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Chemico-Biological Interactions 148 (2004) 11–18

Chemico-Biological Interactions

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Age-associated decreased activities of mitochondrial electron transport chain complexes in heart and skeletal muscle: role of L-carnitine

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Accepted 18 October 2003

Abstract

The mitochondrial respiratory chain is a powerful source of reactive oxygen species (ROS), considered as the pathogenic agent of many diseases and aging. L-Carnitine (4-*N*-trimethylammonium-3-hydroxybutric acid) plays an important role in transport of fatty acid from cytoplasm to mitochondria for energy production. Previous studies in our laboratory reported L-carnitine as a free radical scavenger in aged rats. In the present study we focused the effect of L-carnitine on the activities of electron transport chain in young and aged rats. The activities of electron transport chain complexes were found to be significantly decreased in aged rats when compared to young control rats. Supplementation of carnitine to young and aged rats for 14 and 21 days improved the electron transport chain complexes levels in aged rats when compared with young rats in duration dependent manner. No significant changes were observed in young rats. Our result suggested that L-carnitine improved the activities of electron transport chain enzymes there by improving the energy status in aged rats.
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Keywords: Aging; Mitochondria; Electron transport chain; Carnitine

1. Introduction

Aging is characterized by a general decline in physiological functions that affects many tissues and increases the risk of death. The role of mitochondria in the process of the age-dependent deterioration of tissues has become the focus of many studies with the gradually accepted idea that mitochondrial decay is a major contributor to aging [1]. The age-dependent changes in mitochondria are characterized by a high rate of generation of oxidants, a decline in the activity of electron transport complexes, a decrease in amount and fatty acid composition [2].

The free radical theory of aging was based on the idea that cells, continuously exposed to reactive

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0009-2797/$ – see front matter © 2004 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.cbi.2003.10.010

oxygen species (ROS), are progressively damaged in their most vital macromolecules [3]. The implication of mitochondria both as producers and as targets of ROS has been the basis for the mitochondrial theory of aging; the theory postulates that random alterations of mitochondrial DNA in somatic cells are responsible for the energetic decline accompanying senescence [4]. It was proposed that accumulation of somatic mutations of mtDNA, induced by exposure to ROS, leads to errors in the mtDNA-encoded polypeptides; these errors are stochastic and randomly transmitted during mitochondrial division and cell division. The consequence of these alterations, which affects exclusively the four-mitochondrial complexes involved in energy conservation, would be defective electron transfer and oxidative phosphorylation. Respiratory chain defects may become associated with increased ROS production, thus, establishing vicious circle [5].

Two tissues that are particularly prone to oxidative damage are muscle and the central nervous system. Both tissues contain post mitotic cells, which are liable to accumulate oxidative damage over time and both account for a large share of the body's total oxygen consumption at rest [6].

L-Carnitine has been described as a conditionally essential nutrient for humans. L-Carnitine is a betaine required for the transport of long chain fatty acids into the mitochondria for fuel. It also facilitates the removal from the mitochondria of excess short and medium chain fatty acids that accumulate during metabolism [7]. L-Carnitine and its acetyl derivative, acetyl L-carnitine, affects other cellular functions, including maintenance of key proteins and lipids of the mitochondria at sufficient levels, proper membrane orientation and maximum energy production [8]. L-Carnitine is present in high concentration in brain as well as muscle and provides acetyl-equivalents for the production of the neurotransmitter acetyl choline [9]. Experimental data have demonstrated an age-associated decrease in L-carnitine at tissue levels of animals, including humans and an associated decrease in the integrity of the mitochondrial membrane [10]. Hence, the present study was designed to evaluate the effect of carnitine supplementation on the status of electron transport chain activity in heart and skeletal muscle mitochondria of aged rats.

2. Materials and methods

2.1. Source of chemicals

L-Carnitine, bovine serum albumin and all substrates and inhibitors were purchased from Sigma Chemical Company (St. Louis, MO, USA).

All other chemicals used were of analytical grade and were obtained from Glaxo Laboratories, CDH division, Mumbai, India and Sarabhai M. Chemicals, Baroda, India.

2.2. Animals

Male Albino rats of *Wistar* strain were used in this study. They were healthy animals maintained and bred for more than two decades at King's Institute of Preventive Medicine, Chennai. The animals were housed in large spacious cages and were given food and water ad libitum. The animal room was well ventilated with a 12 h light: 12 h dark cycle, throughout the experimental period.

2.3. Grouping of animals

The animals were divided into two major groups, such as

Group I: Young rats (3–4 months old weighing approximately 130–150 g),
Group II: Aged rats (above 24 months old weighing about 380–410 g).

These groups were further sub-divided into three groups: one control group (Groups Ia, IIa) and two experimental groups based on the duration of carnitine administration for 14 days (Groups Ib, IIb), and 21 days (Groups Ic, IIc). Each group consisted of six animals.

Group Ia: Control young rats,
Group Ib: Young rats (L-carnitine administration for 14 days),
Group Ic: Young rats (L-carnitine administration for 21 days),
Group IIa: Control aged rats,
Group IIb: Aged rats (L-carnitine administration for14 days),
Group IIc: Aged rats (L-carnitine administration for 21 days).

L-Carnitine (300 mg/kg body weight per day) was dissolved in 0.89% physiological saline and administrated intraperitoneally. Control animals received physiological saline alone. On completion of experimental period, animals were killed by cervical decapitation. Heart and skeletal muscle was excised immediately and immersed in physiological saline.

2.4. Isolation of mitochondria

Heart mitochondria were isolated by the method of Takasawa et al. [11]. The heart tissue was put into ice cold 50 mM Tris–HCl (pH 7.4) containing 0.25 M sucrose and homogenized. The homogenates were centrifuged at 700 × *g* for 20 min, and then the supernatants obtained were centrifuged at 9000 × *g* for 15 min. Then the pellets were washed with 10 mM Tris–HCl (pH 7.8) containing 0.25 M sucrose and finally resuspended in the same buffer.

Skeletal muscle mitochondria were isolated by the method of Ernster and Nordenbrand [12]. A 10% tissue homogenate was prepared in Chappel-Perry medium (0.1 M KCl; 0.05 M Tris–HCl pH 7.4; 0.001 M Na-ATP; 0.005 M $MgSO_4$, and 0.001 M EDTA) and then centrifuged at 600 × *g* for 5–10 min. The resulting supernatant was again subjected to centrifugation at 14,000 × *g* for 10 min. The resulting mitochondrial pellet was then rinsed in 0.15 M KCl and finally suspended in 0.15 M KCl. The purity of mitochondria was assessed by the assay of specific marker enzyme; succinate dehydrogenase was assayed by the method of Slater and Bonner [13]. Mitochondrial protein was estimated by the method of Lowry et al. [14].

2.5. Measurement of electron transport chain activity

2.5.1. Activity assays for complex I and II

All assays were performed at 30 °C with a Shimadzu UV-1601 spectrophotometer. Complex **I** and **II** specific activities were assayed as described by Brich-Machin et al. [15] with slight modifications. Mitochondrial samples were subjected to three cycles of fast freeze–thaw in hypotonic buffer (20 mM potassium buffer, pH 7.2) before the assay. For the complex **I** assay, the assay mixtures containing 25 mM potassium phosphate buffer (pH 7.2), 5 mM $MgCl_2$, 2 mM KCN, 2.5 mg/ml bovine serum albumin, 0.13 mM NADH, 65 μM coenzyme Q_1 and 2 μg/ml antimycin A were incubated at 30 °C for 1 min. Mitochondria were added to initiate the reaction and the initial rate of NADH oxidation was monitored at 340 nm (ε 6.81 $mM^{-1}\,cm^{-1}$) for 1 min. The complex **I** specific activity was inhibited by 2 μg/ml of rotenone. For the complex **II** assay, mitochondria were preincubated in the medium containing 25 mM potassium phosphate buffer (pH 7.2), 5 mM $MgCl_2$ and 20 mM succinate for 10 min at 30 °C. Antimycin A (2 μg/ml), rotenone (2 μg/ml), KCN (2 mM) and 2,6-dichlorophenol (50 mM) were added and further incubated for 1 min. The reaction was initiated with coenzyme Q_1 (65 μM) and the enzyme-catalyzed reduction of 2,6-dichlorophenolindophenol was recorded for 3 min at 600 nm (ε 19.1 $mM^{-1}\,cm^{-1}$).

2.5.2. Activity assay for complex III

The assay procedure for complex **III** specific activity was modified from Brich-Machin et al. [15] and Ragan et al. [16]. Complex **III** specific activity was determined by measuring the rate of reduction of cytochrome *c* (**III**) by the reduced form of coenzyme Q_2 (ubiquinol) at 550 nm (ε 20 $mM^{-1}\,cm^{-1}$). Ubiquinol was prepared as described by Ragan et al. [16]. Ten milligrams of coenzyme Q_2 was reduced with sodium borohydride, extracted with ethar/cyclohexane mixtures, dried with nitrogen gas and dissolved in 1.6 ml of absolute ethanol as the stock for ubiquinol. Mitochondria were added to the assay medium containing 25 mM potassium phosphate buffer (pH 7.2), 5 mM $MgCl_2$, 2.5 mg/ml bovine serum albumin, 2 mM KCN, 0.6 mM dodeacyl-β-D-maltoside and 50 μM cytochrome *c* (**III**) and incubated for 1 min at 30 °C. The reaction was initiated by 3 μl of ubiquinol stock solution and the initial rate at 550 nm was monitored for 1 min. The nonenzymatic reduction of cytochrome *c* was recorded in the same reaction mixtures without the sample and was subtracted. The complex **III** specific activity was inhibited by 2 μg/ml of antimycin A.

2.5.3. Activity assay for complex IV

The specific activity of cytochrome *c* oxidase (complex **IV**) was determined by a modification of the method of Wharton and Tzagoloff [17]. To prepare ferrocytochrome *c*, 1% ferricytochrome *c* was reduced completely by dithionate and excess dithionate was removed by passing the solution through of Sephadex

G-25. Potassium phosphate buffer (2.67 ml; 50 mM, pH 7.0) and 30 μl 10% Triton X-100 were added to 200 μl ferrocytochrome *c* solution. Immediately after the addition of 100 μl mitochondrial suspension (0.5 mg/ml), the reaction was followed for 15 s by recording the rate of decrease in absorbance at 550 nm.

2.6. Statistical analysis

Values are mean ± S.D. for six rats in each group, and significance of the differences between mean values were determined by one-way analysis of variance (ANOVA) followed by the Duncan test for multiple comparison. Values of $P < 0.05$ were considered to be significant.

3. Results

Fig. 1 represents the activity of complex **I** in young and aged rats of heart and skeletal muscle mitochondria before and after supplementation of L-carnitine. The activity of complex **I** was significantly decreased in heart and skeletal muscle mitochondria of aged rats. The decrease being 42% in heart 43% in skeletal muscle. After supplementation of L-carnitine for 14 and 21 days to aged rats the activity of complex **I** enzyme was found to be increased in heart and skeletal muscle compared to young control rats. A significant increase of complex **I** was observed in 21 days supplementation of carnitine. The increase being 73% in heart and 69% in skeletal muscle. There was no significant change being observed in young rats supplemented with carnitine.

Figs. 2 and 3 shows the activities of complex **II** and **III** in heart and skeletal muscle mitochondria of young and aged rats. The activities of these enzymes were found to decrease in heart and skeletal muscle mitochondria of aged rats when compared with young control rats. The decrease being 22% in heart and 34% in skeletal muscle for complex **II** and 32% heart and 28% in skeletal muscle for complex **III**. After supplementation of L-carnitine to aged rats the activities of complex **II** and III were reverted to near activities of young control rats. The increase was dose dependent on carnitine supplementation. A significant increase of these enzyme activities were observed on 21 days supplementation of L-carnitine.

Fig. 4 represents the activity of complex **IV** in young and aged rats with supplementation of carnitine for 14 and 21 days. The activity of complex **IV** was found to be significantly decreased in aged rats. The decrease being 31% in heart and 35% in skeletal muscle. Supplementation of carnitine to aged rats the activity of complex **IV** was significantly increased when compared with aged control rats. The increase being 29% in heart 23% in skeletal muscle for 14 days supplementation of carnitine and 42% in heart and 50% in skeletal muscle for 21 days supplementation

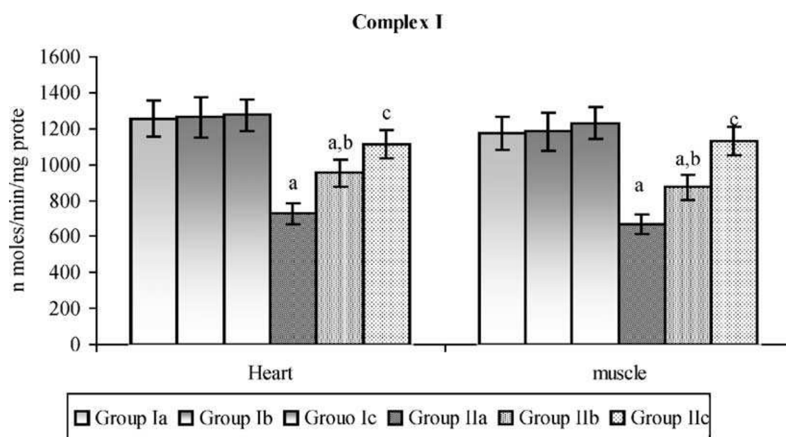

Fig. 1. Specific activity of mitochondrial complex **I** (NADH-coenzyme Q oxidoreductase) from heart and skeletal muscle of young rats (3–4 months old) and aged rats (above 24 months old). Bars represent mean ± S.D. $P < 0.05$. (a) Compared with Group Ia, (b and c) compared with Group IIa.

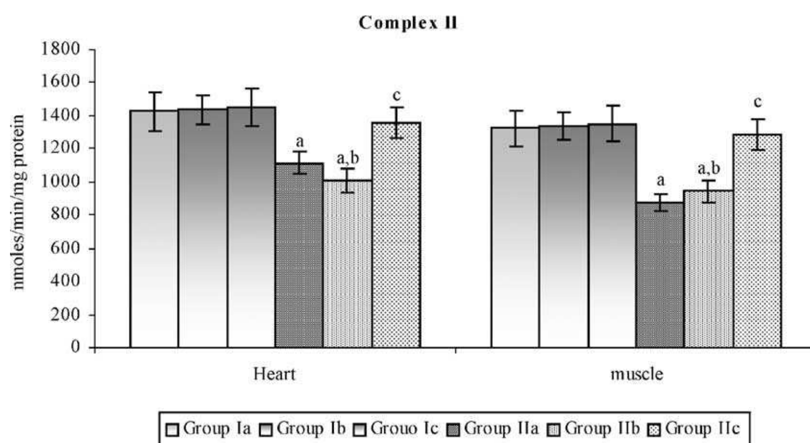

Fig. 2. Specific activity of mitochondrial complex **II** (succinate dehydrogenase-coenzyme Q oxidoreductase) from heart and skeletal muscle of young rats (3–4 months old) and aged rats (above 24 months old). Bars represent mean ± S.D. $P < 0.05$. (a) Compared with Group Ia, (b and c) compared with Group IIa.

of carnitine. There was no significant a change was observed in young rats supplemented with carnitine.

4. Discussion

The mitochondrial respiratory chain is the major source of superoxide, and therefore, mitochondria accumulate oxidative damage more rapidly than the rest of the cell, contributing to mitochondrial dysfunction, cell death in degenerative diseases and in aging [18].

The present study shows that decrease in mitochondrial respiratory complex in heart and skeletal muscle mitochondria of aged rats when compared with young rats. Several studies suggest that oxidative damage to mitochondrial DNA may be responsible for decrease in activities of electron transport chain enzyme complex in aged rats [19] and these changes are more prevalent in post mitotic cells such as central nervous system, heart and skeletal muscle [20]. Mitochondrial DNA may be particularly susceptible to oxidative stress owing to its lack of protective histones,

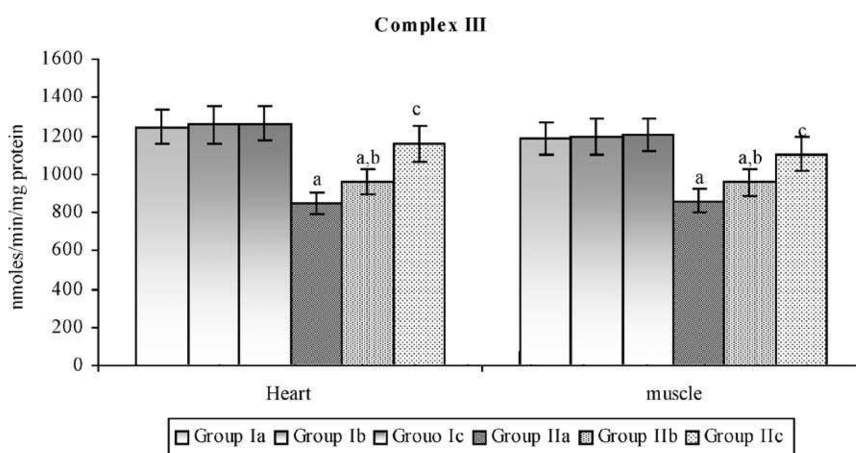

Fig. 3. Specific activity of mitochondrial complex **III** (coenzyme Q cytochrome *c* oxidoreductase) from heart and skeletal muscle of young rats (3–4 months old) and aged rats (above 24 months old). Bars represent mean ± S.D. $P < 0.05$. (a) Compared with Group Ia, (b and c) compared with Group IIa.

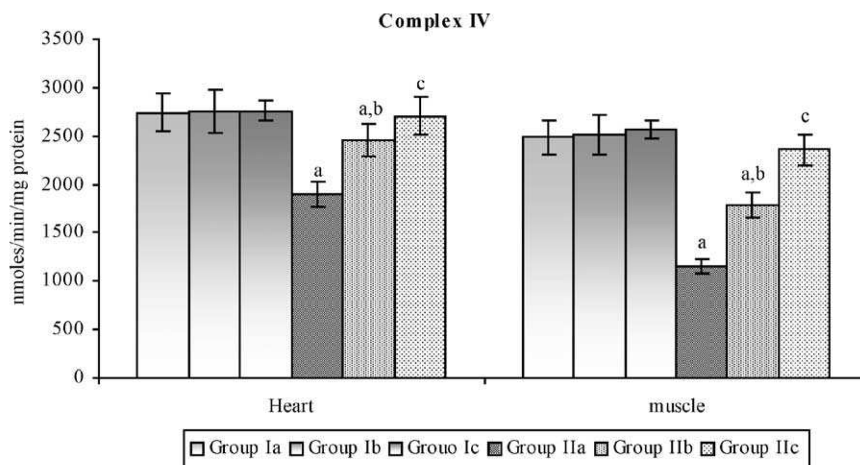

Fig. 4. Specific activity of mitochondrial complex **IV** (cytochrome *c* oxidase) from heart and skeletal muscle of young rats (3–4 months old) and aged rats (above 24 months old). Bars represent mean ± S.D. $P < 0.05$. (a) Compared with Group Ia, (b and c) compared with Group IIa.

its proximity to inner mitochondrial membrane, where reactive oxygen species are produced and its lack of DNA repairing mechanisms [21]. Moreover, mtDNA mutation is greater than that of nuclear DNA. In addition, marked increases of age-dependent deletion of mtDNA are found in post mitotic cells [22].

A progressive increase in the production of free radicals with aging has been correlated with decrease in the number of functional mitochondria per cell, as well as a decline in the production of ATP, protein synthesis and increase in peroxide leakage [23]. NADH-dehydrogenase, a flavin-linked dehydrogenase, constitutes complex **I** of the electron transport chain, which passes electrons from NADH to coenzyme Q. In this present study a decrease in the activity of NADH-dehydrogenase was observed in heart and skeletal muscle mitochondria of aged rats. The significant decline in the activities of this enzyme in aged rats would result in the inhibition of electron flow from NADH to oxygen. The decrease in the activity of NADH-dehydrogenase. This may be due to the depletion of reducing equivalents like NADH and NADPH, which are necessary for the formation of reduced glutathione (GSH) from oxidized glutathione [24]. Mitochondrial reduced glutathione plays a key role in the protection against the oxidative damage to mitochondrial components. Indeed, the oxidative damage to mitochondrial DNA that occurs upon aging is directly related to an oxidation of mitochondrial GSH. GSH oxidation increases with age in mitochondria from liver, kidney, and brain of rats [25]. The mtDNA is highly vulnerable to free radical damages [26] leading to decrease in the activity of the complex **I**. Supplementation of carnitine to aged rats significantly increased the activity of NADH dehydrogenase in the present study. This may be due to antioxidant effect of carnitine, which may prevent free radical mediated mtDNA damage and increases the level of GSH in aged rats.

The activity of complex **II** and **III** being decrease in heart and skeletal muscle mitochondria of aged rats when compared with young rats, but the decrease was less than that of complex **I** and **IV**. Because all the components of complex **II** and one subunit of complex **III** are encoded by nuclear DNA which appears to be more resistant to the oxidative stress [27] than mtDNA. Drouet et al. [28] have suggested that decline in complex **II** activity with aging could be secondary to a decline in the levels of active enzyme molecules per mitochondrion, or due to accumulation of altered molecules in the organelle. The decreased production of mitochondrial energy, associated with a chronic increase of oxidative stress with aging, can activate the mitochondrial permeability transition pore and initiate apoptosis [28]. It has also been proved recently that reactive oxygen species exposure inactivate the iron–sulfur centers of complex **I**, **II**, and **III** [29]. Supplementation of carnitine to aged rats increased the

activities of complex **II** and **III** of electron transport chain. The possible mechanism may be carnitine preventing DNA damage by improving glutathione levels in aged rats [30] and also prevent oxidation of iron–sulfur molecules present in complex **I**, **II**, and **III** of electron transport chain.

Cytochrome *c* oxidase is the terminal enzyme complex of the inner electron transport chain (complex **IV**) and catalyzes electron transfer from reduced cytochrome *c* to molecular oxygen. This enzyme is a vital component of cellular energy transduction responsible for virtually all oxygen consumption in mammals [31]. Cytochrome *c* oxidase activity was to be decreased in aged rats. Decline in cytochrome *c* oxidase activity can cause an increase in H_2O_2 production. It may be speculated that decline in cytochrome *c* oxidase activity results in partial blockage of electron flow, which alters reducing potentials of some electron carriers favoring their autoxidation and consequent generation of O_2^- [32]. The maximal activity of this enzyme is dependent on the levels of cardiolipin, is phospholipid present in mitochondrial membrane. Cardiolipin was particularly prone oxidative damage, which leads to loss of functional activity. The level of cardiolipin was found to be decreased in aged rats [33]. Supplementation of L-carnitine to aged rats increase the activity of cytochrome *c* oxidase. This may possible to carnitine prevents free radical generation and increases the caridiolipin levels in aged rats.

In conclusion of our study, carnitine may prevents free radical mediated mitochondrial membrane damages and increases the electron flow through the electron transport chain and thereby increasing energy production in aged rats.

Acknowledgements

The authors gratefully acknowledge Defence Research & Development Organization, Life Sciences Research Board, Ministry of Defence, Government of India, New Delhi, India for financial assistance in carrying out this work.

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