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Effects of Exercise on Mitochondrial Content and Function in Aging Human Skeletal Muscle

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Skeletal muscle mitochondria are implicated with age-related loss of function and insulin resistance. We examined the effects of exercise on skeletal muscle mitochondria in older (age = 67.3 ± 0.6 years) men ($n = 5$) and women ($n = 3$). Similar increases in ($p < .01$) cardiolipin (88.2 ± 9.0 to 130.6 ± 7.5 μg/mU creatine kinase activity [CK]) and the total mitochondrial DNA (1264 ± 170 to 1895 ± 273 copies per diploid of nuclear genome) reflected increased mitochondria content. Succinate oxidase activity, complexes 2–4 of the electron transport chain (ETC), increased from 0.13 ± 0.02 to 0.20 ± 0.02 U/mU CK ($p < .01$). This improvement was more pronounced ($p < .05$) in subsarcolemmal (127 ± 48%) compared to intermyofibrillar (56 ± 12%) mitochondria. NADH oxidase activity, representing total ETC activity, increased from 0.51 ± 0.09 to 1.00 ± 0.09 U/mU CK ($p < .01$). In conclusion, exercise enhances mitochondria ETC activity in older human skeletal muscle, particularly in subsarcolemmal mitochondria, which is likely related to the concomitant increases in mitochondrial biogenesis.

AGING has been associated with a reduced capacity for oxidative phosphorylation in muscle (1,2), most likely due to a decline in mitochondria content and/or function (3). A poor capacity for oxidative metabolism within skeletal muscle is also associated with insulin resistance (4) and type 2 diabetes mellitus (5,6). Recent studies further indicate that muscle mitochondria of patients with type 2 diabetes are smaller and may also be less functional than are mitochondria of those persons without diabetes (7). Petersen and colleagues (8) have further suggested that a lower oxidative capacity in muscle is an essential feature of age-associated insulin resistance. However, it is not clear from these cross-sectional studies whether this lower oxidative capacity of muscle may be due to deficiencies in mitochondria content, a reduced mitochondrial function, or both. It also raises the important question of whether mitochondrial defects observed in normal aging and in metabolic disorders are the result of an acquired problem, and accordingly, whether they can be restored with intervention. Although it is known that young healthy muscle is quite plastic in its ability to increase its capacity for oxidative metabolism in response to chronic exercise, less is known about whether muscle in pathophysiological conditions, or even muscle of healthy older adults, is able to respond accordingly to intervention.

Given the strong evidence linking mitochondrial dysfunction with aging, insulin resistance, and type 2 diabetes, it is important to more precisely define specific loci of these defects and, perhaps more important, to determine whether clinical interventions may correct these insufficiencies. Previous studies (9–11) have demonstrated the presence of two distinct mitochondrial populations within skeletal muscle. Subsarcolemmal (SS) mitochondria reside near the sarcolemma, and intermyofibrillar (IMF) mitochondria are located between the myofibrils. It has been suggested that SS mitochondria provide energy for membrane-related events including cell signaling and substrate and ion transport, and IMF mitochondria supply adenosine triphosphate to contracting myofibrils (12). SS mitochondria generally represent only 25%–30% of the total amount of skeletal muscle mitochondria, but appear to be more responsive to increased physical activity in rat muscle (10,13), as well as in young human skeletal muscle (14–16). Whether there are improvements in specific mitochondrial subpopulations in older adults with reduced mitochondria content and function has yet to be determined.

These prior observations question whether there are specific populations of mitochondria that may be more responsive to intervention, and moreover, whether improvements are simply due to increased mitochondria content. In the present study we examined the effects of moderate exercise training in older adults on changes in mitochondria content and function located within distinct locations within skeletal muscle.

Methods

Participants

Eight healthy elderly (67.3 ± 0.6 years) volunteers (three women and five men), recruited using community advertisements, participated in this study after providing written informed consent. None of the volunteers was previously engaged in regular (>1 time/week) exercise, nor had any gained or lost more than 2 kg of body weight within the past

6 months prior to the study. None of the volunteers had type 2 diabetes. Those with coronary heart disease, peripheral vascular disease, untreated hypertension, or clinically significant hyperlipidemia (plasma triglycerides greater than 3.95 mmol/L or total cholesterol levels greater than 7.76 mmol/L) were excluded. The research project was reviewed and approved by the University of Pittsburgh Institutional Review Board.

Intervention

Participants completed a 12-week exercise training program, which has been previously described in detail for a similar group of older adults (17), and will be summarized briefly. Participants were asked to complete a minimum of four and a maximum of six exercise sessions weekly, with at least three supervised sessions weekly. Most exercise, performed by using treadmills or stationary bicycles, or by walking outdoors, was individually prescribed based on time and intensity and was progressive. For the first 4 weeks, the participants exercised for 30 minutes at a heart rate corresponding to 50%–60% of maximal aerobic capacity (VO_{2max}). For the next 4 weeks, they increased exercise time to 40 minutes at the same intensity, and for the last 4 weeks they increased the intensity to ≈70% of VO_{2max} for at least 40 minutes per session.

Study Protocol

Before and after 12 weeks of exercise, participants had a percutaneous muscle biopsy, a test for physical fitness (VO_{2max}), and a blood sample during fasting conditions to determine markers of insulin resistance (glucose and insulin).

VO_{2max}

Participants performed a graded exercise test on an electronically braked cycle ergometer (SensorMedics Ergoline 800S; Yorba Linda, CA) to determine changes in physical fitness (VO_{2max}). Expired air was collected via open-circuit spirometry (SensorMedics 2900) to determine VO_2. Heart rate, blood pressure, and electrocardiogram were recorded prior to, during, and immediately following this test. Heart rate–VO_2 relationships obtained during this graded exercise test were also used to prescribe intensity during each exercise training session.

Insulin Resistance

To determine the training effects on insulin resistance, we calculated homeostasis model assessment of insulin resistance (HOMA-IR), based on fasting glucose and insulin. Plasma glucose was measured using an automated glucose oxidase reaction (YSI 2300 Glucose Analyzer; Yellow Springs, OH). Serum insulin was determined using commercially available radioimmunoassay kits (Pharmacia, Uppsala, Sweden).

Muscle Biopsies

Percutaneous biopsies of the vastus lateralis were obtained in the General Clinical Research Center (GCRC) on a morning after an overnight fast as described previously in more detail (17,18). Participants were given a standard 10 kcal/kg meal consisting of 50% carbohydrate, 30% fat, and 20% protein the night before the biopsy. Participants were instructed not to perform physical exercise 48 hours before the muscle biopsy procedure to help prevent acute effects of exercise on muscle mitochondrial function. Muscle specimens were trimmed, frozen in liquid nitrogen, and stored at –80°C. Baseline and postintervention biopsy specimens from each participant were prepared and analyzed together to avoid any interassay variability in isolation of mitochondria or biochemical analysis.

Preparation of Mitochondrial Fractions

A portion of muscle biopsy samples of (≈10–15 mg wet weight) were homogenized in ice-cold basic medium (100 mM mannitol, 80 mM gluconate–K; 20 mM potassium fluoride, 1 mM $MgCl_2$, 0.2 mM EGTA, 10 mM histidine, 10 mM glucose, 10 mM triethylamine-morpholino-hydroxypropanesulfonic acid (TEA-MOPSO), pH 7.6 at 21°C) containing bovine serum albumin at 5.0 mg/ml, 100 μM deferoxamine mesylate, and antiprotease cocktail III, using a Polytron homogenizer according to the procedures described by Krieger and colleagues (10). All procedures were performed at 4°C. Soluble and particulate fractions were prepared as previously described (19), by centrifugation (45,000 *g* for 20 minutes), to pellet a particulate (SS + IMF mitochondria) fraction containing >95% of tissue mitochondria. SS and IMF mitochondrial fractions were prepared as described earlier (20). SS mitochondria were isolated from skeletal muscle following gentle extraction procedures, and after the subsequent extraction of myosin, IMF mitochondrial fraction was collected in two subfractions, a free fraction (IMF1) and another fraction more tightly bound to myofibrils (IMF2). Mitochondrial preparations were suspended in 500 μl of medium, containing 0.5 mM EGTA, 0.1 mg/ml bovine serum albumin, 25 mM potassium phosphate buffer, pH 7.0 at 21°C, and were kept at –80°C until assay.

Mitochondrial DNA Determinations

DNA (mitochondrial and nuclear) was extracted from tissue samples using a QIAamp DNA Mini Kit (QIAGEN, Chatsworth, CA). The concentration of each sample was determined using a GeneQuant spectrophotometer (Pharmacia Biotech). Mitochondrial DNA (mtDNA) content was measured using real-time polymerase chain reaction (PCR) as described earlier (20,21). Detection of a 69 bp fragment of mtDNA (nucleotides 14918–14986) and a 77 bp fragment of β-globin, both based on markers published by Miller and colleagues (22), were used to determine relative copy number of mtDNA per diploid nuclear genome. Primers and 6-carboxyfluorescein (FAM)-labeled Taqman 6-carboxy-tetramethyl-rhodamine (TAMRA) probes (450025; Applied Biosystems, Foster City, CA) were designed using Primer Express software, version 1.5 (Applied Biosystems). Detection of mtDNA and β-globin was performed as two separate reactions, but within the same run for each sample. All samples were run in duplicate for each gene. Reactions were carried out in the presence of 1X Taqman Universal PCR Master Mix (4304437; Applied Biosystems), 1 μM each forward and reverse primer, 0.25 μM (FAM) labeled Taqman/TAMRA probe, and 20 ng of sample DNA to a

final volume of 25 μl. Amplification reactions were performed in an ABI Prism 7700 spectrofluorometric thermal cycler (Applied Biosystems) with the following cycle conditions: 50°C for 2 minutes of uracil-DNA-glycosylase (UNG) incubation, 95°C denaturation and enzyme activation step for 10 minutes followed by 40 cycles of 95°C denaturation for 15 seconds, and 60°C annealing and elongation for 60 seconds. Fluorescence spectra were recorded during the annealing phase of each PCR cycle. The Sequence Detection System software (SDS v1.7) of the ABI-Prism 7700 was used to generate the FAM fluorescence.

Threshold Cycle Calculations

The threshold cycle number (Ct) was calculated using SDS software v1.7 and an automatic setting of the baseline. The baseline value was the average fluorescence value of PCR cycles 3–15 plus 10 times its standard deviation. These values were used for the relative copy number (Rc) calculations by expressing Ct differences of the β-globin and mtDNA PCR as described earlier (21):

$$Rc = 2^{\Delta Ct}\Delta Ct = Ct_{\beta\text{-globin}} - Ct_{mtDNA}$$

Cardiolipin

Cardiolipin is a phospholipid specific to mitochondria, thus reflecting mitochondria content. Cardiolipin was quantified in each mitochondrial subfraction of previously frozen skeletal muscle biopsies by high performance liquid chromatography (HPLC) analysis of a fluorescence-labeled derivative of cardiolipin (23). Cardiolipin content was normalized to the amount of creatine kinase (CK) activity as the amount of active skeletal muscle.

Electron Transport Chain Activity

Activity of NADH oxidase (rotenone-sensitive NADH:O_2 oxidoreductase) was determined in total mitochondria fractions by an HPLC-based assay, as described previously to represent total (complexes I–IV) electron transport chain (ETC) activity (24,25). Succinate oxidase (succinate:O_2 oxidoreductase) activity was measured in total mitochondria fractions and separately in each SS, IMF1, and IMF2 mitochondrial subfraction according to the separation scheme outlined above. Succinate oxidase is a reaction starting from succinate dehydrogenase (SDH; complex II), and is based on assay of the accumulation of fumarate, the end-product of succinate oxidation as described earlier (20,25). This procedure is a modification of a previously developed assay (7,26). Briefly, the assay couples fumarase and malic dehydrogenase reactions to oxidize fumarate and reduce NAD^+, with HPLC and fluorescence detection used to measure NADH (19,25). Activity of CK was measured an index of muscle fiber content in biopsy samples as previously described (7,20,25), and ETC activity is expressed normalized to CK activity.

Statistics

Data are presented as mean ± standard error of the mean, unless otherwise indicated. Paired *t* tests were used to determine effects of exercise intervention on changes in mitochondria content, ETC activity, physical fitness, and markers of insulin resistance. Two-way analysis of variance was used to compare subfractions of mitochondria and their differential response over time (mitochondrial subfraction × time).

Table 1. General Body Composition, Physical Fitness, and Markers of Insulin Resistance Before and After the 12-Week Exercise Program

Variable	Before Training	After Training
BMI, kg/m^2	28.0 ± 1.6	27.7 ± 1.5
Percent body fat	32.2 ± 2.2	32.4 ± 2.3
VO_{2max}, L/min	1.64 ± 0.14	1.88 ± 0.15*
Fasting glucose, mM	5.20 ± 0.13	5.12 ± 0.16
Fasting insulin, μU/ml	13.0 ± 2.6	10.2 ± 1.7*
HOMA-IR	3.05 ± 0.63	2.32 ± 0.40*

Notes: Results presented as means ± standard error of the mean; $N = 8$ (3 women, 5 men).

*Significant change from before to after intervention, $p < .05$ (paired *t* test).

BMI = body mass index; VO_{2max} = physical fitness assessed by maximal oxygen consumption; HOMA-IR = homeostatic model estimate of insulin resistance.

Results

Body Composition, Physical Fitness, and Insulin Sensitivity

Body composition, physical fitness, and markers of insulin resistance before and after the intervention are shown in Table 1. At baseline, participants were overweight but not obese, and were sedentary. The intervention improved physical fitness (VO_{2max}) significantly ($p < .01$) by 15 ± 4% and without a change in body weight or percent body fat. There was a significant ($p < .05$) improvement in insulin sensitivity estimated by HOMA-IR, reflected mainly by a decrease in fasting plasma insulin (Table 1).

Mitochondria Content

Skeletal muscle mtDNA content was determined in biopsy samples before and after the intervention, and the results are shown in Figure 1. At baseline, muscle mtDNA was lower in these elderly men and women in comparison to younger adults recently reported in our laboratory (24), which is also consistent with prior studies (3). Changes in mtDNA reflect changes in mitochondria volume. As illustrated in Figure 1, there was a robust increase (53 ± 15%) in mtDNA content with training ($p < .01$). Cardiolipin, a mitochondria-specific phospholipid that reflects mitochondria content, increased by 56 ± 13% (Figure 2), corresponding to the increase in total mitochondria content assessed by mtDNA.

Mitochondrial ETC Activity

Cardiolipin and mtDNA provide complementary assessments of mitochondria content. To further assess the potential effect of exercise training, ETC activity, a functional measure, was assessed (specifically that of NADH oxidase and succinate oxidase, which represents mitochondria ETC activity from complexes I–IV and II–IV,

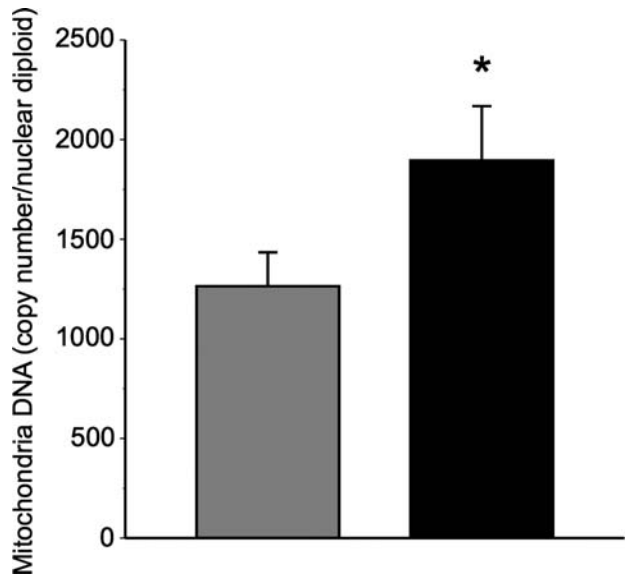

Figure 1. Effects of exercise on skeletal muscle mitochondrial DNA content. Mitochondrial DNA copy number in total mitochondria fraction relative to diploid nuclear genome before (gray bar) and after (black bar) exercise training. *Significant ($p < .01$) change determined by paired t test ($n = 7$). Data are presented as means ± standard error of the mean.

respectively). In response to intervention, activity of NADH oxidase in the total mitochondrial fraction was approximately doubled (Figure 3). Succinate oxidase in the total mitochondria fraction increased ($p < .01$) by 62 ± 13% (Figure 3) from 0.13 ± 0.02 to 0.20 ± 0.02 U·mU CK^{-1}.

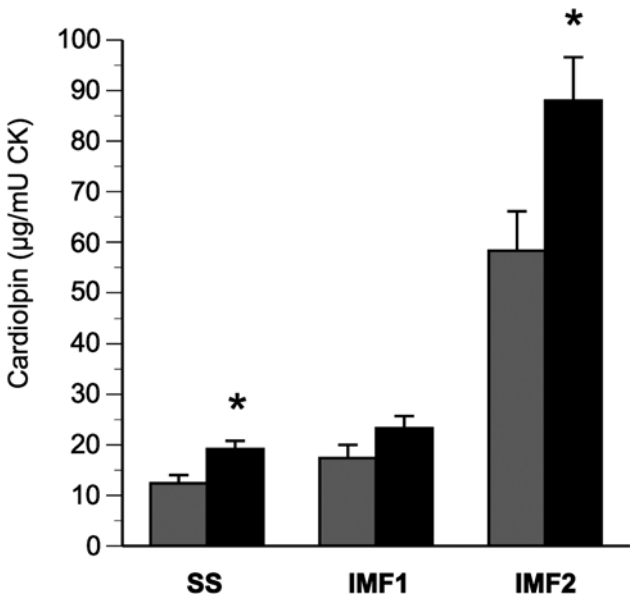

Figure 2. Effects of exercise on skeletal muscle cardiolipin content in subsarcolemmal (SS) and intermyofibrillar (IMF1 and IMF2) mitochondrial fractions. Cardiolipin content before (gray bar) and after (black bar) exercise training. *Significant ($p < .01$) change determined by paired t test ($n = 8$). Data are presented as means ± standard error of the mean. CK = enzymatic activity of skeletal muscle creatine kinase.

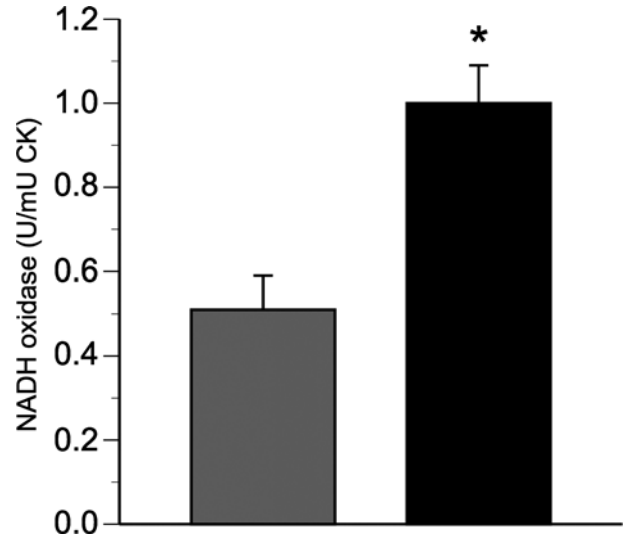

Figure 3. Effects of exercise on total mitochondrial electron transport chain activity. Total skeletal muscle NADH oxidase activity before (gray bar) and after (black bar) exercise training. Data are presented as means ± standard error of the mean ($n = 5$). CK = activity of creatine kinase. *Significant change from preintervention assessed by paired t test, $p < .01$.

The magnitude of these increases corresponded with the increase in the content of both total mtDNA and cardiolipin. This result suggests that the increased mitochondria content corresponded with the overall increase in mitochondrial function in skeletal muscle.

Cellular Distribution of Mitochondria

As described in Methods, mitochondria were separated into three fractions, and cardiolipin content and ETC activity were assessed in each fraction. The results are shown in Figures 2 and 4. At baseline, the IMF2 fraction, which contains the mitochondria most tightly bound to myofibrils, contained the majority of cardiolipin (≈65%), whereas the SS fraction contained a much smaller proportion of cardiolipin (≈14%). Following intervention, there was a highly significant increase in cardiolipin content in the IMF2 and SS fractions, and the relative proportion of cardiolipin content among the three remained essentially unchanged.

ETC (succinate oxidase) activity in the SS and IMF fractions is shown in Figure 4. Prior to intervention, the distribution of ETC activity was not symmetric, such that there was a relative deficiency in the SS fraction (21 ± 3% of total ETC activity). Exercise training increased ($p < .01$) the ETC activity in both SS and IMF2 mitochondrial fractions of skeletal muscle (Figure 4). This increase in ETC activity was more pronounced in SS (127 ± 48%) than in IMF2 (65 ± 14%) mitochondria ($p < .05$). There was not a statistically significant increase of ETC activity in the IMF1 fraction.

Discussion

Impaired oxidative phosphorylation by skeletal muscle mitochondria has been postulated to contribute to age-associated insulin resistance and fat accumulation within

skeletal muscle (8). This impaired mitochondrial functional capacity in aging has been attributed to a reduced mitochondria content, as reflected by lower mtDNA content (3). The current study was therefore undertaken to assess the impact of physical activity on muscle mitochondria in elderly men and women. As reflected in three independent and complementary parameters—mtDNA, cardiolipin, and ETC activity—there was a substantial response of mitochondria, with improvements of at least 50% in each of these parameters. These improvements were further assessed within distinct muscle mitochondrial subpopulations. At baseline, a relatively low fraction of ETC activity and cardiolipin was contained in the SS fraction in these elderly volunteers, a finding that is entirely consistent with their overweight and sedentary status (24). In response to training, there was a robust improvement in the SS fraction, but there was also a substantial improvement in the IMF2 fraction, the subpopulation of mitochondria that most directly provides energy for contracting muscle. Thus, our main finding is that there is a robust improvement in skeletal muscle mitochondria content and function in elderly men and women in response to an achievable program of moderate intensity physical activity.

One of the classic responses to exercise is an increase in the oxidative capacity of skeletal muscle (27–29). Relatively few of the many human studies of exercise intervention, however, have focused on elderly persons. A few earlier studies have demonstrated that chronic endurance training increased the amount of mitochondrial protein (30) and mitochondrial volume (31) in skeletal muscle of older men and women concomitant with enhanced overall physical fitness. In the current study, we have expanded upon this important earlier work by broadening the scope of biochemical assessments of mitochondrial content and function in response to increased physical activity in older men and women.

Although the response observed in the participants of the current study was quite positive, it was not clear that this would in fact occur. Many age-related declines in physiological function can be partially attributed to mitochondrial dysfunction (32,33). There is a significant loss in the number of muscle fibers and also biochemical and morphological abnormalities in aging skeletal muscle (34,35). The specific mechanisms leading to the age-related changes are currently unknown. Mitochondria are primary sites of reactive oxygen species formation that cause progressive damage to mtDNA and proteins (35,36). The analysis of human muscle mitochondria has revealed a progressive decline in mitochondrial respiratory chain function with age (1,3,37,38), which may be related to reduced mtDNA content (3). These studies collectively raise the question of whether age-related mitochondrial defects are the result of normal aging or, conversely, whether they are at least partially acquired through lifestyle and factors other than aging per se.

An important area for investigation is to more fully evaluate whether aging limits or alters the response of mitochondria to intervention. Previously, our laboratory (7,20) has observed an impaired bioenergetic capacity of skeletal muscle mitochondria in type 2 diabetes and obesity,

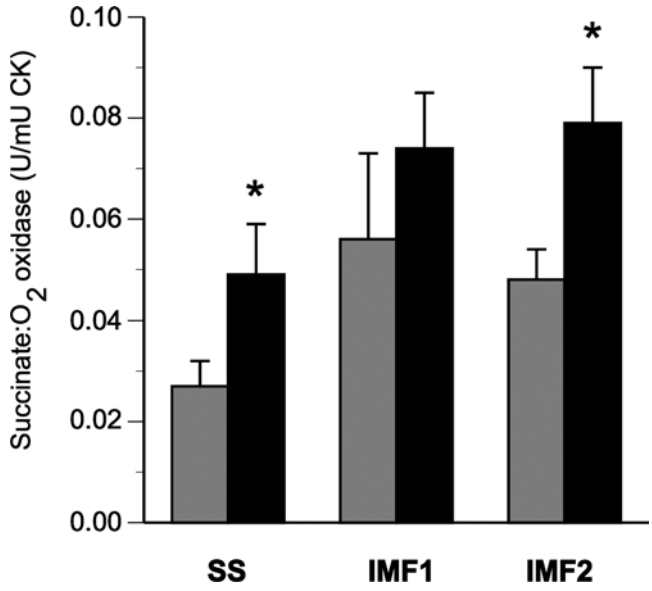

Figure 4. Effects of exercise on electron transport chain activity in subsarcolemmal (SS) and intermyofibrillar (IMF1 and IMF2) mitochondrial fractions. Skeletal muscle succinate oxidase activity in SS and IMF fractions of skeletal muscle before (gray bars) and after (black bars) exercise. Data are presented as means ± standard error of the mean ($n = 8$). CK = activity of creatine kinase. *Significant change from preintervention assessed by paired t test, $p < .01$.

including smaller mitochondria and reduced ETC activity (7). The ETC activity in the healthy older participants in this study at baseline was 3-fold less than that observed in younger lean individuals, but similar to that seen in middle-aged obese participants without type 2 diabetes (20). In particular, the lower ETC activity in these older men and women was pronounced in SS mitochondria compared to IMF mitochondria; there was an approximately 4-fold lower succinate oxidase activity in the SS mitochondria in these elderly participants in comparison with lean younger individuals (24). Similarly, Ritov and colleagues (20) reported a greater deficiency in the SS mitochondria in persons with type 2 diabetes and obesity. In contrast, the ETC activity in skeletal muscle of younger individuals was distributed evenly across the fractions, with approximately one third of overall activity in the SS fraction compared to 21% in these older adults (20). This finding suggests that there may be age-related reductions in oxidative capacity of muscle and, specifically, deficiencies in certain mitochondrial subpopulations in aging. Alternatively, mitochondrial subpopulations could be affected differently by physical activity related to aging. Although the response of these generally healthy older volunteers was quite robust, the limited sample size of our study prevents us from generalizing these results to older persons who may have functional impairments, more severe insulin resistance, or type 2 diabetes.

Few studies have examined whether improvements in mitochondrial function and/or content are related to the improvements in insulin resistance and risk for the development of metabolic syndrome or type 2 diabetes. HOMA-IR as a marker of insulin sensitivity (39) improved in

parallel with improved mitochondria content and function. These results are consistent with the observations that higher oxidative capacity is related to higher insulin sensitivity (40), and that an exercise-enhanced reliance on fat oxidation predicts improved insulin sensitivity in obese (41) and in elderly (42) persons. These results, however, are in apparent contrast to the study by Short and colleagues (43), who reported increased oxidative capacity in older men and women despite little improvement in insulin sensitivity. Thus, the relationship between increased mitochondrial function and insulin resistance should be examined further.

To our knowledge, this is the first study examining the effects of exercise on the function of distinct mitochondrial subpopulations within aging skeletal muscle. The increase in ETC activity of complex II–IV (succinate oxidase) was more pronounced in SS mitochondria than in IMF mitochondria. The *total* ETC assessed by NADH oxidase activity, as well as total succinate oxidase activity, paralleled the increase in total mitochondria content measured as mtDNA content and cardiolipin, all increasing by more than 50%. The degree of response in cardiolipin within the SS and IMF mitochondrial subfraction was closely matched to the increases in ETC within the same fraction. Moreover, the ratio of ETC activity to mitochondria content (cardiolipin) did not change with intervention. Higher mitochondria content in young endurance-trained individuals compared to untrained participants was more prominent for the SS mitochondrial population than for the IMF population (29). Chilibeck and colleagues (15) reported that endurance training resulted in greater increases in SS SDH activity compared to IMF mitochondria. However, Krieger and colleagues (10) reported similar increases in SDH activities in the SS and IMF mitochondrial populations of rat skeletal muscle in response to chronic endurance training. SS mitochondria likely provide energy for cellular processes of substrate transport and cell signaling in skeletal muscle (12), and exhibit higher rates of fatty acid oxidation (44). Thus, SS mitochondria may be specifically linked to physical inactivity, low oxidative capacity, and insulin resistance. Further inquiry into the functional significance of how different mitochondrial subpopulations in human skeletal muscle respond to various interventions might provide insight into mitochondria as potential therapeutic targets for prevention and treatment of insulin resistance and type 2 diabetes.

Summary

Mitochondrial function, as assessed by ETC activity, and content of mitochondria improved similarly with exercise training. However, there were distinct differences in the response of ETC activity within specific populations of skeletal muscle mitochondria. Additional studies are clearly needed to determine the specific function of these mitochondrial subpopulations and, further, to investigate whether there are specific components of mitochondrial function that are implicated in age-associated and obesity-associated disorders. These findings could have implications for designing specific interventions, including exercise, in the treatment and prevention of skeletal muscle functional changes with aging.

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