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Exercise alters SIRT1, SIRT6, NAD and NAMPT levels in skeletal muscle of aged rats

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ABSTRACT

Silent information regulators are potent NAD^+-dependent protein deacetylases, which have been shown to regulate gene silencing, muscle differentiation and DNA damage repair. Here, changes in the level and activity of sirtuin 1 (SIRT1) in response to exercise in groups of young and old rats were studied. There was an age-related increase in SIRT1 level, while exercise training significantly increased the relative activity of SIRT1. A strong inverse correlation was found between the nuclear activity of SIRT1 and the level of acetylated proteins. Exercise training induced SIRT1 activity due to the positive effect of exercise on the activity of nicotinamide phosphoribosyltransferase (NAMPT) and thereby the production of sirtuin-fueling NAD^+. Exercise training normalized the age-associated shift in redox balance, since exercised animals had significantly lower levels of carbonylated proteins, expression of hypoxia-inducible factor-1alpha and vascular endothelial growth factor. The age-associated increase in the level of SIRT6 was attenuated by exercise training. On the other hand, aging did not significantly increase the level of DNA damage, which was in line with the activity of 8-oxoguanine DNA glycosylase, while exercise training increased the level of this enzyme. Regular exercise decelerates the deleterious effects of the aging process via SIRT1-dependent pathways through the stimulation of NAD^+ biosynthesis by NAMPT.

1. Introduction

Studies have revealed common regulatory mechanisms, including maintenance of genomic integrity, insulin growth factor-like signaling and silent information regulators, interact in concert with and influence complex pathways implicated in senescence/aging processes. Recent evidence suggests that among these factors, sirtuins are prominent regulators of aging from single cell organisms to mammals (Imai, 2009). Seven mammalian homologues of yeast Sir2 have been identified and shown to be dependent on nicotinamide adenine dinucleotide (NAD^+), and thus closely linked to cell metabolism, energy production and DNA repair (Imai et al., 2000; Lombard et al., 2008). In support of these roles of NAD^+ in the sirtuin pathway, the level/activity of nicotinamide phosphoribosyltransferase (NAMPT, also known as PBEF or Visfatin), a NAD^+ biosynthetic enzyme, has been shown to extend the replicative lifespan of vascular smooth muscle cells via activation of SIRT1 (van der Veer et al., 2007). The NAD^+/NADH ratio can also reflect the redox status (Ying, 2008) and it has been proven that ROS readily modify the activity of sirtuins (Furukawa et al., 2007).

SIRT1 can influence aging processes and many of the major diseases of aging, including metabolic disorders such as diabetes, or neurodegenerative diseases (Alzheimer's and Parkinson's), cancer and osteoporosis. Aging processes are orchestrated in part by powerful deacetylators SIRTs (Porcu and Chiarugi, 2005). For example, deacetylation of lysine residues of the histone tails by SIRT induces closed chromatin configuration and transcriptional silencing (Shahbazian and Grunstein, 2007). Besides histone deacetylation, SIRT1 targets a number of transcription factors such as nuclear factor κB, p53, peroxisome proliferator-activated receptor gamma coactivator-1α and MyoD, which are involved in inflammation, apoptosis, mitochondrial biogenesis, and skeletal muscle differentiation (Fulco et al., 2003; Lavu et al., 2008; Radak et al., 2004).

The age-associated shift in cellular redox state to an oxidized milieu can be characterized by the NAD^+/NADH ratio, which has been shown to affect the activity of NAD^+-dependent sirtuins. Indeed, increased ROS levels modulate SIRT1 expression (Fulco et al., 2003; Hipkiss, 2008) and the age-associated, organ-

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dependent changes in SIRT activities (Kwon and Ott, 2008). SIRT1, as well as SIRT6, are regulators of DNA repair (Mostoslavsky et al., 2006; Oberdoerffer et al., 2008; Wang et al., 2008). For example, SIRT6-deficient mice show degenerative processes which overlap with age-associated abnormalities due to deficiencies in maintaining genomic stability (Mostoslavsky et al., 2006).

Skeletal muscle, along with other tissues, accumulates increased levels of oxidative damage with aging (Radak et al., 2007, 2008). Regular exercise decreases the level of oxidative damage via increasing antioxidant potential of muscles and this change could be modulated by the activity and levels of SIRT1 (Ferrara et al., 2008; Radak et al., 2008; Suwa et al., 2008). To counteract increased generation of ROS, hypoxic conditions develop in muscle, resulting in increased expression of hypoxia-inducible factor-1α (HIF-1α). This expression is important in maintaining physiological redox conditions in skeletal muscle, especially in older mammals (Clanton, 2007; Mayr et al., 2008; Moller et al., 2001; O'Hagan et al., 2009). Regular endurance exercise-induced alteration in HIF-1α levels is associated with mitochondrial biogenesis and angiogenesis, the latter in consort with vascular endothelial growth factor (VEGF) (O'Hagan et al., 2009).

The present investigation has been designed to test the hypotheses that exercise training re-establishes physiologically relevant activity of SIRT1, which has been attenuated with aging. SIRT1's total activity was significantly increased with training. Similarly, exercise training increased NAD^+, NAMPT and mitochondrial uncoupling protein-3 (UCP3) levels/activities to levels comparable to those seen in skeletal muscle of young animals. These data suggest that regular exercise decelerates aging processes of skeletal muscle via SIRT1-dependent pathways.

2. Methods

2.1. Animals and training protocol

Twelve young (3 mo) and 12 old (26 mo) male Wistar rats were used in the study and grouped into young control (YC), young exercised (YE), old control (OC) and old exercised (OC). The investigation was carried out according to the requirements of The Guiding Principles for Care and Use of Animals, EU, and approved by the local ethics committee. Exercised rats were introduced to treadmill running for 3 days, then for the next 2 weeks the running speed was set to 10 m/min, on a 5% incline for 30 min. The running speed and duration of the exercise were gradually increased to 60% of VO2max of the animals. Therefore, on the last week of the 6 weeks training program, young animals ran at 22 m/min, at a 10% incline, for 60 min, whereas old animals ran at 13 m/min, and a 10% incline for 60 min. The animals were killed 2 days after the last exercise session to avoid the metabolic effects of the final run. Gactrocnemius muscle was carefully excised, homogenized in buffer containing 137 mM NaCl, 20 mM Tris-HCl pH 8.0, 2% NP 40, 10% glycerol and protease inhibitors. For some measurements, nuclear extracts were separated as described earlier (Radak et al., 2009). In brief, part of the gastrocnemius muscle samples were homogenized in buffer (HB) containing 20 mM Tris (pH 8.0), 1 mM EDTA, 1 mM dithiothereitol, 0.5 mM spermidine, 0.5 mM spermine, 50% glycerol, and protease inhibitors. The nuclear fraction was separated by differential centrifugation. To prepare nuclear fractions, the homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C, and the pellet was suspended in HB and recentrifuged. The pellet was then re-suspended in HB with 0.5% NP40 and centrifuged. Next, the pellet was washed twice in HB. After centrifugation, the final nuclear pellet was rocked for 30 min after the addition of a 1/10 (vol/vol) of 2.5 M KCl and centrifuged at 14,000 rpm for 30 min. The supernatant was divided into aliquots and stored at −80 °C. The protein levels were measured using the BCA method.

2.2. Western blots

Ten to 50 μg of protein were electrophoresed on 8–12% (v/v) polyacrylamide SDS-PAGE gels. Proteins were electrotransferred onto PVDF membranes. The membranes were subsequently blocked and after blocking, PVDF membranes were incubated at room temperature with antibodies (1:500 #ab53517 Abcam SIRT1, 1:500 #S4322 Sigma SIRT6, 1:500 #ab37299 Abcam NAMPT (PBEF), 1:1000 #ab193 Abcam Acetylated protein, 1:200, #U7757 Sigma UCP3, 1:4000 #H 6536 Sigma HIF-1alpha, 1:500 #MAB1665 Chemicon VEGF, 1:200 #sc-7159 Santa-Cruz Cytocrome C, 1: 15000 #T6199 Sigma alpha-Tubulin, 1:150 #S7150 Chemicon anti-DNPH, and 1:500 #ab204 Abcam OGG1). After incubation with primary antibodies, membranes were washed in TBS-Tween-20 and incubated with HRP-conjugated secondary antibodies. After incubation with secondary antibody, membranes were repeatedly washed. Membranes were incubated with an ECL plus reagent (RPN 2132, Amershham) and protein bands were visualized on X-ray films. The bands were quantified by ImageJ software, and normalized to tubulin, which served as an internal control.

2.3. Detection of carbonylated proteins

Changes in oxidized protein levels were determined using an Oxyblot kit (Chemicon/Millipore Temecula, CA) according to the manufacturer recommendations. Briefly, proteins were derivatized with 4-dinitrophenylhydrazine (DNPH) for 15 min followed by incubation at room temperature with a neutralization buffer (Chemicon/Millipore). Derivatized proteins were electrophoresed on a 10% SDS-PAGE and blotted on Hybond PVDF membranes (Amersham, Piscataway, NJ). Blots were blocked with 5% non-fat dry milk (blocking buffer) in Dulbecco's PBS containing 0.05% Tween 20 (PBS-T) for 3 h and incubated with anti-DNP primary antibody (1:150) (Chemicon/Millipore) overnight at 4 °C. After three washes with PBS-T, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:300) (Amersham, Piscataway, NJ). Immunocomplexes were visualized by chemiluminescence using the ECL kit (Amersham).

2.4. Assessment of SIRT1 activity

To measure SIRT1 deacetylase activity, Cyclex SIRT1/Sir2 Deacetylsase Fluorometric Assay Kit (Cyclex, CY-1151) was used according to the established protocol, including the separation of nuclear extract. In brief, 10 μl of cytosolic and nuclear extracts of quadriceps muscle were mixed with a reaction mixture (40 μl) containing 50 mM Tris–HCl pH 8.8, 4 mM $MgCl_2$, 0.5 mM DTT, 0.25 mAU/ml Lysyl endpeptidase, 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 (ex. 355 nm, em. 460 nm) was read for 2 h every 10 min and normalized by the protein content.

2.5. Measurement of NAD^+/NADH level

Proteins were filtered through a 10 kD Microcon filter and applied to a NAD^+/NADH Quantification kit (Bio Vision, K337-100) according to the given protocol. First, total NAD^+ level was measured then NAD was decomposed by heating to 60 °C for 30 min, then cooled on ice and transferred to the microplate. Next, a 10 μl NADH developer was added to each well, mixed, and the optical density read at 450 nm every 30 min for 5 h. The NAD^+ levels were calculated according to the manufacturer's directions.

2.6. DNA damage and repair assays

The measurement of 8-hydroxy-2′-guanine (8-oxo-Gua) was done as previously described (Radak et al., 2002). The OGG1 assay was carried out according to the protocol outlined by (Radak et al., 2007). In brief, 20 pmol of synthetic substrate containing 8-oxo-Gua (Trevigen, Gaithersburg, MD, USA) were labeled with ^{32}P at the 5′ end using polynucleotide T4 kinase (Boeringer Mannheim, Germany). For the nicking reaction, protein extract (2 μg) was mixed with 20 μl of a reaction mixture containing 0.5 M of N-[2-hydroxyethel]piperazine-N′-[ethanesulfonic acid], 0.1 M EDTA, 5 mM of dithiolthreitol, 400 mM KCl, purified BSA and labeled probe (approximately 2000 cpm). The reaction was carried out at 30 °C for 15 min and stopped by placing the mixture on ice. Next, 30 μl of chloroform were added, samples were centrifuged, and 15 μl of the aqueous layer were taken and mixed with loading buffer containing 90% formamide, 10 mM NaOH, and blue-orange dye. After three min heating at 95 °C, samples were chilled and loaded into a polyacrylamide gel (20%) with 7 M urea and 1 × TBE and run at 400 mV for 2 h. Radioactive signals of the cleavage product of the labeled substrate were quantified using a STORM Bioimaging Analyzer (Molecular Dynamics, USA). Radioactivity in the separated, cleaved product, and intact oligo bands was quantified with a PhosphpoImager (Molecular Dynamics) loaded with Image Quant software. The activity to repair 8-oxo-Gua was determined and expressed as a percentage of the substrate cleaved (Radak et al., 2007).

2.7. Statistical analyses

Statistical significance was assessed by one-way ANOVA, followed by Tukey's post hoc test. The significance level was set at $p < 0.05$.

3. Results

3.1. Exercise increases the activity of SIRT1

SIRT1 is localized in the cytosol and the nucleus. Therefore, the level and activity in both cell compartments were measured. Aging significantly ($p < 0.01$) increased the level of SIRT1 in the cytosol,