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Ameliorating effects of lifelong physical activity on healthy aging and mitochondrial function in human white adipose tissue

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ABSTRACT

Growing old is patently among the most prominent risk factors for lifestyle related diseases and deterioration in physical performance. Aging in particular affects mitochondrial homeostasis and maintaining a well-functioning mitochondrial pool is imperative in order to avoid age-associated metabolic decline. White adipose tissue (WAT) is a key organ in energy balance and impaired mitochondrial function in adipocytes has been associated with increased low-grade inflammation, altered metabolism, excessive ROS production and an accelerated aging phenotype. Exercise training improves mitochondrial health but whether lifelong exercise training can sufficiently maintain WAT mitochondrial function is currently unknown. Therefore, to dissect the role and dose-dependence of lifelong exercise training on aging WAT metabolic parameters and mitochondrial function, young and older untrained, as well as moderately and highly exercise trained older male subjects were recruited and abdominal subcutaneous (s)WAT biopsies and venous blood samples were obtained to measure mitochondrial function and key metabolic factors in WAT and plasma. Mitochondrial intrinsic respiratory capacity was lower in sWAT from older than in young subjects. In spite of this, maximal mitochondrial respiration per wet weight, markers of oxidative capacity, and mitophagic capacity were increased in sWAT from lifelong highly exercise trained than all other groups. Furthermore, ROS emission was generally lower in sWAT from lifelong highly exercise trained than older untrained subjects. Taken together, aging reduces intrinsic mitochondrial respiration in human sWAT, but lifelong high volume exercise training increases oxidative capacity by increasing mitochondrial volume likely contributing to healthy aging.

KEY WORDS: ADIPOSE TISSUE, EXERCISE TRAINING, MITOCHONDRIA, ROS, METABOLISM
INTRODUCTION

Aging inevitably brings about a gradual deterioration of both physiological and metabolic performance. While the exact causal mechanisms remain elusive, there is mounting evidence for a sedentary lifestyle being a catalyst for an age-associated decline in overall health parameters. In skeletal muscle, atrophy is a hallmark of old age likely mediated by increased activation of pro-inflammatory and apoptotic pathways leading to increased production of reactive oxygen species [1;2] further exacerbated by increased deposition of lipids in white adipose tissue (WAT) [3]. An associated decline in WAT function may well be a largely overlooked but important contributor to derangement of health factors such as insulin resistance [4], chronic low grade inflammation [5;6], loss of metabolic flexibility and reduced metabolic capacity [7]. With a rapidly increasing older population where people in general are less and less physically active, the importance of elucidating the role of WAT in healthy aging through regular physical activity is warranted more than ever.

WAT has traditionally been regarded solely as a regulator of energy homeostasis through basic storing and release of lipids in correspondence to supply and demand. However, research in recent years has highlighted the need to redefine and extend the physiological role of adipose tissue as a true endocrine organ able to modulate metabolic function of other organs through release of adipokines [8;9]. Several studies have suggested that mitochondrial function plays a central role in adipose tissue biology and metabolism [10;11], and although mitochondrial content in WAT is much lower than in skeletal muscle, essential adipocyte processes still rely heavily on mitochondrial bioenergetic output [11;12]. Moreover, the observation that oxygen consumption in adipose tissue was lower in older men and women than in young individuals supports that aging modifies WAT function [13]. Recent studies have indicated that mitochondria in adipocytes might play a pivotal role in proper regulation of whole-body energy homeostasis, insulin sensitivity and substrate metabolism [14;15].
However, human studies addressing the impact of aging on WAT mitochondrial function are limited.

Impairment in the mitochondrial machinery is thought to lead to increased production of mitochondrially derived reactive oxygen species (ROS) [16] and excessive ROS production in parallel with diminished antioxidant capacity is generally thought to be one of the main drivers of cumulative oxidative damage in the free radical theory of cellular aging [17]. Hence, aging mitochondria likely become a chronic source of elevated ROS, increasing oxidative damage, which ultimately can trigger impairment of adipocyte function and a pathological phenotype. Excessive ROS production may affect mitochondrial oxidative capacity over time by impairing both OXPHOS respiration and efficient exchange of cytosolic ADP and mitochondrially derived ATP. This exchange is mediated by the Adenine Nucleotide Translocase (ANT) and the Voltage Dependent Anion Channel (VDAC), across the inner and outer mitochondrial membrane, respectively. ANT2 and ANT3 have previously been reported to be the predominant isoforms in human adipose tissue based on mRNA abundance [18], but no previous studies have reported measurement of ANT mRNA or protein in white adipose tissue in humans with aging and exercise training. Previous studies have reported that the exchange capacity of ANT is reduced with old age in rodent heart and skeletal muscle mitochondria [19;20], but whether a similar effect is present in human WAT with old age remains to be established. A measure of mitochondrial function by assessing absolute respiration over a range of physiologically relevant ADP concentrations, termed ADP sensitivity, can yield valuable information on mitochondrial ADP affinity and nucleotide transport capacity. Exercise training has been reported to improve ADP sensitivity in aging rodent skeletal muscle [21]. However, the effect of aging and lifelong exercise training on mitochondrial respiration in human WAT is unresolved.
Mitochondrial network formation is a dynamic process facilitated by fission and fusion proteins. The fusion protein machinery comprises mitofusin (MFN) 1 and 2 and optic atrophy (OPA) 1 [22] and mitochondrial membranes undergo fusion processes for optimum membrane potential distribution. Furthermore, autophagic maintenance of the mitochondrial pool, termed mitophagy, assures targeting of damaged mitochondria for mitophagic degradation [23;24]. Together, these processes are needed to maintain a healthy mitochondrial population, but the impact of aging and lifelong exercise training on key factors in mitochondrial fusion, fission and mitophagy in human WAT remains to be elucidated.

An effective mean to ameliorate decline in oxidative capacity and mitochondrial structure, function and biogenesis in skeletal muscle is regular physical exercise. Recent findings have shown a positive effect on insulin sensitivity and increase in OXPHOS content in human subcutaneous WAT (sWAT) with short-term exercise in young adults [25] but how exercise training influences adipose tissue health as well as mitochondrial content and quality with old age has currently not been elucidated.

The question remains whether an age-related loss of mitochondrial capacity leads to elevated ROS production in human sWAT and whether exercise training can ameliorate a proposed age-associated decline in WAT mitochondrial function, and avert an increasing risk of developing chronic metabolic disorders. Therefore, the objective of the present study was to investigate effects of aging and lifelong physical activity on mitochondrial function and capacity in aging human sWAT.
MATERIALS AND METHODS

Subjects

Young untrained male subjects (20-32 years of age; n=10) and healthy, older male subjects (62-73 years of age) either lifelong untrained (n=10) or lifelong exercise trained (n=12) were initially recruited for the present study. The study was approved by the Ethics Committee of Copenhagen (H-18051845) and was conducted in accordance with the guidelines of The Declaration of Helsinki. All subjects were given oral and written information and provided written informed consent prior to the study.

Inclusion criteria

Based on self-report questionnaires both young and older untrained subjects were defined as leisurely physically active without having participated in regular endurance exercise training. Lifelong exercise trained subjects were defined by having practiced minimum two hours of endurance exercise training per week from late adolescence and on (as per self-report). Moreover, Body Mass Index (BMI) had to be below 30, and the subjects were recruited based on being unmedicated and otherwise healthy.

All subjects underwent a medical examination with recording of electrocardiography (ECG) and blood sample analyses including hemoglobin concentration (HbA1c), LDL cholesterol, HDL cholesterol, total cholesterol, triglyceride concentration, and C-reactive protein (CRP). Results were evaluated and approved, by a medical doctor, to be within normal reference ranges.

It was decided to divide the exercise trained subjects into older moderately exercise trained (n=6) and older highly exercise trained (n=6) defined by an exercise endurance test (see protocol) and skeletal muscle citrate synthase (CS) activity as a measure of muscle oxidative capacity [26]. Similarly, skeletal muscle CS activity was also used as an additional exclusion.
criterion in young untrained and older untrained resulting in exclusion of one young and one older untrained, because the CS activity of both subjects was higher than mean+2SD for their allocated group. This resulted in n=9 in both of these groups.

Experimental protocol

The subjects participated on two separate days at least three days apart. On the first day, the subjects arrived at the laboratory in the morning. Body composition was measured by a full dual-energy X-ray absorptiometry (DXA) scanning (Prodigy, GE Healthcare, Chalfont St. Giles, UK). Exercise performance was determined by an incremental exercise test using a bicycle ergometer (Oxycon Pro, VIASYS Healthcare, Hoechberg, Germany). The subjects started out with 5 minutes of warm-up at 120 W followed by a graded increase in load of 20 W every two minute until the subjects rated the intensity as 16 on the Borg Rating of Perceived Exertion scale [27]. From this load, the subjects continued cycling until exhaustion. Duration and total energy expenditure of each subject were recorded and calculated and used as in vivo measures of exercise endurance capacity.

On the second day, the subjects arrived in the morning after an overnight fast. Arm venous blood samples were obtained and either immediately placed in EDTA tubes or drawn into a heparin containing syringe (PICO50, Radiometer, Denmark). The blood samples were placed on ice and the EDTA tubes were centrifuged at 2600 g for 15 minutes to obtain plasma, which was stored at -80 °C. Fasting blood glucose was determined in the heparin treated blood using an ABL 700 (Radiometer, Denmark).

Biopsies were obtained from abdominal subcutaneous adipose tissue in the umbilical region and muscle biopsies from vastus lateralis using the Bergström biopsy needle and suction, under local anesthesia (Lidocaine, AstraZeneca, Sweden). The muscle biopsies were
allocated to another study (Ringholm et al., in preparation) and in the present study only used to measure muscle CS activity. A portion of the adipose tissue was immediately placed in Biopsy Preservation Solution (BIOPS) buffer (50 mM MES, 7.23 mM Ca-EGTA, 2.77 mM CaK2EGTA, 20 mM imidazole, 20 mM taurine, 5.7 mM ATP, 14.3 mM phosphocreatine, and 6.56 mM MgCl₂, pH 7.1) for respirometry analyses, while the remaining adipose tissue was rinsed and quickly frozen in liquid nitrogen and stored at -80 °C until further analyses.

**Plasma measurements**

Plasma non-esterified fatty acids (NEFA) were determined using a HR series NEFA-HR(2)-kit (WAKO Chemicals, Neuss, Germany) according to manufacturer’s protocols. Plasma adiponectin, TNFα, and insulin were determined using the Human Adiponectin ELISA kit (#RAB0005, Sigma-Aldrich, Merck), Human TNFα ELISA kit (#RAB0476, Sigma-Aldrich, Merck), and Human Insulin ELISA kit (#RAB0327, Sigma-Aldrich, Merck), respectively, according to manufacturer’s protocols.

**DNA isolation, RNA isolation and reverse transcription**

It is common to use mtDNA as mitochondrial normalization in adipose tissue [51;52] and the present study, mtDNA/nDNA content from the samples used for respirometry was determined in sWAT and used for normalization to mitochondrial content to obtain specific values of mitochondrial content from the sample that was run in respirometry. Total DNA was isolated from sWAT samples used in respirometry as previously described [28]. The DNA pellet was resuspended in 20 μL sterile filtered Millipore H₂O and the DNA samples were subsequently further diluted 12x to prevent inhibition in the PCR. The isolated DNA
was later used to determine the ratio between mitochondrial (mt)DNA and nuclear (n)DNA content by real-time PCR.

Total RNA was isolated from 40-60 mg sWAT by a modified guanidinium thiocyanate-phenol- chloroform extraction method as previously described [29], except that the tissue was homogenized for 2 min at 30 oscillations sec\(^{-1}\) in a tissue lyser (TissueLyserII, Qiagen, Valencia, CA, USA). The final RNA pellets were resuspended in DEPC-treated H\(_2\)O containing 0.1 mM EDTA and the RNA concentration as well as sample purity were determined by spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific, Rockford, IL). Reverse transcription was performed using Superscript II RNase H\(^{-}\) and Oligo-dT (Invitrogen, Carlsbad, CA, USA) as previously described [30].

**Real-time PCR**

Real-time PCR was performed to determine mRNA content of selected genes as previously described [31] using an ABI Prism 7900HT (Applied Biosystems, Waltham, MA, USA) see supplemental methods for details).

**SDS- PAGE and western blotting**

Lysate was generated from 50-80 mg sWAT by homogenization in ice-cold MG buffer as previously described [32] except that the tissue was homogenized for 3 minutes at 30 oscillations s\(^{-1}\) in a TissueLyser II (Qiagen, Valencia, CA, USA) (see supplemental methods for further details).
**Citrate synthase activity**

CS was measured on a subset of samples due to tissue shortage. Adipose tissue samples (10-20 mg) were homogenized for 3 minutes at 30 oscillations s\(^{-1}\) in a TissueLyser II (Qiagen, Valencia, CA, USA) in ice-cold buffer (10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES, 1% NP-40, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 20 μg/mL aprotinin, 10 μg/mL leupeptin, 2 mM Na\(_3\)VO\(_4\), 3 mM benzamidine). Maximal citrate synthase (CS) activity in sWAT was determined spectrophotometrically using a citrate synthase assay kit following the guidelines of the manufacturer (Sigma-Aldrich, Missouri, USA). CS activity was normalized to protein content measured in homogenate samples using the bicinchoninic acid method (Thermo Fischer Scientific, USA).

**Mitochondrial respirometry and H\(_2\)O\(_2\) emission**

High-resolution respirometry and fluorometric H\(_2\)O\(_2\) emission measurements were simultaneously performed on freshly excised sWAT tissue (see supplemental methods for details).

**Protein carbonyl content**

Protein carbonyl content of total protein in lysate samples was determined using a fluorometric assay kit according to the manufacturer's instruction (OxiSelect STA-307, Cell Biolabs, CA, USA). Shortly, adipose tissue lysates were mixed with fluorophore solution and incubated overnight. Proteins were then precipitated in trichloroacetic acid and washed in acetone. Protein pellets were solubilized in guanidine hydrochloride and protein carbonyl content was determined fluorometrically using freshly prepared fluorophore standards and expressed relative to total protein content.
Statistical analyses

Results are presented as means ± SE. The effects of age and lifelong exercise training were examined by performing a one-way analysis of variance (ANOVA) and, in case of an overall significant effect, (P<0.05), Student-Newman-Keuls post hoc test was applied to localize differences. t-tests were applied to localize between-group differences (denoted in the results section). In cases where more than one t-test was conducted on the same dependent variable in a data set (fig. 4L), a Bonferroni correction was applied. Furthermore, in data sets where no effect between older groups were detected, older groups were pooled into one group to increase power and a t-test was used to test for a difference between young and older subjects (denoted by brackets in figures). Shapiro-Wilk and Brown-Forsythe tests were applied to assess normal distribution and equal data variance, respectively. In case the ANOVA prerequisite for equal variance was not met for a given data set, the data were log10 transformed. Statistical analyses were performed using SigmaPlot 14.0 (Systat, San Jose, CA, USA) and figures were made using Graphpad Prism 9 (Graphpad Software, San Diego, CA, USA).

RESULTS

Anthropometrics

There was no difference in height, body weight, or body mass index (BMI) between young untrained, older untrained, older moderately exercise trained, and older highly exercise trained subjects (Table 1).

Total lean, fat, and gynoid fat mass were not different between young untrained, older untrained, and older moderately exercise trained subjects. However, there was a tendency for more (0.05≤P≤0.1) android fat mass in older untrained than in young untrained subjects. In
addition, there was a tendency for higher (0.05≤P≤0.1) total lean mass and lower 
(0.05≤P≤0.1) total fat mass and lower (P<0.05) android fat mass in older highly exercise 
trained than young untrained subjects. Furthermore, total lean mass were higher (P<0.05), 
while total fat and android fat mass was lower (P<0.05) in older highly exercise trained than 
in older untrained subjects. Moreover, android fat mass was lower (P<0.05) in older highly 
exercise trained than in older moderately exercise trained subjects (Table 1).

Time to exhaustion and total energy expenditure during the exercise endurance test were 
lower (P<0.05) in older untrained than in young untrained subjects. There was a tendency for 
longer (0.05≤P≤0.1) time until exhaustion and total energy expenditure in older moderately 
exercise trained than in older untrained. In addition, time to exhaustion and total energy 
expenditure were higher (P<0.05) in older highly exercise trained than in young untrained, 
older untrained and older moderately exercise trained subjects (Table 1).

CS activity in vastus lateralis was not different in young untrained, older untrained and older 
moderately exercise trained subjects. However, CS activity was higher (P<0.05) in older 
highly exercise trained than in young untrained, older untrained subjects, and older 
moderately exercise trained subjects (Table 1).

**Plasma parameters**

Plasma non-esterified fatty acids (NEFA) were higher (P<0.05) in older than in young subject 
whereas triglyceride levels were lower (P<0.05) in older highly exercise trained than in 
young untrained subjects (Table 2).

Plasma HDL cholesterol level was not different between young untrained and older untrained 
subject, but HDL cholesterol was higher (P<0.05) in older moderately exercise trained and 
older highly exercise trained than in young and older untrained. Moreover, HDL cholesterol 
was not different between older moderately exercise trained and older highly exercise trained
subjects (Etable 2). On the other hand, LDL cholesterol and total cholesterol levels were not
different between young untrained, older untrained, older moderately exercise trained and
older highly exercise trained subjects (Etable 2). The LDL/HDL ratio was lower (P<0.05) and
tended to be lower (0.05≤P≤0.1) in older highly exercise trained than in young untrained and
older untrained subjects, respectively (Etable 2).
Fasting plasma glucose concentration was higher (P<0.05) in older untrained than in young
untrained subjects, while there was no difference in plasma glucose concentrations between
the other groups. Moreover, there was no difference in Hemoglobin A1c (HbA1c) between
young untrained, older untrained, older moderately, and older highly exercise trained subjects
(Etable 2).
Fasting insulin concentration was lower (P<0.05) in older highly exercise trained exercise
trained than in young untrained, older untrained, older moderately exercise trained subjects
(Etable 2).

**Lipolytic markers**

ATGL, HSL, Perilipin protein content in sWAT was not different between young untrained,
older untrained, older moderately, and older highly exercise trained subjects (Etable 2).

**Inflammation and adiponectin**

TNFα mRNA content in sWAT was not different between young untrained, older untrained,
older moderately exercise trained and older highly exercise trained subjects (eFig. 1A).
CD68 mRNA content in sWAT was higher (P<0.05) in older than in young untrained
subjects, when older subjects were pooled (see statistics section) (eFig. 1B). CD11c mRNA
and CD206 mRNA content in sWAT was not different between young untrained, older
untrained, older moderately exercise trained and older highly exercise trained subjects (Table 2).

Plasma C-reactive protein concentration was not different between young untrained, older untrained, older moderately exercise trained and older highly exercise trained subjects (eFig. 1C).

Adiponectin mRNA content in sWAT was not different between young untrained, older untrained, older moderately exercise trained and older highly exercise trained subjects (eFig. 1D).

Plasma adiponectin concentrations tended to be higher (0.05≤P≤0.1) in older untrained subjects than young untrained subjects. Furthermore, plasma adiponectin concentrations were higher (P<0.05) in older moderately exercise trained and older highly exercise trained than young untrained subjects. Moreover, plasma adiponectin concentration tended to be higher (0.05≤P≤0.1) in older moderately exercise trained and older highly exercise trained subjects, respectively, than in older untrained subjects, while there was no difference in plasma adiponectin concentration between older moderately exercise trained and older highly exercise trained (eFig. 1E).

**Mitochondrial ADP/ATP carriers**

ANT1 and ANT3 mRNA content in sWAT was not different between young untrained, older untrained, older moderately exercise trained and older highly exercise trained subjects (eFig. 2A and C). However, ANT2 mRNA content in sWAT was lower (P<0.05) in older untrained than in young untrained subjects (eFig. 2B).

VDAC protein content in sWAT was not different between young untrained, older untrained, older moderately exercise trained but tended to be higher (0.05≤P≤0.1) in older highly exercise trained than in young untrained subjects (eFig. 2D).
Autophagy, mitophagy and mitochondrial fusion

Parkin protein content tended to be higher (0.05≤P≤0.1) in older than in young untrained subjects, when older subjects were pooled (t-test) (see statistics section) (Fig. 1A).

Monomeric BNIP3 protein content in sWAT was not different between young untrained and older untrained subjects. However, monomeric BNIP3 protein content tended to be higher (0.05≤P≤0.1) in older moderately exercise trained and highly exercise trained than in young untrained and older untrained subjects (Fig. 1B).

OPA1 protein content in sWAT was not different between young untrained, older untrained, older moderately exercise trained and older highly exercise trained subjects (Fig. 1C).

MFN2 protein content in sWAT was not different between young untrained and older untrained subjects. However, MFN2 protein content was higher (P<0.05) in older moderately exercise trained than in young untrained and older untrained subjects. Furthermore, MFN2 protein content was lower (P<0.05) in older highly exercise trained than in older moderately exercise trained subjects (Fig. 1D).

Oxidative stress and antioxidant defense

SOD2 and catalase content in sWAT was not different between young untrained, older untrained, older moderately exercise trained and older highly exercise trained subjects (Fig. 2A and B).

Carbonyl content, and 4-HNE modified protein content in sWAT was not different between young untrained, older untrained, older moderately exercise trained and older highly exercise trained subjects (Fig. 2C and D).
Mitochondrial oxidative markers

GLUT4 protein content in sWAT was not different in young untrained, older untrained and older moderately exercise trained subjects. However, GLUT4 protein content was higher (P<0.05) in older highly exercise trained than in young untrained, older untrained and older moderately exercise trained subjects (Fig. 3A).

HKII protein content in sWAT tended to be lower (0.05≤P≤0.1) in older untrained than in young untrained subjects. Moreover, HKII protein content tended to be higher (0.05≤P≤0.1) and was higher (P<0.05) in older moderately exercise trained than in young untrained and older untrained, respectively. In addition, HKII protein content was higher (P<0.05) in older highly exercise trained than in older untrained subjects (Fig. 3B).

Total OXPHOS protein content in sWAT was not different between young untrained and older untrained subjects. However, total OXPHOS protein content in sWAT tended to be higher (0.05≤P≤0.1) in older moderately exercise trained and was higher (P<0.05) in older highly exercise trained than in young and older untrained subjects (Fig. 3C).

PDH-E1α protein content in sWAT was not different in young untrained, older untrained and older moderately exercise trained subjects. However, PDH-E1α protein content was higher (P<0.05) in older highly exercise trained than in young untrained, older untrained and older moderately exercise trained subjects (Fig. 3D).

Although CS activity was measured on only a subset of subjects the same overall relationship in CS and mtDNA between groups was evident. The CS activity in sWAT was not different in young untrained, older untrained and older moderately exercise trained subjects. However, CS activity was higher (P<0.05) in older highly exercise trained than in young untrained, older untrained subjects, and older moderately exercise trained subjects (Fig. 3E).
The mtDNA/nDNA ratio in sWAT was not different between young untrained, older untrained, older moderately exercise trained and older highly exercise trained subjects (Fig. 3F).

SIRT3 protein content in sWAT was not different in young untrained, older untrained and older moderately exercise trained subjects. However, SIRT3 protein content was higher (P<0.05) and tended to be higher (0.05≤P≤0.1) in older highly exercise trained than in young untrained and older untrained subjects, respectively (Fig. 3G).

**Mitochondrial respiratory capacity and H$_2$O$_2$ emission**

Complex I-linked LEAK respiration in sWAT normalized to wet weight was not different between young untrained, older untrained, older moderately exercise trained and older highly exercise trained subjects (Fig. 4A). Similarly, complex I-linked and complex I+II-linked OXPHOS respiration in sWAT normalized to wet weight was not different between young untrained, older untrained and older moderately exercise trained subjects (Fig. 4B and C). However, complex I-linked and complex I+II-linked OXPHOS respiration in sWAT normalized to wet weight was higher (P<0.05) in older highly exercise trained than in young untrained, older untrained and older moderately exercise trained subjects (Fig. 4B and C).

Complex I-linked LEAK respiration in sWAT further normalized mtDNA/nDNA ratio was not different between young untrained, older untrained, older moderately exercise trained and older highly exercise trained subjects (Fig. 4D). On the other hand, complex I-linked and complex I+II-linked OXPHOS respiration in sWAT further normalized to mtDNA/nDNA ratio was lower (P<0.05) in older pooled groups than in young untrained subjects (t-test) (Fig. 4E and F).

ADP-sensitivity followed apparent Michaelis-Menten kinetics (Fig. 4G), thus Km and Vmax
values were estimated with the Lineweaver-Burk equation revealing that apparent Km in sWAT was not different between young untrained, older untrained, older moderately exercise trained and older highly exercise trained subjects (Fig. 4H). Vmax in sWAT higher (P<0.05) in older highly exercise trained than in young untrained, older untrained and older moderately exercise trained subjects (Fig. 4I).

H$_2$O$_2$ emission in sWAT normalized to oxygen flux was not different during complex I-linked LEAK respiration between young untrained, older untrained, older moderately exercise trained and older highly exercise trained subjects (Fig. 4J).

H$_2$O$_2$ emission in sWAT normalized to oxygen flux during complex I+II-linked LEAK respiration was not different between young untrained, older untrained, and older moderately exercise trained. However, H$_2$O$_2$ emission was lower (P<0.05) in sWAT from older highly exercise trained than older untrained subjects (t-test) (Fig. 4K).

H$_2$O$_2$ emission in sWAT normalized to oxygen flux during complex I+II-linked OXPHOS respiration was higher (P<0.05) in older untrained than in young untrained subjects (t-test), and lower (P<0.05) in older highly exercise trained than in older untrained subjects (t-test) (Fig. 4L).

**DISCUSSION**

The main novel findings of the present study were that older subjects had decreased intrinsic WAT mitochondrial function but lifelong high volume exercise training ostensibly compensated for this loss by an increase in mitochondrial content resulting in markedly elevated respiratory capacity. Furthermore, high volume exercise training led to lowered ROS emission in both LEAK and OXPHOS respiratory states.

The present observation that complex I-linked OXPHOS respiration as well as complex I+II-linked OXPHOS respiration was higher in older highly exercise trained but when normalized to mtDNA content no longer showed effect of exercise training suggests that the age-
associated reduction in intrinsic mitochondrial respiratory capacity in human sWAT was irreversible despite lifelong exercise training. Therefore, the observed exercise training effects on mitochondrial respiration were solely due to higher mitochondrial content rather than maintained, or improved, intrinsic properties.

The elevated ROS emission in sWAT from older untrained subjects during both LEAK and OXPHOS respiration in the present study indicates excess mitochondrial ROS production. Although neither carbonylation nor 4-HNE protein modifications as a proxy for ROS-induced damage was increased, the elevated ROS emission indicates potentially undesirable effects on ROS handling with physically inactive aging. Moreover, the present observation that SOD2 and catalase protein in sWAT was not different in older and young subjects, suggests that there was no upregulation of the antioxidant defense mechanisms in sWAT with aging, which appears in accordance with the lack of signs of oxidative stress. In this context, SIRT3 has been shown to deacetylate and increase SOD2 activity [33;34] and positively regulates glutathione balance by activation of isocitrate dehydrogenase (IDH) 2 [35;36], enhancing ROS scavenging and thereby promoting mitochondrial ROS balance and maintaining mitochondrial integrity. In the present study, SIRT3 protein content in sWAT did not decrease with age in untrained individuals but lifelong exercise led to higher levels in older than in young subjects. This indicates that the apparent training effects observed on ROS emission may be mediated indirectly by increased SIRT3 content in human sWAT. The functional effects of increased ROS remain elusive as markers of oxidative damage do not appear to accumulate, at least not in adipose tissue. It could be speculated that antioxidant enzyme activity may be different between group, or that increased deacetylation by SIRT3 in the highly trained subjects are the cause for lowered ROS as observed in both LEAK and OXPHOS respiration states. Thus, it would be interesting to focus future studies on elucidating the mechanistic effects behind these observations.
Measures of ADP sensitivity in human sWAT followed Michaelis-Menten kinetics, but the observation that aging did not affect sWAT Km and Vmax values suggests that aging does not affect ADP sensitivity in human sWAT. Furthermore, the lack of effect of exercise training on Km suggests that lifelong exercise training independent of exercise level does not alter ADP sensitivity in sWAT. On the other hand, the present finding is in line with a previous study showing no difference in apparent Km in sWAT in response to 6 weeks of high intensity exercise training in healthy young subjects [37]. The present observation that Km was unchanged does not necessarily exclude that there may have been effects of lifelong exercise training on ADP sensitivity in the highly exercise trained subjects at low ADP concentrations. The present finding that ANT2 mRNA in sWAT was lower in older untrained than young untrained, while ANT1 and ANT3 mRNA was unchanged with aging, indicates an ANT isoform specific regulation in sWAT with aging. VDAC protein has been reported to be reduced in skeletal muscle of older compared to young subjects [38]. Together, this suggests that both exercise and aging have slight but possibly negligible effects on VDAC and ANT expression and that sWAT ADP sensitivity is not affected by aging or lifelong exercise training.

In the present study, aging was not associated with systemic low-grade inflammation albeit slightly increased adipose tissue macrophage infiltration. Similarly, the lack of change in CRP is not in accordance with previous studies showing increased plasma CRP in older individuals relative to young [39;40;41]. However, it must be stipulated that the subset of untrained older subjects selected for this study were submitted to strict criteria of being healthy and medicine free to avoid confounding factors in metabolic parameters, while matching activity level to the young subjects. Therefore, they may not objectively represent the general inactive population of this age group, often suffering from age- and lifestyle-
related metabolic complications [42], but rather individuals that represent older, healthy “versions” of the young untrained control group.

The observation that aging tended to increase (P=0.056), and lifelong exercise training increased, circulating adiponectin relative to young untrained individuals without an increase in sWAT adiponectin mRNA may suggest that the bulk of adiponectin is released from other adipose tissue depots than sWAT as previously suggested [43]. The finding is also in line with previous studies showing increased circulating adiponectin in older subjects subjected to 12-week exercise programs [44,45]. Furthermore, the increased circulating adiponectin, this is in line with the undetectable fasting insulin levels in the older highly exercise trained subjects, along with glucose parameters indicating improved insulin sensitivity. Efficient degradation of malfunctioning mitochondria is necessary for the preservation of a healthy mitochondrial population. The present observation that Parkin protein content in adipose tissue tended to increase with aging indicates an age-related upregulation of the capacity of the Parkin-mediated mitophagy pathway, which has not previously been reported in aging human adipose tissue. In line with this observation, Parkin has been shown to increase in skeletal muscle with age in mice and to be activated in order to elevate mitophagic flux [48]. Accordingly, it may be speculated that age increases Parkin content to accommodate the need to remove older mitochondria. The observation that monomeric BNIP3 protein tended to increase with exercise training in the present study further indicates an augmented mitophagic capacity in sWAT with lifelong exercise training. Skeletal muscle BNIP3 protein content has been reported to increase with exercise training in young subjects [49], while no studies until now have reported on the effect of exercise training on mitophagic markers in white adipose tissue. However, the present study may indicate that the BNIP3-mediated pathway is exercise-regulated in the same manner as seen in skeletal muscle. Taken together, the findings in the present study suggest that aging and lifelong exercise training have both
distinct and convergent effects on the capacity for parkin-mediated and BNIP3-mediated mitophagy pathway in human sWAT.

While fusion proteins are expressed in human sWAT, the question whether the dynamics and extent of mitochondrial network formation is analogous to skeletal muscle is completely unresolved. While exercise training in older adults has been reported to increase protein content of both MFN2 and OPA1 in human skeletal muscle [50]. As the moderately exercise trained group was quite heterogeneous in terms of exercise type, the finding that MFN2 increased only in this group could indicate that exercise modality, or intensity, may play a yet unknown role in upregulation of network formation in sWAT.

The observation that CS, GLUT4 and HK II protein content was higher in sWAT from lifelong highly exercise trained individuals indicates that capacity for glucose uptake and glycolysis are entrainable metabolic characteristics in older human sWAT in line with sWAT in young individuals [25]. In addition, the higher basal PDH-E1α, OXPHOS protein as well as CS activity content in sWAT in older highly exercise trained than in all other groups indicate a clear benefit of high volume exercise training in older individuals. Thus, while lifelong moderate exercise training was insufficient, high-volume exercise training upregulated mitochondrial proteins and oxidative capacity in older sWAT. This may further indicate that sWAT adaptations in older subjects necessitates a higher volume of exercise training to attain similar exercise adaptations as young subjects. Taken together, parallel increases in key glucose metabolic markers and mitochondrial proteins that enhance oxidative capacity occur in sWAT with lifelong high volume exercise training.

Aging and lifelong exercise training did not alter basal ATGL and HSL protein content in sWAT, and although lipid mobilization was not challenged beyond overnight fasting in the present study, it would appear that basal lipolytic capacity in response to regular exercise
training could be blunted with age. Although NEFA levels were increased with aging, indicating increased lipid availability, this could possibly be due to reduced utilization.

In conclusion, while aging was not associated with changes in oxidative stress in sWAT, an age-associated decline in intrinsic mitochondrial respiration was evident. High volume exercise training seemed to compensate for this decline in function by increasing mitochondrial content allowing markedly elevated absolute mitochondrial respiration. Furthermore, inactive aging led to increased ROS emission while high volume exercise training led to lowering of mitochondrial ROS emission in human sWAT. Taken together, this suggests that human sWAT function can be beneficially regulated by lifelong exercise training in a volume-dependent manner leading to improvements in mitochondrial respiratory capacity and metabolic health with aging.
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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS’ CONTRIBUTIONS

Conceived and planned the experiments: HP; AG; AQ; PP and JW. Carried out the experiments: HP; AG; PP; AQ and SR. Contributed to sample preparation: HP; AG; PP; AQ; SR. Contributed to analytical measurements: HP; AG; PP; AQ and SR. Contributed to data analysis: HP; AG; AQ and SR. AG and AQ took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon request.

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Table 1. Age, height, body weight, body mass index (BMI), total lean mass, total fat mass, android fat mass (total and percentage of total fat), gynoid fat mass (total and percentage of total fat), time to exhaustion and total energy expenditure in performance test in young untrained (Y-UT), older untrained (A-UT), older moderately exercise trained (A-T) and older highly exercise trained subjects (A-HT). Values are given as mean ± SD, except for citrate synthase (CS) activity in vastus lateralis, which is given as mean ± SE; n=6-9. *: Significantly different from young untrained, (P<0.05). #: Significantly different from older untrained, (P<0.05). ¤: Significantly different from older moderately exercise trained, (P<0.05). (*): Tends to be different from young untrained, (0.05≤P≤0.1). (#): Tends to be different from older untrained, (0.05≤P≤0.1).

Figure 1. A) Parkin protein content, B) monomeric BNIP3 protein content, C) OPA1 protein content, D) MFN2 protein content in sWAT from young untrained (Y-UT), older untrained (A-UT), older moderately exercise trained (A-T) and older highly exercise trained (A-HT) subjects. Values are given as mean ± SE; n=6-9. The protein content is given in arbitrary units (AU). *: Significantly different from young untrained, (P<0.05). #: Significantly different from older untrained, (P<0.05). ¤: Significantly different from older moderately exercise trained, (P<0.05). (*): Tends to be different from young untrained, (0.05≤P≤0.1). (#): Tends to be different from older untrained, (0.05≤P≤0.1).

Figure 2. A) SOD2 protein content, B) catalase protein content, C) protein carbonyl content, D) 4-HNE modified proteins in sWAT from yung untrained (Y-UT), older untrained (A-UT), older moderately exercise trained (A-T) and older highly exercise trained (A-HT) subjects. Values are mean ± SE; n=6-9. The protein content is given in arbitrary units (AU).

Figure 3. A) GLUT4 protein content, B) HKII protein content, C) total OXPHOS protein content, D) PDH-E1α protein content, E) CS activity, F) mitochondrial (mt) DNA/nuclear (n)
DNA (mtDNA/nDNA), G) SIRT3 protein content, and H) representative western blots of OXPHOS complex II-V protein content in sWAT from young untrained (Y-UT), older untrained (A-UT), older moderately exercise trained (A-T) and older highly exercise trained (A-HT) subjects. Values are given as mean ± SE; n=6-9 (Fig.3E n=4-8). The protein content is given in arbitrary units (AU). *: Significantly different from young untrained, (P<0.05). #: Significantly different from older untrained, (P<0.05). ¶: Significantly different from older moderately exercise trained, (P<0.05). (*): Tends to be different from young untrained, (0.05≤P≤0.1). (#): Tends to be different from older untrained, (0.05≤P≤0.1). (¤): Tends to be different from older moderately exercise trained, (0.05≤P≤0.1).

**Figure 4.** Oxygen consumption rate in sWAT normalized to wet weight (A-C) and mitochondrial DNA/nuclear DNA (mtDNA) (D-F) during A+D) complex I-linked LEAK respiration, B+E) complex I-linked OXPHOS respiration, C+F) complex I+II-linked OXPHOS respiration; G) Michaelis-Menten plot, H) apparent Km in sWAT, I) Vmax in sWAT; (J-L) H₂O₂ emission normalized to oxygen consumption during G) Complex I-linked LEAK respiration, H) complex I+II-linked LEAK respiration, I) complex I+II-linked OXPHOS respiration in sWAT from young untrained (Y-UT), older untrained (A-UT), older moderately exercise trained (A-T) and older highly exercise trained (A-HT) subjects. Values are mean ± SE; n=6-9. *: Significantly different from young untrained, (P<0.05). #: Significantly different from older untrained, (P<0.05). ¶: Significantly different from older moderately exercise trained, (P<0.05).
Table 1: Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Young Untrained</th>
<th>Older Untrained</th>
<th>Older Moderately Trained</th>
<th>Older Highly Trained</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anthropometry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>26.3 ± 3.2</td>
<td>67.1 ± 2.6 *</td>
<td>69.1 ± 2.6 *</td>
<td>66.4 ± 2.6 *</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>184.4 ± 4.9</td>
<td>180.7 ± 5.8</td>
<td>178.8 ± 6.1</td>
<td>179.3 ± 5.2</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>86.7 ± 11.4</td>
<td>87.7 ± 14.4</td>
<td>80.1 ± 6.0</td>
<td>75.8 ± 2.8</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>25.5 ± 3.3</td>
<td>26.8 ± 3.7</td>
<td>25.0 ± 1.9</td>
<td>23.6 ± 0.9</td>
</tr>
<tr>
<td><strong>Body composition/ Dual-Energy X-ray absorptiometry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lean mass total (kg)</td>
<td>60.3 ± 6.0</td>
<td>57.3 ± 7.6</td>
<td>56.0 ± 4.1</td>
<td>58.1 ± 4.2</td>
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<tr>
<td>Lean mass total (%)</td>
<td>70.1 ± 8.4</td>
<td>65.8 ± 4.9</td>
<td>69.9 ± 3.1</td>
<td>76.2 ± 4.8 (*) #</td>
</tr>
<tr>
<td>Fat mass total (kg)</td>
<td>23.2 ± 9.6</td>
<td>27.3 ± 8.2</td>
<td>21.0 ± 3.3</td>
<td>15.1 ± 3.9 #</td>
</tr>
<tr>
<td>Fat mass total (%)</td>
<td>26.2 ± 8.6</td>
<td>30.7 ± 5.1</td>
<td>26.2 ± 3.3</td>
<td>19.8 ± 5.0 (*) #</td>
</tr>
<tr>
<td>Android fat mass (% fat)</td>
<td>31.0 ± 11.9</td>
<td>40.5 ± 6.7 (*)</td>
<td>34.1 ± 5.2</td>
<td>19.8 ± 9.1 * # II</td>
</tr>
<tr>
<td>Gynoid fat mass (% fat)</td>
<td>28.4 ± 9.4</td>
<td>29.5 ± 7.4</td>
<td>25.0 ± 3.9</td>
<td>20.8 ± 5.9</td>
</tr>
<tr>
<td><strong>Group allocation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to exhaustion (min)</td>
<td>15.3 ± 2.9</td>
<td>9.7 ± 3.1 *</td>
<td>13.2 ± 3.9 (#)</td>
<td>20.8 ± 3.4 * # II</td>
</tr>
<tr>
<td>Total energy expenditure (kJ)</td>
<td>148.4 ± 39.5</td>
<td>77.9 ± 27.7 *</td>
<td>117.0 ± 42.2 (#)</td>
<td>228.3 ± 51.9 * # II</td>
</tr>
<tr>
<td>Vastus lateralis CS activity(µmol·min$^{-1}$·mg$^{-1}$·protein)</td>
<td>49.2 ± 3.8</td>
<td>54.9 ± 6.2</td>
<td>61.2 ± 4.7</td>
<td>126.7 ± 4.6 * # II</td>
</tr>
</tbody>
</table>
Figure 1

A

![Bar chart showing Parkin protein expression levels](image)

B

![Bar chart showing BNIP3 protein expression levels](image)

C

![Bar chart showing OPA1 protein expression levels](image)

D

![Bar chart showing MFN2 protein expression levels](image)
Figure 2

A

SOD2 protein (AU)

B

Catalase protein (AU)

Y-UT O-UT O-T O-HT

25 kDa

20 kDa

C

Protein carbonyls (nM/mg protein)

D

4-HNE modified proteins (AU)

Y-UT O-UT O-T O-HT

75 kDa

50 kDa

4-HNE

50 kDa
Figure 3

A

GLUT4 protein (AU)

Y-UT O-UT O-T O-HT

5.0
4.0
3.0
2.0
1.0
0.0

* #

GLUT4

50 kDa
37 kDa

B

HKII protein (AU)

Y-UT O-UT O-T O-HT

12.0
10.0
8.0
6.0
4.0
2.0
0.0

(*) (#) *

HKII

150 kDa
100 kDa

C

Total OXPHOS protein (AU)

Y-UT O-UT O-T O-HT

12.0
10.0
8.0
6.0
4.0
2.0
0.0

(*) (#) *

D

PDH-Eα protein (AU)

Y-UT O-UT O-T O-HT

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0

* #

PDH-Eα

50 kDa
37 kDa

E

Citrate synthase activity (μmol·min⁻¹·g protein⁻¹)

Y-UT O-UT O-T O-HT

100
60
20
0

* #

Citrate synthase

F

mDNAmRNA ratio (AU)

Y-UT O-UT O-T O-HT

2.5
2.0
1.5
1.0
0.5
0.0

G

SIRT3 protein (AU)

Y-UT O-UT O-T O-HT

0.0
1.0
2.0
3.0
4.0
5.0
6.0

* (#)

SIRT3

37 kDa
25 kDa

H

Complexes

Complex V

Complex III

Complex IV

Complex II

75 kDa
50 kDa
37 kDa
25 kDa