the interventions (Post). The total activities of β-HAD (A), citrate synthase (B), and rotenone-sensitive-NADH oxidase (C) in biopsies were normalized to the Creatine Kinase activity. Citrate Synthase, ß-HAD, and NADH:O_2 oxidoreductase activities were normalized to CK activity to control for muscle fiber content. Data represent Mean ± SEM. φ = P<0.05, Significant Time Effect. * = P<0.05, Significant within-group differences between Pre- and Post-intervention. ** = P < 0.05, Significant between-group difference at the Pre-intervention time point. Abbreviations: CK; Creatine Kinase activity, NADH; Nicotinamide adenine dinucleotide.

Figure 4. Ratios between activity of mitochondrial electron transport chain (NADH-oxidase), β-oxidation (β-HAD), and TCA cycle (Citrate Synthase) in skeletal muscle before (Pre) and after (Post) the Exercise and Calorie Restriction interventions. Total NADH-oxidase (A) or β-HAD (B) activities in biopsy were normalized to Citrate Synthase activity to examine changes in the relative capacity for mitochondrial electron transport chain/TCA or ß-oxidation/TCA. Data represent Mean ± SEM. * = P<0.05, Significant within-group differences between Pre- and Post-intervention. Abbreviations: CS; Citrate Synthase activity, NADH; Nicotinamide adenine dinucleotide.

REFERENCES

1. Facchini F, Hua N, Abbasi F, Reaven G. Insulin resistance as a predictor of age-related diseases. J Clin Endocrinol Metab. 2001;**86**:3574–3578.
2. DeFronzo R, Tripathy D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care. 2009;**32**:S157–S163.
3. Schrauwen P. High-fat diet, muscular lipotoxicity and insulin resistance. Proc Nutr Soc. 2007;**66**:33-41.
4. Amati F, Dubé J, Coen P, Stefanovic-Racic M, Toledo F, Goodpaster B. Physical inactivity and obesity underlie the insulin resistance of aging. Diabetes Care. 2009;**32**:1547-1549.
5. Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, *et al*. Mitochondrial dysfunction in the elderly: possible role in insulin resistance. Science. 2003;**300**:1140-1142.
6. Johnson ML, Robinson MM, Nair KS. Skeletal muscle aging and the mitochondrion. Trends Endocrinol Metab. 2013;**24**:247-256.
7. Menshikova E, Ritov V, Toledo F, Ferrell R, Goodpaster B, Kelley D. Effect of Weight Loss and Physical Activity on Skeletal Muscle Mitochondrial Function in Obesity. Am J Physiol Endocrinol Metab 2005;**288**:E818-E825.
8. Megeney L, Neufer P, Dohm G, Tan M, Blewett C, Elder G, *et al*. Effects of muscle activity and fiber composition on glucose transport and GLUT-4. Am J Physiol. 1993;**264**:E583-593.
9. Iversen N, Krustrup P, Rasmussen H, Rasmussen U, Saltin B, Pilegaard H. Mitochondrial biogenesis and angiogenesis in skeletal muscle of the elderly. Exp Gerontol. 2011;**46**:670-678.
10. Wright L, Brandon A, Hoy A, Forsberg G, Lelliott C, Reznick J, *et al*. Amelioration of lipid-induced insulin resistance in rat skeletal muscle by overexpression of Pgc-1β involves reductions in long-chain acyl-CoA levels and oxidative stress. Diabetologia. 2011;**54**:1417-1426.
11. Kulkarni S, Salehzadeh F, Fritz T, Zierath J, Krook A, Osler M. Mitochondrial regulators of fatty acid metabolism reflect metabolic dysfunction in type 2 diabetes mellitus. Metabolism. 2011;**61**:175-185.
12. Hwang H, Bowen B, Lefort N, Flynn C, De Filippis E, Roberts C, *et al*. Proteomics analysis of human skeletal muscle reveals novel abnormalities in obesity and type 2 diabetes. Diabetes. 2010;**59**:33-42.
13. Ritov V, Menshikova E, Azuma K, Toledo F, Wood R, Goodpaster B, *et al*. Deficiency of Electron Transport Chain in Human Skeletal Muscle Mitochondria in Type 2 Diabetes Mellitus. Am J Physiol Endocrinol Metab. 2010;**298**:E49-E58.
14. Civitarese A, Carling S, Heilbronn L, Hulver M, Ukropcova B, Deutsch W, *et al*. Calorie restriction increases muscle mitochondrial biogenesis in healthy humans. PLos Medicine. 2007;**4**:e76.
15. Coen PM, Menshikova EV, Distefano G, Zheng D, Tanner CJ, Standley RA, *et al*. Exercise and Weight Loss Improve Muscle Mitochondrial Respiration, Lipid Partitioning and Insulin Sensitivity Following Gastric Bypass Surgery. Diabetes. 2015.
16. Koster A, Ding J, Stenholm S, Caserotti P, Houston D, Nicklas B, *et al*. Does the Amount of Fat Mass Predict Age-Related Loss of Lean Mass, Muscle Strength, and Muscle Quality in Older Adults? Gerontol A Biol Sci Med Sci. 2011;**66A**:888-895.
17. Flegal K, Carroll M, Ogden C, Curtin L. Prevalence and trends in obesity among US adults, 1999-2008. JAMA. 2010;**303**:235-241.

18. Chomentowski P, Dubé J, Amati F, Stefanovic-Racic M, Zhu S, Toledo F, *et al*. Moderate exercise attenuates the loss of skeletal muscle mass that occurs with intentional caloric restriction-induced weight loss in older, overweight to obese adults. J Gerontol A Biol Sci Med Sci. 2009;**64**:575-580.
19. Amati F, Dube J, Shay C, Goodpaster B. Separate and combined effects of exercise training and weight loss on exercise efficiency
and substrate oxidation. J Appl Physiol. 2008;**105**:825–831.
20. Ritov V, Menshikova E, Kelley D. High-Performance Liquid Chromatography-Based Methods of Enzymatic Analysis: Electron Transport Chain Activity in Mitochondria from Human Skeletal Muscle. Analytical Biochemistry 2004;**333**:27-38.
21. Ritov V, Menshikova E, Kelley D. Analysis of cardiolipin in human muscle biopsy. Chromatography B. 2006;**831**:63-71.
22. Chi M, Hintz C, Coyle E, Martin Wr, Ivy J, Nemeth P, *et al*. Effects of detraining on enzymes of energy metabolism in individual human muscle fibers. Am J Physiol Endocrinol Metab. 1983;**244**:C276-287.
23. Toledo F, Menshikova E, Ritov V, Azuma K, Radikova Z, DeLany J, *et al*. Effects of physical activity and weight loss on skeletal muscle mitochondria and relationship with glucose control in type 2 diabetes. Diabetes. 2007;**56**:2142-2147.
24. Toledo F, Menshikova E, Azuma K, Radiková Z, Kelley C, Ritov V, *et al*. Mitochondrial capacity in skeletal muscle is not stimulated by weight loss despite increases in insulin action and decreases in intramyocellular lipid content. Diabetes. 2008;**57**:987-994.
25. Menshikova E, Ritov V, Ferrell R, Azuma K, Goodpaster B, Kelley D. Characteristics of skeletal muscle mitochondrial biogenesis induced by moderate-intensity exercise and weight loss in obesity. J Appl Physiol. 2007;**103**:21-27.
26. Legros F, Malka F, Frachon P, Lombes A, Rojo M. Organization and dynamics of human mitochondrial DNA. J Cell Sci. 2004;**117**:2653-2662.
27. Rardin MJ, Newman JC, Held JM, Cusack MP, Sorensen DJ, Li B, *et al*. Label-free quantitative proteomics of the lysine acetylome in mitochondria identifies substrates of SIRT3 in metabolic pathways. Proc Natl Acad Sci U S A. 2013;**110**:6601-6606.
28. Hirschey MD, Shimazu T, Capra JA, Pollard KS, Verdin E. SIRT1 and SIRT3 deacetylate homologous substrates: AceCS1,2 and HMGCS1,2. Aging (Albany NY). 2011;**3**:635-642.
29. Hebert AS, Dittenhafer-Reed KE, Yu W, Bailey DJ, Selen ES, Boersma MD, *et al*. Calorie restriction and SIRT3 trigger global reprogramming of the mitochondrial protein acetylome. Mol Cell. 2013;**49**:186-199.
30. Anderson EJ, Lustig ME, Boyle KE, Woodlief TL, Kane DA, Lin CT, *et al*. Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. The Journal of clinical investigation. 2009;**119**:573-581.
31. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, *et al*. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell metabolism. 2008;**7**:45-56.
32. Lauritzen HP, Galbo H, Toyoda T, Goodyear LJ. Kinetics of contraction-induced GLUT4 translocation in skeletal muscle fibers from living mice. Diabetes. 2010;**59**:2134-2144.
33. Li J, Romestaing C, Han X, Li Y, Hao X, Wu Y, *et al*. Cardiolipin remodeling by ALCAT1 links oxidative stress and mitochondrial dysfunction to obesity. Cell metabolism. 2010;**12**:154-165.

34. Chicco AJ, Sparagna GC. Role of cardiolipin alterations in mitochondrial dysfunction and disease. American journal of physiology Cell physiology. 2007;**292**:C33-44.
35. Claypool SM. Cardiolipin, a critical determinant of mitochondrial carrier protein assembly and function. Biochim Biophys Acta. 2009;**1788**:2059-2068.
36. Noel H, Pande SV. An essential requirement of cardiolipin for mitochondrial carnitine acylcarnitine translocase activity. Lipid requirement of carnitine acylcarnitine translocase. Eur J Biochem. 1986;**155**:99-102.
37. Schlame M, Ren M, Xu Y, Greenberg ML, Haller I. Molecular symmetry in mitochondrial cardiolipins. Chem Phys Lipids. 2005;**138**:38-49.
38. Haines TH, Dencher NA. Cardiolipin: a proton trap for oxidative phosphorylation. FEBS Lett. 2002;**528**:35-39.
39. Shah KB, Duda MK, O'Shea KM, Sparagna GC, Chess DJ, Khairallah RJ, *et al*. The cardioprotective effects of fish oil during pressure overload are blocked by high fat intake: role of cardiac phospholipid remodeling. Hypertension. 2009;**54**:605-611.
40. Lanza IR, Short DK, Short KR, Raghavakaimal S, Basu R, Joyner MJ, *et al*. Endurance exercise as a countermeasure for aging. Diabetes. 2008;**57**:2933-2942.
41. Wang L, Mascher H, Psilander N, Blomstrand E, Sahlin K. Resistance exercise enhances the molecular signaling of mitochondrial biogenesis induced by endurance exercise in human skeletal muscle. J Appl Physiol. 2011;**111**:1335-1344.
42. Sandri M, Lin J, Handschin C, Yang W, Arany ZP, Lecker SH, *et al*. PGC-1alpha protects skeletal muscle from atrophy by suppressing FoxO3 action and atrophy-specific gene transcription. Proceedings of the National Academy of Sciences of the United States of America. 2006;**103**:16260-16265.

Table 1. Changes in weight, body composition, maximal aerobic capacity, blood chemistry and exercise intervention adherence.

	EXERCISE		WEIGHT LOSS	
	Pre-INT	**Post-INT**	**Pre-INT**	**Post-INT**
N	10		7	
Gender (F/M)	6/4		4/3	
Metabolic status (IGT/NGT)	4/6		7/0 ‡	
Age	68.0 ± 1.5		67.6 ± 2.0	
Weight (kg)	84.2 ± 3.2	82.2 ± 3.0*	87.4 ± 3.8	79.7 ± 3.5*
BMI	30.4 ± 0.8	29.8 ± 0.8*	31.7 ± 1.3	28.9 ± 1.3*
Fat Mass (kg)	35.6 ± 2.1	32.4 ± 2.0*	38.0 ± 2.3	32.4 ± 2.7*
FFM (kg)	45.7 ± 2.7	46.3 ± 2.9	45.7 ± 3.2	43.9 ± 2.9*
Fasting glucose (mg/dL)	92.7 ± 5.1	91.6 ± 3.5	104.0 ± 5.5**	98.1 ± 2.5
HBA1c (%)	5.66 ± 0.09	5.84 ± 0.12	6.03 ± 0.13**	5.81 ± 0.13
Fasting insulin (μU/mL)	5.31 ± 0.51	5.37 ± 0.89	8.08 ± 3.05	4.62 ± 1.45*
VO_2max (mL/kgFFM/min)	35.9 ± 2.0	38.7 ± 2.3*	28.00 ± 2.32**	30.8 ± 1.98
Time per exercise session (min/session)		39.3 ± 0.8		
Sessions per week		3.68 ± 0.3		
Exercise energy expenditure per session (Kcal/session)		273 ± 28.2		
Exercise energy expenditure per week (Kcal/week)		1016 ± 144		

Values are means ± SEM. *Significant within-group differences between Pre- and Post-Intervention; P<0.05. **Significant between-group difference at the Pre-Intervention time point; P<0.05. ‡ Significant difference in baseline ratio as determined by Pearson's chi-squared test; P<0.05.

Table 2. Changes in cardiolipin species.

Cardiolipin (CL) (% of total)	Pre EX	Post EX	Pre CR	Post CR
N	10		7	
Tetralinoleoyl CL	76.5 ± 1.1	78.2 ± 1.1* P=0.02	74.8 ± 1.3	71.5 ± 1.3* P=0.05
Trilinoleoyl-monooleoyl CL	15.4 ± 0.7	14.6 ± 0.7 P=0.06	16.7 ± 1.1	19.2 ± 1.2* P=0.05
Dilinoleoyl-dioleoyl CL	4.9 ± 0.3	4.5 ± 0.3* P=0.05	4.7 ± 0.3	5.5 ± 0.4 P=0.06
Trilinoleoyl-monostearoyl CL	1.9 ± 0.2	1.5 ± 0.1* P<0.01	2.4 ± 0.1	2.6 ± 0.2

Values are means ± SE. *Significant differences between Pre- and Post-Intervention.

Figures legends

Figure 1: Effect of exercise or calorie restriction interventions on skeletal muscle insulin sensitivity in older sedentary individuals. Glucose clamps were performed before (pre) and after the interventions (post). Data represent Mean ± SEM. *p < .05, Significant within-group differences between pre- and post-intervention. Rd = Rate of disappearance of glucose; FFM = Fat free

mass.

Figure 2: Effect of exercise or calorie restriction interventions on mitochondrial volume density (A) or cardiolipin content (marker of mitochondrial mass) (B) in skeletal muscle biopsy from older sedentary individuals. Biopsies were analyzed before (pre) and after the interventions (post). Cardiolipin content was normalized to creatine kinase (CK) activity to control for muscle fiber content. Data represent Mean ± SEM. *p < .05, Significant within-group differences between pre- and post-intervention.

Figure 3: Effect of exercise or calorie restriction interventions on markers of mitochondrial mass and electron transport chain activity in skeletal muscle biopsy from older sedentary individuals. Biopsies were analyzed before (pre) and after the interventions (post). The total activities of β-HAD (A), citrate synthase (B), and rotenone-sensitive- Nicotinamide adenine dinucleotide (NADH) oxidase (C) in biopsies were normalized to the creatine kinase (CK) activity. Citrate Synthase, β-HAD, and NADH:O2 oxidoreductase activities were normalized to CK activity to control for muscle fiber content. Data represent Mean ± SEM. ϕp < .05, Significant time effect. *p < .05, Significant within-group differences between pre- and post-intervention. **p < .05, Significant between-group difference at the pre-intervention time point.

Figure 4: Ratios between activity of mitochondrial electron transport chain (Nicotinamide

adenine dinucleotide; NADH-oxidase), β-oxidation (β-HAD), and tricarboxylic acid (TCA) cycle (Citrate Synthase; CS) in skeletal muscle before (pre) and after (post) the exercise and calorie restriction interventions. Total NADH-oxidase (A) or β-HAD (B) activities in biopsy were normalized to CS activity to examine changes in the relative capacity for mitochondrial electron transport chain/TCA or β-oxidation/TCA. Data represent Mean ± SEM. *$p < .05$, Significant within-group differences between pre- and post-intervention.

Figures

Figure 1

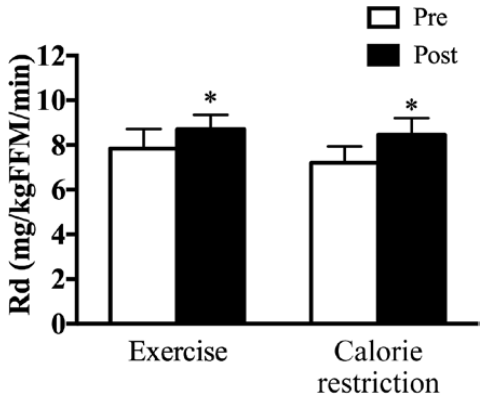


Figure 2

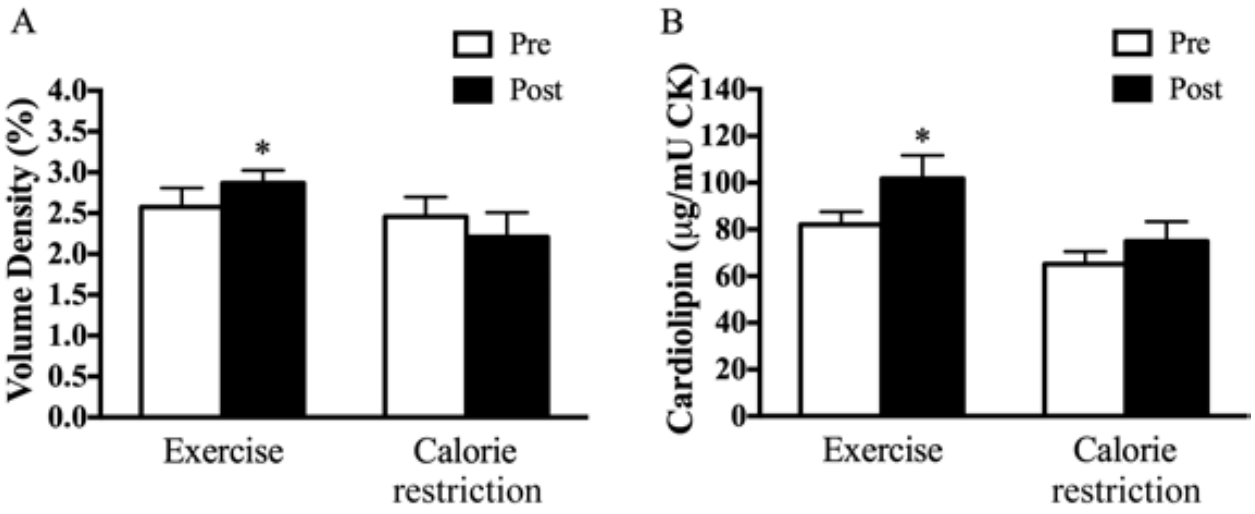


Figure 3

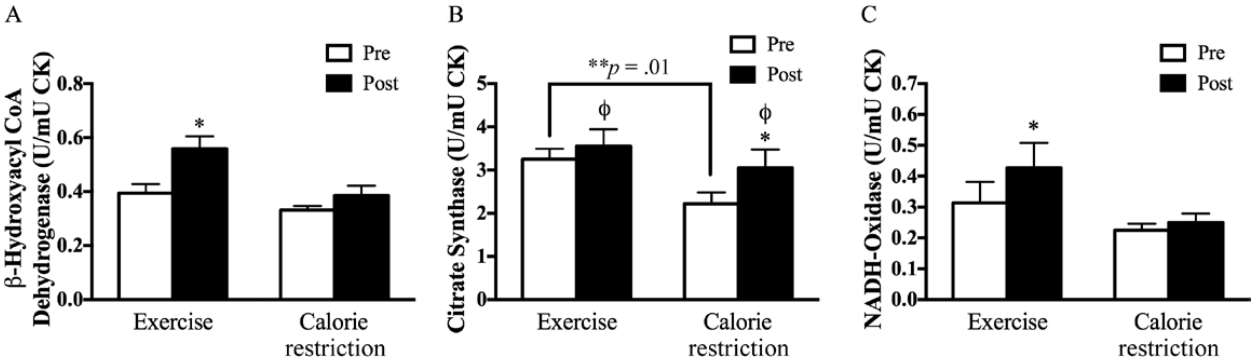
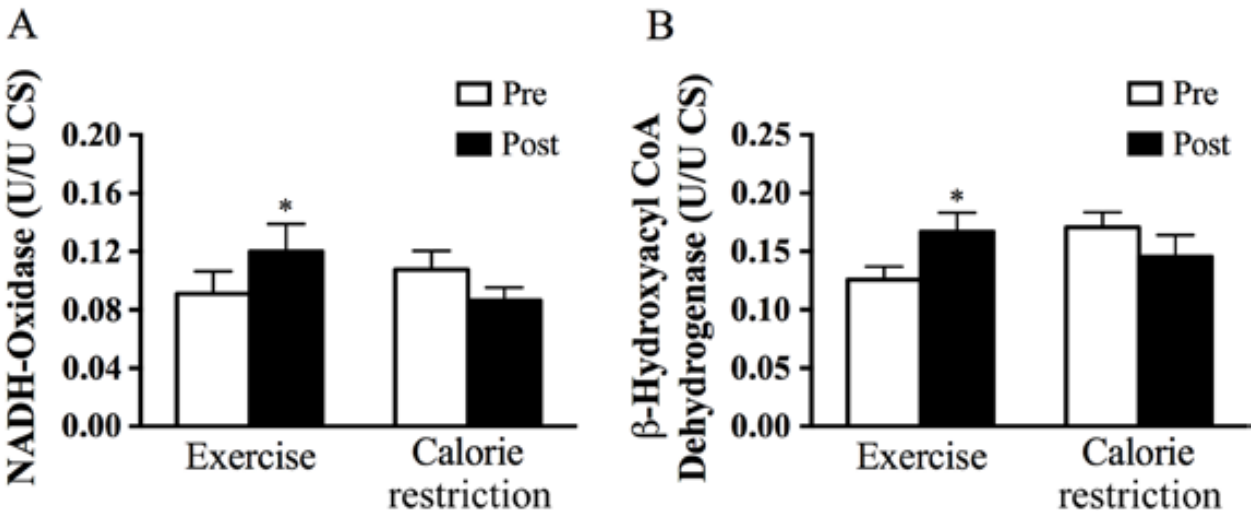


Figure 4


A
NADH-Oxidase (U/U CS)
0.20
0.16
0.12
0.08
0.04
0.00
Pre
Post
*
Exercise
Calorie restriction
B
β-Hydroxyacyl CoA Dehydrogenase (U/U CS)
0.25
0.20
0.15
0.10
0.05
0.00
Pre
Post
*
Exercise
Calorie restriction