Endurance exercise causes mitochondrial and oxidative stress in rat liver: Effects of a combination of mitochondrial targeting nutrients

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Aims: Endurance exercise causes fatigue due to mitochondrial dysfunction and oxidative stress. In order to find an effective strategy to prevent fatigue or enhance recovery, the effects of a combination of mitochondrial targeting nutrients on physical activity, mitochondrial function and oxidative stress in exercised rats were studied.

Main methods: Rats were subjected to a four-week endurance exercise regimen following four weeks of training. The effects of exercise and nutrient treatment in rat liver were investigated by assaying oxidative stress biomarkers and activities of mitochondrial complexes.

Key findings: Endurance exercise induced an increase in activities of complexes I, IV, and V and an increase in glutathione (GSH) levels in liver mitochondria; however, levels of ROS and malondialdehyde (MDA) and activities of complexes II and III remained unchanged. Exercise also induced a significant increase in MDA and activities of glutathione S-transferase and NADPH-quinone-oxidoreductase 1 (NQO-1) in the liver homogenate. Nutrient treatment caused amelioration of complex V and NQO-1 activities and enhancement of activities of complex I and IV, but had no effect on other parameters.

Significance: These results show that endurance exercise can cause oxidative and mitochondrial stress in liver and that nutrient treatment can either ameliorate or enhance this effect, suggesting that endurance exercise-induced oxidative and mitochondrial stress may be either damaging by causing injury or beneficial by activating defense systems.

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Introduction

High-intensity sports and exercise training, such as long distance running and soccer, cause fatigue and muscle injury. Increasing evidence suggests that endurance exercise may increase reactive oxygen species (ROS) generation that may in turn impair mitochondrial respiration. Effects of such impairment include free radical generation, increases in oxidative damage biomarkers such as protein carbonyls and thiobarbituric acid reactive substances, effects on mitochondrial function, and changes in levels of antioxidants and antioxidant enzymes in the heart, blood, lung, liver, brain, and muscles (Alessio 1993; Alessio et al. 1997; Davies et al. 1981; Ji 1995a; Liu et al. 2000). Therefore, oxidative damage and mitochondrial dysfunction play a key role in sports and exercise-induced fatigue and injury. A previous study of ours (Liu et al. 2000) investigated the responses to oxidative stress induced by chronic exercise (eight-week treadmill running) or acute exercise (treadmill running to exhaustion) in the brain, liver, heart, kidney, and muscles of rats. Various biomarkers of oxidative stress were measured, namely, lipid peroxidation [malondialdehyde (MDA)], protein oxidation (protein carbonyl levels and glutamine synthetase activity), oxidative DNA damage (8-hydroxy-2′-deoxyguanosine), and endogenous antioxidant levels (ascorbic acid, alpha-tocopherol, glutathione, ubiquinone, ubiquinol, and cysteine). The most notable changes due to exercise were in MDA, ascorbic acid, glutathione, cysteine, and cystine. Acute exercise induced an increase in MDA and a decrease in glutamine synthetase both in liver homogenate and in mitochondria and an increase in ascorbic acid and decreases in ubiquinol, cysteine, and cystine in liver. Taken together, these changes suggest that exercise causes oxidative and mitochondrial stress in this organ.

People have tried various strategies to improve the body's strength and enhance recovery from fatigue, including the use of stimulants, which cause long-term harm to the body. Because acute strenuous exercise and chronic exercise training increase the consumption of various antioxidants and a deficiency of antioxidant nutrients can severely hamper proper functioning of the corresponding antioxidant system and exacerbate exercise-induced oxidative stress and tissue damage, it has been suggested that dietary supplementation with...
specific antioxidants may be beneficial (Ji 1995b). Various dietary antioxidant supplements have been marketed to and used by athletes as a means of countering oxidative stress due to exercise (Urso and Clarkson 2003). Well-known antioxidants, such as vitamin E (Goldfarb et al. 1994; Kumar et al. 1992), a mixture of vitamin E, vitamin C and beta-carotene (Kanter et al. 1993), glutathione (GSH) (Johnson and Klueber 1991), N-acetylcysteine (Sen et al. 1994), and polysaccharides (Yu et al. 2006) have been tested and have shown detectable effects. The antioxidative activities of antioxidant compounds against exercise-induced oxidative stress include reducing the production of ROS, inhibiting lipid peroxidation, and enhancing antioxidant defenses such as increasing the production of antioxidant enzymes and endogenous antioxidants. Antioxidants may be involved in glycogen metabolism so as to meet the energy requirements of working skeletal muscles and may act by terminating the chain reaction of lipid peroxidation so as to maintain the morphological stability of mitochondria in spinal motor neurons (Yu et al. 2006). Because of the complexity of exercise-induced cell damage, more comprehensive strategies should be explored. Mitochondria are the major sites of cellular ROS production and are also targets of ROS (Beckman and Ames 1998; Echty 2007), and mitochondrial dysfunction appears to play a key role in exercise. Therefore, it is rational to target mitochondria for ameliorating the cellular damage caused by exhaustive and strenuous exercise.

Our studies (Liu and Ames 2005) have focused on a group of micronutrients, termed mitochondrial targeting nutrients, that are either mitochondrial components or the metabolites of which that influence the structure and function of mitochondria. Such nutrients can perform a number of beneficial functions: 1) prevent oxidative production or scavenge free radicals so as to eliminate oxidative stress in mitochondria; 2) enhance antioxidant defenses, such as phase 2 enzyme inducers; 3) enhance mitochondrial metabolism by repairing and degrading mitochondria or by increasing mitochondrial biogenesis; 4) protect mitochondrial enzymes and/or stimulate mitochondrial enzyme activity by elevating substrate and cofactor levels. Deficiency of mitochondrial nutrients may cause increases in ROS and oxidative stress in mitochondria, leading to mitochondrial dysfunction and age-associated diseases. On the other hand, keeping sufficient mitochondrial nutrients in mitochondria may be effective in reducing oxidative stress and mitochondrial dysfunction. Therefore, a promising combination of mitochondrial nutrients was designed, and this combination was fed to rats subjected to endurance exercise. This nutrient treatment significantly increased endurance in platform running, stimulated mitochondrial biogenesis and fusion, improved mitochondrial function, and inhibited oxidative damage in muscle (Shen et al., unpublished).

Because the liver is one of the most important organs affected by exercise, the present study investigated the effects of the combination of mitochondrial nutrients on mitochondrial function and oxidative stress biomarkers in the liver of rats subjected to a four-week-long endurance exercise regimen. The parameters measured include the activities of mitochondrial complexes I, II, III, IV and V, MDA as an index of lipid peroxidation, endogenous antioxidant GSH, and antioxidant enzymes, including glutathione S-transferase (GST), NADPH-quinone-oxidoreductase 1 (NQO-1), and glutathione peroxidase (GPx).

Materials and methods

Materials

Cytochrome c, coenzyme Q10, coenzyme Q9, antimony A, rotenone, p-iodonitrotetrazolium violet (INT), dithiothreitol, thiamine pyrophosphate, and lipoamide dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO); Tris base and NADH from Amersco Inc. (Palm Harbor, FL); 2,6-dichlorophenolindophenol (DCPIP) from Merck & Co., Inc. (USA), and BCA kit for protein quantification from Pierce Biotechnology, Inc. (Pierce, Rockford, IL). Other chemicals were all analytical purity reagents from local vendors.

Animals and treatments

Exhaustive endurance exercise was performed by rats on a treadmill. Wistar rats at 4 weeks of age (Shanghai SLAC Experimental Animal Center, Shanghai, China) were divided into 3 groups, 12 animals per group: control (sedentary), endurance exercise (EE), and EE + nutrient-dosed. Nutrients and doses (mg/kg/day) were as follows: R-lipoic acid (50), acetyl-l-carnitine (100), biotin (0.1), nicotinamide (15), riboflavin (6), B6 (6), creatine (50), CoQ10 (5) and resveratrol (5). The nutrients were administered by gavage for 12 weeks. After a 4-week pretreatment with nutrient gavage, the animals were trained for 4 weeks (weeks 5–8), 5 times a week (every day except Saturday and Sunday). Treadmill running times were recorded during weeks 9–12, 5 times a week (every day except Saturday and Sunday). Nutrient gavage was performed on each animal each day in 45 min before the start of the exercise program.

Exercise program

Rats were exercised for the entire eight-week experimental period on a treadmill, at room temperature, six days/week (Mon–Sat). The exercise program consisted of a four-week endurance training period followed by a four-week period of high-intensity exercise. After being familiarized with a motor-driven rodent treadmill (Hangzhou, China) for 2 days, animals ran on a treadmill at 10 m/min, 0% slope for 10 min/day during the first week. The intensity and duration of the exercise training were progressively increased until at week 4 the animals were running at 25 m/min, 0% slope for 40 min/day. During the 5–8-week period, exercise capacity was evaluated by beginning with a treadmill speed of 15 m/min, and increasing it 5 m/min every 20 min until a speed of 30 m/min was reached (10° incline throughout). Rats were run until they could no longer maintain the pace of the treadmill, i.e., until they were completely exhausted, defined as the inability to continue running despite contact with a shock bar located at the rear of the treadmill belt (Bedford et al. 1979; Liu et al. 2005; Pan 2008; Yatabe et al. 2003). The animals were sacrificed 24 h after the last exercise session by an intraperitoneal injection of sodium pentobarbital (60 mg/kg). The liver was dissected out, rinsed in PBS and used immediately for isolating mitochondria.

Mitochondrial preparation

Mitochondria were isolated as described (Krahenbuhl et al. 1991) with slight modification. Briefly, tissues were rinsed with saline, weighed, and put into ice-cold isolation buffer containing 0.25 M sucrose, 10 mM Tris, 0.5 mM EDTA, pH 7.4. Tissues were sheared carefully to mince, rinsed to get rid of residual blood, and then homogenized in 2.5 vol of isolation buffer. The homogenate was adjusted to 8 vol with isolation buffer and centrifuged at 1000 g for 10 min; the supernatant fraction was decanted and saved. The pellet was washed once with 2 vol of isolation buffer. The supernatant fractions were combined and centrifuged at 10,000 g for 10 min. The mitochondrial pellet was washed twice with isolation buffer. The supernatant fractions were combined and centrifuged at 10,000 g for 10 min. The mitochondrial pellet was washed twice with isolation buffer. All the above operations were carried out at 4 °C. The mitochondrial protein concentration was determined using the BCA™ Protein Assay kit (Pierce 23225) using bovine serum albumin (BSA) as a standard. Freshly isolated mitochondria were either used immediately for respiration and permeability transition assays or stored at −80 °C until enzyme analysis.
Assays for mitochondrial enzyme activities

NADH-CoQ oxidoreductase (Complex I) activity was assayed by monitoring the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm upon addition of assay buffer (10× buffer containing 0.5 M Tris–HCl, pH 8.1, 1% BSA, 10 μM antimycin A, 2 mM NaN₃, 0.5 mM coenzyme Q₁₀) (Trounce et al. 1996). The final concentration of mitochondrial protein was 25 μg/ml. The reaction was started by adding 200 μM NADH and scanned at 600 nm for 2 min. Rotenone (3 μM) was added to the reaction system as blank control.

Assays of succinate-CoQ oxidoreductase (complex II), CoQ–cytochrome c reductase (complex III), and cytochrome c oxidase (complex IV) were performed as described (Calon et al. 2004; Humphries and Szweida 1998; Picklo et al. 1999). Briefly, complex II was assayed in assay buffer (10× buffer containing 0.5 M phosphate buffer, pH 7.8, 1% BSA, 1 mM antimycin A, 2 mM NaN₃, 0.5 mM coenzyme Q₁₀) with mitochondria (final concentration 25 μg/ml). The reaction was started with 10 mM succinate and scanned at 600 nm for 2 min at 30 °C. Complex III was assayed by monitoring the reduction of cytochrome c at 550 nm upon the addition of assay buffer (10× buffer containing 0.5 M Tris–HCl, pH 7.8, 2 mM NaN₃, 0.8% Tween-20, 1% BSA, 2 mM decylubiquinol) with mitochondria (final concentration 10 μg/ml) and 40 μM cytochrome c. The reaction was started by addition of 1× assay buffer and scanned at 550 nm for 2 min. For the analysis of complex IV, assay buffer contained 50 mM phosphate buffer, pH 7.0, 0.1% BSA, 0.2% Tween-20, and 40 μM reduced cytochrome c. The reaction was initiated by addition of 3 μg/ml mitochondria and scanned at 550 nm for 2 min.

Complex V activity was measured as oligomycin-sensitive, Mg²⁺-ATPase activity (Picklo and Montine 2001). The process was performed by measuring the increase of NADPH at 340 nm upon the addition of 10 mM HEPES, pH 8.0, 20 mM succinate, 20 mM glucose, 3 mM MgCl₂, 11 mM AMP, 0.75 mM NADP⁺, 10 mM K₂HPO₄, 4 U/ml hexokinase, 1 mM ADP. All assays were performed at 30 °C.

Reactive oxygen species (ROS) assay

ROS generation was detected using dichlorodihydrofluorescein diacetate (H₂DCF-DA) (Royall and Ischiropoulos 1993). Fluorescence was determined with a fluorescence spectrometer (FlexStationII 384, Molecular Devices) at 488 nm (excitation) and 525 nm (emission).

MDA assay

MDA levels were measured by the thiobarbituric acid method (Liu et al. 1997) using a kit (Jiancheng Biochemical Inc, Nanjing, China).

GSH assay

Reduced glutathione (GSH) content was determined in mitochondria using the commercially available Glutathione (GSH) Detection Kit (Jiancheng Biochemical Inc., Nanjing, China) using an assay based on the reaction with the thiol-specific reagent dithionitrobenzoic acid. The adduct was measured spectrophotometrically at 412 nm with a spectrophotometer (Hao et al. 2008).

GST activity assay

GST activity was measured with a spectrometric method as previously described (Fabst et al. 1974). The reaction mixture contained 5 mg mitochondrial protein, 1 mM GSH, 1 mM chloro-2,4-dinitrobenzene, and 3 mg/ml BSA in 10 mM sodium phosphate buffer. The mixture was scanned at 340 nm for 5 min at 25 °C.

NQO-1 activity assay

NQO-1 activity was measured as previously described (Jaiswal 2000) with minor modifications. The reaction mixture (200 μl final volume) consisted of 25 mmol/L Tris–HCl (pH 7.4), 80 μmol/L 2,6-dichlorophenolindophenol (DCPIP), 0.2 mg/ml BSA and 0.01% (v/v) Tween-20, with or without 10 μmol/L dicoumarol, and 10 μg mitochondrial protein. The reaction was started by the addition of 180 μmol/L NADPH. Reduction of DCPIP was measured at room temperature for 1–2 min at 600 nm (ε = 21 × 10³ M⁻¹ cm⁻¹). NQO-1 activity was considered to be the dicoumarol-inhibitable part of the DCPIP reduction.

GPx assay

Glutathione peroxidase (GPx) activity was measured by the method described by Wendel (Wendel 1981). The reaction mixture contained 0.25 M potassium phosphate buffer (pH 7.0; 0.4 ml), liver mitochondria (1 mg protein in 0.2 ml), 10 mM GSH (0.1 ml), 2.5 mM NADPH (0.1 ml), and glutathione reductase (6 U/ml, 0.1 ml). The reaction was started by adding 12 mM hydrogen peroxide (0.1 ml). The absorbance was measured at 366 nm at 1 min intervals for 5 min using a molar extinction coefficient of 6.22 × 10³ M⁻¹ cm⁻¹.

Results

Mitochondrial complex activities are the most commonly assayed parameters of mitochondrial function. Activities of all five complexes of the electron transfer chain in rat livers were measured. As shown in Fig. 1, exercise induced an increase in activities of complexes I, IV and V, but there was no change in complex II and III activities. Although the changes in complexes I and IV were marginal, the increase in complex V was very prominent, about two fold. The mitochondrial nutrient treatment had no effect on the unchanged activities of complexes II and III (Fig. 1).

Mitochondrial ROS was then measured, since it is a possible cause of inactivation of the mitochondrial complexes. As shown in Fig. 2, endurance exercise did not affect ROS levels compared with the control group. Nevertheless, nutrient treatment caused a small but significant decrease in ROS levels in liver mitochondria.

MDA has been the most widely used parameter for evaluating oxidative damage to lipids, although it is known that oxidative damage to amino acids, proteins and DNA also release MDA. Therefore, MDA is a marker of oxidative damage to macromolecules.

![Fig. 1. Activities of mitochondrial complexes I, II, III, IV, and V in liver mitochondria of rats after four weeks of endurance exercise on the treadmill. Values are means ± SEM of 12 rats in the sedentary group, 9 in the EE group, and 11 in the EE + Nutrients group.](image-url)
MDA levels in liver mitochondria and tissue homogenate were assayed. Endurance exercise caused no change in MDA in the mitochondria but did cause a significant increase in the liver tissue homogenate (Fig. 3). Nutrient treatment did not have a significant effect on MDA levels either in the mitochondria or in the tissue homogenate (Fig. 3).

GSH is one of the most important endogenous antioxidants. GSH levels in both isolated mitochondria and tissue homogenate were measured. Endurance exercise caused a significant increase in GSH in the mitochondria but no change in liver tissue homogenate (Fig. 4). Nutrient treatment did not have any effect on GSH levels either in the mitochondria or in tissue homogenate (Fig. 4).

NQO-1, GST, and GPx are important antioxidant enzymes. Endurance exercise caused a significant increase in the activity of GST and NQO-1, but no change in the activity of GPx (Fig. 5). Nutrient treatment restored NQO-1 activity to the control level, but had no effect on the activities of GST or GPx (Fig. 5).

**Discussion**

There are various types of exercise and each type of exercise, whether acute or endurance, impacts aerobic capacity differently. Acute exercise is a single bout of exercise, while endurance exercise consists of performing low- to medium-intensity exercise for long periods of time. It has been shown that endurance exercise training promotes mitochondrial biogenesis in skeletal muscle and enhances muscle oxidative capacity (Freyssenet et al. 1996; Wu et al. 2002). In the present study the effects of endurance exercise on liver mitochondria were studied. Endurance exercise was found to cause mitochondrial stress by activating complexes I, IV and V. The increase in activities of these complexes might be a compensatory mechanism for coping with the enhanced production of ROS during endurance exercise. It often has been seen that endurance exercise results in a large increase in enzyme activities and this activation can be viewed as a defensive mechanism of the cell in the face of an increased oxidative challenge (Ji 1995b). For example, Somani and colleagues (Somani et al. 1995) found that acute exercise caused increases in mitochondrial superoxide dismutase, catalase, and glutathione peroxidase in rat heart subcellular fractions. Levels of antioxidant enzymes in moderate–mild exercise are also increased as a defense against oxidative damage (Vina et al. 2006). Treatment with nutrients enhanced this stress response by further increasing the activities of complexes I and IV, but it inhibited complex V. The varying effects of nutrient treatment on different complexes needs to be studied further, but these results clearly suggest these nutrients may target mitochondria so as to enhance antioxidant defenses and reduce ROS generation and oxidative stress.

GSH, one of the most important endogenous antioxidants, exists in both the cytosol and mitochondria. Liver is the major organ for de novo GSH synthesis; it supplies 90% of the circulating GSH and exports GSH into plasma during prolonged exercise. It has been reported that
In conclusion, the effects of a four-week long endurance exercise regimen on mitochondrial and oxidative stress in rat liver and the effects of a combination of mitochondrial targeting nutrients were investigated. The results showed clearly that long-term endurance exercise generally increased oxidative and mitochondrial stress in the liver, and that treatment with the combination of mitochondrial targeting nutrients caused amelioration of complex V and NQO-1 activities and enhancement of activities of complexes I and IV, but had no effect on other parameters. These results suggest that endurance exercise-induced oxidative and mitochondrial stress may be either damaging by causing injury or beneficial by activating defense systems. Although the combination of mitochondrial nutrients improves running endurance and mitochondrial biogenesis and function in muscle (Sun et al., unpublished), the present results suggest that supplementation with mitochondrial nutrients may have limited impact on the liver, possibly due to a more active metabolism and stronger recovery in liver than in muscle.

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References


