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α-LIPOIC ACID SUPPLEMENTATION PREVENTS SYMPTOMS OF VITAMIN E DEFICIENCY

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α-Lipoic acid, an essential cofactor in mitochondrial dehydrogenases, has recently been shown to be a potent antioxidant in vitro, as well as being capable of regenerating vitamin E in vitro. In this study, using a new animal model for rapid vitamin E deficiency in adult animals and a new technique for tissue extraction of oxidized and reduced α–lipoic acid, we examined the antioxidant action of α–lipoic acid in vivo.
Vitamin E-deficient adult hairless mice displayed obvious symptoms of deficiency within five weeks, but if the diet was supplemented with α-lipoic acid the animals were completely protected. At five weeks on a vitamin E-deficient diet animals exhibited similar decreases in tissue vitamin E levels, whether supplemented or unsupplemented with α-lipoic acid: vitamin E levels in liver, kidney, heart, and skin decreased 70 to 85%; levels in brain decreased only 25%. These data show that there was no effect of α–lipoic acid supplementation on vitamin E tissue concentrations, arguing against a role for α-lipoic acid in regenerating vitamin E in vivo.

α-Lipoic acid, as lipoamide, is a cofactor in several mitochondrial dehydrogenase complexes (1). It has recently been shown to act as antioxidant in vitro and in vivo. In mammalian cells it is readily converted to its reduced form, dihydrolipoic acid (2). In vitro experiments have shown that both α-lipoic acid and dihydrolipoic acid are potent scavengers of reactive oxygen species. α-Lipoic acid quenches singlet oxygen (3), hydroxyl radicals (4), and hypochlorus acid (5), while dihydrolipoic acid scavenges hydroxyl radicals (4), hypochlorus acid (5), superoxide anion radicals (4), peroxyl radicals (6), and hydrogen peroxide (7).

A number of in vitro studies also suggest that α-lipoic acid is able to recycle other natural antioxidants such as vitamin C or vitamin E. Scholich et al. found that protection against microsomal lipid peroxidation by α-lipoic acid was vitamin E

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Abbreviations: EPR, electron paramagnetic resonance; LDL, low density lipoproteins; PUFA, polyunsaturated fatty acids.

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dependent (8), and Kagan et al. found EPR evidence in dioleoylphosphatidylcholine (DOPC) liposomes that dihydrolipoate protects membranes by recycling vitamin C, which then recycles vitamin E. Dihydrolipoate recycling of vitamin E through vitamin C has also been observed in LDL and erythrocyte membranes in vitro (9, 10).

However, until now no experimental design has addressed the question of whether there is vitamin E recycling by α-lipoic acid in vivo. In 1959 Rosenberg and Culik (11) observed that α-lipoic served as a replacement for vitamin E in vitamin E-deficient rats; α-lipoic acid was almost equivalent to α-tocopherol in ensuring normal growth and breeding performance. Furthermore, they found that a combination of α-lipoic acid and α-tocopherol was as effective as a considerably larger amount of α-tocopherol alone. These results are consistent with either recycling of vitamin E by lipoate, or a separate antioxidant action of lipoate in vivo. However, no measurements of tissue levels of vitamin E or α-lipoic acid were made in these experiments.

In the present study, we sought to determine if α-lipoic acid was able to replace vitamin E in another deficiency model and to measure levels of vitamin E, α-lipoic acid, and dihydrolipoic acid levels in different tissues. Such measurements would help to distinguish the antioxidant role of α-lipoic acid in vivo. Hairless mice were fed a diet deficient in vitamin E, with or without α-lipoic acid supplementation, and their weights and tissue levels of vitamin E, α-lipoic acid, and dihydrolipoic acid were assessed.

METHODS

Chemicals. dl-α-Tocopherol and dl-α-tocopheryl acetate were kindly provided by Hoffmann-La Roche, New Jersey. α-Lipoic acid was kindly provided by ASTA-Medica, Frankfurt. HPLC grade water, ethanol, and methanol were purchased from Fisher Scientific, all other chemicals were purchased from Sigma.

Diets. The diets are designated as N (normal diet, control), -E (vitamin E deficient diet), and -E+LA (vitamin E deficient, α-lipoic acid supplemented diet). The N diet was prepared as previously described (12) using tocopherol-stripped corn oil as a fat source and 30 IU/kg diet dl-α-tocopheryl acetate. The -E diet was the same diet lacking dl-α-tocopheryl acetate. The -E+LA diet was the same as the -E diet, but supplemented with 1.65 g/kg diet racemic α-lipoic acid.

Animals. Adult hairless mice, having reached a constant body weight, (6-8 week old, HRS/hr hr, Simonsen laboratory) were divided into three groups of 8 animals each, designated according to the diets N, -E, -E+LA. Four animals from each group were sacrificed after 5 weeks of feeding, and tissues were analyzed for tocopherol and lipoate/dihydrolipoate. The other animals were kept on their respective diets to determine the weights and the occurrence of vitamin E deficiency symptoms. Animals showing vitamin E deficiency symptoms were histologically examined by a veterinary pathologist (Office of Laboratory Animal Care, University of California, Berkeley). Animals were sacrificed by neck dislocation, blood was drained by cutting the vena cava, and heart, liver, kidney, and skin were removed. Tissues were immediately frozen in liquid nitrogen and kept at -80°C until extracted.

Assays. Tissues were extracted and analyzed for α-tocopherol using a modification of the procedure previously described by Lang et al. (13). Briefly, depending on the organ, 10-100 mg wet weight of tissue were homogenized while thawing, extracted by ethanol/hexane, dried down under nitrogen, and resuspended in ethanol/methanol 1:1 before injection into the HPLC. After separation on a 25 cm 5μm C18 Beckman

Ultrasphere column, α-tocopherol was detected electrochemically using a glassy carbon electrode.
A new method was developed for the extraction of α-lipoic acid and dihydrolipoic acid from tissues. The tissues (100-500 mg) were homogenized for 1 min in 1.5 ml of 3.3% 5-sulfosalicylic acid and 5 mM EDTA. Then the same volume of ethanol was added, the mixture was vortexed for 1 min, and centrifuged at 3,000 x g for 3 min. The supernatant was either directly injected into the HPLC or stored under liquid nitrogen for a maximum of one week. Skin was ground under liquid nitrogen before homogenization. The lipoate/dihydrolipoate couple was detected by HPLC utilizing a method developed by Handelman et al (2) which uses electrochemical detection with a dual gold/mercury electrode. During the extraction and directly before injection, special care was taken to remove all oxygen, which interferes with the reduction, by constantly bubbling with nitrogen or helium. A Microsorb 10 cm 3μm C18 column (Rainin Instruments) was used with a water/methanol/acetonitrile mobile phase (50/30/20) with 5 g/l monochloroacetic acid.

Statistics. In tables or text data are expressed as mean ± standard deviation, in the figures as mean ± standard error of the mean. Statistical significance was assessed by ANOVA and if significant difference was obtained ($p<0.05$), Tukey posttests were performed.

RESULTS

It is widely accepted that the dietary requirement for vitamin E depends on the concentration of polyunsaturated fatty acids in the tissues (PUFA), which depends on the PUFA content of the diet (14). The diet used in this study was high in PUFA, and after three weeks on this diet, the -E animals started to lose weight. After 5 weeks on the diet they showed clear symptoms of vitamin E deficiency, as can be seen in photograph b of figure 1. The N animals and the -E+LA animals showed no weight loss and no changes in health status (Figure 1 and 2).

All tissues showed a large decrease of α-tocopherol in the animals on -E and -E+LA diet (Figure 3). Liver α-tocopherol showed the greatest depletion, to 15% of control levels ($p < 0.01$). Skin, kidney, and heart decreased to 20-30% of control levels ($p < 0.01$ for skin, $p <0.001$ for kidney and heart). The brain showed a much smaller, but still significant ($p < 0.01$), decrease to 73%. This data confirms recent observations in vitamin E deficient dogs and rats, where the CNS retained higher percentages of α-tocopherol than other, non-nervous tissues (15, 16)

The decrease of tissue α-tocopherol levels was independent from the supplementation with α-lipoic acid, since there were no significant differences in the tissue α-tocopherol levels between the -E and the -E+LA groups (Fig. 3)

α-Lipoic acid and dihydrolipoic acid were detectable only in -E+LA animals, where they were detectable in heart, kidney, liver, and skin (Table 1); 20-45% of the total lipoate was present in its reduced form. Other metabolites of α-lipoic acid or bound lipoate were not determined. In brain, dihydrolipoic acid was detectable in two tissue samples, but was undetectable in the other two samples. This could be due to different degrees of contamination of brain tissue samples with blood. Therefore, it is not possible to evaluate if lipoate crosses the blood brain barrier.

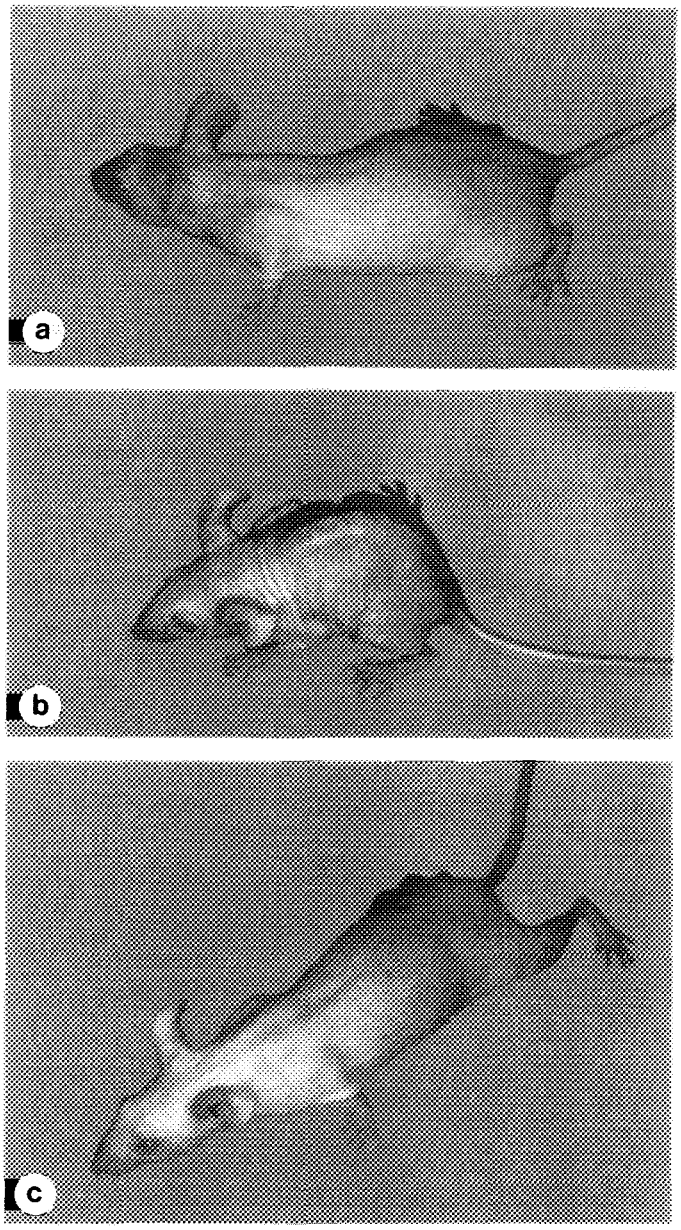
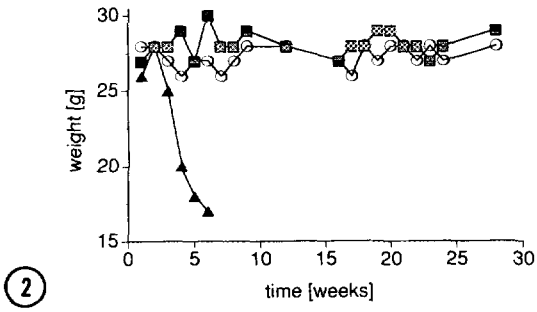


Fig. 1. Adult 12-week-old hairless mice after 6 weeks of **a** normal control diet (N); **b** Vitamin E deficient diet (-E); **c** Vitamin E deficient, α-lipoic acid supplemented diet (-E+LA). The animal on the -E diet shows symptoms of vitamin E deficiency with muscular dystrophy and weight loss.

DISCUSSION

Adult hairless mice fed a vitamin E-deficient diet high in PUFA start to lose weight after 3 weeks and show clear symptoms of vitamin E deficiency, more obvious due to the lack of fur, with muscular dystrophy and neurologic changes, after six weeks



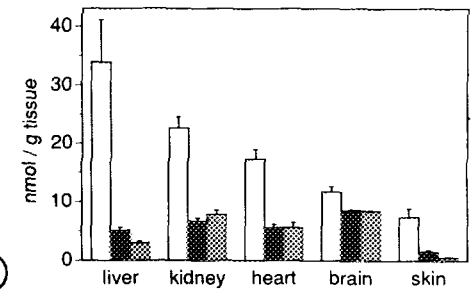


Fig. 2. Mean weight of animals on various diets (each group n=4). ○ Normal diet; ▲ vitamin E-deficient diet ; ▒ vitamin E deficient, α-lipoic acid supplemented diet. The vitamin E deficient animals lost weight rapidly after the third week, whereas the vitamin E deficient, α-lipoic acid supplemented maintained their weight and health status for the entire time monitored (28 weeks).

Fig. 3. α-Tocopherol levels in various animal tissues after 5 weeks diet.
White bars control, black bars vitamin E deficient, shaded bars vitamin E deficient, α-lipoic acid supplemented (n=4). All tissues show a large decrease in α-tocopherol concentration in the -E and -E+LA animals compared to N animals (liver, brain, skin $p<0.01$; kidney, heart $p<0.001$). The vitamin E levels in the -E and -E+LA group are not significantly different.

(Figure 1). Histological analysis of symptomatic animals shows muscular changes typical of vitamin E deficiency. Because of the rapidity of the onset and ease of detection of symptoms, we chose this new model to assess the effect of α-lipoic acid on vitamin E deficiency.

Previous studies have reported that synthetic antioxidants structurally unrelated to vitamin E can replace vitamin E in E-deficient animals. Thus, diphenyl-p-phenylenediamine (DPPD) and ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline) were found to be effective in the prevention of nutritional encephalomalacia and muscular dystrophy in the vitamin E-deficient chick (17) (18), and

Table 1: α-Lipoic-acid and dihydrolipoic acid concentrations in various tissues of α-lipoic acid supplemented, vitamin E deficient animals (n=4).
α-lipoic or dihydrolipoic acid was not detectable in tissues of non-α-lipoic acid supplemented animals.

tissue	α-lipoic acid (nmol/ g wet weight)			ratio
	oxidized	reduced	total	reduced / oxidized
liver	0.32±0.29	0.27±0.09	0.60±0.33	0.84
kidney	0.85±0.77	0.34±0.43	1.19±0.77	0.40
heart	2.70±3.33	0.71±0.64	3.42±3.96	0.26
skin	2.42±1.42	0.94±0.78	3.36±2.20	0.38

butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA), widely used as food antioxidants, have also been recognized to have some biological activity as vitamin E substitutes (19). However, all these synthetic substances have either toxic or carcinogenic potential in different organs (20, 21).

If α-lipoic acid acts as antioxidant in vivo, we would expect to see a delayed occurrence, or no occurrence at all, of vitamin E deficiency symptoms in vitamin E deficient animals fed α-lipoic acid. This was the case in the present study, confirming in the mouse model the observations of Rosenberg and Culik (11), and confirming in vivo the in vitro observations of the potent antioxidant activity of α-lipoic acid.

Measurements of tissue concentrations of α-tocopherol, α-lipoic acid, and dihydrolipoic acid allow a further refinement in our understanding of how α-lipoic acid may act in vivo as an antioxidant which spares vitamin E. Previous studies in cell culture showed a conversion of α-lipoic acid to dihydrolipoic acid by a variety of mammalian cells (2, 22). In the present experiment we extended these findings. This is the first demonstration of the conversion of α-lipoic acid to its reduced form dihydrolipoic acid in vivo.

It is possible that α-lipoic acid and/or dihydrolipoic acid act to recycle vitamin E, as has been shown in vitro in several systems (8-10), thus serving to keep vitamin E concentrations high, so that vitamin E performs its antioxidant functions adequately. On the other hand, α-lipoic acid and dihydrolipoic acid have also been shown in vitro to possess potent antioxidant activity in their own right (4), and may substitute for vitamin E. If we measure vitamin E levels in tissues when we start to see deficiency symptoms in the vitamin E deficient animals and no symptoms in the E-deficient, α-lipoic acid supplemented animals, we may distinguish between the two possibilities. In the first case, tissue E concentrations would be low in the E-deficient animals and higher in the E-deficient, α-lipoic acid supplemented animals. In the second case, E concentrations would be low in both sets of animals.

Vitamin E levels in all selected tissues were greatly decreased in the -E and -E+LA groups compared to control, but not different between the two groups. Therefore, the present study indicates that α-tocopherol concentrations were equally reduced in the presence or absence of dietary α-lipoic acid. This finding supports the idea that the major effect of α-lipoic acid in E-deficient animals is to replace vitamin E, not to recycle it.

However, it cannot be ruled out that α-lipoic acid antioxidant activity may be acting through other antioxidants such as ubiquinol or ascorbate, where in vitro recycling has been described. Further studies should address this alternative.

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