Exercise and Weight Loss Improve Muscle Mitochondrial Respiration, Lipid Partitioning and Insulin Sensitivity Following Gastric Bypass Surgery

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ABSTRACT

Both Roux-en-Y gastric bypass (RYGB) surgery and exercise can improve insulin sensitivity ($S_I$) in individuals with severe obesity. However, the impact of RYGB with or without exercise on skeletal muscle mitochondria, intramyocellular lipids and $S_I$ is unknown. We conducted a randomized exercise trial in patients (N=101) who underwent RYGB surgery and completed either a 6-month moderate exercise (EX) or a health education control (CON) intervention. $S_I$ was determined by intravenous glucose tolerance test (IVGTT). Mitochondrial respiration, intramyocellular triglyceride, sphingolipid and diacylglycerol content were measured in vastus lateralis biopsies. We found that EX provided additional improvements in $S_I$, and only EX improved cardiorespiratory fitness, mitochondrial respiration and enzyme activities and cardiolipin profile with no change in mitochondrial content. Muscle triglycerides were reduced in type-1 fibers in CON, and sphingolipids decreased in both groups, with EX further reducing a number of ceramide species. In conclusion, exercise superimposed on bariatric surgery-induced weight loss enhanced mitochondrial respiration, induced cardiolipin remodeling, reduced specific sphingolipids and provided additional improvements in insulin sensitivity.

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INTRODUCTION

Roux-en-Y Gastric Bypass (RYGB) is the most commonly performed bariatric procedure in the United States and provides large and sustained weight loss, increased insulin sensitivity in multiple organs, and leads to diabetes remission in a significant percentage of patients\(^1\). Improvements in glycemic control and tissue-specific insulin sensitivity following RYGB surgery occur over two discrete phases\(^2\). The first phase is immediately (weeks to months) after surgery wherein glycemic control is normalized, whereas peripheral tissue (principally muscle) insulin sensitivity slowly improves over a later phase (up to ~18 months). However, even after substantial postoperative weight loss, peripheral tissue insulin sensitivity typically remains much lower than in metabolically healthy lean individuals\(^3\). Exercise is another treatment option that we have recently demonstrated to be a feasible and effective adjunct therapy to improve insulin sensitivity and glucose effectiveness in severely obese bariatric surgery patients\(^4\).

Although a heavily studied area over the past decade, the precise mechanisms that mediate skeletal muscle insulin resistance in human obesity remain unclear. Impairments in mitochondrial oxidation have been implicated\(^6\) and evidence points to reduced capacity for mitochondria to oxidize fatty acids which in turn leads to incomplete fatty acid oxidation\(^6\) and accumulation of long chain acyl coA, sphingolipids and diacylglycerol (DAG)\(^7\). Experiments in cell culture and animal models suggest that intramyocellular sphingolipids and DAGs contribute to insulin resistance by inhibition of Akt/PKB and IRS-1/2 signaling\(^8\). Studies in human muscle are equivocal, with some showing that muscle ceramide content is elevated in insulin resistance and obesity\(^9,10\), while others report no association\(^11,12\). The extent to which muscle DAG content is related to insulin resistance is also unclear\(^12,13\), with one report of elevated muscle DAG in insulin sensitive athletes\(^14\). There is also a lack of clarity regarding the nature of mitochondrial
dysfunction that contributes to muscle insulin resistance in obesity and whether calorie restriction interventions that improve insulin sensitivity also improve mitochondrial function. Weight loss by dietary caloric restriction alone has been reported to either induce mitochondrial biogenesis, reduce mitochondrial respiration, or have no effect on mitochondrial content and electron transport chain activity. Weight loss induced by bariatric surgery procedures induces greater changes in weight and insulin sensitivity than those observed with diet-based weight loss. However, whether surgery-induced weight loss improves peripheral issue insulin sensitivity via favorable changes in mitochondria and muscle lipid has not been extensively studied. Moreover, whether adding exercise training modulates the response in this setting is unknown.

It has been previously demonstrated that exercise improves muscle insulin sensitivity concomitant with improved mitochondrial function and reduced intramyocellular DAG and ceramide. To date, there have been no studies on the effects of exercise on muscle mitochondria, intramuscular lipids and insulin sensitivity in the context of bariatric surgery-induced weight loss. To address this paucity in the literature, we conducted a 6-month prospective randomized exercise trial with percutaneous skeletal muscle biopsies and intravenous glucose tolerance tests (IVGTT) to examine the effects of a moderate exercise intervention on muscle mitochondria respiration, intramyocellular lipids (sphingolipids, diacylglycerols, cardiolipin) and insulin sensitivity during bariatric surgery-induced weight loss. We hypothesized that RYGB surgery induced weight loss with and without exercise would affect mitochondrial energetics and intramyocellular lipid partitioning, and that this would be associated with improved insulin sensitivity. The exercise intervention was specifically focused on the period following surgery when significant weight loss improves peripheral tissue insulin sensitivity (~3 to 9 months post surgery).
RESEARCH DESIGN AND METHODS

**Patient Recruitment.** The study volunteers were a subset of RYGB-surgery patients enrolled in a larger randomized controlled exercise trial\(^4\), who completed the study and who had muscle biopsies (n = 101). Participants were recruited from two academic bariatric surgery practices in Pittsburgh, PA and Greenville, NC. The study protocol was approved by the human ethics committees of the University of Pittsburgh and East Carolina University. All participants provided written informed consent to participate in the study. Male and female patients were eligible if they were between the age of 21 and 60 years, had a body mass index (BMI) below 55 kg/m\(^2\) and underwent Roux-en-Y gastric bypass surgery, were not diabetic, and who volunteered to have muscle biopsies before and after the intervention. Other aspects of the study design, patient recruitment and inclusion/exclusion criteria are described in detail elsewhere\(^4\).

**Intervention Groups.** Between 1-3 months following bariatric surgery, participants were randomized to a 6-month intervention of health education control (CON) or an exercise program\(^4\) (EX, n = 50; CON = 51). Study measurements were made over separate clinic visits before and after the 6-month interventions. The exercise program started following completion of baseline metabolic and body composition assessments and the initial muscle biopsy. Participants were required to participate in 3-5 exercise sessions per week, with at least 1 directly supervised session per week to assure that the target exercise intensity (60-70% heart rate max) and duration was achieved and to document progress. Participants progressed over three months to a minimum of 120 mins/wk of exercise, which was maintained for the final 3 months of the program. The health education control group was asked to attend 6 health education sessions. The sessions were held once per month and included lectures, discussions and demonstrations providing up-to-date information on health topics such as medication use, nutrition and
communicating with health care professionals. Physical activity habits were also reported and documented at the health education session.

**Intravenous Glucose Tolerance Test (IVGTT).** A 3-hour insulin-modified IVGTT was performed in the morning hours following an overnight fast and at least 48hrs removed from the last exercise session to measure insulin action based on the Bergman minimal model calculations\(^2^4\), as previously described\(^4\).

**Body Composition and Cardiorespiratory Fitness.** Fat and lean mass was determined by dual-energy x-ray absorptiometry (DXA) using a GE Lunar (GE Healthcare). Cardiorespiratory fitness (VO\(_{2}\)peak) was measured by indirect calorimetry (Moxus, AEI) during a 5-12 minute graded exercise test on a cycle ergometer (Lode), as previously described\(^4\). Twelve-lead ECG recordings were monitored by the study physician and interpreted for contraindications to exercise. Body weight, waist circumference, BP, and plasma lipids were measured by standard clinical protocols.

**Muscle Biopsy and Specimen Collection Procedures.** Percutaneous muscle biopsy samples were obtained following an overnight fast and at least 48hrs removed from the last exercise session as described previously\(^1^3\). Briefly, muscle biopsies were obtained under local anesthesia (2 % buffered lidocaine) from the medial vastus lateralis, 15cm above the patella using a 5 mm muscle biopsy Bergström cannula with suction (Stille Surgical instruments, Eskilstuna, Sweden). Immediately after the biopsy procedure the specimen was blotted dry and trimmed of visible adipose tissue using a standard dissecting microscope (Leica EZ4, Leica Microsystems, Switzerland). Three portions of the specimen (~30mg each) were snap frozen in liquid nitrogen and stored at -80 °C for lipidomics analysis, immunoblotting, and assays of mitochondrial enzyme activity. A separate portion (~10 mg) was placed in relaxing and biopsy preserving
solution (BIOPS media; Oroboros, Innsbruck, Austria) for high-resolution respirometry. For immuno-histochemistry, a portion (~30mg) was mounted on a small piece of cork with mounting medium (Shandon Cryochrome; Thermo Electron, Pittsburgh, PA), frozen in isopentane cooled with liquid nitrogen for 2–3 min (-160 °C), and then placed into liquid nitrogen. All frozen samples were stored at -80 °C until analysis.

**Mitochondrial Biochemistry.** A portion of frozen muscle (20-30mg) was used to measure NADH-oxidase, citrate synthase, and creatine kinase activities, and cardiolipin as described in the supplemental section.

**High Resolution Respirometry.** Immediately following the muscle biopsy procedure, muscle fiber bundles (~1-3mg each) were prepared as described in the supplemental section. The fiber bundles were then gently placed into the respirometer chambers (Oroboros, Oxygraphy-2K, Oroboros Instruments, Innsbruck, Austria) and after a stable baseline was reached, two assay protocols were run in duplicate at 37°C and between 230-150 nmol of O₂ in Buffer Z with blebbistatin (25 µM) (supplemental figure 1). **Protocol 1:** Complex I supported LEAK (CIₖ or State 4) respiration was determined through the addition of glutamate (5mM) and malate (2mM). ADP (4mM) was added to elicit complex I supported OXPHOS (CIₚ or State 3) respiration. Succinate (10mM) was then added to elicit complex I&II supported OXPHOS (CI&IIₚ). Cytochrome c (10 µM) was added to assess the integrity of the outer mitochondrial membrane. Finally, FCCP (2uM) was added to determine complex I&II supported electron transfer system capacity (CI&IIₑ) or maximal uncoupled respiration.

**Protocol 2:** Fatty acid oxidation supported LEAK (FAOₖ) respiration was determined through the addition of palmitoyl-carnitine (25uM) and Malate (2mM). ADP (4mM) was added to elicit fatty acid oxidation supported OXPHOS (FAOₚ). Glutamate (5mM) was then added to elicit CI &
FAO$_P$ respiration. Succinate (10mM) was added to stimulate CI&II & FAO$_P$ respiration. Finally, cytochrome c (10µM) was added to assess mitochondrial integrity. After the assay protocols, the fiber bundles were retrieved from the respirometry chambers, dried and weighed on an analytical balance (XS105, Mettler Toledo, Columbus, OH). Oxygen flux was normalized to dry weight of the fiber bundle.

**Analysis of Sphingolipid and Diacylglycerol (DAG) Species.** Intramuscular sphingolipids and DAGs were quantified by high-pressure liquid chromatography (HPLC)-tandem mass spectrometry as described previously$^{25}$ and in the supplemental section. Intramuscular DAG and ceramide content was normalized to tissue-wet weight (pmol/mg tissue).

**Fiber Type and Intramyocellular Triglyceride (IMTG) Content.** Determination of IMTG content was performed using a modified version of methods previously used in our laboratory$^{13}$, and as described in the supplemental section. Oil red O staining intensity and cross sectional area was determined in type I and type II myocytes. Analysis is based on $>200$ fibers per section.

**Protein Content by Immunoblot.** A portion of frozen muscle (~30mg) was prepared for immunoblot for GLUT protein expression as described in the supplemental section. Gel-to-gel variation was controlled for by using a standardized sample on each gel. Protein loading was controlled by normalizing bands of interest to $\alpha$-Tubulin.

**Statistical Analysis.** Group differences in baseline characteristics were determined using 2-sample Student’s t test (2-tailed) or $\chi^2$ or Fisher exact tests. Any variables with high skew were log- or square-root transformed to achieve a normal distribution. The general linear mixed model with repeated measures (PROC MIXED) was performed to detect group and time effects in the outcome variables. Group, time, and group*time were treated as fixed effects and subjects nested within each group as random effect. Age, sex, and race were covariates. P-values for post-hoc
tests were adjusted by false discovery rate (FDR). A P value of less than 0.05 was considered significant. Analyses were performed using SAS base 9.1 or JMP Pro 11 for Mac (SAS Institute Inc.).
RESULTS

Study Participants. The characteristics of the study groups before and after the interventions are shown in Table 1. There were no baseline differences in age, sex, race, mass, fat mass, or BMI between groups. The average time from the date of RYGB surgery to randomization into study groups was $77 \pm 24$ days. There was no difference between groups in average time from surgery to randomization. All muscle biopsies and IVGTT’s were conducted one day prior to randomization. There were no serious adverse events in either study group, nor were there differences in reported adverse events between groups. By design, all participants completed the interventions and participants in the EX group performed an average $\pm$ SEM of $154 \pm 17$ min/wk of structured exercise. See Supplemental Table 1 for further exercise compliance data. Both groups reported similar medication use at baseline, and there was no difference in medication use following intervention (data not shown).

Weight, Body Composition and Cardiorespiratory Fitness. Both groups experienced similar reductions in mass, fat mass, BMI, and waist circumference following the interventions (Table 1). Changes in blood pressure (systolic and diastolic) and blood lipids (total, LDL, and HDL cholesterol and TG) were reduced to a similar degree in the EX and CON groups, similar to that reported in the parent trial$^4$. EX, but not CON, significantly improved VO$_2$ peak (Table 1), an index of cardiorespiratory fitness and an effective predictor of future morbidity and mortality.

Intravenous Glucose Tolerance Test. Baseline $S_I$ measurement for both groups were similar (Table 1). Baseline fasting insulin, glucose and HOMA-IR were slightly higher in the CON group, compared to EX. Following the interventions, $S_I$ was improved in both groups. The EX group, however, had a significantly greater improvement in $S_I$ compared to the CON group. The intervention effects on weight, body composition, fitness and $S_I$, reported here for these
subgroups, were the same or similar as those reported for the parent randomized controlled trial study groups.

**Muscle Cardiolipin and OXPHOS content.** Cardiolipin and OXPHOS protein expression were measured as surrogates of mitochondrial content. Total cardiolipin did not change in either intervention group, indicating no change in inner mitochondrial membrane content (Figure 1, Panel A). However, exercise altered individual molecular cardiolipin species (based on fatty acyl carbon chain length and degree of saturation). Exercise increased the (C18:2)₄ species and decreased (C18:2)(C18:1)₁, (C18:2)(C18:1)₂, and (C18:2)(C18:0)₁ species. Cardiolipin (C18:2)(C18:3)₁ was not altered by EX (Figure 1, Panel B). These data indicate that the exercise intervention remodeled cardiolipin profile in the mitochondria independent of changes in total content. We also found that neither the total OXPHOS content nor each of the five electron transfer system (ETS) subunit proteins were significantly changed by either CON or EX (Figure 1, Panel C,D,E). Relative change of each ETS subunit did not change for either intervention (Figure 1, Panel F).

**Mitochondrial respiration.** Measuring mitochondrial respiration is a widely employed approach to assess mitochondrial ETS function. In the first assay protocol, which used carbohydrate-derived substrates, we found that EX improved CI and CI&II supported OXPHOS respiration. ETS capacity was also improved with EX. In contrast, the CON group had no change in steady state respiration (Figure 2, Panel A). The second assay protocol used fatty acid (palmitoyl-carnitine) and carbohydrate-derived substrates. EX improved FAOₗ respiration and CI&II & FAO supported OXPHOS respiration. There was also an increase for both groups (time effect) for FAOᵢ respiration and CI&II&FAOᵢ respiration (Figure 2, Panel B), suggesting an independent effect of RYGB surgery-induced weight loss. A benefit of the mitochondrial
respiration protocol is that ratios of oxygen flux in different respiratory states can be examined. Flux control ratios (FCR) provide an internal normalization and qualitative assessment of coupling and substrate control independent of mitochondrial content, quality of the fiber bundle preparation, and inadvertent variation in assay conditions. We found that there was an increase in both groups (time effect) for CI phosphorylation system control ratio (Figure 2, Panel C). This ratio is an expression of the limitation of CI ETS capacity by the phosphorylation system that increases with increasing capacity of the phosphorylation system. Similarly, there was an increase in both groups for CI&II phosphorylation system control ratio (Figure 2, Panel D). When we examined the ratio of CI OXPHOS/CI&II OXPHOS (CI control ratio), we found an increase for both groups (Figure 2, Panel E). This observation indicates that the relative contribution of CI to maximal OXPHOS respiration increases following RYGB surgery induced weight loss, with or without exercise. There was no change in the LEAK control ratio with either intervention (Figure 2, Panel F) indicating no change in inner mitochondrial membrane uncoupling. Finally, the cytochrome C response for both groups was <10%, indicating maintenance of mitochondrial membrane integrity.

**Mitochondrial Enzyme Assays.** Activities of both succinate dehydrogenase (SDH), and citrate synthase (CS), enzyme complexes of the TCA cycle, tended to increase in EX but not in CON (Table 2). NADH oxidase, representing the activity of the entire ETC, also tended to be higher in EX. Moreover, the ratio of NADH oxidase to cardiolipin significantly increased in EX, reflecting an exercise-induced increase in the ETC activity per unit of mitochondrial mass. Creatine kinase activity did not change with the interventions (Table 2).

**Intramyocellular Triglyceride (IMTG), and Diacylglycerol Content.** There was no change in fiber type proportion or fiber cross sectional area (Table 3). IMTG content in type I myofibers
was decreased in the CON group, but not in the EX group, indicating an exercise induced preservation of higher IMTG during weight loss (Figure 3, Panel B). The exercise effect was not seen in type IIA fibers, for which both groups decreased (time effect) IMTG content (Figure 3, Panel C). We found no significant change in any species or total content of diacylglycerol (DAG) in either group.

**Intramyocellular sphingolipid.** We quantified a profile of sphingolipid species, purported lipotoxic mediators of insulin resistance. We found a significant decrease for the EX group and a time effect for total sphingolipid, total ceramide and total unsaturated ceramide. There was also a group effect for total sphingolipid, ceramide, saturated and unsaturated ceramide, largely driven by the higher content in EX group at Pre-intervention (Figure 4, Panel A). Similar observations were made for many other ceramide species (Figure 4, Panel B&C). Finally, there were no intervention effects on sphingosine species (Figure 4, Panel D).

**Glut transporter expression.** We measured protein expression of insulin dependent (Glut4) and independent (Glut1 and 12) glucose transporters as potential mediators of improved S1 following the interventions. There was no change in expression of Glut transporters with either EX or CON (Figure 5).
DISCUSSION

Despite being recognized as a key element in the etiology of type 2 diabetes, skeletal muscle insulin resistance lacks a unifying mechanism that can be consistently translated to human disease or pathobiology. Moreover, it is not clear whether improvements in insulin sensitivity with weight loss and exercise share common underpinnings. In this context, bariatric surgery-induced weight loss, with or without concomitant exercise training, provides a conceptual and practical framework to examine important questions regarding treatment for insulin resistance. We recently reported that insulin resistance is not generally normalized in the weeks to months following RYGB surgery, and that regular exercise can further improve peripheral insulin sensitivity\(^4\). Now, we examined whether alterations in mitochondrial energetics or intramyocellular lipids correspond with improved insulin sensitivity following RYGB surgery with or without regular exercise.

A key finding was that surgery-induced weight loss, particularly with adjunct exercise, imparted distinct effects on mitochondrial energetics in the absence of any notable alterations in mitochondrial content. There is much controversy regarding the role of mitochondria in muscle insulin resistance and how weight loss affects this relationship\(^15\). Calorie restriction-induced weight loss in humans, an intervention that improves peripheral insulin resistance\(^15\), has been reported to elicit muscle mitochondrial biogenesis\(^16\), reduce mitochondrial respiration\(^17\), or have no effect on mitochondrial content and electron transport chain activity\(^18\). In the context of bariatric surgery-induced weight loss only a handful of studies have examined skeletal muscle. These studies show changes in gene expression relating to glucose metabolism and mitochondrial function in muscle biopsy and myotubes derived from biopsy specimens\(^26\), changes that may be mediated by an altered epigenome\(^27\). Others have shown that the expression
of genes associated with mitochondrial biogenesis in skeletal muscle were enhanced and related to improved insulin sensitivity after bariatric surgery\textsuperscript{28}. The few studies that have examined mitochondrial respiratory performance following bariatric surgery produced equivocal results\textsuperscript{19,20}.

We employed a number of analytical approaches to more comprehensively assess mitochondrial function. Our observation that surgery induced weight loss did not alter markers of mitochondrial content is in line with a growing consensus that weight loss alone does not induce mitochondrial biogenesis\textsuperscript{15,18}. Surgery induced weight loss did, however, induce a robust increase in CI and CI&II OXPHOS control ratios, indicating increased capacity for oxidative phosphorylation without increased ETS capacity. Weight loss in the CON group also increased the CI/CI&II control ratio, indicative of a relative increase in the contribution of electron flow from complex I to maximal OXPHOS respiration. Taken together these data suggest a unique remodeling of mitochondrial respiratory parameters with surgery induced weight loss that occur along with improved insulin sensitivity.

The exercise program further improved $S_I$ compared with surgery-induced weight loss alone, concomitant with distinct improvements in mitochondrial respiration. There is abundant evidence that aerobic exercise induces muscle mitochondrial biogenesis and improves oxidative capacity\textsuperscript{23,29}. However, few exercise studies with a focus on mitochondria have been conducted in obese patients\textsuperscript{29,30}, and none in patients following bariatric surgery. We found no increase in OXPHOS protein or total cardiolipin content following exercise, a surprising observation that may be due to massive weight loss modulating the effect of exercise on mitochondria, while mitochondrial enzymes, including citrate synthase and NADH oxidase activities increased robustly.

Exercise induced a remodeling of cardiolipin species, a phospholipid lipid that is
primarily localized to the inner mitochondrial membrane and plays a key role in maintaining
OXPHOS complex integrity and function\textsuperscript{31}. We observed a distinct pattern of remodeling, with
species containing the polyunsaturated linoleoyl-CoA (CL-(C18:2)\textsubscript{4}) becoming more abundant,
while CL-(C18:2)\textsubscript{3}(C18:1)\textsubscript{1}, CL-(C18:2)\textsubscript{2}(C18:1)\textsubscript{2}, and CL-(C18:2)\textsubscript{3}(C18:0)\textsubscript{1} were decreased with
exercise. The increase in tetra linoleic CL may reflect lower exposure of cardiolipin to oxidative
stress, as polyunsaturated acyl chains in CL are particularly vulnerable to oxidative modification
by reactive oxygen species (ROS)\textsuperscript{32}. This would be in line with the increase in mitochondrial
antioxidant capacity that has been reported with regular exercise\textsuperscript{33}. The increase in tetra linoleic
CL also reflects a more “mature” cardiolipin pool, meaning that the immature form of cardiolipin
is characterized by a random assortment of attached acyl chains that are saturated and variable in
length. A mature cardiolipin pool has structural uniformity and contains predominantly tetra
linoleoyl acyl chains\textsuperscript{31} features that are essential to its function to maintain integrity of the
OXPHOS capacity. The importance of cardiolipin to mitochondrial function is highlighted by
studies of patients of with mutations in the tafazzin gene (Barth syndrome patients)\textsuperscript{34} and studies
in rodents where relatively small changes in cardiolipin profile reduce mitochondrial respiratory
capacity in cardiomyocytes\textsuperscript{35}. Further investigations are needed to interrogate the role of
cardiolipin in improved insulin resistance.

The differential response of various indices of mitochondrial content and cardiolipin
remodeling to exercise may relate to the fact that obese individuals may not adapt to contractile
activity in a manner consistent with the plasticity of the mitochondria\textsuperscript{36,37}. The impact of varying
intensity and volume of exercise on mitochondrial remodeling, particularly in obese individuals,
may also play a role\textsuperscript{38}. Overall, these data suggest that exercise induced remodeling of the
various components of the mitochondria may occur to different degrees in obese individuals\textsuperscript{39}. 
Congruent with a more mature cardiolipin pool, we observed an increase CI\(_P\), CI&II\(_P\) and CI&II&FAO\(_P\) respiration following the exercise program. While these effects have been previously reported for exercise\(^{29,33}\), this is the first report in obese bariatric surgery patients. Our data, for the first time, link exercise induced cardiolipin remodeling with improved mitochondrial oxidative phosphorylation and insulin sensitivity during RYGB surgery induced weight loss.

Surgery induced weight loss reduced IMTG in type I and II fibers, a finding in line with that of Grey et al.,\(^{21}\) and others who have shown that calorie restriction induced weight loss also reduces IMTG\(^{18,40}\). This is the first study to comprehensively compare skeletal muscle sphingolipid and DAG species post bariatric surgery. The sphingolipid ceramide has been touted as a key mediator of insulin resistance through inhibition of Akt/PKB signaling and mediation of inflammation (TNF\(\alpha\)). Associations between muscle ceramide content and insulin resistance in obesity have been reported in some\(^9,13,41\), but not all studies\(^{11}\). A limitation in the current literature is that many previous studies only measure total ceramide\(^{42}\) or a small number of molecular species\(^9\). However, the identity of sphingolipid species that are associated with improvements in insulin sensitivity with weight loss has not been adequately examined. In this study, we used state-of-the-art lipidomics to quantify individual molecular species of sphingolipid (17 total, including ceramide) in muscle of RYGB surgery patients during weight loss, and provide strong evidence that ceramide is reduced concomitant with improved insulin sensitivity. Interestingly, the reduction in ceramide may also be involved in the observed alterations in mitochondria respiration as ceramide can act to suppress the ETS at Complexes I and III\(^{43}\), leading to oxidative stress\(^{44}\), and also modulate mitochondrial permeability transition pore (MPTP)\(^{45}\). However, in two previous studies, diet induced calorie restriction\(^{46}\) and RYGB-
and laproscopic gastric banding-induced weight loss (20%)\textsuperscript{47} did not alter intramyocellular ceramide. While it is difficult to reconcile our data to these findings, they underscore the need for further studies to evaluate the importance of intramyocellular ceramide in improved insulin sensitivity with weight loss.

Exercise also induced distinct intramyocellular lipid partitioning and prevented the RYGB surgery induced decrease in IMTG in type I myofibers, an observation that echo’s the athletes paradox\textsuperscript{48} and is consistent with observations made during calorie induced weight loss with exercise\textsuperscript{18}. Exercise also resulted in greater selective reductions in a number of individual ceramide species with long acyl chains (C18, C18:1, C24:1, C22:1) and total sphingolipid and ceramide content. This is consistent with our previous reports of individual ceramide species being elevated in obesity\textsuperscript{41} and insulin resistance\textsuperscript{13,14} and being reduced with exercise\textsuperscript{22}, further supporting a role for ceramide in human muscle insulin resistance.

A significant body of research in animal models supports a role for DAG as a mediator of muscle insulin resistance\textsuperscript{49}. However, evidence from studies of human muscle remain equivocal. Here, we did not observe changes in intramyocellular DAG during RYGB surgery induced weight loss, with or without exercise, indicating no relationship with improved insulin sensitivity, a result in line with our previous reports that whole muscle DAG does not play a role in insulin sensitivity in human muscle\textsuperscript{13,14,41} and a recent report that intramyocellular DAG does not change following surgery induced weight loss\textsuperscript{47}. Interestingly, Jocken et al. paradoxically reported lower levels of DAG in obese subjects compared to lean\textsuperscript{50}. While the consensus in human studies thus far suggests that whole muscle levels of DAG are not associated with insulin resistance, this does not preclude the possibility that organelle/lipid membrane specific accumulation of DAG species plays an important role in mediating insulin resistance in human obesity\textsuperscript{51}. 
Finally, the expression of glucose transporter (GLUT) proteins did not appear to explain the exercise-induced improvements in S1. GLUT4 is the key glucose transporter responsible for insulin- and contraction-stimulated glucose transport in skeletal muscle52. Expression of GLUT4 is not different between healthy, insulin resistant and individuals with type 2 diabetes53 and caloric restriction has no effect on GLUT expression in muscle54. However, many studies have demonstrated that increased GLUT4 expression is a key adaptation to chronic exercise55,56 and that GLUT4 expression increases in muscle of obese patients with type 2 diabetes following exercise57. Fewer studies have examined the effect of exercise on GLUT1 and 12 isoforms, which are expressed at lower levels than GLUT4. It is possible, that, similar to the lack of effect on mitochondria biogenesis or content in our study, that the dose of exercise in our study was insufficient to elicit changes in glucose transporter proteins (exercise-induced expression of GLUT4 is regulated in parallel with mitochondrial biogenesis via the same signaling pathways58). It is also possible that, for reasons not explored in this study, these patients are resistant to these exercise improvements.

A distinguishing feature of our study was that we employed a randomized exercise trial to determine the additional effects of exercise post surgery, so we did not capture changes that could have initially occurred following surgery. Pre-surgery data would have allowed us to examine potentially important changes in myocellular metabolism during the initial period after surgery. Another limitation was that nutritional intake was not controlled or monitored and may represent an important factor that affects outcome measures, including intramyocellular lipids, mitochondrial function and S1. Moreover, future studies should examine other potential mediators of insulin resistance and their response to surgery-induced weight loss and exercise, e.g., acylcarntines, amino acids, gut microbiome.
In summary, this is the first evidence from a randomized exercise intervention study in RYGB surgery patients showing distinct weight loss and exercise effects on mitochondrial respiration and cardiolipin remodeling independent of mitochondrial content, and reductions in intramyocellular triglyceride and ceramide, but not diacylglycerol. RYGB surgery-induced weight loss enhanced specific aspects of mitochondrial oxidative phosphorylation, a novel observation that may prove to be a valuable target for therapy. Exercise further improved S1i along with improved respiratory capacity, remodeling of cardiolipin profile and further reductions in specific ceramide species. Thus several facets of myocellular energetics are not rectified with substantial weight loss from bariatric surgery alone and further support the implementation of an adjunct exercise program following bariatric surgery. These data provide valuable mechanistic insight into how both surgery-induced weight loss and exercise are complimentary therapies to improve metabolic profile in severe obesity.
AUTHORS CONTRIBUTIONS

PMC researched the data; contributed to study execution, researched the data and interpretation of the data; and wrote the manuscript. GD, DZ, CJT, RAS, NLH, GSD, MD, EM, and VBR contributed to study execution and researched the data. XH and PMC contributed to the statistical analyses. FGST contributed to study execution and researched data. BHG and JAH contributed to the study concept and design and the analysis and interpretation of the data and reviewed the manuscript. BGH and PMC are the guarantors of the data.

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DISCLOSURE STATEMENT

No potential conflicts of interest relevant to this article were reported. A subset of this data was presented during a short talk at the Cell Symposia on Exercise Metabolism, July 12-14, 2015, Amsterdam, The Netherlands (Abstract #0116).
REFERENCES


Figure 1. Exercise remodels cardiolipin profile without altering total cardiolipin or OXPHOS content in vastus lateralis following RYGB-surgery. Panel A, total cardiolipin is not altered with RYGB-surgery induced weight loss, with or without exercise (n = 21 EX, n = 20, CON). Panel B, exercise increases the relative proportion of CL-(C18:2)₄, while CL-(C18:2)₃(C18:1)₁, CL-(C18:2)₂(C18:1)₂, CL-(C18:2)₃(C18:0)₁ all decreased. Panel C, representative western blot for five OXPHOS proteins and α-tubulin. Panel D & E, OXPHOS content is not altered with RYGB-surgery induced weight loss, with or without exercise. Panel F, when data from both CON and EX groups are combined, the change in NDUFB8 (CI) is significantly greater compared to the change in SDHB (CII) protein content (n = 19 EX, n = 17 CON). The letters A and B denote significant differences between group/time points (P < 0.05, ANOVA). MWM; molecular weight marker. Data presented are Mean±SEM.

Figure 2. Exercise improves mitochondrial respiratory capacity, while RYGB-surgery induced weight loss improves phosphorylation system control ratio. Panel A, exercise improves ADP (4mM) stimulated respiration supported by CI (5mM glutamate & 2mM Malate), CI&II (10mM succinate) and ETS capacity (2µM FCCP). Panel B, exercise improves LEAK respiration and ADP (4mM) stimulated respiration supported by FAO & CI&II (25µM palmitoyl carnitine, 2mM malate, 5mM glutamate, and 10mM succinate). There is also a time effect for ADP (4mM) stimulated respiration supported by FAO (25µM palmitoyl carnitine, 2mM malate), and FAO & CI&II (25µM palmitoyl carnitine, 2mM malate, 5mM glutamate, and 10mM succinate). Panel C, there was an increase in both groups (time effect) for CI phosphorylation system control ratio. Panel D, there was an increase in both groups (time effect) for CI&II phosphorylation system control ratio. Panel E, there was an increase in both groups (time effect) for CI control ratio. Panel F, there was no change in the LEAK control ratio. (n = 30 EX, n = 41
Figure 3. **RYGB-surgery reduces IMTG content in type I and type II myofibers without altering whole muscle diacylglycerol content.** Panel A, Representative images of immunofiber typing and oil red o staining generated from the muscle biopsy of one of the study participants. Panel B, The CON group, but not EX, reduced IMTG in type I myofibers. Panel C, IMTG content was significantly reduced in type 2A myofibers for both groups (time effect). Panel D, There was no change in IMTG content in type 2X myofibers. (n = 34 EX, n = 33 CON). Panels E-G, There were no EX or CON effects on any diacylglycerol species quantified. (n = 20 EX, n = 16 CON). The letters A and B denote significant differences between group/time points (P < 0.05, ANOVA). * = P < 0.05, indicate a group x time interaction. ** = P<0.05, indicate a time effect. Data presented are Mean±SEM.

Figure 4. **Ceramide content in muscle decreases with RYGB-surgery induced weight loss.** **Exercise may further reduce content of certain molecular species of ceramide.** Panel A, Total Sphingolipid, ceramide and unsaturated ceramide all decrease in both groups (time effect) with the decreases being more pronounced in the EX group. Ceramide levels before intervention were higher in EX than CON. Panel B, C16, C18:1, and C24:1 ceramides are decreased in both groups (time effect) with the decreases being more pronounced in the EX group. There is also a group effect for C18 and C18:1, influenced mainly by higher levels of ceramide at EX Pre. Panel C, There was a number of group effects (EX higher) for many of the low abundance ceramides. Panel D, There were no EX or CON effects on any sphingosine species quantified. (n = 20 EX, n = 16 CON). The letters A and B denote significant differences between group/time points (P <
0.05, ANOVA). * = P < 0.05, indicate a group x time interaction. ** = P<0.05, indicate a time effect. # = P<0.05, indicates a group effect. Data presented are Mean±SEM.

**Figure 5. Glucose transporter (GLUT) protein expression did not change with EX or CON.**

Panel A, representative western blots for GLUT1, GLUT4, GLUT12 and the housekeeping protein, α-tubulin. GLUT 1 (Panel B), GLUT4 (Panel C), GLUT 12 (Panel D) protein expression in muscle did not change with either CON or EX interventions. (n = 19 EX, n = 17 CON). Data presented are Mean±SEM.
### Table 1. Participant Characteristics

<table>
<thead>
<tr>
<th></th>
<th>CON (n=51)</th>
<th>EX (n=50)</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pre Post</td>
<td>Pre Post</td>
<td>Group Time Group*Time</td>
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<tr>
<td>Age, years</td>
<td>42.1±9.9</td>
<td>41.6±9.3</td>
<td>0.830</td>
</tr>
<tr>
<td>Race, Ratio</td>
<td>5AA/46C</td>
<td>8AA/42C</td>
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<tr>
<td>Sex, Ratio</td>
<td>8M/43F</td>
<td>6M/44F</td>
<td></td>
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<tr>
<td>Weight, Kg</td>
<td>106.8±25.4</td>
<td>84.7±21.9</td>
<td>0.910 &lt;0.001 0.710</td>
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<td>BMI, Kg/m2</td>
<td>38.1±6.9 H</td>
<td>30.3±5.8 A</td>
<td>0.970 &lt;0.001 0.690</td>
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<tr>
<td>Waist, cm</td>
<td>112.2±15.0</td>
<td>94.7±12.8</td>
<td>0.850 &lt;0.001 0.620</td>
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<td>Fat Mass, Kg</td>
<td>49.4±14.5 A</td>
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<tr>
<td>Lean Mass, Kg</td>
<td>51.0±10.3 A</td>
<td>50.1±10.7 A</td>
<td>0.880 0.380 0.930</td>
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<tr>
<td>VO2max(ml/min)</td>
<td>1935.3±468</td>
<td>1859.8±552</td>
<td>0.030 0.940 0.120</td>
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<tr>
<td>VO2max(ml/min/KgFFM)</td>
<td>18.4±3.5 A</td>
<td>22.2±5.0 B</td>
<td>0.012 &lt;0.001 0.027</td>
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<td>IVGTT</td>
<td></td>
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<tr>
<td>S&lt;sub&gt;1&lt;/sub&gt;, µIU/ml</td>
<td>2.29±1.43 A</td>
<td>3.76±2.19 B</td>
<td>0.110 &lt;0.001 0.052</td>
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<tr>
<td>AIRg, pmol/l</td>
<td>392.9±350.1</td>
<td>295.6±238.6</td>
<td>0.170 0.030 0.760</td>
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<tr>
<td>D&lt;sub&gt;1&lt;/sub&gt;, x 10&lt;sup&gt;-5&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>726.2±593.4</td>
<td>1103.3±1044.2</td>
<td>0.058 0.002 0.730</td>
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<tr>
<td>Fasting Insulin, µIU/ml</td>
<td>6.4±4.3 A</td>
<td>4.1±2.4 B</td>
<td>0.140 &lt;0.001 0.300</td>
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<tr>
<td>Fasting Glucose, mg/dl</td>
<td>88.2±12.8 A</td>
<td>88.6±11.3 A</td>
<td>0.054 &lt;0.001 0.390</td>
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<tr>
<td>HOMA-IR, mg/dl x µIU/ml</td>
<td>1.4±1.1 A</td>
<td>0.9±0.6 B</td>
<td>0.7±0.3 B</td>
</tr>
</tbody>
</table>

Data: Mean±SD for all subjects. A, B, C indicate significant differences between group/time points with fdr p<0.05. Abbreviations: BMI, body mass index; SD, standard deviation; IVGTT, intravenous tolerance test; FFM, fat free mass; S<sub>1</sub>, insulin sensitivity index; AIRg, acute insulin response to glucose; D<sub>1</sub>, disposition index; homeostasis model assessment of insulin resistance (HOMA-IR): (fasting glucose x fasting insulin)/22.5. SI conversion factors: To convert glucose values from mg/dl to mmol/l, by 0.0555; and insulin values from µIU/ml to pmol/l, by 6.945.
Table 2. Muscle Mitochondria Enzyme Activity

<table>
<thead>
<tr>
<th></th>
<th>CON (n=20)</th>
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<th>P Value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>Succinate Dehydrogenase, A.U.</td>
<td>3321±7242</td>
<td>3278±8293</td>
<td>3331±8019</td>
</tr>
<tr>
<td>Citrate Synthase, U/mU CK</td>
<td>3.39±0.88&lt;sup&gt;A,B&lt;/sup&gt;</td>
<td>3.67±0.94&lt;sup&gt;A,B&lt;/sup&gt;</td>
<td>3.39±0.69&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>NADH Oxidase, U/g w-w</td>
<td>1.39±0.49&lt;sup&gt;A,B&lt;/sup&gt;</td>
<td>1.67±0.63&lt;sup&gt;A,B&lt;/sup&gt;</td>
<td>1.55±0.72&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>NADH Oxidase, U/mg CL</td>
<td>2.76±1.03&lt;sup&gt;A,B&lt;/sup&gt;</td>
<td>3.20±1.06&lt;sup&gt;A,B&lt;/sup&gt;</td>
<td>2.76±0.99&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Creatine Kinase, U/g w-w</td>
<td>5509±897</td>
<td>5634±850</td>
<td>5930±971</td>
</tr>
</tbody>
</table>

Data: Mean+SD for all subjects. <sup>A,B</sup> indicate significant differences between group/time points with fdr p<0.05. Abbreviations: CK, creatine kinase; CL, cardiolipin; w-w, wet weight of tissue.
## Table 3. Muscle Fiber Type Distribution and Cross Sectional Area in Vastus Lateralis

<table>
<thead>
<tr>
<th></th>
<th>CON (n=33)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
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<tr>
<td><strong>Type 1 CSA, µm²</strong></td>
<td>3268±940</td>
<td>3357±1605</td>
<td>3499±1435</td>
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<tr>
<td><strong>Type 2A CSA, µm²</strong></td>
<td>2790±1073</td>
<td>2640±1848</td>
<td>3007±1317</td>
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<tr>
<td><strong>Type 2X CSA, µm²</strong></td>
<td>3562±920</td>
<td>3871±1767</td>
<td>3657±893</td>
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<tr>
<td><strong>Type 1 content, %</strong></td>
<td>44±13</td>
<td>47±12</td>
<td>49±11</td>
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<tr>
<td><strong>Type 2A content, %</strong></td>
<td>45±11</td>
<td>43±11</td>
<td>44±10</td>
</tr>
<tr>
<td><strong>Type 2X Content, %</strong></td>
<td>13±11</td>
<td>20±33</td>
<td>10±6</td>
</tr>
</tbody>
</table>

Data: Mean+SD for all subjects. Abbreviations: CSA, cross sectional area; FT, fiber type; SD, standard deviation.
**FIGURE 3**

*Diabetes*
FIGURE 4

A

Concentration (pmol/mg ww)

Total Sphingolipid
Total Ceramide
Total Saturated Ceramide
Total Unsaturated Ceramide

B

Concentration (pmol/mg ww)

C16
C18
C18:1
C24
C24:1

High Abundance Ceramide Species

C

Concentration (pmol/mg ww)

C14
C20
C20:1
C22
C22:1
C26
C26:1
dhC-16

Low Abundance Ceramide Species

D

Concentration (pmol/mg ww)

dhSph
dhSph-1P
Sph
Sph-1P

Sphingosine Species
FIGURE 5

A

<table>
<thead>
<tr>
<th>Glut 1 ~ 50kD</th>
<th>Tabulin 50kD</th>
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</thead>
<tbody>
<tr>
<td>Pre</td>
<td>Post</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glut 4 ~ 55kD</th>
<th>Tabulin 50kD</th>
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</thead>
<tbody>
<tr>
<td>Pre</td>
<td>Post</td>
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</table>

<table>
<thead>
<tr>
<th>Glut 12 ~ 50kD</th>
<th>Tabulin 50kD</th>
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</thead>
<tbody>
<tr>
<td>Pre</td>
<td>Post</td>
</tr>
</tbody>
</table>

B

![Bar Graph]

- **Glut 1**
  - CON Pre
  - CON Post
  - EX Pre
  - EX Post

C

![Bar Graph]

- **Glut 4**
  - Protein Content (A.U.)

D

![Bar Graph]

- **Glut 12**
  - Protein Content (A.U.)
SUPPLEMENTAL

Mitochondrial Biochemistry. NADH-oxidase, citrate synthase, and creatine kinase activities, and cardiolipin were measured as described in our previous publications\(^1\)-\(^3\). To prepare soluble and particulate fractions, homogenates were centrifuged at 45,000\(\times\)g for 20 min\(^1\). The soluble fraction was mixed (1:1) with buffer containing 50\% glycerol\(^1\) and saved for analysis of creatine kinase and citrate synthase released from mitochondria. The pellet (particulate fraction) containing mitochondria was suspended in storage medium containing 0.1 mg/ml BSA, 20\(\mu\)mol/l leupeptin, and 25\% glycerol\(^1\). To release mitochondria trapped by the myofibrillar matrix, the particulate fraction was treated with the KCl/pyrophosphate medium method\(^1,2,4\). Samples were stored at -80\(^\circ\)C before analyses. The activity of creatine kinase was measured in the soluble fraction at 30\(^\circ\)C by HPLC monitoring of the generation of NADPH in a coupled enzymatic reaction (hexokinase/glucose-6P dehydrogenase)\(^3,5\). The activity of citrate synthase was determined by high-performance liquid chromatography monitoring of the generation of CoA-SH after conversion to a fluorescent adduct in a reaction with ThioGlo-1\(^3,6\). NADH-oxidase activity and cardiolipin content in the particulate fraction were measured with high-performance liquid chromatography\(^3,7,8\). Cardiolipin peaks were identified from retention time and relative intensity as previously described\(^9,10\). Citrate synthase and NADH oxidase were expressed relative to creatine kinase activity to normalize for minor variations in muscle biopsy fiber content\(^1\). Succinate dehydrogenase activity was measured using histochemical methods as described previously\(^11\).
**Fiber Bundle Preparation**

Fiber bundles were prepared under magnification, by gently teasing apart individual fibers with two sharp tweezers, submerged in ice-cold BIOPS media in a petri dish. The fiber bundles were permeabilized with saponin (2ml of 50 µg/ml) for 20 minutes and then washed twice in Buffer Z (105mM K-MES, 30 mM KCl, 10mM KH$_2$PO$_4$, 5mM MgCl$_2$-6H$_2$O, 5mg/ml BSA, 1mM EGTA, pH 7.4 with KOH) with blebbistatin (25µM) to inhibit contraction.

**Analysis of Sphingolipid and Diacylglycerol (DAG) Species.** Intramuscular sphingolipids and DAGs were quantified by high-pressure liquid chromatography (HPLC)-tandem mass spectrometry as described previously$^{12}$ and in the supplemental section. (Lipidomics Core, Medical University of South Carolina). Briefly, liquid nitrogen-frozen samples (~30mg) were homogenized in ice-cold buffer (250mM sucrose, 25 mM KCl, 50 mM Tris, and 0.5 mM EDTA, pH 7.4) and were fortified with internal standards and extracted into a one-phase neutral organic solvent system (ethyl acetate/isopropyl alcohol/water; 60:30:10 v/v/v), evaporated and reconstituted in methanol and quantified by a surveyor/TSQ 7000 liquid chromatography/mass spectrometry system (Thermo Finigan, Thermo Fisher Scientific Inc., Waltham, MA). Quantitative analysis was performed in a positive multiple-reaction monitoring mode, based on calibration curves generated by adding to an artificial matrix known amounts of target analytes, synthetic standards, and an equal amount of internal standard. Intramuscular DAG and ceramide content was normalized to tissue-wet weight (pmol/mg tissue).

**Fiber Type and Intramyocellular Triglyceride (IMTG) Content.** Determination of IMTG content was performed using a modified version of methods previously used in our laboratory$^{11}$. Briefly, serial transverse sections (10 µm) of mounted biopsy samples were generated using a cryostat (Cryotome E; Thermo Shandon, Pittsburgh, PA) at -20 °C and placed on a cleaned glass...
slide (Fisherfinest, Fischer Scientific, Pittsburgh, PA). Sections were then stained in a filtered solution of Oil Red O (300 mg/ml in 36 % triethylphosphate) for 30 minutes at room temperature. Thereafter, sections were incubated with primary antibodies for anti-human myosin heavy chain (MYH)7 (type I myocytes) and MYH2 (type IIa myocytes) overnight at room temperature and subsequently incubated with fluorescein (FITC) (type IIa myocytes) and Rhodamine (type I myocytes) conjugated secondary antibodies (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Type IIx fibers remained unstained. Images were visualized using a Leica microscope (Leica DM 4000B; Leica Microsystems, Bannockburn, IL), digitally captured (Retiga 2000R camera; Q Imaging, Surrey, Canada), and semi-quantitative image analysis was conducted using specialized software (Northern Eclipse, v6.0; Empix Imaging, Cheektowaga, NY). Oil red O staining intensity and cross sectional area was determined in type I and type II myocytes. Analysis is based on >200 fibers per section.

**Protein Content by Immunoblot.** A portion of frozen muscle (~30mg) was prepared for immunoblot for GLUT protein expression as described in the supplemental section. The frozen muscle sample was homogenized (T8 Ultra Turrax; IKA Inc., Wilmington, NC) in ice-cold cell lysis buffer (Cell signaling Technology, Danvers, MA) including a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The homogenates were incubated with gentle rocking (1 hr, 4°C) and then centrifuged (10 min, 12000 xg, 4°C) and the supernatant removed. Protein content of the supernatant was determined using the bicinchoninic acid assay (BCA) (Thermo Scientific, Rockford, IL). Aliquots of supernatant were mixed with 5x Laemmli buffer (10 % SDS, 10 % glycerol, 10 mM beta-mercaptoethanol, 0.05 % bromophenol blue, 0.2 M Tris-HCl pH 6.8) and denatured by heating (5 min, 100 °C). Samples were separated on a 4-20% SDS-PAGE gel followed by transfer onto polyvinylidene difluoride membranes (Bio-Rad
Laboratories, Hercules, CA). Membranes were blocked in 5 % non-fat milk, and incubated with the following primary antibodies: Anti-GLUT4 (#2213, Cell Signaling, Danvers, MA), anti-GLUT1, anti-GLUT12 (#GT12-A and #GT122-A, Alpha Diagnostics International, San Antonio, TX), anti-OXPHOS antibody cocktail (#MS601, Mitosciences, Eugene, OR), and anti-α tubulin (Mouse #DM1A and Rabbit #11H10, Cell Signaling, Danvers, MA). Membranes were then incubated in appropriate species-specific secondary antibodies (IRDye 800CW anti-Rabbit #926-32211 and IRDye 680RD anti-Mouse #926-68070, Li-Cor Biosciences, Lincoln, NE). Protein bands were visualized using a Li-Cor Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) and analyzed with Image Studio v2.1 software (Li-Cor Biosciences, Lincoln, NE). Gel-to-gel variation was controlled for by using a standardized sample on each gel. Protein loading was controlled by normalizing bands of interest to α-Tubulin.
SUPPLEMENTAL TABLE 1.

Exercise Compliance Data for participants in the EX group.

<table>
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<tr>
<th></th>
<th>Sessions/wk</th>
<th>Min/Session</th>
<th>Min/week</th>
<th>HR/session</th>
<th>% max HR</th>
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<tbody>
<tr>
<td>Mean</td>
<td>3.5</td>
<td>44.2</td>
<td>154.4</td>
<td>132.5</td>
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<td>SD</td>
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<td>2.1</td>
<td>17.0</td>
<td>2.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Abbreviations: wk, week; Min, minutes; HR, heart rate
SUPPLEMENTAL REFERENCES


