Effect of Alpha-lipoic Acid Supplementation on Trace Element Levels in Serum and in Postmitotic Tissue in Aged Rats

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Abstract: Redistribution of redox-active divalent metal ions (e.g. copper, zinc, and iron) in postmitotic tissues of lipoic acid supplemented aging rats has been proposed to contribute to metal-catalyzed protein oxidation. DL-alpha lipoic acid (LA) (100 mg/kg body wt/day) was administered intraperitoneally to the Sprague-Dawley rats for 14 days. Serum copper levels lowered in the aged rats with LA supplementation compared to the rats without LA supplementation. On the other hand, serum zinc and iron levels increased in the aged rats with LA supplementation compared to the rats without LA supplementation. Copper levels of the postmitotic tissues were not changed in the aged rats with LA supplementation compared to the controls. The heart zinc levels detected in LA supplemented rats were significantly lower than controls. Similarly, the iron levels of the heart were found to be significantly lower in LA supplemented rats when compared to control rats. LA supplementation did not affect brain and muscle iron levels. The brain and muscle zinc levels remained the same in both group of rats. Based on the findings of our study, we have concluded that LA may exhibit prooxidant effect depending on the altered trace element homeostasis. Therefore, our results emphasize the importance of monitoring the dose of LA supplementation, duration of treatment and its potential harmful effects in the postmitotic tissues of aged rats.

Key Words: Lipoic acid, trace elements, free radicals, postmitotic tissues.

INTRODUCTION

The redox state of the cell is an important determinant of cell function and survival. It is largely linked to transition metals and is maintained under strict physiological limits. It is also known that several essential transition metals, such as zinc, iron, copper, cobalt, selenium and manganese participate in the control of various metabolic and signalling pathways [1]. Dysregulation of trace element homeostasis and consequent oxidative damage have been implicated in the pathogenesis of age-related disorders [2,3]. Metal-mediated formation of free radicals causes various modifications to DNA bases, enhanced lipid peroxidation, and protein oxidation [1]. The free radical generating activity of redox-active metal ions suggests that chelation to remove excess metal ions *in vivo* may be beneficial, e.g. in the treatment of age-related disorders [4-6].

DL-alpha lipoic acid (LA) is relatively a small molecule (mwt:206). LA is synthesized *de novo* in mitochondria by lipoic acid synthase, although adequate amounts are normally found in human diets. LA exists in two isomeric configurations, R, the naturally occurring form, and S, which is the synthetic form. It is known that (R)-LA is transformed by lipoamide dehydrogenase to DHLA with a higher rate than (S)-LA. Also, (R)-LA is more effectively incorporated into mitochondrial enzyme complexes. Therefore, (S)-LA which is converted into DHLA at a slower rate, is also less efficiently incorporated into the enzyme complexes and might be fully available for radical scavenging or other actions which would explain the observed pharmacological results [7]. So far the studies have shown that when taken up by eukaryotic cells, both enantiomers are converted to dihydrolipoic acid (DHLA) in all cells and tissues. Owing to its low reduction potential (-320 mV), DHLA effectively reduces other endogenous antioxidants, including ascorbate and GSH. The chemical reactivity of LA is mainly centered in its dithiolane ring [6]. The chemical structure of the dithiolane ring in LA is active enough to function as the oxidizing compound without the need for endogenous reactive oxygen species (ROS) production. In addition, LA and DHLA have been reported to exhibit metal-binding properties, and the use of LA to remove metal ions *in vivo* has been proposed previously [6]. Structurally, the two thiol groups and the carboxyl group of DHLA allow binding of various divalent metals. Such binding by DHLA appears to be effective in slowing iron- or copper-mediated oxidation of lipids in various biological model systems [1,6]. On the other hand, LA has been reported to have prooxidant effects when given in various concentrations [8-10], thus making its antioxidant effects controversial. Tissues of high energy demand are at greater risk of being damaged by ROS , which is consistent with the notion where signs of aging usually start to appear at these body sites [11]. The tissues such as brain, heart, and skeletal muscles, which have postmitotic cells and one of the highest oxygen consumption rates in the body, have a slow turnover of antioxidant enzymes and are therefore, highly susceptible to free radical generating activity of redox-active metal ions [9,12,13].

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Although antioxidants, in general, have been shown to have prooxidant potential, the prooxidant chemistry of LA and DHLA have not been fully characterized. Over the past years, in addition to the antioxidant role of LA, a limited number of studies have explored prooxidant properties of LA [8-10,14,15]. Some researchers have raised concerns, mainly over potentially deleterious transition-metal ion-mediated prooxidant effects of LA in aging [8-10,14,15]. Substantial evidence suggested that especially Cu^{2+},Zn^{2+}, and Fe^{2+} participate in the oxidation process of the proteins and lipids [1,2]. According to our knowledge based on our searches in literature, redox-active transition metals such as Cu, Zn, and Fe levels have not been determined previously in homogenates of the postmitotic tissues in LA-supplemented aged rats in any study. In the present study, we have investigated if LA supplementation have affected the serum and postmitotic tissue trace element levels in aged rats and whether this change in the levels may cause a prooxidant effect of LA.

MATERIALS AND METHODS

Inbred male Sprague-Dawley rats, supplied by Center of Reproduction and Research of Experimental Animals, Cerrahpaşa Faculty of Medicine, Istanbul University, Istanbul, Turkey were used. Aging rats were randomly divided into two groups: Group I consisted of normal aged rats (Control group, 24 months, n= 10), and Group II consisted of LA-supplemented aged rats (24 months, n= 10). DL-α-Lipoic acid (Sigma, St Louis, MO, USA; 100 mg/kg body wt/day) was mixed with sterile saline in a dark bottle and 1 M NaOH was added until the suspension dissolved. The pH was then brought to 7.4 with 1 M HCl. Fresh LA solutions were administered intraperitoneally to the experimental animals for 14 days [9,14,15]. We selected the effective dosage (100 mg/kg body wt/day) on the basis of the concentration capable of prooxidant effect [9,14,15]. Control rats received vehicle alone in the similar manner.

Rats were monitored daily for changes in body weight, fluid intake, and physical appearance. After 14 days of LA supplementation, blood samples were collected in acid-washed glass tubes at 90 ^{o}C in a drying oven by cardiac puncture and the tissues were obtained from the heart, brain and the skeletal muscle under deep anesthesia with intraperitoneally administered sodium pentothal (Abbott, Campoverde di Aprilia, Italy, 100 mg/kg). The serum was separated by centrifugation and stored at –80 ^{o}C until the trace elements were analyzed. These rats were housed in conventional wire-mesh cages, four rats per cage, in a room with the temperature regulated at 21 ± 1 °C, humidity 45-50%, and light/dark cycles (12h). All rats were given *ad libitum* access to food, and tap water by drinking bottle throughout the course of the experiment. They were fed a standard laboratory diet. Interventions concerning experimental animals were performed according to "*Principles of Laboratory Animal Care*" (NIH publication No. 85-23, revised 1985).

The aging rats were terminated between 9 and 10 a.m., and tissue samples for trace element analysis were collected.

Preparation of Tissue Samples

Hearts were excised immediately and immersed in physiological saline. The major heart vessels, valves, and atria were trimmed away and the ventricles were cut open and rinsed free of blood. Heart muscles were separated from connective tissues. Muscle sample (190-200 mg) was weighed and diluted 20% (w/v) in 20 mM ice-cold Tris-HCl, pH 7.4, and homogenized with a Bosch Scintilla SA, Switzerland. The homogenate was centrifuged at 5000 x g for 10 min, and various trace elements and protein determinations were performed in the supernatant fraction [9].

The brain was quickly removed, washed in cooled 0.15 M NaCl, placed on an ice-cold plate. The brain tissue (except cerebellum, pons, and medulla oblongata) was immediately frozen in liquid N_2 until assay. Brain tissue (200 mg) was homogenized in 2 ml of homogenizing buffer (100 mM KH_2PO_4-K_2HPO_4, pH 7.4, plus 0.1% (w/v) digitonin) in a glass homogenizer [14].

Muscle samples were obtained from gluteus maximus. 190-200 mg of muscle sample was weighed and diluted 20% w/v in 20 mM ice-cold tris-HCl, pH 7.4, and homogenized with a Bosch Scintilla SA, Switzerland. The homogenate was centrifuged at 5000x g for 10 minutes, and various analyte determinations were performed in the supernatant fraction [14].

Trace Element Determinations

The trace elements such as zinc, copper, and iron were measured by atomic absorption spectrophotometry (Shimadzu, Kyoto, Japan) in the serum and postmitotic tissues of the aging rats.

Protein Determinations

Protein determinations were carried out by using the Sigma Protein Assay Kit (P5656).

Data Analysis

The statistical program Instat was used for data analysis. All data was not normally distributed. The plasma and tissue trace element levels in two groups of Sprague-Dawley rats were compared using the Mann-Whitney U-test. Results were expressed as the mean ± SEM. A p-value of less than 0.05 was considered significant.

RESULTS

No significant difference could be observed in the body weight and daily food intake among different experimental rat groups. Serum trace element levels in the aged rats with and without LA supplementation are given in Fig. (**1**). Serum copper levels decreased in the aged rats with LA supplementation compared to the rats without LA supplementation ($p<0.05$). Serum iron and zinc levels were increased in the aged rats with LA supplementation compared to the rats without LA supplementation ($p<0.05$, $p<0.001$, respectively).

On the other hand, tissue copper levels were not changed in the aged rats with LA supplementation compared with those of the rats without LA supplementation Fig. (**2A**). Heart zinc levels detected in LA supplemented rats were significantly lower than controls Fig. (**2B**). The brain and muscle zinc levels remained the same in both group of rats. Similarly, heart iron level was found to be significantly lower ($p<0.05$) in LA supplemented rats when compared to

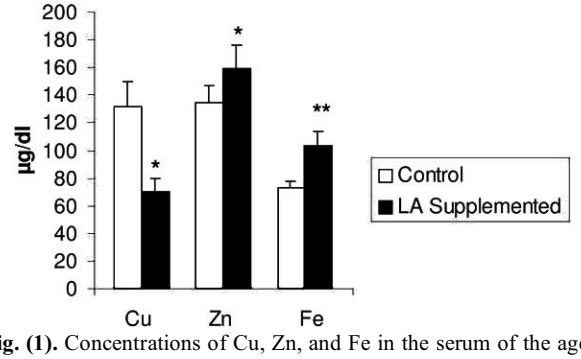

Fig. (1). Concentrations of Cu, Zn, and Fe in the serum of the aged rats with (n=10) and without (n=10) LA supplementation. Results are expressed as mean ± SEM. *Data are statistically different between aged rats with and without LA supplementation.

control rats Fig. (**2C**). LA supplementation did not affect brain and muscle iron levels.

DISCUSSION

Since metal ions are integral part of many proteins necessary for biological functioning, the role of redox-active transition metal ions such as Cu^{2+}, Fe^{2+}, Zn^{2+}, and Mn^{2+} in metal-catalyzed protein oxidation was intensively studied. The role of transition metals in oxidative damage has been implicated in age related disorders [1,3,16]. There is strong evidence that LA supplement is a good insurance and can markedly improve human health [8]. While beneficial roles for LA and DHLA in augmenting antioxidant defenses have been well characterized, much less is known about their potential role in modulating trace element status *in vivo*. LA can be a double-edged sword for the cell. The *in vivo* potential prooxidant effects of DHLA raise the question of whether LA and DHLA chelate transition metal ions from antioxidant enzymes, such as Cu, Zn superoxide dismutase, catalase and aconitase, thereby lowering enzyme activity. On the other hand, Suh *et al*., [5] suggest that DHLA chelates and inactivates redox-active transition metal ions in small-molecular and biological complexes without affecting iron-or copper-dependent enzyme activities *in vitro*. There are no established data or explanation in the current literature why the transition metal ions can not be efficiently balanced in postmitotic tissues and serum in LA-supplemented aged rats. This imbalance is probably due to different LA/DHLA ratio in these tissues and serum.

Since there is increasing evidence that zinc ions are involved as inter- and intra-cellular messengers, the homeostasis of zinc has to be controlled tightly. Zinc also has significant cardioprotective effects. In the present study, the decrease in iron and zinc in heart tissues of LA supplemented aged rats compared with non-supplemented aged rats may suggest that metal binding capacity of LA is increased in LA supplemented aged rats. The metabolic fates of iron and copper are closely related. On the other hand, myocardial copper levels would have been expected to be slightly decreased during LA supplementation, however, we found them to be unchanged. As a matter of fact, there are no studies in the literature contradicting these findings.

Selection of appropriate pharmacological doses of LA for use in oxygen-related diseases is critical. On the other hand,

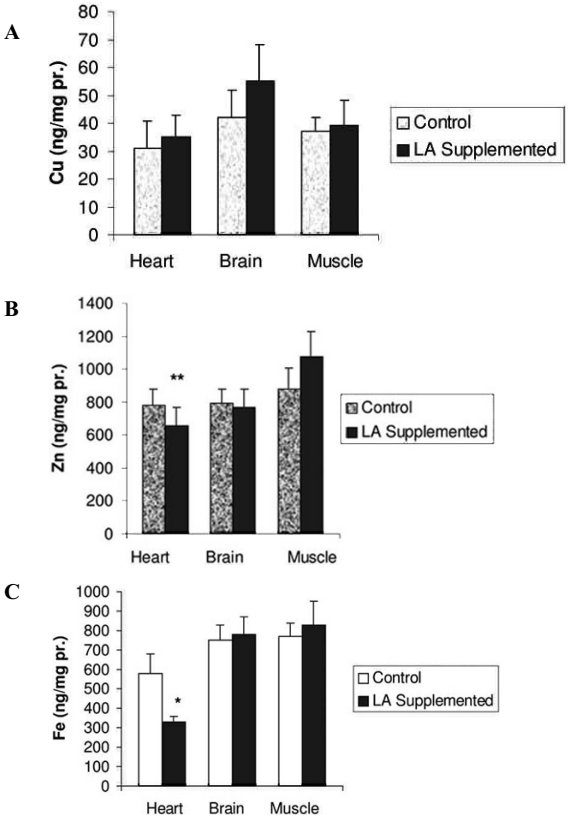

Fig. (2). Cu (A), Zn (B), and Fe (C) levels were determined in postmitotic tissues of the aged rats with (n=10) and without (n=10) LA supplementation. *Significant ($p<0.05$) difference between the aged rats with (n=10) and without (n=10) LA supplementation; dagger denotes differences between controls and LA supplemented rats.

much of the discussion in clinical studies has been devoted to the prooxidant role of LA [8]. A wide range of daily recommended doses of LA has been reported and used in human trials, ranging from 100-1800 mg/day [17]. In experimental animal models, LA has been used in the range of 40-150 mg/kg/day [9,18,19]. It is quite intriguing, however, that none of these animal studies have reported the effect of different doses of LA in the postmitotic tissues of aging rats. The detrimental effects of LA on redox status of the proteins and the altered homeostatic regulations of trace elements may be dose-related. We have previously reported that 100mg/kg body wt/day dose of LA also causes an increased in myocardial [9] and plasma protein oxidation [15] in aging rats, confirming that LA, most often, behaves as prooxidant. As a hypothesis, we suggest that increased protein oxidation levels in the heart muscles of the LA supplemented old rats may be due to the impaired antioxidant activity of the metalloenzymes.

Most interestingly, our present study shows that the LA supplementation is associated with an increase in serum iron and zinc levels. Iron excess is believed to generate oxidative stress, understood as an increase in the steady-state concentration of reactive oxygen and nitrogen species. It has been

suggested that iron regulation ensures there is no free intracellular iron; however, *in vivo*, under stress conditions, an excess of superoxide releases "free iron" from iron-containing molecules. It causes severe damage to biological membranes, proteins, and DNA [20]. There are no established data or explanation in the current literature why the redox state cannot be efficiently balanced in postmitotic tissues of aging rats. This situation is probably due to different oxygen consumption and/or altered homeostatic regulations of trace elements in these tissues.

This data suggest that LA and DHLA may remove protein-bound redox-active transition metals in postmitotic tissues of aging rats. Based on the findings of our study, we have conclude that LA may exert prooxidant effects depending on the altered trace element homeostasis. Thus, our results emphasize on the importance of monitoring the dose of LA supplementation, the duration of the treatment and its potential harmful effects on the postmitotic tissues. Further studies are needed to determine the biochemical mechanisms of the redox-active transition metal-chelating properties of LA in postmitotic tissues of aged rats.

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