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Effect of DL-α-lipoic acid on the status of lipid peroxidation and antioxidants in mitochondria of aged rats

Palaniyappan Arivazhagan, Kadirvel Ramanathan, Chinnakkannu Panneerselvam*

Department of Medical Biochemistry, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai - 600 113, India

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Abstract

The life span of a species is thought to be determined by the rate of mitochondrial damage which in turn is inflicted by free radicals in the mitochondria during the course of normal metabolism. The level of lipid peroxidation and antioxidants were measured in liver and kidney mitochondria of young and aged rats before and after DL-α-lipoic acid supplementation. In both liver and kidney, mitochondrial lipid peroxidation increased with age and a decrease in the enzymatic and non-enzymatic antioxidants were observed. DL-α-lipoic acid treated aged rats showed a decrease in the level of lipid peroxides and an increase in the antioxidant status. Our results conclude that supplementation of lipoic acid restores the depleted mitochondrial antioxidant status and suggest that it could be an effective therapeutic agent in treatment of age-associated disorders where free radicals are the major causative factor.

Keywords: DL-α-lipoic acid; Aging; Mitochondria; Lipid peroxidation; Antioxidants

1. Introduction

Aging is a complex biological process that leads to gradual loss of ability of an individual to maintain homeostasis. It is accompanied by a gradual decline in biochemical and physiological functions of most organs, ultimately leading to an increase in the susceptibility of age-associated diseases [1]. The life span of a species is thought to be determined by the rate of mitochondrial damage inflicted by oxygen free radicals in the mitochondria during the course of normal cellular metabolism [2]. Mitochondria are the most important intracellular source and target of reactive oxygen species. The production of $O_2^{\cdot -}$ normally the tendency of univalent reduction of O_2 in respiring cells is restricted by cytochrome oxidase of the mitochondrial electron transport chain, which reduces O_2 by four electrons to H_2O without releasing either $O_2^{\cdot -}$ or H_2O_2. However, $O_2^{\cdot -}$ is invariably produced in respiring cells. This is due to the probable leak of single electron at the specific site of the mitochondrial electron transport chain, resulting in inappropriate single electron reduction of oxygen to $O_2^{\cdot -}$ [3]. The phagocytic cells when activate during phagocytosis, generates free radicals through activation of NADPH oxidase. The rate of mitochondrial superoxide anion radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) generation increases in the later part of life [4]. This in turn affects the balance between pro-oxidants and antioxidants in biological systems leading to modifications in vital biomolecules [5]. Accumulation of these free radicals may also lead to increase of mitochondrial DNA damage and impair respiratory functions as well as ATP synthesis [6].

During aging antioxidant functions decline in almost all mammals [7]. The protection rendered by antioxidants in various free radical mediated pathological conditions has been already reviewed [8]. α-Lipoate has long been known for its role in oxidative metabolism; as lipoamide, an essential cofactor in mitochondrial α-ketoacid dehydrogenase complexes [9]. Depletion of this cofactor has been manifested during the process of aging [10]. Recent reports indicate that lipoate and its reduced form dihydrolipoate act as potent antioxidants [11,12]. Lipoate has been reported to exert its therapeutic efficacy in pathological conditions involving free radicals [13–15]. Studies on the antioxidant

* Corresponding author.

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effect of lipoate on aging are sparse and is yet to be elucidated. Hence the present study was delineated to explore the role of lipoate as an antioxidant and to shed light on its effect on other antioxidants in the mitochondria of aged rats.

2. Materials and methods

DL-α-lipoic acid was purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were of reagent grade. Male albino rats of Wistar strain weighing approximately 130–160 g (young) and 380–410 g (old) were used. The animals were divided into two major groups namely, Group I: Normal young rats (3–4 months old) and Group II: Normal aged rats (above 22 months old). Each group was further sub-divided into three groups: one control group (Groups Ia, IIa) and two experimental groups based on the duration of lipoic acid administration for 7 days (Groups Ib, IIb) and 14 days (Groups Ic, IIc). The animals were maintained on a commercial rat feed which contained 5% fat, 21% protein, 55% nitrogen free extract and 4% fiber (wt/wt) with adequate mineral and vitamin contents. Each group consisted of six animals and had access to food and water *ad libitum.* Various concentrations of DL-α-lipoic acid (50 mg, 100 mg, 150 mg and 200 mg) were dissolved in alkaline saline (0.5%) and administered intraperitoneally to the animals and the effective dosage (100 mg/kg body weight/day) was selected on the basis of the concentration, capable of inhibiting lipid peroxidation. Control animals received vehicle alone in the similar manner. Body weight on both the young and aged rats were monitored throughout the duration of DL-α-lipoic acid therapy and the changes were found to be insignificant.

On completion of 7 and 14 days of lipoic acid administration, the animals were killed by cervical decapitation. Liver and kidney were excised immediately and immersed in physiological saline and mitochondria were isolated with fresh tissues [16]. The purity of the mitochondria was checked using specific marker enzymes. Protein was estimated by the method of Lowry et al. [17]. Lipid peroxidation (LPO) of tissue fractions was measured by the method of Okawa et al. [18]. The activity of whole kinds of superoxide dismutase (SOD) was measured at the degree of inhibition of autooxidation of pyrogallol at an alkaline pH [19]. The activity of catalase (CAT) was measured by the method of Sinha [20]. The method based on the fact that dichromate in acetic acid was reduced to chromic acid, when heated in the presence of H_2O_2, with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus produced was measured at 570 nm. Glutathione peroxidase (GPx) was assayed by the method of Rotruck et al. [21]. The activity was expressed on the basis of inhibition of GSH. Reduced glutathione (GSH) was estimated by the method of Moron et al. [22]. Virtually all the nonprotein sulfhydryl content of cells is in the form of reduced glutathione. 5,5′-dithio(2-nitrobenzoic acid) (DTNB) is a disulfide compound that is readily reduced by sulfhydryl compounds forming a highly colored yellow anion. The optical density of this yellow substance is measured at 412 nm. Ascorbic acid was measured by the method of Omaye et al. [23]. Ascorbic acid is oxidized by copper to form dehydroascorbic acid which reacts with 2,4-dinitrophenyl hydrazine to form bis-2,4-dinitrophenyl hydrazine. This undergoes further rearrangement to form a product with an absorption maximum at 520 nm. Thiourea provides a reducing medium which helps to prevent interference from non ascorbic acid chromogens. Vitamin E was estimated by the method of Desai [24]. This method reduces ferric ions to ferrous ions in the presence of tocopherol and the formation of pink colored complex with a more sensitive reagent such as bathophenanthroline. Orthophosphoric acid is added as a chelating agent to reduce carotene interference by preventing its oxidation and stabilization of color, by binding excess ferric ions, thus preventing their photochemical reduction.

3. Statistical analyses

Values are expressed as mean ± SD for six rats in each group, and significance of the differences between mean values was determined by one-way analysis of variance (ANOVA) coupled with Student-Newman-Kuel multiple comparison test. Values of $P < 0.05$ were considered to be significant.

Statistical significance of differences between the young control (Group Ia) and aged control (Group IIa) was determined by Student's t-test. Levels of significance were evaluated with p-values.

4. Results

Table 1 shows the lipid peroxidation and antioxidant status in the liver mitochondria of control and DL-α-lipoic acid treated young and aged rats. The level of lipid peroxide was considerably high ($p < 0.001$) whereas the levels of GSH, vitamins C and E and the activities of SOD, CAT and GPx were remarkably low ($p < 0.001$) in aged rats (Group IIa) when compared to young control rats (Group Ia). DL-α-lipoic acid administration brought down the level of lipid peroxidation while elevated the levels/activities of non-enzymatic and enzymatic antioxidants in aged rats (Group IIc) compared to aged control rats (Group IIa). DL-α-lipoic acid administration also lowered the level of lipid peroxide considerably in young rats (Group Ic) compared to their respective controls (Group Ia).

Table 2 presents the kidney mitochondrial lipid peroxidation, enzymatic and non-enzymatic antioxidant status in young and aged rats before and after lipoate administration. The level of lipid peroxidation was found to be significantly high ($p < 0.001$) whereas the antioxidant status (both en-

Table 1
Effect of DL-α-lipoic acid on LPO, SOD, CAT, GPx, GSH, vitamins C and E in young and aged rat liver mitochondria

Parameters	Young rats			Aged rats		
	Group Ia (Control)	Group Ib (7 days)	Group Ic (14 days)	Group IIa (Control)	Group IIb (7 days)	Group IIc (14 days)
LPO	1.73 ± 0.13	1.61 ± 0.11	1.53 ± 0.10[a]	2.51[b] ± 0.17*	2.17 ± 0.15[c]	1.87 ± 0.14[d,e]
SOD	3.21 ± 0.22	3.29 ± 0.31	3.40 ± 0.25	2.42[b] ± 0.19*	2.97 ± 0.25[c]	3.18 ± 0.27[d]
CAT	5.11 ± 0.31	5.36 ± 0.41	5.58 ± 0.50	3.07[b] ± 0.22*	3.96 ± 0.27[c]	4.89 ± 0.36[d,e]
GPx	6.31 ± 0.45	6.49 ± 0.40	6.71 ± 0.52	4.36[b] ± 0.29*	5.09 ± 0.33[c]	6.11 ± 0.49[d,e]
GSH	11.97 ± 0.90	12.50 ± 0.87	13.12 ± 0.98	9.21[b] ± 0.73*	10.87 ± 0.85[c]	11.59 ± 0.89[d]
Vitamin C	9.71 ± 0.79	9.93 ± 0.83	10.57 ± 0.75	7.10[b] ± 0.62*	7.99 ± 0.61[c]	8.86 ± 0.70[d,e]
Vitamin E	4.43 ± 0.31	4.45 ± 0.37	4.61 ± 0.41	3.14[b] ± 0.25*	3.61 ± 0.29[c]	4.21 ± 0.33[d,e]

Values are expressed as mean ± SD for six rats in each group.

[LPO nmoles of MDA released/mg protein, SOD Units/min/mg protein; CAT μmoles of H_2O_2 consumed/min/mg protein; GPx μmoles of GSH oxidized/min/mg protein; GSH μg/mg protein; vitamin C μg/mg protein and vitamin E μg/mg protein].

[a] Group Ia compared with Ib and Ic; [b] Group Ia compared with Group IIa; [c] Group IIa compared with IIb; [d] Group IIa compared IIc; [e] Group IIb compared with IIc; Group Ia versus Group IIa.

* $p < 0.001$.

zymatic and non-enzymatic) were found to be significantly lowered ($p < 0.001$) in aged rats (Group IIa) when compared to young control rats (Group Ia). Administration of lipoate significantly reduced the level of lipid peroxidation, while elevated both the non-enzymatic and enzymatic antioxidant status in aged rats (Group IIc) when compared to their age matched controls (Group IIa). Lipoic acid administration also showed a decrease in the level of lipid peroxidation in young rats (Group Ic) when compared to their younger controls (Group Ia).

5. Discussion

The mitochondrial DNA is one of the major targets of reactive oxygen species. The mitochondrial theory of aging proposes that accumulation of mitochondrial genomic mutations as a major cause of aging and age-associated degenerative diseases [25]. Mitochondrial aging is characterized by destruction of structural integrity of membrane, leading to a decrease in mitochondrial membrane fluidity and activities of enzymes associated with membrane lipids [26–28]. As the activities of most enzymes are regulated by the physicochemical state of the lipid environment of the membrane, it seems likely that impaired mitochondrial membrane function brought about by aging could be related to a decrease in membrane fluidity due to free radical reactions such as lipid peroxidation. Free radicals and their metabolites are generated by components of electron transport chain in the membrane during mitochondrial respiration. This potential for self-destruction renders the mitochondrial membrane, more vulnerable to damage than any other cellular membrane [29].

Lipid peroxidation in vivo has been identified as the basic deteriorative reaction in cellular mechanisms of the aging process. The free radical mediated lipid peroxidation

Table 2
Effect of DL-α-lipoic acid on LPO, SOD, CAT, GPx, GSH, vitamins C and E in young and aged rat kidney mitochondria

Parameters	Young rats			Aged rats		
	Group Ia (Control)	Group Ib (7 days)	Group Ic (14 days)	Group IIa (Control)	Group IIb (7 days)	Group IIc (14 days)
LPO	1.65 ± 0.11	1.56 ± 0.13	1.47 ± 0.10[a]	2.43[b] ± 0.15*	2.15 ± 0.16[c]	1.83 ± 0.14[d,e]
SOD	2.87 ± 0.16	2.93 ± 0.20	3.05 ± 0.27	1.61[b] ± 0.11*	2.09 ± 0.19[c]	2.65 ± 0.26[d,e]
CAT	4.71 ± 0.33	4.85 ± 0.36	4.99 ± 0.30	2.81[b] ± 0.20*	3.52 ± 0.25[c]	4.63 ± 0.33[d,e]
GPx	5.91 ± 0.40	5.99 ± 0.51	6.20 ± 0.53	4.10[b] ± 0.33*	4.86 ± 0.45[c]	5.63 ± 0.52[d,e]
GSH	10.17 ± 0.91	10.36 ± 0.87	10.49 ± 0.95	7.07[b] ± 0.59*	8.67 ± 0.63[c]	9.88 ± 0.68[d,e]
Vitamin C	8.14 ± 0.69	8.29 ± 0.75	8.67 ± 0.60	6.02[b] ± 0.45*	6.95 ± 0.50[c]	7.99 ± 0.68[d,e]
Vitamin E	4.10 ± 0.27	4.21 ± 0.32	4.37 ± 0.29	3.17[b] ± 0.21*	3.57 ± 0.27[c]	3.90 ± 0.30[d]

Values are expressed as mean ± SD for six rats in each group.

[LPO nmoles of MDA released/mg protein, SOD Units/min/mg protein; CAT μmoles of H_2O_2 consumed/min/mg protein; GPx μmoles of GSH oxidized/min/mg protein; GSH μg/mg protein; vitamin C μg/mg protein and vitamin E μg/mg protein].

[a] Group Ia compared with Ib and Ic; [b] Group Ia compared with Group IIa; [c] Group IIa compared with IIb; [d] Group IIa compared with IIc; [e] Group IIb compared with IIc; Group Ia versus Group IIa.

* $p < 0.001$.

has been proposed to be critically involved in disease states including immune function decline, brain dysfunction, cataract, cardiovascular disease, cancer as well as in the degenerative processes associated with aging [30]. In the present investigation, levels of lipid peroxide in mitochondria of liver and kidney of aged rats were significantly high when compared with young rats. Earlier investigations reveal that the average content of lipid peroxides in the mitochondria of tissue from aged subjects are higher than those of younger subjects [31]. The age-dependent increase in the production of free radicals in mitochondria inevitably elevates the oxidative damage in the mitochondria as well as in the entire cell [32].

The present study illustrates that lipoate administration prevents lipid peroxidation during aging. This may be attributed to the bioactivity of lipoic acid to directly react with various reactive oxygen species as well as its ability to interfere with oxidation processes in the lipid and in the aqueous cellular compartment [33,9]. The two thiol residues of dihydrolipoic acid enables this biomolecules to prevent Fenton-type reactions by chelating free iron [14].

Superoxide dismutase is a major intracellular enzyme, which protects against oxygen free radicals by catalyzing the removal of the superoxide radical. Catalase has been shown to be responsible for the detoxification of significant amounts of H_2O_2. Glutathione peroxidase metabolizes peroxides such as H_2O_2 and protects cell membranes from lipid peroxidation. In normal cells, a balance exists between oxidative products and antioxidant protection. Increased free radicals affect pro-oxidant antioxidant balance during the process of aging [34]. Hence in the present study we have observed an increase in lipid peroxidation and a decrease in antioxidant enzymes.

The age-related increase in peroxidation and decrease in protective enzymes observed in our study is in agreement with earlier investigations [35–39]. Administration of lipoate to aged rats prevents lipid peroxidation and enhances antioxidant status due to its antioxidant nature [40]. In general enzymes are proteins, a reduction in protein synthesis during aging may be due to decrease in ATP production [41]. This may also be the cause for the reduction in the activities of various antioxidant enzymes. α-Lipoic acid is a natural constituent which is involved in mitochondrial energy metabolism, where it cycles between the oxidized and reduced form [14,42]. In addition lipoate by virtue of its ability to enhance ATP production [43], might have improved the overall protein synthesis (and thus enzymes) in cells.

Apart from enzymatic antioxidants, the non-enzymatic antioxidants like glutathione, ascorbic acid and α-tocopherol were also found to be lowered during aging. Mitochondrial GSH plays a critical role in maintaining cell viability through the regulation of mitochondrial inner membrane permeability by maintaining sulfhydryl groups in the reduced state [44]. Glutathione is responsible for the regulation of intracellular level of lipid peroxidation during aging. It thwarts peroxidative damage as indicated by earlier investigation [28]. Decrease in the level of glutathione with advancing age [45] supports our present study. The observed decrease in glutathione concentration in the aged rats may be due to the enhanced oxidative damage due to free radicals. Loss of thiol groups in key proteins may adversely affect transport of substrates necessary for mitochondrial function. DL-α-lipoic acid plays an important role in improving GSH status [46]. Reduced GSH maintains cell membrane sulfhydryl groups and other structural proteins in stable form. The effect of lipoic acid is associated with protection against oxidation induced depletion of cellular GSH content, confirming its antioxidant activity.

Ascorbic acid is the most widely cited form of water soluble antioxidant that prevents oxidative damage to cell membrane induced by aqueous radicals. During aging, a significant reduction in ascorbic acid levels has been noticed which is in corroboration with earlier investigation [47]. Glutathione plays a vital role in the reduction of dehydroascorbate to ascorbate [48]. The decreased ascorbic acid content in the mitochondria of aged rats may be due to the decreased availability of glutathione.

α-Tocopherol is an antioxidant that prevents biological membranes from undergoing oxidative damage. This effect is due to its ability to quench lipid peroxides thereby protecting the cellular structures from the attack of free radicals [49]. A significant reduction in the vitamin E level observed in the present investigation is in accordance with the study of Verical et al. [50]. Ascorbic acid regenerates α-tocopherol from its oxidized form, as a result of which tocopherol continues to scavenge the free radicals [51]. The decreased ascorbic acid content in the aged rats might be one of the reason for the observed decrease in α-tocopherol level.

Lipoate administered aged rats replenishes glutathione, ascorbic acid and α-tocopherol. This may be due to its capacity to enhance the levels of these antioxidants (GSH, vitamins C and E) via the reduction of their radicals to repair oxidative damage [11]. In addition to the radical scavenging property of lipoate it is also known for its metal chelating effect [40]. Data of this study, illustrate the superadditive effect of lipoic acid in preventing peroxidation of biomembrane and thereby suggest it as a possible therapeutic approach to attenuate the pathological consequences associated with aging.

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