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Endurance training increases the expression of mitochondrial and nuclear encoded cytochrome c oxidase subunits and heat shock proteins in rat skeletal muscle

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**Abstract** Cytochrome c oxidase (CCO) is an enzyme complex found on the inner mitochondrial membrane and serves as the final electron acceptor in mitochondrial electron transport. Heat shock proteins (HSPs) are involved in the import of nuclear encoded protein subunits into the mitochondria and induce conformational changes to form active enzyme complexes. As both the nuclear and mitochondrial encoded subunits of CCO have been shown to increase in activity and expression in muscle subsequent to artificial loading, and as exercise has been shown to induce HSPs, we sought to determine whether 16–20 weeks of treadmill exercise would result in enhanced CCO subunit expression, and to determine if there was a relationship between this expression and HSP content in medial gastrocnemius muscle of Fischer 344 rats. Our results indicated that endurance training resulted in a 53%, 87% and 80% increase (P < 0.05) in the levels of HSP 60, CCO subunit II and CCO subunit VI, respectively. Enzymatic activity of CCO was 84% greater (P < 0.05) after endurance training. Mann Whitney U analyses showed that CCO subunit II and VI increased to the same extent as HSP 60 after endurance training. It appears that 16–20 weeks of endurance training leads to uniform increases in CCO subunits and parts of the transport and assembly mechanisms required for CCO enzyme assembly. The similarity among the increases in CCO subunits II and VI protein levels and the increase in CCO enzyme activity suggest that this increase in activity is due to an increase in the amount of CCO enzyme.

**Key words** Exercise · Heat shock protein · Cytochrome c oxidase

**Introduction**

The heat shock or stress response has been identified in all organisms studied to date. This condition was first described as puffs on salivary gland DNA of Drosophila bruchii after heat shock (Ritossa 1962) and this phenomenon has subsequently been revealed to include the rapid expression of a number of proteins, later termed, heat shock proteins (HSPs; Tissières et al. 1974). Enhanced expression of HSPs has been associated with an assortment of physiological stresses ranging from the classically described heat shock to amino acid analogue treatment and more recently, exercise training (Locke et al. 1990, 1995; Morimoto et al. 1994).

Previous data indicate that pre-treatment of Chinese hamster fibroblasts with a non-lethal thermal shock protects cells when cultured at otherwise lethal temperatures and this has been attributed to an increase in HSP expression (Li and Hahn 1978). Conversely, cell death has been associated with a lack of an appropriate heat shock response (Johnston and Kucey 1988; Riabowol et al. 1988). These studies have shown that antibodies raised against HSP 70, in addition to competitive inhibition of the HSP 70 promoter, render cultured cells intolerant to thermal stress, which increases the likelihood of damage by heat shock.

In addition to the classic heat shock response, HSP function in a variety of routine cellular processes. Under normal conditions, HSP facilitate conformational changes and stabilisation of intermediate species during protein assembly and degradation, which have been shown to be essential for cell viability (Ellis and Hemmingsen 1989; Gething and Sanbrook 1992). HSP 60 and HSP 75 are stress-induced proteins and have been
shown to be involved in the trafficking and processing of nuclear encoded peptides within the mitochondria (Kang et al. 1990; Martin et al. 1992).

In mammalian tissues, the cytochrome c oxidase (CCO) complex [European Commission no. (EC) 1.9.3.1] has been described as being composed of at least 13 distinct subunits (Hood 1990). Subunits I through III have been described as being encoded by mitochondrial DNA and the remaining subunits by nuclear DNA (Hood 1990). Catalytic properties, such as electron transport and protein translocation, have been associated with the mitochondrial subunits, whereas regulatory functions have been associated with nuclear-encoded subunits (Kadenbach and Merle 1981).

Nuclear encoded precursor polypeptides are required for the assembly of most mitochondrial proteins. It is therefore plausible that HSPs provide the link between protein translation, transport and functional structure within the mitochondria. Specifically, an increase in HSP levels may facilitate an increase in CCO subunit levels and hence CCO activity.

In support of this, it has been shown that the physiological stress of exercise (Locke et al. 1990, 1995; Salo et al. 1991) and exercise-like activities (Neuf er et al. 1996, Ornatsky et al. 1995) acutely elevate HSP expression. Locke et al. (1990, 1995) have shown that rodent skeletal and cardiac muscle express increased amounts of HSP 70 following treadmill runs. The level of HSP 60 in rodent muscle has also been shown to increase following both acute exercise and chronic contractile activity (Locke et al. 1990; Ornatsky et al. 1995).

It is well established that endurance training results in increased activity of a number of oxidative enzymes (Brooks and Fahey 1985). Chronic stimulation of rodent skeletal muscle has been found to increase the oxidative activity of CCO and cause a concomitant increase in HSP 60 and 75 (Ornatsky et al. 1995). However, these increases were considered to be independent of one another. Some, if not all, of the noted increase in CCO activity may be due to an increase in the levels of this protein. Indeed, Akiyama et al. (1994) have demonstrated increased CCO subunit expression following chronic loading in sheep diaphragm muscle.

Previous studies have shown that acute exercise increases the expression of HSP (Locke et al. 1990, 1995; Salo et al. 1991) and increased CCO subunit expression after chronic loading (Akiyama et al. 1994); however, the effects of endurance exercise on these parameters are, for the most part, unknown. Therefore, the purposes of the present study were to determine the effect of 16–20 weeks of treadmill running on:

1. The expression of CCO subunit II and VI and HSPs 60, 70, and 75 in the medial gastrocnemius (MG) muscle
2. CCO activity in the MG muscle after endurance training and
3. To describe the relationship between the different HSPs and CCO subunits II and VI.

**Methods**

**Animals and exercise**

Sixteen 10-month-old male Fischer 344 rats were equally and randomly assigned to either control (CON) or exercised (EX) groups. The EX rats were trained five times per week for 16–20 weeks on a motorised treadmill. Training sessions began with running at 10 m · min⁻¹ at 0% gradient for 10 min and progressed to 15 m · min⁻¹ at a 10% gradient for 1 h at the end of the training period. The rats were sacrificed 48 h after the last exercise session and the MG muscles were removed, immediately frozen in liquid nitrogen, and stored at ~80°C for further analysis.

**CCO activity (EC 1.9.3.1)**

The CCO activity in MG muscles was measured using a procedure modified from Wharton and Tzagoloff (1967). Briefly, muscle samples were homogenised in five volumes of 0.1% Triton-X 100 in 0.1 mol · l⁻¹ TRIS buffer; pH 8.35. Cytochrome c was reduced with l-ascorbic acid and dialysed in three changes of 0.1 mol · l⁻¹ sodium phosphate buffer; pH 7.0. Photopulscrometer readings were obtained every 4 s for 2 min at 550 nm and 30°C. The CCO activity was determined from the linear portion of the slope and presented as micromoles oxidised cytochrome c per minute per milligram.

**Electrophoresis and Western blotting – HSP**

Muscle samples from the enzyme activity assay were mixed with equal volumes of dissociation buffer (0.1 mol · l⁻¹ TRIS-HCl, 5% sodium dodecylsulfate, SDS, 8 mol · l⁻¹ urea, 1% β-mercaptoethanol, and 1% glycerol; pH 6.2) and incubated at 37°C for 45 min. A small quantity (5 µl) of dye solution containing 0.5% methyl green in 30% sucrose was added to each sample to provide a visual indication of migration. Samples (20 µl per lane) were loaded on to an 11% SDS-PAGE gel as previously described (Laemmli 1970). Gels were processed at 150 V for 1.25 h and transferred to 10% nitrocellulose membranes (Bio-Rad trans blot cell, Richmond, Calif., USA).

Membranes were blocked overnight using tris-buffered saline (TBS) (20 mmol · l⁻¹ TRIS-HCl, 150 mmol · l⁻¹ NaCl; pH 7.6) containing 0.5% bovine serum albumin and immunoblotted to quantify HSP 60, HSP 70 and HSP 75 (Sigma, St. Louis, Mo., and Stressgen, British Columbia, Canada). Blots were developed in nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) according to the manufacturers recommendations (Promega, Madison, Wis., USA). Relative optical densities were determined from digital photographs with a Bio Image Advanced Quantifier (B.I. Systems, Ann Arbor, Mich., USA).

**Electrophoresis and Western blotting – CCO**

Immunoblotting for CCO followed the procedure described by Estey et al. (1990). Briefly, MG samples and CCO standard were prepared as described above. Samples were electrophoresed at 10 mA · per gel for 3 h through a stacking gel composed of 5% acrylamide and 8 mol · l⁻¹ urea above a running gel composed of 16% acrylamide and 8 mol · l⁻¹ urea. Proteins were subsequently blotted to nitrocellulose (Bio-Rad Trans blot cell) and were blocked in TBS-milk buffer (50 mmol · l⁻¹ TRIS, 5% non-fat dry milk, and 170 mmol · l⁻¹ NaCl; pH 7.5) for 3 h with slow shaking. Membranes were then incubated in fresh buffer containing primary polyclonal antibody for bovine CCO (anti-CCO was a gift from D.M. Medeiros and provided by L. Prochaska). Blots were developed and relative optical densities were determined in the same manner as the HSP blots.