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Age-associated declines in mitochondrial biogenesis and protein quality control factors are minimized by exercise training

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Koltai E, Hart N, Taylor AW, Goto S, Ngo JK, Davies KJ, Radak Z. Age-associated declines in mitochondrial biogenesis and protein quality control factors are minimized by exercise training. Am J Physiol Regul Integr Comp Physiol 303: R127–R134, 2012. First published May 9, 2012; doi:10.1152/ajpregu.00337.2011.—A decline in mitochondrial biogenesis and mitochondrial protein quality control in skeletal muscle is a common finding in aging, but exercise training has been suggested as a possible cure. In this report, we tested the hypothesis that moderate-intensity exercise training could prevent the age-associated deterioration in mitochondrial biogenesis in the gastrocnemius muscle of Wistar rats. Exercise training, consisting of treadmill running at 60% of the initial VO2max, reversed or attenuated significant age-associated (detrimental) declines in mitochondrial mass (succinate dehydrogenase, citrate synthase, cytochrome-c oxidase-4, mtDNA), SIRT1 activity, AMPK, pAMPK, and peroxisome proliferator-activated receptor gamma coactivator 1-α, UCP3, and the Lon protease. Exercise training also decreased the gap between young and old animals in other measured parameters, including nuclear respiratory factor 1, mitochondrial transcription factor A, fission-1, and PNPase expression (21), but the effects of exercise on PNPase levels are currently unknown.

During high-intensity exercise, the metabolic rate of skeletal muscles can increase up to 100-fold, compared with that of cells at rest, and the mitochondrial network in the skeletal muscle exhibits a high degree of plasticity as a result of exercise, including biogenesis, fission, and fusion (3, 6, 12, 24, 28). Successful mitochondrial biogenesis requires a well-orchestrated signaling process, in which many factors are involved.

Mitochondrial transcription factor A (TFAM) is a major regulator of the mitochondrial genome, while nuclear respiratory factors (Nrf1, Nrf2) regulate the expression of many mitochondrial proteins whose genes are located in the cell nucleus (23, 49); TFAM, Nrf1, and Nrf2 are all encoded by nuclear genes. The coordination and activation of TFAM, Nrf1, and Nrf2, in turn, are controlled by peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1α) (39). Indeed, the coactivator PGC-1α induces mitochondrial biogenesis, once it has been activated (by phosphorylation) by the mitogen-activated protein kinase (p38 MAPK) or AMP-activated protein kinase (AMPK) (23, 39, 60). In addition, it has been shown that SIRT1 catalyzes PGC-1α deacetylation, which also activates this coactivator (36, 62). It is known that PGC-1α is readily induced by acute and regular exercise (25, 56).

Recent work indicates that the type 1 IFN-β inducible polynucleotide phosphorylase (PNPase), an evolutionarily conserved 3’5’ exoribonuclease with an expanding repertoire of functions, also plays a crucial role in mitochondrial biogenesis (61). PNPase localized to the mitochondrial intermembrane space (also present in the cytosol), is vital for mitochondrial homeostasis (9) and can induce cellular senescence (52). Free radicals, and other reactive oxygen species, can downregulate PNPase expression (21), but the effects of exercise on PNPase are currently unknown.

The ability for mitochondria to fuse with one another enables them to mix their contents, thereby minimizing the effects of dysfunctional mitochondria. Mitofusins, Mfn1 and Mfn2, are located in the outer membrane, and regulate the initial steps of mitochondrial fusion (7, 8). These steps are critical and have been suggested to protect the function and integrity of mitochondrial DNA (14, 57). Fission of mitochondria segregates them from the mitochondrial network (2). Mitochondrial fission downregulates mitochondrial elongation and may have a protective role in cellular senescence (31). However, the effects of exercise on fusion and fission of aged skeletal muscle is not well known.

Oxygen radicals, and oxidants in general, seem to play a role in modulating mitochondrial biogenesis (13), mitochondrial fragmentation (15), and oxidative damage to mitochondrial proteins (4, 5, 37). To maintain mitochondrial function under normal and stressful conditions, the integrity of proteins is under constant surveillance by mitochondrial chaperones. Lon is the major protease responsible for the degradation of oxidized proteins in mitochondria and is part of the oxidative stress adaptive response repertoire (4, 5, 37, 58). Interestingly, Lon is required to maintain the integrity of mitochondrial DNA and has also been correlated with...
enhanced mitochondrial biogenesis (34). The molecular chaperone Hsp78 has been shown to reinitate mitochondrial DNA replication following thermal inactivation (17). HSP78 may also be required for the efficient Lon-mediated proteolysis of damaged proteins (48).

Pioneering work by Holloszy (22) showed that exercise can significantly increase mitochondrial enzyme activity in skeletal muscle, and work by Davies and colleagues (11, 13) demonstrated that endurance exercise training increases the mitochondrial mass of skeletal muscles. In aging, there is a marked decrease in muscle endurance, force production, and muscle mass. There is also a marked decline in Lon mRNA and protein levels in skeletal muscles (4, 30) and increased oxidative damage (29). These properties are associated with the attenuated mitochondrial function observed in inactive humans and in laboratory rodents maintained in standard housing without exercise “facilities”.

We have suggested that the exercise-induced adaptation decreases the aging-associated impairment of mitochondrial biogenesis and quality control of mitochondrial proteins.

MATERIALS AND METHODS

Exercise training. Twelve young (3 mo) and twelve old (26 mo) male Wistar rats were used in the study, randomly assigned into young control (YC), young-exercised (YE), old control (OC) and old-exercised (OE) groups. The investigation was carried out according to the requirements of “The Guiding Principles for Care and Use of Animals, EU”, and was approved by the Semmelweis University Ethics Committee. Exercise-trained rats were first introduced to treadmill running for 3 days. Then for the next 2 wk, they were trained for 30 min daily at a running speed of 10 m/min, on a 5% incline. The mill running for 3 days. Then for the next 2 wk, they were trained for the intensity of running significantly alters the biochemical profile of the final run. Gastrocnemius muscles were carefully excised, homogenized in buffer containing 137 mM NaCl, 20 mM Tris·HCl (pH 8.0), 2% NP 40, 10% glycerol, and protease inhibitors.

Western blots. Ten to 50 μg of protein were electroophoresed on 8–12% vol/vol polyacrylamide SDS-PAGE gels. Proteins were electrophoretically transferred onto PVDF membranes. The membranes were subsequently blocked and after blocking, were incubated at room temperature with the following antibodies: 1:500, sc-13067 [Santa Cruz PGC-1 (H-300); Santa Cruz Biotechnology, Santa Cruz, CA], 1:1400, 2532 (Cell Signaling, Beverly, MA) AMPK, 1:500, 2535 Cell Signaling p-AMPKα (Thr172) (40H9), 1:1,000, sc-33771 Santa Cruz NRF-1 (H-300), 1:500 sc-30963 Santa Cruz mTFa (E-16) /TFAM, 1:500, sc-98900 Santa Cruz Fis1 (F1-152), 1:10,000, sc-50330 Santa Cruz Mnfs1 (H-65), 1:1,000, sc-99006 Santa Cruz PPNpase (H-124), 1:200, U7757 Sigma-Aldrich UCP3, 1:5,000 ab87253 Abcam CLPB/HSP78, 1:5,000, sc-98253 Santa Cruz SDH (H-204), 1:1,000, sc-69359 Santa Cruz COX4 (D-20), 1:500, ab53517 Abcam SIRT1, and 1:15,000, T6199 Sigma α-tubulin. The antibody for Lon protease was generated by us, as described previously (38). After incubation with primary antibodies, membranes were washed in TBS-Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies. After incubation with secondary antibody, membranes were repeatedly washed. Membranes were incubated with an ECL plus reagent (RPN 2132; Amersham, Piscataway, NJ), and protein bands were visualized on X-ray films. The bands were quantified by Imager software, and normalized to tubulin, which served as an internal control.

Estimation of oxidant levels and redox active iron. Intracellular oxidant and redox-active iron levels (26) were estimated using modifications of the dichlorodihydrofluorescein diacetate (H2DCFDA) staining method (44). The oxidative conversion of stable, nonfluorescent, DCF-DA to highly fluorescent 2′,7′-dichlorofluorescein (DCF) was measured in the presence of esterases, as previously reported (43). This assay approximates levels of reactive species, such as superoxide radical, hydroxyl radical, and hydrogen peroxide. The method has been widely used in the literature but does have the problem of not being particularly specific, and results can be strongly affected by release of labile iron or copper (26). Thus, the DCF fluorescence assay measures the production of (unknown) oxidants, probably including peroxide, and the release of redox-active iron that together cause the fluorescence (26). Briefly, the H2DCFDA (Invitrogen-Molecular Probes, D399) was dissolved at a concentration of 12.5 mM in ethanol and kept at –80°C in the dark. The solution was freshly diluted with potassium phosphate buffer to 125 μM before use. For fluorescence reactions, 96-well black microplates were loaded with potassium phosphate buffer (pH 7.4) to a final concentration of 152 μM/well. Then 8 μl of diluted tissue homogenate and 40 μl 125 μM dye were added to achieve a final dye concentration of 25 μM. The change in fluorescence intensity was monitored every 5 min for 30 min with excitation and emission wavelengths set at 485 nm and 538 nm (Fluoroskan Ascent FL, respectively). The fluorescence intensity unit was normalized with the protein content and expressed in relative unit production per minute.

Assessment of SIRT1 activity. The Cyclex SIRT1/Sir2 deacetylase fluorometric assay kit (Cyclex, CY-1151) was used to measure SIRT1 deacetylase activity, according to the manufacturer’s established protocol, including the separation of nuclear extract. For our assays, 10 μl of cytosolic and nuclear extracts of quadriceps muscle was mixed with a reaction mixture (40 μl) containing 50 mM Tris-HCl pH 8.8, 4 mM MgCl2, 0.5 mM DTT, 0.25 mM dATP, 1 μM trichostatin A, 20 μM fluoro-substrate peptide, and 200 μM NAD+ in a microplate. The samples were mixed well and incubated for 10 min at RT, and the fluorescence intensity (excitation: 355 nm, emission: 460 nm) was read for 2 h every 10 min and normalized by the protein content.

Citrate synthase activity assay. To appraise the effect of aging and physical exercise, citrate synthase activity was measured as described by Shepard and Garland (54).

Mitochondrial DNA measurement. To estimate the mtDNA content, we quantified the mtDNA to nuclear DNA (nDNA) ratio (mtDNA/nDNA). Total DNA was extracted (fast DNA kit, 6540–400, BIO 101 Systems Qbiogene) and quantitated spectrophotometrically. The mtDNA content was measured by PCR (Rotor-Gene 6000; Corbett Research, Sydney, Australia) and corrected by the simultaneous measurement of a single copy nuclear BDNF gene. Primers used for the analysis of mtDNA were R-CYTB-F (5′-CCC CAG AGG ATT AAA CTC CAA CGC A-3′), and R-CYTB-R (5′-GGG TGG GGT CAG GGG GT-3′). Primers used for the analysis of nDNA were R-BDNF-genome-exon-IV-F (5′-TTG GGA TGG CAA AGA TGG G-3′) and R-BDNF-genome-exon-IV-R (5′- CAG AGT AGG GAA CAA GTG TGA C-3′). Then the mtDNA content was normalized to nDNA. Data are expressed as the means of three measurements.

Statistical analyses. Statistical significance was assessed by one-way ANOVA, followed by Tukey’s post hoc test. Correlation matrices were applied to evaluate the relationship between different variables. The significance level was set at P < 0.05.
RESULTS

Exercise training modulates many factors in the PGC1-α repertoire. An acute bout of exhaustive exercise increases production of reactive oxygen species, but this response is ameliorated by exercise training (11, 12, 50). Factors, such as AMP-K and SIRT1, are also affected by exercise training. These factors are upstream of PGC1-α, and can regulate the activity of this master gene. PGC-1α, in turn, can initiate the transcription of NRF1, which can then initiate the transcription of proteins that regulate mitochondrial biogenesis, including nuclear DNA-coded TFAM.

We first estimated intracellular oxidant levels and reactive iron release levels in young and old rats that were either exercised or not exercised-trained. Young rats were tested at 3 mo old, while old rats were tested at 26 mo old. The gastrocnemius muscle was isolated and stained for 2′,7′-dichlorofluorescein oxidation. Although a trend toward higher DCF fluorescence was seen in older animals, no statistically significant difference was found between samples (Fig. 1A), possibly suggesting that neither aging nor exercise training had consistently strong effects on intracellular oxidant levels and reactive iron release levels. Alternative explanations for the negative results of Fig. 1A are that the 2-day rest between final exercise session, and euthanasia of the animal was sufficient to reverse most evidence of tissue oxidation or that, as previously reported (50), exercise training actually causes cellular adaptations that include a higher threshold than the training stimulus for stress responses to be activated. Uncoupling protein 3 (UCP3) has been shown to be protective against aging and protein damage through the modulation of reactive oxygen species. We analyzed UCP3 levels in our rats and found that UCP3 decreased with aging and that this decline could not be completely reversed by exercise training (Fig. 1B).

Fig. 2. Relative activity of SIRT1. SIRT1 deacetylase activity was analyzed in YC, YE, OC, and OE rat skeletal muscle. In both age groups, exercise training significantly increased SIRT1 activity. SIRT1 activity was measured fluorometrically (see MATERIALS AND METHODS) and divided by sample protein content. Values are means ± SD for six animals per group (*P < 0.05, **P < 0.01).

Fig. 3. PGC-1α levels. Western blots of YC, YE, OC, and OE skeletal muscles show significant reductions in PGC-1α levels with age, which was prevented by exercise training. Values for PGC-1α reported as “relative densities,” which have corrected for loading level differences, as judged by α-tubulin levels. Values are expressed as means ± SD for six animals per group (**P < 0.01).
SIRT1 and AMPK are both capable of activating PGC1α, a master regulator of mitochondrial protein expression in the nuclear genome. SIRT1 can activate PGC1α by deacetylation. We analyzed SIRT1 activity in young and old rats, with and without exercise training. We found that SIRT1 activity tended to decrease with aging; however, exercise training increased the activity in both the young and the old age groups (Fig. 2). The PGC-1α content, on the other hand, decreased with aging, and this was reversed by exercise training (Fig. 3). We next studied AMPK levels. Because AMPK phosphorylation results in AMPK activation, we studied both unphosphorylated and phosphorylated AMPK. We found that total and phosphorylated AMPK content decreased with aging; however, regular exercise reversed this decrease (Fig. 4). We also studied the factors downstream of PGC1α, the mitochondrial TFAM, and NRF1. We found that the levels of TFAM and NRF1 were significantly increased by aging in murine skeletal muscles, but that these increases were blocked in rats that were exercise trained (Fig. 5). We have apprised the level of mitochondrial biogenesis by mtDNA content, the activity of citrate synthase, and the contents of succinate dehydrogenase, as well as cytochrome-c oxidase-4, and all of these markers showed age-associated decline ($P < 0.05$), which was reversed by exercise training (Fig. 6).

Mitochondrial fission and fusion is maintained with exercise training. Mitochondrial plasticity depends on the ability of mitochondria to fuse and then separate from one another rapidly, especially during high-intensity exercise. The two most important proteins responsible for mitochondrial fusion and fission are mitofusin-1 (Mfn1) and fission-1 (Fis1). Our results indicate that both Mfn1 and Fis1 protein levels increased with aging, but that this increase was prevented by exercise training (Fig. 7).

PNPase is also an important regulator of mitochondrial biogenesis. Interestingly, PNPase protein levels showed a similar profile to Mfn1 and Fis1: in other words, PNPase exhibited an age-associated increase of that was attenuated by exercise training (Fig. 8).

Mitochondrial quality control proteins are rescued with exercise training. We also wanted to look at mitochondrial protein quality control by assessing both the Lon protease and HSP78, also known as ClpB. We found that Lon levels decreased significantly with age, but that this reduction was, in part, rescued with exercise training (Fig. 9A). HSP78, previously...
ously suggested to work in coordination with Lon protease, was also elevated as a result of exercise training (Fig. 9B).

DISCUSSION

One of the deficits associated with aging is a loss of mitochondrial function, leading to higher levels of superoxide and hydrogen peroxide production, which cause mitochondrial damage and, eventually, perpetuate the process (27). Interestingly, oxidative stress has been proposed as one of the regulators of mitochondrial biogenesis (11, 13, 27). In this study, we aimed to look at the effects of exercise training on mitochondrial biogenesis and aging. We found that steady-state oxidant levels and reactive iron release levels do not seem to be affected by exercise training or aging significantly. It seems that although single bouts of exhaustive exercise do transiently increase oxidative stress (11, 13), exercise training causes adaptations that minimize the stress (19, 45, 46, 50).

It should also be noted that the animals in the present study were given 2 days to recover from their last exercise training session before being studied. These results are also in accordance with some previous reports (1, 10, 20, 42).

Since exercise training did not significantly increase steady-state oxidant levels and reactive iron release levels, we did not expect to see a concomitant induction in UCP3 with the exercise training used for these rats. In fact, we actually found a decrease in UCP3 that was not reversed by exercise training. We were interested in studying the PGC-1α/PGC1α repertoire, as this coactivator is one of the major controllers of mitochondrial protein expression and biogenesis. AMPK and SIRT are two known regulators of PGC-1α, and it has been reported previously that aging decreases the levels of both of these factors (27, 47). Our results here are in accordance with these findings, and we show that aging is associated with decreased levels of PGC-1α in skeletal muscle of rats, as well as its upstream factors, Fis1 and Mfn1. Levels of mitochondrial fusion and fission proteins were assessed in YC, YE, OC, and OE rats. Values for Fis1 and Mfn1 are reported as relative densities that have been corrected for loading-level differences, as judged by α-tubulin levels. In both the mitochondrial fusion regulator Fis1, as depicted in A, and the fusion controller Mfn1, as depicted in B, there was an age-associated increase that was attenuated with exercise training. Values are means ± SD for six animals per group (***P < 0.01).
regulators AMPK and SIRT1, which confirms earlier observations (27, 47). We also look at two downstream factors of the PGC1α master regulator that is NRF1 and TFAM (18). Although there was an age-associated downregulation of AMPK, pAMPK, SIRT1, and PGC-1α, it was not accompanied by the same trend in NRF1 and TFAM. On the contrary, the levels of NRF-1 and TFAM actually increased with age, which is in line with previous observations that were also performed in human skeletal muscle. It is suggested that the upregulation of both NRF1 and TFAM results in the upregulation of mtDNA content (33). Indeed, the increase in mitochondrial DNA content (35) might be part of an adaptive response that will help the cell compensate for the age-associated loss of mitochondrial function. Our data suggest that the observed increases in both NRF1 and TFAM are independent of PGC-1α activation and reactive oxygen species, suggesting the involvement of other regulatory mechanisms.

To study mitochondrial biogenesis more directly, we also investigated the two main regulatory proteins of mitochondrial fusion and fission, Mfn1 and Fis1. The fission and fusion of mitochondria are not only protective against the accumulation of mitochondrial damage, but are a major part of the continuous reorganization of the mitochondrial network, essential in providing efficient structure for energy production and signaling (40). As observed with NRF1 and TFAM, Mfn1 and Fis1 also increased with age. This might be a compensatory mechanism toward the increasing levels of dysfunctional mitochondria, as it is known that fission and fusion can dilute out mitochondrial damage. Exercise training, however, seemed to block the age-associated increase in all of these factors. Since exercise training significantly decreased the gap between young and aged muscles in the levels of various mitochondrial markers, NRF1, TFAM, Mfn1 and Fis1, we propose that regular exercise could help delay many of the age-associated changes in muscle mitochondria.

PNPase is an exoribonuclease that is localized to the mitochondrial intermembrane space. PNPase plays a crucial role in mitochondrial RNA import (61); however, the upregulation of PNPase could induce inflammation by interferon-mediated degradation of specific mRNAs and small noncoding RNAs (51). Interestingly, NRF1 overexpression has also been associated with inflammation (59). Aging is associated with increased levels of inflammation (41), and there is limited data suggesting that PNPase could play a role in cellular senescence (32). The correlation between our NRF1 and PNPase data ($r = 0.6115, P = 0.009$) further support this suggestion. We found that exercise training partially prevented the induction of PNPase that occurred in old animals. The effects of regular exercise on PNPase levels may be even more pronounced, since exercise also decreased PNPase levels in young animals ($P = 0.0798$). Whether NRF1 and PNPase are actually working together to modulate age-associated inflammation needs further investigation.

The Lon protease is important for the quality control of mitochondrial proteins (4, 5, 37, 58), and it has been proposed that HSP78 may assist Lon in the degradation of damaged proteins (48). The levels of Lon decline with age, which could result in the accumulation of oxidized or dysfunctional proteins...
in the mitochondria (4, 5, 30), as well as result in the loss of mitochondrial function with age. We present novel data that suggest that regular exercise can prevent the age-related decline in Lon and, therefore, rejuvenate proper quality control of proteins in older cells (58). We also present the first study in which exercise training induced HSP78 levels in both young and old groups. The induction of both Lon and HSP78 indicates that regular exercise has beneficial effects on mitochondrial protein quality control. Although not directly studied in this paper, it is quite clear that Lon plays a major role in maintaining mitochondrial quality control (4, 5, 37). It has also been reported that Lon levels and activity decline with age in skeletal muscles (5, 58) and that the inducibility of Lon and its ability to cope with stress-induced protein damage, declines with senescence (37). Thus, our finding that the normal age-associated decline in Lon may be reversed by exercise training gives hope that exercise therapy may be able to help older individuals correct mitochondrial pathologies. Clearly, however, much more detailed and specific research will be needed to properly address this exciting possibility.

Taken together, our data suggest that regular exercise training stimulates mitochondrial biogenesis through the PGC1α system, a rejuvenation of the mitochondrial network via fission and fusion, and an improved quality control of mitochondrial proteins by the Lon protease. All of these properties, working in conjunction with one another, would improve the overall functionality of mitochondria in aged cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: E.K., N.H., and Z.R. performed experiments; E.K., S.G., J.K.N., K.J.A.D., and Z.R. approved final version of manuscript; A.W.T. and N.H., A.W.T., and Z.R. analyzed data; E.K., S.G., J.K.N., K.J.A.D., and Z.R. contributed to the writing of the manuscript.

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