Effects of age and dietary antioxidants on cerebral electron transport chain activity

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Received 30 November 2000; received in revised form 12 February 2001; accepted 12 February 2001

Abstract

Aging is a pleiotropic process involving genetic and environmental factors. Recently it has been demonstrated that dietary constituents may affect senescence. In the present study, adult (3 month-old) mice were fed diets supplemented with ubiquinone (coenzyme Q10), α-lipoic acid, melatonin or α-tocopherol for a six-month period to determine if antioxidants may reverse or inhibit the progression of certain age-associated changes in cerebral mitochondrial electron transport chain (ETS) enzyme activities. The control consisted of a group of mice maintained on a basal diet for the same period. The activity of cytochrome c oxidase (Complex IV) increased with age but melatonin supplementation restored the activity to levels of 3 month-old animals. The activity of succinate dehydrogenase (Complex II) showed no age-related changes. However, this enzyme complex was elevated, in animals supplemented with coenzyme Q10, α-lipoic acid and α-tocopherol, above corresponding values obtained with basal diet. NADH-ubiquinone oxidoreductase (Complex I) and ubiquinol:ferricytochrome-c oxidoreductase (Complex III) activities remained unchanged. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Diet; Aging; Brain; Melatonin; Lipoic acid; Tocopherol; Ubiquinone; Coenzyme-Q10

1. Introduction

The mitochondrial, where aerobic eukaryotes generate most of their energy, has been reported to undergo significant functional changes with aging [4,13,36]. Of particular interest are the changes in activity of the enzymes constituting the mitochondrial’s electron transport system (ETS) and the role such changes may play in the progression of neurodegenerative diseases and the overall aging process. Given the brain’s high energy requirements, any decline in brain ETS enzyme activity could have a significant impact on brain function and particularly on the etiology of neurodegenerative disease. It has been shown that ETS enzymatic activity is reduced by excess levels of reactive oxygen species (ROS) [36]. Recent studies have also shown that oxidatively modified proteins are found not only in the CNS of aged subjects but also in the brains of subjects affected by a range of neurological disorders including Alzheimer’s and Parkinson’s diseases [5]. Long term dietary administration of various plant extracts has been found to retard the onset of age-related deficits in neural functioning and this has been attributed to the antioxidant properties of such extracts [18,19]. Similar remediation by melatonin, lipoic acid and α-tocopherol has been proposed [17,30,33,40].

Dietary supplementation with antioxidants capable of entering brain mitochondria and reacting with the ROS generated there, may maintain the ETS enzyme activity level by preventing ROS-induced damage to these enzymes. To the extent that maintaining high ETS enzyme activity is critical for preventing age-related decline in brain function and the occurrence of neurodegenerative diseases, antioxidants may prevent or slow the development of these conditions. In order to be effective, it may be necessary to begin supplementation at a fairly young age, before substantial loss of enzyme activity has occurred. Therefore we began feeding modified diets to mice at three months of age and subsequently examined the effects on the activity of the ETS enzymes in cerebrocortical mitochondria. The antioxidants chosen were coenzyme Q10 (ubiquinone), α-lipoic acid (thioctic acid), melatonin, and DL-α-tocopherol. We based the selection of these agents on their reported ability

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to produce changes in the CNS following systemic administration [3,6,7,31,32].

The supplemented animals were compared to two control groups: an older control group was fed unsupplemented minimal basal diet starting at 3 months of age for 6 months, and a younger control group was fed standard rodent lab chow until sacrifice at 3 months of age.

2. Materials and methods

Unless otherwise noted, all reagents were obtained from Sigma (St. Louis, MO), and optical measurements were made with a Spectronic Genesys 5 spectrophotometer.

2.1. Animals

Male B/6C3F1 mice, a hybrid between C57BL/6 and C3H (Harlan Labs, Indianapolis, IN) were housed 4–6 animals per cage at the Animal Resource Facility of the UCI College of Medicine. The animals were maintained on a 12-hour light/dark cycle in a temperature controlled (20 ± 1°C) room. Specified diets and water were provided ad libitum to 6–8 animals per group.

2.2. Diets

The minimal basal diet (#101101, Dyets Inc., Bethlehem, PA) consisted of 50% sucrose and 26% casein (w/w) as well as a minimal salt and vitamin mix including 110 ppm α-tocopheryl acetate. This was supplemented with one of the following: 0.1% DL-α-tocopheryl succinate, 0.02% coenzyme Q10 (ubiquinone), 0.165% DL-lipoic acid (thioctic acid) or 0.004% melatonin. All diets were administered to mice at 3 months of age for a further 6 months.

2.3. Preparation of the cerebrocortical synaptosomal-mitochondrial fraction

Mice were decapitated, the brains were excised quickly on ice, and the cerebrocortex was dissected out. Tissue was weighed and homogenized in 10 volumes of 0.32 M sucrose and centrifuged at 1,800 g for 10 minutes. The resulting supernatant fraction was then centrifuged at 24,000 g for 10 minutes to yield the crude cerebral mitochondrial pellet (P2). The P2 pellet was taken up in HEPES buffer to a concentration of 0.01 gEq/mL and stored at −70°C. The composition of the HEPES buffer was (mM): NaCl, 120; KCl, 2.5; NaH2PO4, 1.2; NaHCO3, 5.0; glucose, 6.0; CaCl2, 1.0; and HEPES, 10, at pH 7.4.

2.4. Complex I (NADH:ubiquinone oxidoreductase) assay

The method of Shults et al., [38] involving the reduction of ubiquinone-1 to the corresponding quinol with the concurrent oxidation of NADH, was utilized. However, the assays were run at room temperature and decylubiquinone was used in place of ubiquinone-1 because the latter has higher rotenone-insensitive activity [15]. A pH 7.4 buffer consisting of 35 mM phosphate, 1.3 mM MgCl2, 250 μM EDTA, and 0.25 mg/ml of BSA was used. 10 μl of 0.125 M KCN solution, 10 μl of 0.1 mg/ml antimycin solution, 10 μl of decylubiquinone solution, and then 50 μl of frozen/thawed P2 fraction were added to buffer. The reaction was initiated by adding 20 μl of 20 mM NADH solution; then the mixture was shaken briefly, and the absorbance was measured immediately at 340 and 400 nm wavelengths with a Beckman DU 640B spectrophotometer. The loss in absorbance at these wavelengths was measured periodically until the rate of change was nearly zero. The mixture was shaken between measurements to facilitate continual air oxidation of decylubiquinol back to the quinone; such agitation prevented a buildup of quinol and thus assured a more accurate measure of the complex I rate. By fitting an exponential function to the (340 nm–400 nm) differential absorbance points, an initial rate could be computed that was little affected by variations in induction times. The complex I rate was calculated from the computed initial rate by utilizing an NADH differential extinction of 5.4 mM−1 cm−1 and adjusting for the amount of protein present. Retonone-insensitive complex I activity was determined by following the above protocol, except that 1.6 μl of 6.4 mM rotenone solution was added just after adding the antimycin solution.

2.5. Complex II (Succinate:ubiquinone oxidoreductase) assay

Complex II was activated by maintaining 20 μl of the P2 suspension, 100 μl of 200 mM succinate, 2 μl of 0.1 mg/ml antimycin, and 737 μl of 100 mM pH 7.4 phosphate buffer at 27°C for 20 minutes. Then 8 μl 0.125 M KCN and 100 μl of 350 μM ferricytochrome c were added; finally the reaction was initiated by addition of 33 μl of 3.3 mg/ml phenazine methosulfate. The increase in absorbance of ferricytochrome c at 551 nm minus the absorbance at 520 nm was measured during the first minute. Although the differential absorbance change was small, a good signal-to-noise level was obtained (Fig. 1). Taking into account that succinate is a two electron reductant, the activity in nanomoles of succinate per minute per mg of protein was determined from these absorbance data, utilizing a measured cytochrome c differential extinction of 9.0 mM−1 cm−1 [1].

2.6. Complex III (Ubiquinol:ferricytochrome-c oxidoreductase) assay

16 μl of 0.125 M KCN solution, 200 μl of 350 μM cytochrome c solution, then 50 μl of frozen/thawed P2 suspension were added to water for a final volume of 2.0 ml. 20 μl of decylubiquinol solution were then added to initiate the reaction. The reaction was followed by measuring the time-course increase in ferricytochrome c absorbance at

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**References:**

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550 nm. Complex III activity was computed as the initial reaction rate per mg of protein [12].

Decylubiquinol was prepared by shaking a suspension of 50 ml of alcoholic decylubiquinone (25 mg/ml in absolute ethanol), 200 ml of n-hexane, and 1 ml of an aqueous solution of 0.25 M sucrose in 0.1 M phosphate pH 7.4 buffer with a few milligrams of sodium dithionite powder. The hexane phase was separated, the hexane was removed under vacuum, and the decylubiquinol was dissolved in 50 ml ethanol and stored at 20°C in the dark prior to use [35].

2.7. Complex IV (Cytochrome-c oxidase) assay

20 µl of ferrocytochrome-c solution were added to 10 mM pH 7.4 phosphate buffer to a final volume of 1.0 ml. Then 10 µl of P2 suspension were added to initiate the reaction. The reaction was followed by measuring the time-course of the absorbance difference: 550 nm–540 nm for 30 seconds at room temperature. The complex IV activity was computed as nanomoles of cytochrome c per minute per mg of protein, utilizing a differential cytochrome c extinction of 15 mM cm⁻¹.

Ferrocytochrome-c was prepared by adding an excess of ascorbic acid to cytochrome-c and purified by using a Sephadex G-25 column. The solution was then stored at −70°C in nitrogen-purged vials [42].

2.8. Protein estimation

Total protein in the mitochondrial P2 fractions was estimated by Bradford’s method [9] with a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

2.9. Statistical analyses

Differences between groups were assessed by one-way Analysis of Variance followed by Fisher’s Least Significant Difference Test. The acceptance level of significance was p < 0.05 using a two-tailed distribution.

3. Results

3.1. Complex I

There were no significant differences among the cortical complex I activities of any of the groups (Table 1).

3.2. Complex II

There was no significant change between the younger and older control complex II activities. However, activities of mice supplemented with coenzyme Q₁₀, lipoic acid, and α-tocopherol were significantly higher than the level of the older mice receiving basal diet. The activity of the melatonin-supplemented mice was also higher, but this was not significant (Fig. 2).

3.3. Complex III

No significant differences were found among the complex III activities of the older control or any of the supplemented mice groups (Table 1).

3.4. Complex IV

The complex IV activity of the older mice, as judged by the rate of cytochrome c oxidation, was considerably higher than that of the younger mice (Fig. 3). Dietary exposure to

| Age at sacrifice (mo) | Diet         | Complex I | Complex II | Complex III | Complex IV |
|-----------------------|--------------|-----------|------------|-------------|------------|
| Younger (3)           | Basal        | 183 ± 22  | 52 ± 5     | 850 ± 139   | *237 ± 31  |
| Older (9)             | Basal        | 221 ± 18  | 45 ± 3     | 675 ± 78    | 383 ± 44   |
| Older (9)             | Ubiquinone   | 207 ± 33  | *63 ± 8    | 690 ± 102   | 435 ± 71   |
| Older (9)             | Lipoate      | 233 ± 40  | *58 ± 7    | 692 ± 88    | 346 ± 42   |
| Older (9)             | Tocopherol   | 231 ± 22  | *59 ± 9    | 656 ± 86    | 434 ± 40   |
| Older (9)             | Melatonin    | 249 ± 8   | 52 ± 6     | 767 ± 117   | *195 ± 25  |

Activities are in units of nmol/min/mg mitochondrial protein ± SE (n = 6).

*: Differs significantly from corresponding value for older animals fed basal diet.
coenzyme Q₁₀, lipoic acid, and α-tocopherol did not modify the complex IV activities of older mice. However, the complex IV activity of the melatonin-supplemented mice was restored from levels consonant with those of older controls, to values statistically indistinguishable from younger mice.

4. Discussion

In order to have a direct effect on ETS enzyme activity within the brain, an orally-administered substance must enter the circulatory system, cross the blood-brain barrier, and be transported into the mitochondrial inner membrane. Several reports show that the penetrance of coenzyme Q into the rodent brain is very marginal [23,26]. In contrast, one study showed that feeding 12 month-old rats 200 mg/kg/day of coenzyme Q for 2 months increases brain mitochondrial content of this cofactor by over 50% [28]. Regardless of whether measurable increases of coenzyme Q are produced by supplementation, sufficient increments appear to be achieved for physiological action within the CNS. In Huntington’s disease patients, increased occipital cortex lactate levels are an indication of compromised oxidative metabolism; following oral coenzyme Q₁₀ administration, lactate fell toward normal levels and increased again following cessation of supplementation [20]. Moreover, supplementation of parkinsonian patient diets with 400 mg/day of coenzyme Q₁₀ increased complex I activity of platelets [39].

The ability of α-tocopherol to cross into the brain is similarly limited and only follows a linear dose/response characteristic under conditions of deficiency [27]. We have
found that repeated daily dosing of rats for 14 days with vitamin E (200 mg/kg body weight) has a minor effect on cortical levels of α-tocopherol. However, this is sufficient to effect significant biochemical changes [6]. Even 2 days of α-tocopherol injection have been reported to be protective against oxidative injury within the CNS [43].

Lipoic acid is readily absorbed from the diet and crosses the blood-brain barrier [31]. Supplementation of rat diets with lipoate has been shown to cause behavioral changes as demonstrated by an increase in ambulatory activity [17].

In the current study, melatonin had the most pronounced effect in preventing an age-related increase in complex IV activity. This agent can readily pass into the CNS and improve motor activity in aged C57BL/6 mice [2]. Melatonin can also suppress iron or paraquat-induced neurodegeneration in rat brain [25]. Its mechanism of action for protecting against such oxidant events may be indirect and involve promotion of antioxidant enzyme expression [21].

A number of studies have measured the change in activity of the ETS enzymes within cerebral tissue with age. The current work was designed to avoid age-related extremes but rather to investigate the changes associated with transition from full maturity to early aging. Thus, comparisons were made between 3 and 9 month-old mice. These ages constitute neither the very young, nor the very old state. Rather they essentially represent the transition from younger to older middle age. It is the differences that emerge during this period that may lay the early foundations underlying senescence. This may account for the lack of significant change in several mitochondrial complex activities in the present study. We found no changes in complex I despite the fact that seven subunits of this complex are encoded by mammalian mitochondrial DNA. However, the enzyme activity of this complex is the most difficult to accurately quantitate [24]. The increase in complex IV reported in the current study, is consistent with increases reported for both ROS generation [16], and state 3 respiration in cardiac muscle of middle-aged rats [11], and in skeletal muscle [22]. In addition, mitochondria from the senescence-accelerated mouse strain (SAMP8) possess higher respiratory rates and redox state than the corresponding senescence-resistant strain (SAMR1) [29]. These data were obtained from 2 month-old mice prior to any observable age-related deficits. However, parallel changes have not been found in human neurodegenerative disorders. In fact, decreased complex IV levels have been reported in brains of Alzheimer’s disease patients [8,10]. While extreme old age may be characterized by sluggish cerebral mitochondrial respiration accompanied by reduced overall metabolic rates [14], there is evidence both from the current study and reports of others, that this may be preceded by a mitochondrial state which may be hyperactive yet inefficient. Future study of the effects of melatonin supplementation on complex IV activities in more aged animals would be useful to elucidate these possibilities.

The results reported here demonstrate that reversal of age-related changes in ETS can be enhanced by melatonin and not by the other agents tested. This is in agreement with our findings that only melatonin can prevent age-related changes in cortical reactive oxygen species and nitric oxide synthase (Bondy et al., submitted for publication), and that only the melatonin-enriched diet can prevent the enhanced responsiveness of cerebral cytokine mRNA to lipopolysaccharide found in aged animals (manuscript in preparation). Thus melatonin appears to possess unique properties not shared by other antioxidants or mitochondrial cofactors. This may have implications for the utility of this agent in slowing progression of age-related neurodegenerative changes, although demonstration of any neuroprotective benefits in truly aged animals requires further investigation.

Acknowledgments

This work was supported in part by grants from the National Institutes of Health (ES 7992 and AG 16794 and AG 14882). We are grateful to Ms. Ellen Y. Yang for technical assistance.

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