Endurance Exercise as a Countermeasure for Aging

Ian R. Lanza, Daniel K. Short, Kevin R. Short, Sreekumar Raghavakaimal, Rita Basu,
Michael J. Joyner, Joseph P. McConnell, and K. Sreekumaran Nair

OBJECTIVE—We determined whether reduced insulin sensitivity, mitochondrial dysfunction, and other age-related dysfunctions are inevitable consequences of aging or secondary to physical inactivity.

RESEARCH DESIGN AND METHODS—Insulin sensitivity was measured by hyperinsulinemic-euglycemic clamp and ATP production in mitochondria isolated from vastus lateralis biopsies of 42 healthy sedentary and endurance-trained young (18–30 years old) and older (59–76 years old) subjects. Expression of proteins involved in fuel metabolism was measured by mass spectrometry. Citrate synthase activity, mitochondrial DNA (mtDNA) abundance, and expression of nuclear-encoded transcription factors for mitochondrial biogenesis were measured. SIRT3, a mitochondrial sirtuin linked to lifespan-enhancing effects of caloric restriction, was measured by immunoblot.

RESULTS—Insulin-induced glucose disposal and suppression of endogenous glucose production were higher in the trained young and older subjects, but no age effect was noted. Age-related decline in mitochondrial oxidative capacity was absent in endurance-trained individuals. Although endurance-trained individuals exhibited higher expression of mitochondrial proteins, mtDNA, and mitochondrial transcription factors, there were persisting effects of age. SIRT3 expression was lower with age in sedentary but equally elevated regardless of age in endurance-trained individuals.

CONCLUSIONS—The results demonstrate that reduced insulin sensitivity is likely related to changes in adiposity and to physical inactivity rather than being an inevitable consequence of aging. The results also show that regular endurance exercise partly normalizes age-related mitochondrial dysfunction, although there are persisting effects of age on mtDNA abundance and expression of nuclear transcription factors and mitochondrial protein. Furthermore, exercise may promote longevity through pathways common to effects of caloric restriction. *Diabetes* **57: 2933–2942, 2008**

Reduced insulin sensitivity is a common factor in the metabolic syndrome, a cluster of clinical conditions that shows increased risk with age (1–3). Mitochondrial dysfunction is also prevalent in the elderly (4,5), with reductions in mitochondrial enzyme activities (6), protein synthesis (7) and expression (5), and DNA (mtDNA) abundance (5,8). A close association between insulin sensitivity and muscle mitochondrial function has been reported in aging (4,5), type 2 diabetes (9), and obesity (10) as well as in offspring of type 2 diabetic individuals (11), prompting a hypothesis that either reduced insulin sensitivity results from muscle mitochondrial dysfunction (4,11) or vice versa (5,12).

Endurance exercise increases insulin sensitivity (13,14) and mitochondrial enzyme activities (15,16). Short-term and longitudinal studies have documented that older populations respond favorably to endurance exercise but that there are persisting age effects that cannot be eliminated by short-term exercise programs (8,17). For practical reasons, most training studies are acute interventions, whereas the effects of aging are chronic. This precludes conclusions regarding whether older adults are less adaptable to exercise training than young adults or if the volume and duration of the training were insufficient to reveal the full potential for adaptation. Furthermore, it remains to be determined whether long-term endurance exercise shares some of the biochemical effects of caloric restriction, which prolongs the lifespan of many species through the DNA-stabilizing actions of sirtuins (18), in particular the mitochondrial localized SIRT3 (19).

The purpose of this study was to determine if age-related declines in insulin sensitivity and mitochondrial function could be prevented by long-term endurance training. Peripheral and hepatic insulin sensitivity were measured by euglycemic-hyperinsulinemic clamp in sedentary and chronically endurance-trained young (18–30 years old) and older (59–76 years old) subjects. Mitochondrial function was assessed by measuring muscle ATP production rates (MAPR) in isolated mitochondria from vastus lateralis. To examine molecular and cellular mechanisms responsible for group differences in mitochondrial function, citrate synthase (CS) activity, mtDNA, large-scale protein expression using mass spectrometry, and expression of key mitochondrial transcription factors, including nuclear respiratory factor-1 (NRF-1), mitochondrial transcription factor A (TFAM), and their coregulator, peroxisomal proliferator–activated receptor γ coactivator 1α (PGC-1α), were measured. We also determined the effects of aging on SIRT3 expression and if chronic endurance training could induce effects similar to caloric restriction by increasing SIRT3 expression. This study was designed as a cross-sectional comparison of sedentary and endurance-trained young and old subjects to circumvent the numerous practical limitations that would complicate a prospective study of sufficient duration to satisfy the aim of the study.

RESEARCH DESIGN AND METHODS

A total of 22 healthy young (18–30 years old) and 20 healthy older (59–76 years old) subjects gave written informed consent, as approved by the Mayo Foundation Institutional Review Board. Subjects were further divided into the following groups: young sedentary (YS) (5 women, 6 men), young trained (YT) (5 women, 6 men), older sedentary (OS) (4 women, 6 men), and older trained (OT) (4 women, 6 men). Sedentary subjects exercised less than 30 min per

day, twice per week. Trained subjects performed at least 1 h of cycling or running 6 days per week over the past 4 years or longer by self-report. Activity levels were confirmed with a leisure-time activity questionnaire. Subjects were screened by medical history, physical exam, graded treadmill test, and comprehensive blood tests, including blood lipids from standard photometric methods, hormones, and glucose. Magnetic resonance spectroscopy was used to measure lipoprotein particle concentrations in plasma (20). Briefly, an EDTA plasma sample diluted 1 to 1 was injected into the flow probe of a Bruker-Biospin 400-MHz ^{1}H-nuclear magnetic resonance (NMR). Lipana software analyzed the data to determine LDL and HDL particle concentration. Exclusion criteria included history of metabolic or cardiovascular disease, plasma glucose >99 mg/dl, BMI >28 kg/m^2, medications affecting outcome measures, anemia, pregnancy, and substance abuse.

Dual X-ray absorptiometry (Lunar DPX-L; Lunar Radiation, Madison, WI) was used to measure fat and fat-free mass (FFM). Abdominal and visceral fat area were measured with single-slice computed tomography scans (Imatron C-150; Imatron, San Francisco, CA), as described previously (21). V_{O_2} peak was determined from expired gas analysis during a graded bicycle test. To ensure that subjects achieved V_{O_2} peak, at least two of the following criteria were confirmed: a plateau in V_{O_2}, respiratory exchange ratio ≥1.1, and attainment of age-predicted maximal heart rate. At least 7 days after the V_{O_2} peak test, subjects were given a meat-free weight-maintaining diet for 3 days before inpatient testing and refrained from exercise. At 1700 h on day 3 of the diet, subjects were admitted to the Mayo Clinic General Clinical Research Center for 48 h. Muscle mass was estimated from 48-h urinary creatinine excretion. Subjects were given a standard meal and snack at 1800 and 2200 h of the second day, after which they remained fasted until the end of the study. Following baseline blood sampling at 0330 h the following morning, a primed (2.4 mg/kg FFM), continuous (0.04 mg · kg FFM^{-1} · min^{-1}) infusion of [6,6-^{2}H$_2$]-D-glucose was initiated. After 0530 h, one hand was placed in a heated box (120°F) with a retrograde IV catheter. At 0600 h, arterialized blood samples were taken every 10 min until 0630 h for [6,6-^{2}H$_2$]-D-glucose isotopic enrichment measurements. At 0630 h, a percutaneous needle muscle biopsy (350–400 mg) was obtained from the vastus lateralis muscle under local anesthesia (22). Approximately 40 mg of muscle was immediately used for measurement of MAPR, as previously described (5), and the remaining tissue was frozen in liquid nitrogen and stored at −80°F for other analyses.

At 0630 h, following the muscle biopsy, an 8-h euglycemic-hyperinsulinemic clamp was started with insulin infused at 1.5 mU · kg FFM^{-1} · min^{-1}. The infusion rate of [6,6-^{2}H$_2$]-D-glucose was varied to mimic the anticipated changes in endogenous glucose production, as described previously (23). Plasma glucose was maintained at ~90 mg/dl during insulin infusion with a variable infusion of 40% dextrose containing 2.15% [6,6-^{2}H$_2$]-D-glucose to minimize the changes in glucose enrichments and maintain constant plasma specific activity (23). The glucose infusion rate was adjusted as needed based on blood samples taken at 10-min intervals; samples were measured with a Beckman glucose analyzer (Beckman Coulter, Fullerton, CA) (24). [6,6-^{2}H$_2$]-D-glucose enrichment of blood samples and infusates were measured using mass spectrometry. As described previously (23), the steady-state equations of Steele (25) were used to calculate glucose appearance (R_a) and disappearance (R_d): $R_a = R_d = F_{Glu}/$SA of [6,6-^{2}H$_2$] glucose (1), where F_{Glu} is the infusion rate of [6,6-^{2}H$_2$]-D-glucose and specific activity (SA) of [6,6-^{2}H$_2$] glucose is the plasma specific activity of [6,6-^{2}H$_2$] glucose. Endogenous glucose production (EGP) was calculated as the difference between total glucose rate of appearance and exogenous glucose infusion rate (23,26). Peripheral insulin sensitivity was assessed from the rate of glucose infusion required to maintain euglycemia during the clamp, while hepatic insulin sensitivity was assessed by the extent to which EGP was suppressed during hyperinsulinemia.

Mitochondrial ATP production. MAPR was measured using a bioluminescent technique, as described previously (5). Briefly, ~40 mg of fresh muscle was homogenized and centrifuged to isolate mitochondria. The mitochondria were suspended in buffer containing 35 μmol/l ADP and a luciferin-luciferase ATP-monitoring reagent (BioThelma, Haninge, Sweden). A BioOrbit 1251 luminometer (BioOrbit Oy, Turku, Finland) was used to monitor bioluminescence in response to the addition of glutamate plus malate, palmitoyl-L-carnitine plus malate, and succinate plus rotenone. All reactions were monitored for 25 min at 25°C, followed by calibration with an ATP standard. CS activity was measured using spectrophotometric analyses in the mitochondrial pellet (7).

Mitochondrial DNA abundance. DNA were extracted from frozen muscle samples using a QIAamp DNA mini kit (QIAGEN, Chatworth, CA). The abundances of mtDNA-encoded NADH dehydrogenase 1 and 4 genes were measured using a real-time quantitative PCR system (PE Biosystems, Foster City, CA). Samples were run in duplicate and normalized to S28 ribosomal DNA, as described previously (5).

Western blotting. Muscle samples were homogenized in cold lysing buffer with protease inhibitors. Following centrifugation for 15 min at 1,000g, the supernatants were diluted to 2 μg/μl, based on protein concentrations determined using the Lowry procedure (DC Protein Assay, BioRad). Next, 10-μl quantities were loaded and separated on SDS-PAGE (Invitrogen) and transferred onto polyvinylidene difluouride membranes (BioRad). Membranes were blocked with Tris-buffered saline with 5% nonfat milk and then washed with primary antibodies for PGC-1α (516557; Calbiochem, San Diego, CA), TFAM (PA1–24435; Affinity Bioreagents, Golden, CO), NRF-1 (33771; Santa Cruz Biotechnology, Santa Cruz, CA), and SIRT3 (ARP32389; Aviva Systems Biology, San Diego, CA). Following incubation with secondary antibody conjugated to horseradish peroxidase, membranes were exposed to enhanced chemiluminescence reagents for 5 min. Densitometric analyses were conducted using a Kodak Image Station 1000.

Proteomics. To determine the abundance of several individual proteins relevant to cellular ATP production, muscle samples from each subject were prepared as previously described, and the relative abundance of single peptides was determined using the iTRAQ approach (27). Samples from YT, YS, OT, and OS subjects were labeled with iTRAQ reagents 114, 115, 116, and 117, separated, and identified using liquid chromatography/tandem mass spectrometry.

Statistical analyses. Data are presented as means ± SEM. Two-way (age and activity level) ANOVA was used to examine the effects of age, activity level, and the interaction between the two on all outcome variables. When significant interactions were found, post hoc pairwise comparisons were conducted using Tukey's procedure. Individual t tests were used for a priori planned comparisons between OS and YS and between OT and YT. Regression analyses were conducted to explore the relationships between insulin sensitivity and predictors such as age, fat mass, activity score, BMI, and abdominal adiposity. Statistical analyses were conducted using SAS software (SAS, Cary, NC).

RESULTS

Subject characteristics. Age was associated with decreased muscle mass, increased adiposity, and decreased V_{O_2} peak (Table 1). In OT and YT subjects, V_{O_2} peak was higher but percent fat and abdominal and visceral fat content were lower than in OS and YS subjects. Total and LDL cholesterol were elevated in OS compared with YS adults, and HDL cholesterol was elevated in endurance-trained individuals in both age-groups. Although LDL cholesterol did not differ between sedentary and endurance-trained individuals, both total and small LDL particle concentrations measured by NMR were significantly lower in individuals who exercise.

Insulin sensitivity. Average plasma insulin levels during the clamp were similarly high in YS (44.7 ± 1.9 μU/ml), OS (43.1 ± 3.2 μU/ml), YT (45.7 ± 2.1 μU/ml), and OT (47.3 ± 2.3 μU/ml) subjects. The glucose infusion rate required to maintain euglycemia during the clamp was similar in young and old subjects, but higher in trained compared with sedentary subjects (Fig. 1A). The area under the glucose infusion rate curve (Fig. 1A, insets) during 0 through 480 min and 240 through 480 min (plateau region) indicate greater glucose infusion per unit FFM in trained compared with sedentary subjects, with no effects of age in either activity group. Likewise, total R_d during hyperinsulinemia was higher in trained compared with sedentary subjects, with no effects of age (Fig. 1B). EGP was lower in trained compared with sedentary individuals at baseline (Fig. 1B). During the clamp, EGP was suppressed in all groups (YS, 69 ± 7%; YT, 100 ± 11%; OS, 78 ± 7%; and OT, 97 ± 10%), although the magnitude of EGP suppression by insulin was greater in trained compared with sedentary subjects (Fig. 1B). No effects of age on EGP or relative suppression of EGP were noted. The relationship between R_d and age was nonsignificant ($R = 0.11$, $P = 0.53$; data not shown). However, R_d during the clamp was inversely related to BMI ($R = -0.37$, $P = 0.026$), fat mass ($R = -0.53$, $P = 0.001$), and abdominal fat ($R = -0.37$, $P = 0.027$) (data not shown).

TABLE 1
Subject characteristics

	YS	YT	OS	OT	P_{age}	$P_{training}$	$P_{interaction}$
n	11	11	10	10			
Physical characteristics							
Age (years)	22.7 ± 0.7	26.0 ± 1.0	65.1 ± 1.5*	65.4 ± 1.8*	<0.01	0.17	0.26
Height (cm)	172.6 ± 2.7	174.4 ± 2.9	170.8 ± 3.0	167.8 ± 3.0	0.16	0.85	0.41
Weight (kg)	69.4 ± 3.6	69.2 ± 3.8	71.5 ± 4.0	69.1 ± 4.0	0.80	0.73	0.79
BMI (kg/m^2)	23.1 ± 0.9	22.7 ± 0.7	24.4 ± 0.7	24.4 ± 1.0	0.09	0.81	0.75
FFM (kg)	48.5 ± 3.1	53.5 ± 3.0	45.5 ± 3.8	50.4 ± 3.1	0.35	0.14	0.98
Body fat (%)	25.8 ± 1.9	17.4 ± 2.2	32.2 ± 2.9	20.8 ± 3.2	0.06	<0.001	0.55
Muscle mass (kg)	33.7 ± 4.5	33.6 ± 2.3	25.4 ± 2.3*	27.1 ± 1.7	0.02	0.80	0.77
Abdominal fat (cm^2)	185 ± 23	101 ± 20	264 ± 31	186 ± 33*	0.005	0.08	0.57
Visceral fat (cm^2)	59.2 ± 11.0	32.1 ± 4.2	127 ± 16.3*	91.9 ± 19.3*	0.001	0.03	0.98
V_{O_2} peak (ml $\cdot$ kg FFM^{-1} $\cdot$ min^{-1})	51.2 ± 2.3	66.7 ± 1.7	36.5 ± 1.6*	54.0 ± 3.6*	<0.001	<0.001	0.67
LTA score (min/week)	198 ± 59	990 ± 110	217 ± 77	$1,114 \pm 206$	0.56	<0.001	0.67
Lipids							
Total cholesterol (mg/dl)	132 ± 6	135 ± 8	182 ± 9*	167 ± 11*	<0.01	0.45	0.27
HDL cholesterol (mg/dl)	32 ± 2	43 ± 3	47 ± 5*	51 ± 3	<0.01	0.04	0.33
LDL cholesterol (mg/dl)	73 ± 5	78 ± 6	116 ± 9*	97 ± 10	<0.01	0.37	0.13
TG (mg/dl)	134 ± 17	74 ± 7†	98 ± 8*	91 ± 11	—	—	0.03
Total HDL particles (μmol/l)	30.4 ± 1.3	29.4 ± 1.8	35.3 ± 1.6*	31.9 ± 1.1	0.02	0.12	0.45
Large HDL particles (μmol/l)	4.67 ± 0.81	8.25 ± 0.40	6.62 ± 1.27	7.66 ± 0.75	0.42	0.01	0.14
Total LDL particles (nmol/l)	966 ± 79	727 ± 63	1178 ± 74*	$1,013 \pm 70$*	0.001	0.008	0.65
Small LDL particles (nmol/l)	582 ± 112	191 ± 48	487 ± 78	363 ± 79	0.64	0.004	0.11
Glucose and hormones							
Glucose (mg/dl)	84.8 ± 1.8	83.2 ± 1.2	89.5 ± 2.0*	92.0 ± 1.7*	<0.01	0.78	0.24
Glucagon (pg/ml)	151 ± 14	142 ± 8	138 ± 6	121 ± 10	0.11	0.23	0.68
Insulin (μU/ml)	6.0 ± 0.5	5.0 ± 0.4	5.1 ± 0.5	5.1 ± 2.0	0.70	0.61	0.60

Data are means $\pm$ SEM unless otherwise indicated. *Unpaired t test indicated values significantly ($P < 0.05$) different from young subjects within the same physical activity group; †in the event of a significant interaction term, Tukey's procedure indicated values significantly ($P < 0.05$) different from untrained subjects within the same age-group. LTA, leisure-time activity questionnaire; TG, triglycerides.

Mitochondrial ATP production capacity. MAPR was reduced in older versus younger people using substrates glutamate plus malate, succinate plus rotenone, and palmotoyl-L-carnitine plus malate but was higher in endurance-trained compared with sedentary subjects ($P_{age} \leq 0.004$) (Fig. 2). Furthermore, paired comparisons revealed age-related declines in MAPR in sedentary ($P \leq 0.006$) but not in trained subjects. Similar results were found for CS activity (Fig. 3A).

Mitochondrial DNA abundance. The abundance of mtDNA based on both the NADH dehydrogenase 1 and 4 gene probes was lower in older compared with young subjects ($P_{age} \leq 0.0004$, Fig. 3B and C). Although mtDNA abundance was significantly higher in trained compared with sedentary subjects ($P_{training} < 0.05$), mtDNA remained significantly lower in OT compared with YT subjects.

Protein expression of mitochondrial biogenesis genes. Protein expression of PGC-1α, NRF-1, and TFAM were similar in YS and OS subjects and higher in trained than in sedentary ($P_{training} < 0.001$, Fig. 4) subjects. PGC-1α and TFAM protein content was significantly lower in OY than in YT subjects ($P < 0.0001$); however, NRF-1 expression was similar in YT and OT subjects.

Proteomic data. Figure 5A–D depicts the relative abundance of several proteins involved in key metabolic processes in skeletal muscle. Comparison between OS and YS subjects revealed 27 oxidative and glycolytic proteins with significantly lower relative concentrations in older adults (Fig. 5A). Endurance-trained young and older individuals exhibited higher expression of numerous proteins involved in oxidative ATP production relative to their sedentary counterparts (Fig. 5B and C). A comparison of YT and OT subjects revealed that, with the exception of three

subunits of cytochrome c oxidase and an aminotransferase enzyme, the age differences in protein abundance that were observed in sedentary individuals were no longer apparent in endurance-trained individuals (Fig. 5D).

SIRT3 expression. Protein expression of SIRT3 was lower with age in sedentary adults and significantly higher in trained than in sedentary subjects, with no effect of age in trained adults (Fig. 6).
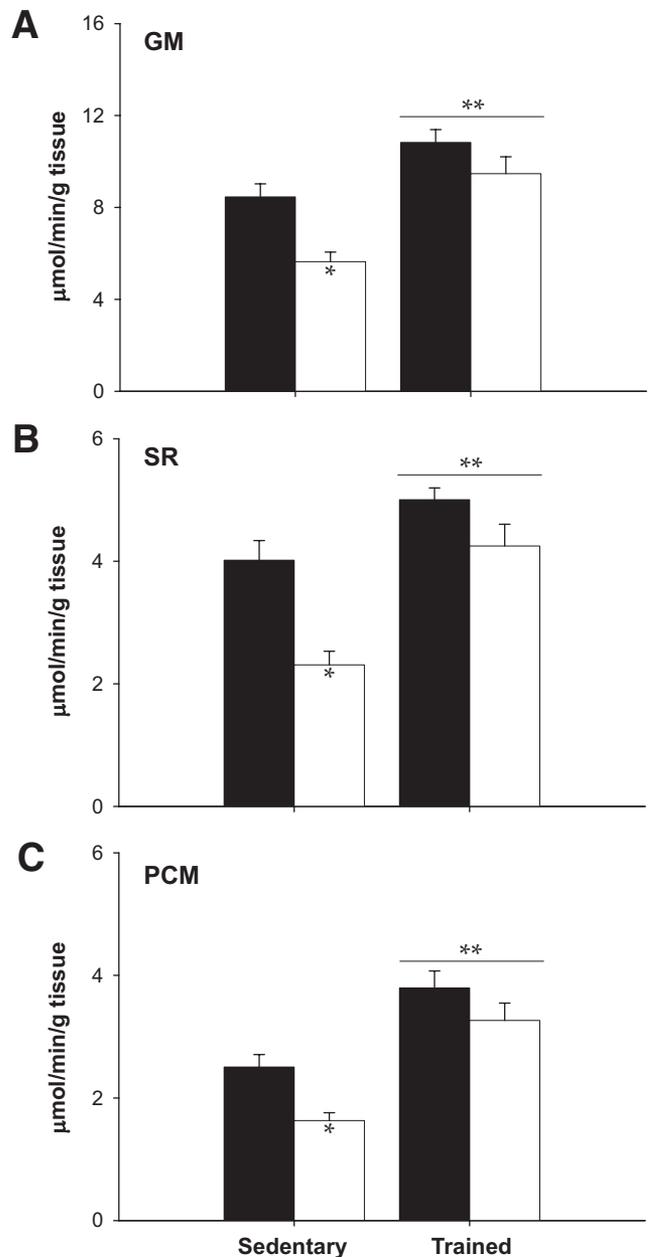
DISCUSSION

The current study examined the effects of aging and endurance exercise on insulin sensitivity and mitochondrial function to further explore whether age-related mitochondrial dysfunction and lowering of insulin sensitivity are inevitable consequences of chronological age. The main findings of the present study are, first, that YT and OT subjects had substantially higher insulin sensitivity than sedentary subjects and that no differences were observed between young and older subjects in either sedentary or exercise-trained groups. Second, in contrast, we found age-related declines in various markers of mitochondrial function in sedentary groups, but these age-related differences were partly (but not completely) abolished in people who practice regular endurance exercise. Finally, we show that endurance exercise may exert similar potentially lifespan-enhancing effects to those of caloric restriction through elevated SIRT3 expression in both young and older adults.

In agreement with previous reports (4,5), the capacity for mitochondrial ATP production declined with age in sedentary adults. As expected, MAPR and CS activity were higher in endurance-trained than in sedentary individuals.
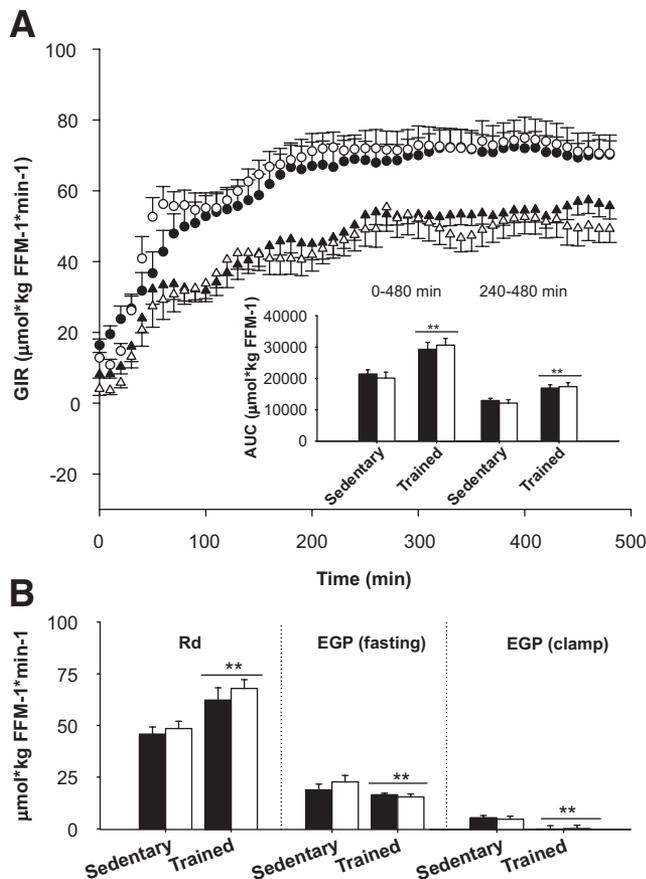
FIG. 1. Hyperinsulinemic-euglycemic clamp results. The rates of glucose infusion (GIR) required to maintain euglycemia during the 8-h clamp were higher in trained compared with sedentary subjects, with no effects of age (*A*). Insets show the area under the curve (AUC) during minutes 240–480, corresponding to a plateau in GIR, and during the entire clamp (0–480). R_d during hyperinsulinemia was higher in trained than sedentary subjects (*B*). No effects of age on R_d were observed. EGP was suppressed during the clamp in all groups (YS, 69 ± 7%; YT, 100 ± 11%; OS, 78 ± 7%; and OT, 97 ± 10%). The extent of suppression did not differ by age, but was greater in trained compared with sedentary individuals ($P = 0.01$). EGP was not significantly different from zero in trained individuals. Data are presented as mean ± SEM. **Significant ($P < 0.05$) differences in relative suppression. ●, YT; ○, OT; ▲, YS; △, OS; ■, young; □, older.

FIG. 2. Mitochondrial ATP production rates. An age-related decline in mitochondrial ATP production was observed using substrates glutamate plus malate (GM) (*A*), succinate plus rotenone (SR) (*B*), and palmitoyl-L-carnitine plus malate (PCM) (*C*) in sedentary subjects. These effects of age were not apparent in endurance-trained subjects. Data are presented as means ± SEM. *Pairwise comparisons revealed significant ($P < 0.05$) effects of age within activity groups; **significant ($P < 0.05$) main effects of training. ■, young; □, older.

Although direct comparisons revealed ~10–15% lower CS activity and MAPR in OT compared with YT subjects, these differences were not statistically significant. Thus, our data underscore the effectiveness of regular endurance exercise in largely preventing age-related declines in mitochondrial oxidative capacity. Similarly, histochemical and enzymatic measurements in young (27 ± 3 years old) and older (63 ± 6 years old) endurance runners showed that despite 11% lower V_{O_2} peak compared with young runners, master's level runners exhibited no signs of decreased mitochondrial enzyme activities or capillary density (28). In vitro measures of oxidative capacity, such as those employed in the present study, provide more distinct information regarding mitochondrial function than in vivo measurements of resting mitochondrial ATP production, such as those measured using magnetic resonance spectroscopy (4). Given that skeletal muscle is relatively metabolically inert in the postabsorptive resting state and that endurance training increases V_{O_2} peak with minimal effects on resting V_{O_2}, it is our belief that the maximal capacity for mitochondrial ATP synthesis is more
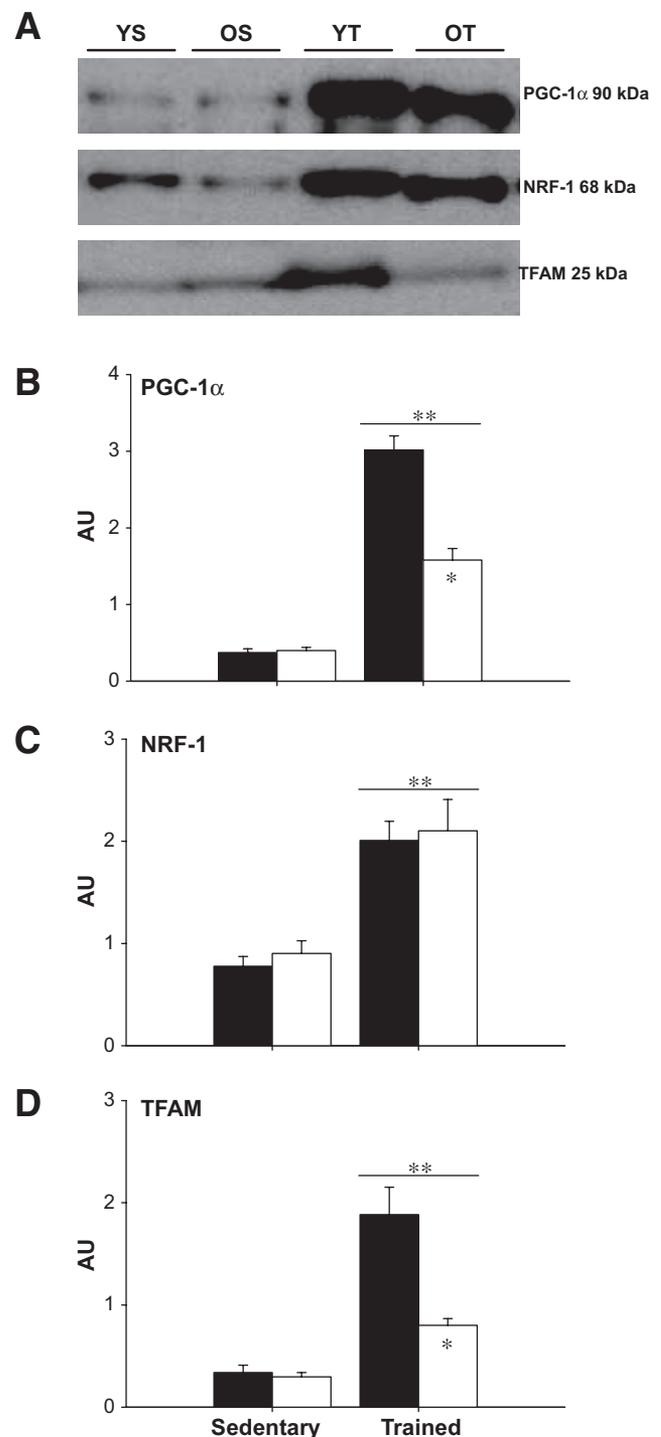
relevant to the study of endurance exercise training and aging.

Several additional mitochondrial markers were also assessed in an effort to determine molecular and cellular mechanisms that would explain our MAPR findings. Using mass spectrometry methods, we demonstrated that numerous proteins involved in the citric acid cycle (isocitrate dehydrogenase, aspartate aminotransferase, malate dehydrogenase, and oxoglutarate dehydrogenase) and electron transport (cytochrome c oxidase, ubiquinol-cytochrome c reductase, NADH dehydrogenase, and ATP synthase) were expressed at lower levels in OS compared with YS subjects. Regardless of age, endurance-trained individuals
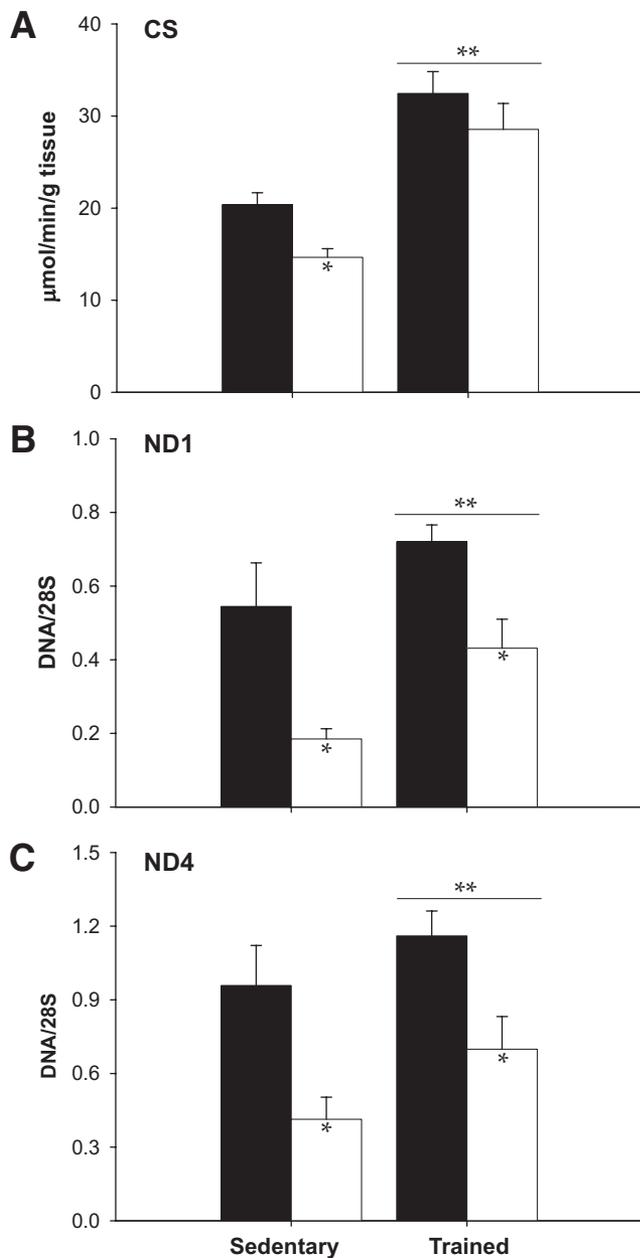
FIG. 3. CS activity and mitochondrial DNA abundance. An age-related decline in CS activity was observed in sedentary but not in endurance-trained subjects (*A*). Mitochondrial DNA copy number for NADH dehydrogenase subunits 1 (ND1) (*B*) and 4 (ND4) (*C*) were lower with age in sedentary and trained subjects. Training was associated with higher mitochondrial DNA abundance in both age-groups, but the effects of age remained apparent. Data are presented as means ± SEM. *Pairwise comparisons revealed significant ($P < 0.05$) effects of age within activity groups; **significant ($P < 0.05$) main effects of training. ■, young; □, older.

FIG. 4. Expression of proteins involved in mitochondrial biogenesis. Protein expression of PGC-1α (*B*), NRF-1 (*C*), and TFAM (*D*) were similar in YS and OS subjects and higher in trained subjects. Representative blots of each protein are showed in panel *A*. Data are presented as means ± SEM. *Pairwise comparisons revealed significant ($P < 0.05$) effects of age within activity groups; **significant ($P < 0.05$) main effects of training. ■, young; □, older.

exhibited higher expression of these proteins. Furthermore, the age-related differences in protein expression evident in sedentary individuals were absent in endurance-trained individuals, with the exception of three subunits of cytochrome c oxidase and aspartate aminotransferase, which remained significantly lower in OT compared with YT subjects. Our proteomic data closely parallel our MAPR data, suggesting that the expression level of key mitochondrial proteins may be a primary determinant of age-related declines in oxidative capacity and of the beneficial effects of regular endurance exercise. An important point is that regular endurance exercise could not

entirely eliminate the effects of aging on protein expression, consistent with the ~15% lower ATP production capacity in OT versus YT subjects.

The abundance of mtDNA plays a role in the expression level of several proteins that were measured using mass spectrometry: specifically, subunits of cytochrome c oxidase and NADH dehydrogenase. Consistent with previous reports (5,8), we found that mtDNA abundance was significantly
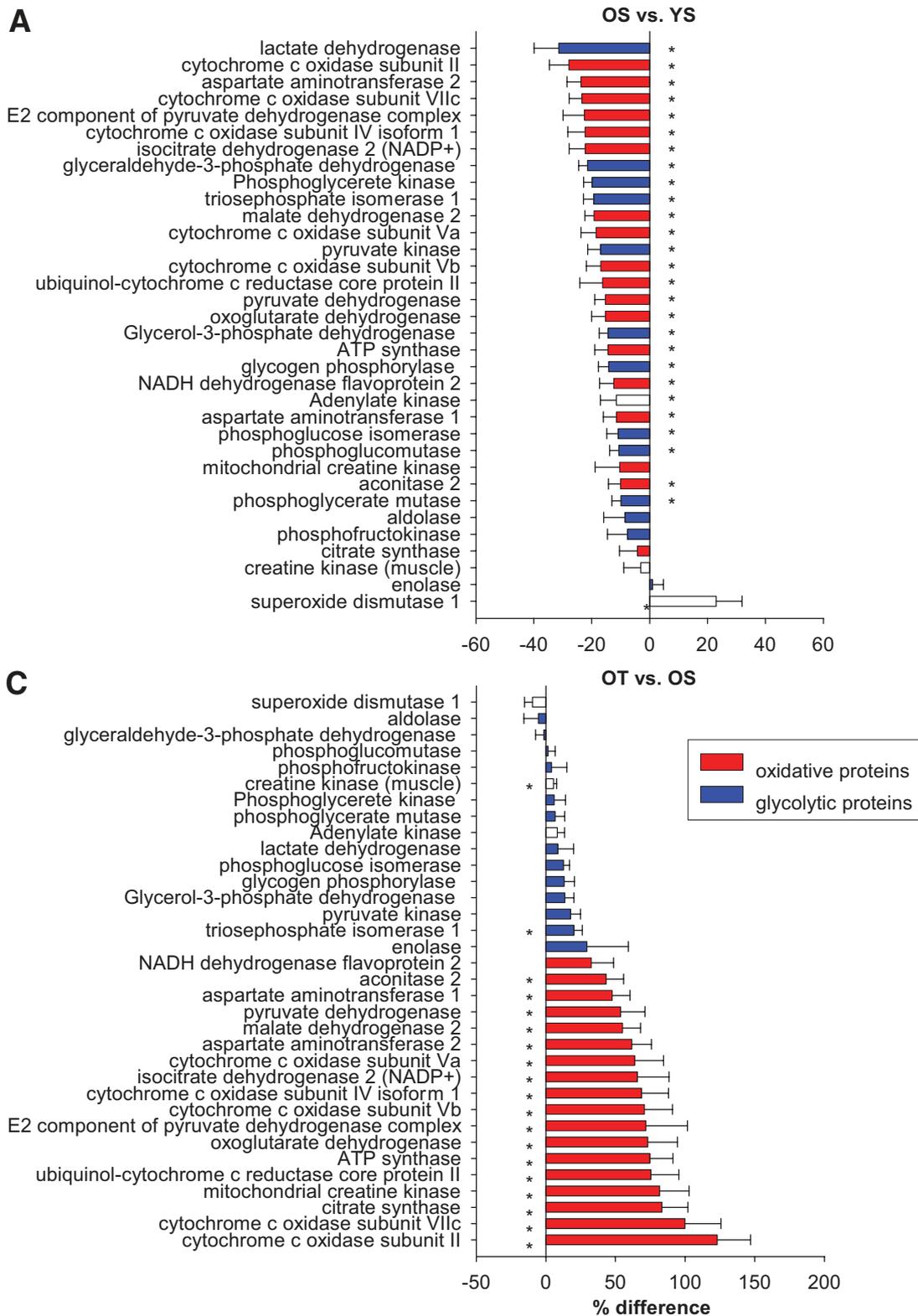
FIG. 5. **Relative abundance of proteins involved in oxidative and glycolytic ATP production in skeletal muscle.** Horizontal bars indicate the percent difference in relative abundance of proteins involved in oxidative (red) and glycolytic (blue) energy metabolism. *A:* Numerous proteins involved in oxidative and glycolytic ATP synthesis are reduced in OS compared with YS individuals. *B* and *C* indicate that endurance training is associated with elevated expression of proteins involved in oxidative ATP synthesis in young (*B*) and older (*C*) subjects. *D:* Long-term endurance exercise normalized the effects of age on the expression of all but four mitochondrial proteins. Data are presented as means ± SEM. *Significant (*P* < 0.05) differences in relative expression.

reduced with age in sedentary individuals and that endurance-trained individuals in both age groups exhibited higher mtDNA abundance. Of interest, the regular endurance exercise did not completely normalize age-related declines in

mtDNA abundance, which we are tempted to posit as a mechanism explaining persisting age-related deficits in the protein expression of mitochondrial-encoded cytochrome c oxidase subunits in endurance-trained individuals.

B

YT vs. YS

D

YT vs. OT

% difference
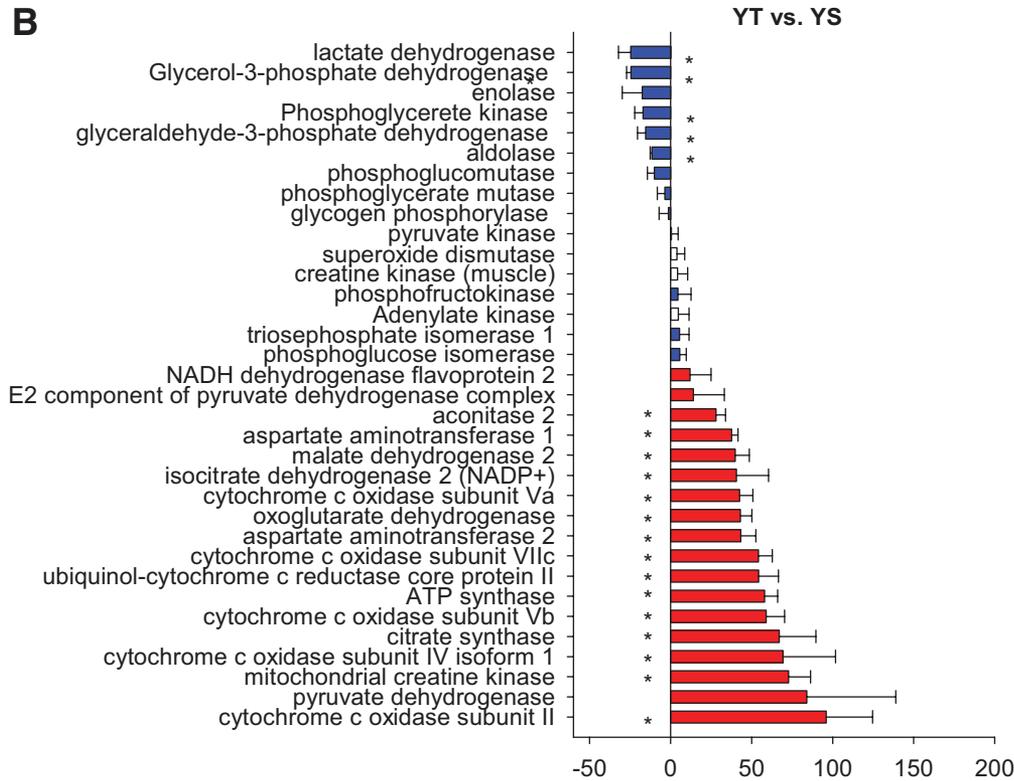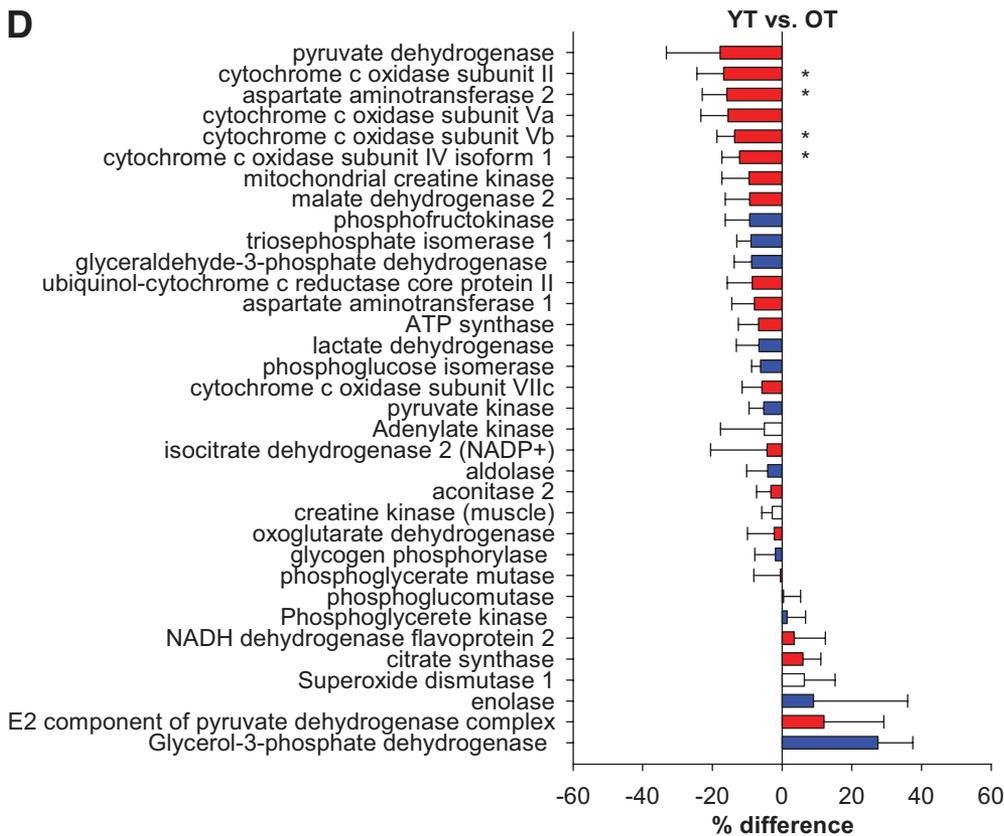
The replication, maintenance, and transcription of mitochondrial DNA are controlled by TFAM. TFAM expression is controlled by upstream nuclear transcription factors (NRF1, NRF2), which also regulate the expression of nuclear genes encoding mitochondrial proteins. PGC-1α plays a key role in activating these downstream transcription factors and, therefore, acts as a control point regulat-

ing mitochondrial biogenesis (29). The protein expression levels of PGC-1α, NRF1, and TFAM were similar in YS and OS subjects and cannot explain our observations of decreased mtDNA abundance in OS subjects. Age-related accumulation of DNA oxidative damage (5) could explain reduced mtDNA in OS subjects in spite of similar expression of mitochondrial transcription factors. PGC-1α,
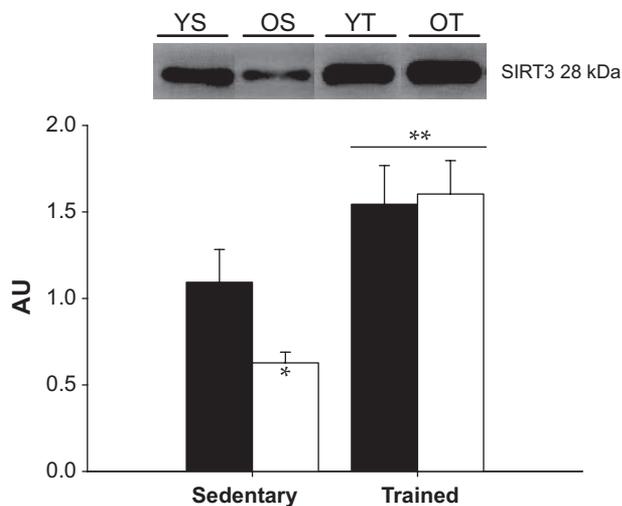
FIG. 6. Protein expression of SIRT3. Protein expression of SIRT3 was lower with age in sedentary adults, with no effect of age in trained adults. Data are presented as means ± SEM. *Pairwise comparisons revealed significant ($P < 0.05$) effects of age within activity groups; **significant ($P < 0.05$) main effects of training. ■, young; □, older.

NRF1, and TFAM were all expressed at higher levels in endurance-trained individuals; however, a key finding is that significant effects of age were evident for PGC-1α and TFAM, but not NRF1. Thus, the persisting effects of age on mtDNA abundance despite endurance exercise training may be explained by blunted expression of upstream transcription factors involved in mitochondrial biogenesis.

A key finding of the current study is related to the effects of aging and endurance exercise on insulin sensitivity. Although decreased insulin sensitivity is reported to occur with age, we found that both hepatic and peripheral insulin sensitivity were similar in YS and OS subjects. While the absence of age-related declines in insulin sensitivity is in contrast with numerous previous reports (4,21,30,31), our results are consistent with a growing body of literature indicating that adiposity and physical activity levels, rather than chronological age, are primary determinants of age-related declines in insulin sensitivity (32–36). In support of this notion, we find that fat mass, abdominal adiposity, and BMI were significant predictors of R_d during the clamp. The OS subjects in our cohort were healthy, relatively lean people with substantially less visceral and abdominal adiposity than what has been reported in older adults with decreased insulin sensitivity (21). Our findings are in keeping with earlier studies that also revealed similarities in insulin sensitivity in relatively lean sedentary young and older adults (34,37). Peripheral and hepatic insulin sensitivity were significantly higher in endurance-trained than in sedentary individuals, and these effects were independent of age. These data are supported by previous investigators who have demonstrated that endurance exercise confers substantial improvements in peripheral and hepatic insulin sensitivity, regardless of age (14,38,39). The results from the current study strongly support the notion that decreased insulin sensitivity may not be an inevitable consequence of aging and that age-related changes in body composition are a key factor contributing to the previously reported decline in insulin sensitivity with aging. Furthermore, in addition to maintenance of normal body weight, endurance exercise should be considered a viable, effective intervention to reverse, delay, or prevent the onset of age-related

declines in insulin sensitivity that is likely to occur at more advanced age.

Mitochondrial dysfunction and decreased sensitivity to insulin are often observed concurrently in type 2 diabetic (9), elderly (4,5), and obese individuals (10), prompting two opposing hypotheses concerning this relationship. It has been proposed that mitochondrial dysfunction plays an etiological role in the development of insulin resistance (11,40), while some evidence supports an alternative hypothesis that mitochondrial dysfunction may result from insulin resistance (41,42). Other studies find a dissociation between mitochondrial capacity and insulin sensitivity (21,43,44). In the present study, OS subjects were as insulin sensitive as YS subjects despite significantly reduced MAPR, casting further doubt on the causal role of mitochondrial dysfunction in decreasing insulin sensitivity. However, we observed significant elevations in both oxidative capacity and insulin sensitivity in endurance-trained individuals regardless of age. These data underscore the fact that, although mitochondrial function has been extensively studied in insulin-resistant populations, our understanding of its role in the development of insulin resistance is far from complete.

We assessed other outcomes relevant to age-related comorbidities that may impact insulin sensitivity and mitochondrial function. Percent body fat, abdominal fat, and visceral fat were elevated in older adults, and although these measures were lower in endurance-trained individuals, the effects of age on body composition were not completely normalized in endurance-trained adults. Similarly, the age-related decline in muscle mass observed in sedentary individuals was also apparent in endurance-trained individuals, suggesting that endurance exercise can partially prevent age-related changes in adiposity but has limited utility in preventing sarcopenia, which is prevented by resistance training. Both the traditional photometric-based clinical lipid panel and NMR-based method of measuring lipoprotein particle concentrations revealed the expected elevations of total and LDL cholesterol in OS compared with YS subjects, as well as the beneficial increase in HDL cholesterol in both age-groups. However, while exercise was not related to total LDL cholesterol when measured photometrically, both total and small LDL particle concentrations measured by NMR were significantly lower in exercising individuals.

We also provide data to link endurance exercise with the lifespan-enhancing effects of caloric restriction (45). Mitochondrial-localized SIRT3 is linked to longevity, possibly by interfering with the release of apoptosis-inducing factor (19,46). In a manner similar to that by which nutrient restriction increases lifespan (19), we found that SIRT3 expression was higher in endurance-trained than in sedentary individuals. Furthermore, SIRT3 expression was lower with age in OS but not OT individuals. Thus, our results support a hypothesis that exercise may confer lifespan-extending effects similar to those of caloric restriction through the actions of mitochondrial SIRT3.

It is important to consider that many detrimental effects of old age are compounded after 80 years of age. It is unclear if similar effects of regular endurance exercise would be evident in individuals at more advanced ages than were studied in the present study. It is theoretically possible that the cross-sectional design of the study may introduce a sampling bias wherein individuals who exercise across their lifespan do so because their mitochondrial function is inherently higher than that of their more

sedentary peers. Notwithstanding, it is becoming increasingly apparent that waning physical activity levels are a primary determinant of age-related mitochondrial dysfunction and may accelerate the aging process through adverse effects on many biochemical and molecular factors that require ATP.

In conclusion, we find that age-related declines in oxidative capacity can be largely ameliorated by regular endurance exercise, highlighting the fact that physical inactivity plays an important role in age-related dysfunctions and underscoring the need for better control of this variable in the literature. However, certain mitochondrial markers, specifically mtDNA, transcription factors, and mitochondrial-encoded proteins, remain depressed with increased age despite endurance exercise. Neither hepatic nor peripheral insulin sensitivity was impaired with age in these healthy, relatively lean individuals, which was in keeping with the notion that adiposity is a primary determinant of age-related reductions in insulin sensitivity. Notwithstanding, we illustrate that compared with sedentary individuals, endurance-trained individuals exhibit elevated insulin sensitivity in a manner independent of age. Finally, we observed an age-related decline in muscle expression of SIRT3 in sedentary but not endurance-trained individuals, suggesting that endurance exercise may exert similar potentially lifespan-enhancing effects to those demonstrated with caloric restriction in other organisms.

REFERENCES

1. Reaven GM: Banting Lecture 1988. Role of insulin resistance in human disease. *Diabetes* 37:1595–1607, 1988
2. Grundy SM, Brewer HB Jr, Cleeman JI, Smith SC Jr, Lenfant C: Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Arterioscler Thromb Vasc Biol* 24:e13–e18, 2004
3. Ford ES, Giles WH, Dietz WH: Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. *J Am Med Assoc* 287:356–359, 2002
4. Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, DiPietro L, Cline GW, Shulman GI: Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science* 300:1140–1142, 2003
5. Short KR, Bigelow ML, Kahl J, Singh R, Coenen-Schimke J, Raghavakaimal S, Nair KS: Decline in skeletal muscle mitochondrial function with aging in humans. *Proc Natl Acad Sci* 102:5618–5623, 2005
6. Coggan AR, Spina RJ, King DS, Rogers MA, Brown M, Nemeth PM, Holloszy JO: Histochemical and enzymatic comparison of the gastrocnemius muscle of young and elderly men and women. *J Gerontol* 47:B71–B76, 1992
7. Rooyackers OE, Adey DB, Ades PA, Nair KS: Effect of age on in vivo rates of mitochondrial protein synthesis in human skeletal muscle. *Proc Natl Acad Sci* 93:15364–15369, 1996
8. Menshikova EV, Ritov VB, Fairfull L, Ferrell RE, Kelley DE, Goodpaster BH: Effects of exercise on mitochondrial content and function in aging human skeletal muscle. *J Gerontol A Biol Sci Med Sci* 61:534–540, 2006
9. Kelley DE, He J, Menshikova EV, Ritov VB: Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* 51:2944–2950, 2002
10. Simoneau JA, Kelley DE: Altered glycolytic and oxidative capacities of skeletal muscle contribute to insulin resistance in NIDDM. *J Appl Physiol* 83:166–171, 1997
11. Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI: Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med* 350:664–671, 2004
12. Short KR, Nair KS, Stump CS: Impaired mitochondrial activity and insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med* 350:2419–2421, 2004
13. Cox JH, Cortright RN, Dohm GL, Houmard JA: Effect of aging on response to exercise training in humans: skeletal muscle GLUT-4 and insulin sensitivity. *J Appl Physiol* 86:2019–2025, 1999
14. Seals DR, Hagberg JM, Hurley BF, Ehsani AA, Holloszy JO: Effects of endurance training on glucose tolerance and plasma lipid levels in older men and women. *J Am Med Assoc* 252:645–649, 1984
15. Holloszy JO: Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J Biol Chem* 242:2278–2282, 1967
16. Short KR, Vittone JL, Bigelow ML, Proctor DN, Nair KS: Age and aerobic exercise training effects on whole body and muscle protein metabolism. *Am J Physiol Endocrinol Metab* 286:E92–E101, 2004
17. Hughes VA, Fiatarone MA, Fielding RA, Kahn BB, Ferrara CM, Shepherd P, Fisher EC, Wolfe RR, Elahi D, Evans WJ: Exercise increases muscle GLUT-4 levels and insulin action in subjects with impaired glucose tolerance. *Am J Physiol Endocrinol Metab* 264:E855–E862, 1993
18. Lamming DW, Wood JG, Sinclair DA: Small molecules that regulate lifespan: evidence for xenohormesis. *Mol Microbiol* 53:1003–1009, 2004
19. Yang H, Yang T, Baur JA, Perez E, Matsui T, Carmona JJ, Lamming DW, Souza-Pinto NC, Bohr VA, Rosenzweig A, de Cabo R, Sauve AA, Sinclair DA: Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival. *Cell* 130:1095–1107, 2007
20. Otvos JD, Jeyarajah EJ, Bennett DW, Krauss RM: Development of a proton nuclear magnetic resonance spectroscopic method for determining plasma lipoprotein concentrations and subspecies distributions from a single, rapid measurement. *Clin Chem* 38:1632–1638, 1992
21. Short KR, Vittone JL, Bigelow ML, Proctor DN, Rizza RA, Coenen-Schimke JM, Nair KS: Impact of aerobic exercise training on age-related changes in insulin sensitivity and muscle oxidative capacity. *Diabetes* 52:1888–1896, 2003
22. Nair KS, Halliday D, Griggs RC: Leucine incorporation into mixed skeletal muscle protein in humans. *Am J Physiol Endocrinol Metab* 254:E208–E213, 1988
23. Basu A, Basu R, Shah P, Vella A, Johnson CM, Nair KS, Jensen MD, Schwenk WF, Rizza RA: Effects of type 2 diabetes on the ability of insulin and glucose to regulate splanchnic and muscle glucose metabolism: evidence for a defect in hepatic glucokinase activity. *Diabetes* 49:272–283, 2000
24. DeFronzo RA, Tobin JD, Andres R: Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol Endocrinol Metab* 237:E214–E223, 1979
25. Steele R, Wall JS, De Bodo RC, Altszuler N: Measurement of size and turnover rate of body glucose pool by the isotope dilution method. *Am J Physiol Endocrinol Metab* 187:15–24, 1956
26. Finegood DT, Bergman RN, Vranic M: Modeling error and apparent isotope discrimination confound estimation of endogenous glucose production during euglycemic glucose clamps. *Diabetes* 37:1025–1034, 1988
27. Wang XL, Fu A, Raghavakaimal S, Lee HC: Proteomic analysis of vascular endothelial cells in response to laminar shear stress. *Proteomics* 7:588–596, 2007
28. Coggan AR, Spina RJ, Rogers MA, King DS, Brown M, Nemeth PM, Holloszy JO: Histochemical and enzymatic characteristics of skeletal muscle in master athletes. *J Appl Physiol* 68:1896–1901, 1990
29. Finck BN, Kelly DP: PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J Clin Invest* 116:615–622, 2006
30. DeFronzo RA: Glucose intolerance and aging: evidence for tissue insensitivity to insulin. *Diabetes* 28:1095–1101, 1979
31. Houmard JA, Weidner MD, Dolan PL, Leggett-Frazier N, Gavigan KE, Hickey MS, Tyndall GL, Zheng D, Alshami A, Dohm GL: Skeletal muscle GLUT4 protein concentration and aging in humans. *Diabetes* 44:555–560, 1995
32. Kohrt WM, Kirwan JP, Staten MA, Bourey RE, King DS, Holloszy JO: Insulin resistance in aging is related to abdominal obesity. *Diabetes* 42:273–281, 1993
33. Szoke E, Shrayyef MZ, Messing S, Woerle HJ, van Haeften TW, Meyer C, Mitrakou A, Pimenta W, Gerich JE: Effect of aging on glucose homeostasis:

accelerated deterioration of beta-cell function in individuals with impaired glucose tolerance. *Diabetes Care* 31:539–543, 2008

34. Seals DR, Hagberg JM, Allen WK, Hurley BF, Dalsky GP, Ehsani AA, Holloszy JO: Glucose tolerance in young and older athletes and sedentary men. *J Appl Physiol* 56:1521–1525, 1984

35. O'Shaughnessy IM, Kasdorf GM, Hoffmann RG, Kalkhoff RK: Does aging intensify the insulin resistance of human obesity? *J Clin Endocrinol Metab* 74:1075–1081, 1992

36. Basu R, Breda E, Oberg AL, Powell CC, Dalla MC, Basu A, Vittone JL, Klee GG, Arora P, Jensen MD, Toffolo G, Cobelli C, Rizza RA: Mechanisms of the age-associated deterioration in glucose tolerance: contribution of alterations in insulin secretion, action, and clearance. *Diabetes* 52:1738–1748, 2003

37. Broughton DL, James OW, Alberti KG, Taylor R: Peripheral and hepatic insulin sensitivity in healthy elderly human subjects. *Eur J Clin Invest* 21:13–21, 1991

38. Kahn SE, Larson VG, Beard JC, Cain KC, Fellingham GW, Schwartz RS, Veith RC, Stratton JR, Cerqueira MD, Abrass IB: Effect of exercise on insulin action, glucose tolerance, and insulin secretion in aging. *Am J Physiol Endocrinol Metab* 258:E937–E943, 1990

39. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM: Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med* 346:393–403, 2002

40. Lowell BB, Shulman GI: Mitochondrial dysfunction and type 2 diabetes. *Science* 307:384–387, 2005

41. Stump CS, Short KR, Bigelow ML, Schimke JM, Nair KS: Effect of insulin on human skeletal muscle mitochondrial ATP production, protein synthesis, and mRNA transcripts. *Proc Natl Acad Sci* 100:7996–8001, 2003

42. Asmann YW, Stump CS, Short KR, Coenen-Schimke JM, Guo Z, Bigelow ML, Nair KS: Skeletal muscle mitochondrial functions, mitochondrial DNA copy numbers, and gene transcript profiles in type 2 diabetic and nondiabetic subjects at equal levels of low or high insulin and euglycemia. *Diabetes* 55:3309–3319, 2006

43. Toledo FG, Menshikova EV, Azuma K, Radikova Z, Kelley CA, Ritov VB, Kelley DE: Mitochondrial capacity in skeletal muscle is not stimulated by weight loss despite increases in insulin action and decreases in intramyocellular lipid content. *Diabetes* 57:987–994, 2008

44. Nair KS, Bigelow ML, Asmann YW, Chow LS, Coenen-Schimke JM, Klaus KA, Guo ZK, Sreekumar R, Irving BA: Asian Indians have enhanced skeletal muscle mitochondrial capacity to produce ATP in association with severe insulin resistance. *Diabetes* 57:1166–1175, 2008

45. Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA: Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 305:390–392, 2004

46. Haigis MC, Guarente LP: Mammalian sirtuins–emerging roles in physiology, aging, and calorie restriction. *Genes Dev* 20:2913–2921, 2006