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The effect of treadmill training and *N*-acetyl-L-cysteine intervention on biogenesis of cytochrome *c* oxidase (COX)

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ABSTRACT

Mitochondrial biogenesis refers to increased content of mitochondria, which has been shown to be promoted by aerobic exercise. During this process, oxidative stress is considered the essential initiator. Even though some studies have addressed the issue as to whether antioxidants would hamper the effects of exercise on mitochondrial biogenesis, no consensus has been achieved. Therefore, the purpose of the present study was to investigate the effects of exercise and antioxidant intervention on mitochondrial biogenesis, as well as COX biogenesis. Thirty-two clean-grade male ICR mice were randomly assigned to a control group (Con), exercise group (Ex), *N*-acetyl-L-cysteine group (NAC), or NAC plus exercise group (NEx). The NAC and NEx groups were injected with NAC (0.1 mg/g/2 days) intraperitoneally for 3 weeks, whereas the Con and Ex groups were administered saline for the same period of time. Mice assigned to Ex and NEx groups started exercise training 1 week before drug intervention was initiated. After 1 week of acclimatization, the mice were allowed to run at a speed of 28 m/min for 60 min, 6 days a week. The results showed that exercise training caused an increase in mRNA and protein levels of COXIV, whereas NAC intervention lowered the two so significantly that even exercise training could not reverse the effect of NAC intervention. Our data suggest that even though antioxidant intervention could alleviate oxidative damage caused by exercise, it was not necessarily beneficial for mitochondrial biogenesis.

1. Introduction

Mitochondria are ubiquitous, genetically semiautonomous, double-membrane-enclosed organelles in eukaryotic cells. Mitochondria are essential for maintaining cellular functions, physiological functions, and overall well-being of mammals. Decreased mitochondrial content or disturbed mitochondrial function is involved in the causes of chronic diseases such as sarcopenia [1], diabetes [2], neurodegenerative disease [3], and cancer [4], as well as in the aging process [5].

Mitochondria content is the overall result of the balance between mitochondrial biogenesis and mitochondrial degradation. Mitochondrial biogenesis refers to increased content of mitochondria in tissues, which involves changes in space conformation, volume, and mitochondrial DNA (mtDNA) content, as well as alterations in mitochondrial activity. Even though the exact process and regulation mechanism of mitochondrial biogenesis remain elusive, PGC-1α[1] has emerged as a potent inducer of this process since being recognized in 1998 [6], owing to its ability to coactivate numerous nuclear transcriptional factors [7,8]. Cytochrome *c* oxidase (COX), also known as respiratory chain complex IV, or complex IV, is an important structural component of the inner membrane of mitochondria. It transfers electrons from reduced cytochrome *c* to oxygen molecules, pumping four protons out of mitochondria and driving F0-F1 ATP synthase at the same time. Therefore, COX is considered a key variable in determining mitochondrial content and activity [9–12].

The mitochondria of skeletal muscle have great plasticity. Endurance training has been suggested to promote mitochondrial biogenesis in skeletal muscle, and oxidative stress is considered the essential initiator inducing mitochondrial biogenesis during exercise [13]. In fact, antioxidant supplements, such as vitamins C and E, could hamper, and even inhibit, the boosting effect of exercise on mitochondrial biogenesis [14,15]. This suggests that maintaining an optimal level of oxidative stress is vital to promote mitochondrial biogenesis. Even though NAC (*N*-acetyl-L-cysteine) administration has been used to investigate the role of reactive oxygen species (ROS) in various biological and pathological processes [16], its effect on COX biogenesis has not been explored

Abbreviations: COX, Cytochrome c Oxidase; GS, Gastrocnemius Muscle; IMF, Intermyofibrillar; NAC, N-acetyl-L-Cysteine; PGC-1α, Peroxisome Proliferator-Activated Receptor-γ Coactivator 1α; PPARγ, Peroxisome Proliferator-Activated Receptor-γ; SS, Subsarcolemmal

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before. Therefore, in the present study, the effects of aerobic exercise training, NAC injection, and the combination of both on COX biogenesis will be examined, with the attempt to explore the tentative underlying mechanism.

2. Materials And Methods

2.1. Animals And Experimental Design

Clean-grade male ICR mice (Shanghai Slac Laboratory Animal Co. Ltd) were housed in groups of eight or nine per cage at 22°C with a 12-h light/dark cycle, with free access to standard chow and water, ad libitum. After 1 week of acclimatization, the mice were randomly assigned to a control group (Con, $n = 8$), an exercise group (Ex, $n = 8$), a NAC group (NAC, $n = 8$), or a NAC group combined with exercise intervention (NEx, $n = 8$). The NAC and NEx groups were injected with NAC (0.1 mg/g/2 days) intraperitoneally for 3 weeks, whereas the Con and Ex groups were administered saline for the same period of time. The body weights were measured every 5 days. All experiments and procedures complied with the Animal Care Guidelines of East China Normal University.

2.2. Exercise training protocol

Mice assigned to the Ex and NEx groups started exercise training 1 week before drug intervention was initiated. Initially, mice were progressively acclimatized to the treadmill for a period of 1 week by running for from 40 to 60 min at the speed of 25–28 m/min. For the subsequent 3 weeks, the mice were allowed to run at a speed of 28 m/min for 60 min, 6 days a week.

2.3. Tissue harvesting and mitochondria isolation

All the mice were sacrificed by cervical dislocation within 48 h of the last training session. The gastrocnemius muscle (GS) of both hind limbs, as well as part of the liver, was carefully isolated, rinsed with saline, and weighed. The right GS was ground in a mortar and homogenized in ice-cold buffer (100 mM KCl, 5 mM $MgSO_4$, 5 mM EDTA, 50 mM Tris-base, 1 mM ATP, *p*H 7.4). Subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria were fractionated by differential centrifugation, as described in detail [17–19]. The pellet was rinsed and resuspended in a mixture of 10 mM Hepes, 0.25 M sucrose, 10 mM Na succinate, 2.5 mM K_2HPO_4, 0.21 mM ADP, and 1 mM dithiothreitol, with *p*H of 7.4. The rest of the tissue was snap-frozen in liquid nitrogen and further stored at −80°C.

2.4. Mitochondrial oxidative stress and activity assays

The level of oxidative stress of mitochondria was measured directly by using assay kits purchased from Nanjing Jiancheng Bioengineering Institute. Malondialdehyde (MDA) derived from polyunsaturated fatty acid peroxides was evaluated with an MDA assay kit (Product A003-1) by the thiobarbituric acid method. Glutathione (GSH) content was determined with a GSH detection kit (Product A006-2) based on its reaction with the thiol-specific reagent dithionitrobenzoic acid. A superoxide dismutase (SOD) assay kit (Product A001-1) was used for the measurement of MnSOD activity by colorimetric analysis. Mitochondrial COX activity (complex IV) was determined with a commercially available assay kit purchased from Shanghai Genmed Gene Pharmaceutical Technology Co. Ltd. (Product GMS50010). Mitochondrial ATP level was determined with an assay kit by Beyotime Institute of Biotechnology (Product S0026). Briefly, muscle samples were lysed in ATP assay buffer, homogenized, and then mixed with reaction mixes. A luminometer was then used to measure RLU or CPM value.

2.5. Measurement of glycogen content

Glycogen contents of skeletal muscle and liver were measured with assay kits purchased from Nanjing Jiancheng Bioengineering Institute (Product A043). Briefly, the tissue sample was mixed with 3 volumes of alkali and incubated in boiled water for 20 min. The glycogen hydrolysis buffer was then mixed with distilled water, glycogen detection buffer, and chromogenic agent, heated in boiled water for 5 min. The adduct was measured spectrophotometrically at 620 nm with a plate reader.

2.6. Measurement of mtDNA content and real-time PCR

About 20 mg of frozen tissue samples was homogenized with a Trizol assay kit for extraction of total RNA and DNA. RNA was quantified by spectrophotometry (260 nm). cDNA was prepared by reverse transcription of 5 μl of total RNA using a ReverTra Ace qPCR RT kit (Toyobo, FSQ-101). Real-time PCR was performed with a Step One instrument (ABI) and software (Applied Biosystems) using a SYBR Green PCR Master Mix (Toyobo, QPK201) for detection. Real-time PCR primers were designed (Supplementary Table S1) for mouse SCO1, SCO2, COXIV, PGC-1α, PGC-1β, PPARγ, ERRα, Tfam, Tfb1m, Tfb2m, Mfn1, and Mfn2. All samples were run in duplicate and data were analyzed according to the $2^{-\Delta Ct}$ method. The identity and purity of the amplified product were checked through analysis of the melting curve carried out at the end of amplification. A value of 1 was arbitrarily assigned to group Con, against which the values of groups Ex, NAC, and NEx were reported.

The measurement of mtDNA content was also performed by real-time PCR, using extracted DNA as the model. Mitochondrial gene-specific primers (such as ATPase6 and mt1000) were designed as in Supplementary Table S1. The relative copy numbers of ATPase6 and mt1000 genes were calculated using the formula $2^{-\Delta Ct}$ and β-actin as the inner reference.

2.7. Western blotting

The GS muscle was homogenized in lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride and 1% NP-40, with *p*H of 7.4. The homogenate was centrifuged at 8000 *g* for 10 min at 4°C. The protein concentration of the supernatant was measured with the BCA method. SDS–PAGE (10%) was used to separate protein that was then blotted on a polyvinylidene difluoride membrane. The blots were incubated with primary antibodies (1:200, Santa Cruz Biotechnology) overnight at 4°C (Table 1). The membranes were incubated with secondary antibodies (1:500, Santa Cruz Biotechnology) for 1 h at room temperature. The membrane was examined with ECL Plus (high-sensitivity ECL chemiluminescence kit). β-Actin was used as a control to ensure equal protein loading on the gel, and the blots were quantified with Alpha-Tech gel imager.

2.8. Statistical analysis

Results are reported as the mean ± SE. The difference between groups was tested for significance with Student's *t* test or one-way ANOVA using SPSS 15.0. The significance threshold was set to $p < 0.05$. Extremely significant difference was set to $p < 0.01$.