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FREE RADICAL-INDUCED LIVER INJURY. I. EFFECTS OF DIETARY VITAMIN E DEFICIENCY ON TRIACYLGLYCEROL LEVEL AND ITS FATTY ACID PROFILE IN RAT LIVER

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Effects of dietary vitamin E deficiency on the fatty acid compositions of total lipids and phospholipids were studied in several tissues of rats fed a vitamin E-deficient diet for 4, 6, and 9 months. No significant differences were observed between the vitamin E deficiency and controls except in the fatty acid profiles of liver total lipids. Triacylglycerol (TAG) accumulation was found in the liver of rats fed a vitamin E-deficient diet. The levels of TAG-palmitate and -oleate increased particularly in the liver from such animals. The fatty acid compositions of hepatic phospholipids were not affected by the diet. Increased TAG observed in the liver of rats fed a vitamin E-deficient diet was restored to normal when the diet was supplemented with 20 mg α-tocopheryl acetate/kg diet. These findings indicate that dietary vitamin E deficiency causes TAG accumulation in the liver and that the antioxidant, vitamin E, is capable of preventing free radical-induced liver injury.

KEY WORDS: Free radical, vitamin E deficiency, α-tocopherol, hepatic phospholipids, triacylglycerol.

INTRODUCTION

It has been generally accepted that free radicals play an important role in the oxidative deterioration of subcellular membranes, and cause pathological changes, such as liver injuries and neurological disorders.[1-4] Some of these changes have been demonstrated in experimental animals with vitamin E deficiency.[5-8] The biological antioxidant, vitamin E, is known to scavenge free radicals and provides protection against injury due to lipid peroxidation.[9-11] In early studies, several investigators showed a decrease of polyunsaturated fatty acids in the tissue lipids of vitamin E-deficient rats,[12-14] while other workers[15-17] found no significant differences between vitamin E deficiency and controls. These findings may be different because it is difficult to control free radical generation *in vivo*,[18] and a number of general homeostatic process occur in all living tissues, even in antioxidant-deficient animals.[12,13] The study presented here was designed to examine the liver cell injury due to free radicals caused by dietary vitamin E deficiency.

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MATERIALS AND METHODS

Materials

Fatty acid standards for GLC analyses were purchased from Nu-Chek-Prep., Inc. (Elysian, MN). α-Tocopherol and tocol as standards for HPLC analysis were gifts from Eisai Co., Ltd. (Tokyo, JPN). A nucleosil column (5 μm, 250 mm × 4.6 mm, i.d.) was purchased from Senshu Pak Co., Ltd. (Tokyo, JPN). All other reagents were obtained from Kanto Chemical Co., Inc. (Tokyo, JPN). and organic solvents from Wako Pure Chemical Ind. (Tokyo, JPN).

Animals and diets

Weanling male Sprague-Dawley rats, 3 weeks old, were purchased from The Tokyo Animal Experimental Laboratory (Tokyo, JPN) and fed a basal diet containing the following by weight %: sucrose, 25.0; glucose, 25.0; vitamin-stripped casein, 20.0; corn starch, 15.0; fiber, 5.0; AIN minerals, 3.5; D,L-methionine, 0.3; AIV vitamins (without vitamin E), 1.0; and choline bitartrate, 0.2. This diet is a slight modification of the American Institute of Nutrition AIN-76 purified diet.[19] The basal diet was mixed with tocopherol-stripped corn oil (Eisai Co. Ltd., JPN) at a 92:8 ratio and prepared every two weeks and stored at −20°C until use. There was no significant change in the fatty acid composition of the diet during storage for two weeks. The control diet was supplemented with 20 mg α-tocopheryl acetate (20 I.U.)/kg diet and had the following fatty acid composition: 12.7% palmitic acid, 2.1% stearic acid, 31.9% oleic acid, 51.9% linoleic acid, and 1.3% α-linolenic acid.

Lipid extraction and analysis

Animals were fasted overnight and ca. 5 ml of blood were collected in heparinized tubes before sacrifice by decapitation. Total lipids were extracted from the plasma and tissue with chloroform/methanol (2:1, v/v) as described by Folch *et al.*[20] Neutral lipids and phospholipids were separated on a precoated thin-layer chromatography (TLC) plate in one dimension with a solvent system of hexane/diethyl ether/acetic acid (70 : 30 1, v/v/v). The TLC plate was kept for dryness under a stream of nitrogen, and the spots of free fatty acids, triacylglycerol, and cholesterol ester were identified from their Rf values on the plate by comparison with authentic standards. The area of the neutral lipids and that of phospholipids, remaining at the origin, were scraped directly into test tubes, and the lipids were esterified under an atmosphere of nitrogen with methanol using 5% anhydrous HC1, as a catalyst, at 85°C for 2 hours. Pentadecanoic acid (25.2 μg) was used as the internal standard. Gas liquid chromatography (GLC) was carried out with a Shimazu 1A gas chromatograph equipped with a flame ionization detector using a column of 15% EGSS-X at 195°C.[21] Nitrogen was used as the carrier gas.

Determination of α-tocopherol

α-Tocopherol was measured with a high performance liquid chromatography (HPLC) using an analytical colum (5 μm Nucleosil, 250 mm × 4.6 mm, i.d.) and JASCO LC-880 variable UV/VIS detector (JASCO UV-870, Tokyo, JPN). The mobile phase

TABLE 1
Body and liver weights, and tissue α-tocopherol levels in rats fed the vitamin E-deficient diet

	Feeding periods of diets								
	4 months		6 months		9 months				
	Group 1 (n = 4)	Group 2 (n = 4)	Group 1 (n = 6)	Group 2 (n = 6)	Group 1 (n = 4)	Group 2 (n = 4)	Group 3 (n = 4)
Body wt (g)	563.5 ± 37.1	527.7 ± 35.7	520.5 ± 65.1	541.0 ± 66.4	664.5 ± 73.5	582.1 ± 76.2	657.5 ± 50.9
Liver (g)	17.8 ± 1.9	17.5 ± 1.5	20.9 ± 3.9	22.1 ± 2.8	18.7 ± 1.8	17.7 ± 1.2	17.6 ± 0.5
Tissue α-tocopherol levels (μg/mg protein)							
Liver	0.05 ± 0.006	0.001 ± 0.0007	0.07 ± 0.02	0.001 ± 0.0002	0.09 ± 0.005	n.d.	0.06 ± 0.006
Heart	0.11 ± 0.01	0.004 ± 0.001	0.10 ± 0.01	0.014 ± 0.005	0.16 ± 0.007	0.006 ± 0.001	0.14 ± 0.006
Lung	0.16 ± 0.01	0.005 ± 0.001	0.12 ± 0.01	0.005 ± 0.002	0.22 ± 0.016	0.005 ± 0.001	0.15 ± 0.025
Kidney	0.06 ± 0.014	0.001 ± 0.0001	0.05 ± 0.001	0.001 ± 0.0005	0.10 ± 0.25	0.003 ± 0.001	0.06 ± 0.005
Spleen	0.11 ± 0.02	0.003 ± 0.0005	0.09 ± 0.01	0.002 ± 0.0005	0.10 ± 0.01	0.003 ± 0.001	0.10 ± 0.009
Testes	0.10 ± 0.011	0.007 ± 0.0005	0.09 ± 0.001	0.005 ± 0.0005	0.12 ± 0.009	0.007 ± 0.001	0.08 ± 0.001
Brain	0.09 ± 0.012	0.027 ± 0.002	0.14 ± 0.001	0.034 ± 0.007	0.09 ± 0.015	0.017 ± 0.002	0.07 ± 0.007

[1] Mean ± S.D.
Group 1 (control) received the basal diet supplemented with the 20 IU α-tocopheryl acetate/kg diet.
Group 2 (vitamin E deficiency) received the diet without the vitamin E-supplement.
Group 3 received the vitamin E-deficient diet for 6 months, and then the control diet for 3 months.

was a solvent system of hexane/2-propanol (98 : 2, V/V) at a flow rate of 1.0 ml/min, and the column effluent was monitored at 295 nm. All samples for the HPLC analysis contained 3.84 μg tocol as the internal standard.

Statistical analysis

All data were expressed as means ± S.D. Statistical analysis was performed by the Student t-test with $p < 0.05$ being considered significant.

RESULTS

Tissue and plasma α-tocopherol of rats fed the vitamin E-deficient and control diets

Body and liver weights and tissue α-tocopherol levels are shown in Table 1. The levels of α-tocopherol in several tissues of rats fed the vitamin E-deficient diet for 4,6, and 9 months were significantly lower than those of control rats in any of the measures ($p < 0.001$; group 1 vs group 2). α-Tocopherol content of the tissues of rats fed the vitamin E-deficient diet for 6 months and then the control diet for 3 months (group 3) was restored to normal (group 1). Plasma α-tocopherol levels decreased from 4.27 ± 0.7 μg/ml, a control level (group 1), to 0.19 ± 0.02 μg/ml at 4 months; 0.17 ± 0.03 μg/ml at 6 months and 0.08 ± 0.01 μg/ml at 9 months (group 2), while the level of μ-tocopherol in group 3 was 1.87 ± 0.56 μg/ml which was significantly lower than that of the control ($p < 0.01$; group 1 vs group 3).

Effects of vitamin E deficiency on liver lipids

Total lipid content of liver in control rats (group 1) was 57.67 ± 7.64 mg/g wet weight of tissue at 4 months, but it slightly decreased thereafter (Table II). In contrast to this, total lipids in the liver of vitamin E-deficient rats increased significantly from the control level to 70.62 ± 8.24 mg/g at 4 months ($p < 0.05$); 79.07 ± 7.44 mg/g at 6 months ($p < 0.01$) and 88.86 ± 10.07 mg/g at 9 months ($p < 0.01$), respectively. Triacylglycerol (TAG) was the most prominent component in the hepatic lipids of the vitamin E-deficient rats. The densitometric detection showed that the level of TAG in the livers increased more than 3.8-fold at 6 months ($p < 0.001$; group 1 vs group 2).

TABLE II
Changes in hepatic total lipids of rats fed the vitamin E-deficient diet

	Total lipids mg/g wet weight		
	Feeding periods of diets		
Diets[1]	4 months ($n = 4$)	6 months ($n = 6$)	9 months ($n = 4$)
Group 1	57.67 ± 7.64[2]	53.73 ± 8.95	50.73 ± 2.12
Group 2	70.62 ± 8.24*	79.07 ± 7.44*	88.86 ± 10.07*
Group 3	-	-	50.06 ± 8.15**

[1] See text for details.
[2] Mean ± S.D.
* Difference between group 1 vs group 2 was significant ($p < 0.01$).
** NS

TABLE III
Levels of fatty acid residues of triacylglycerol in the liver of rats fed the vitamin E-deficient diet

	Major fatty acids mg/g wet weight of liver						
	Feeding periods of diets						
	4 months		6 months		9 months		
	Group 1 (n = 4)	Group 2 (n = 4)	Group 1 (n = 6)	Group 2 (n = 6)	Group 1 (n = 4)	Group 2 (n = 4)	Group 3 (n = 4)
16:0	4.63 ± 1.82[1]	5.93 ± 1.55	3.52 ± 0.71	11.61 ± 4.83	3.31 ± 0.65	10.47 ± 2.38	4.37 ± 0.58
16:1	0.87 ± 0.42	1.14 ± 0.33	0.51 ± 0.21	2.86 ± 0.85	0.62 ± 0.16	2.13 ± 0.88	0.60 ± 0.31
18:0	0.37 ± 0.13	0.41 ± 0.17	0.13 ± 0.05	0.57 ± 0.36	0.15 ± 0.03	0.59 ± 0.13	0.23 ± 0.08
18:1	5.89 ± 2.11	8.48 ± 2.91	3.77 ± 0.86	10.18 ± 5.62	4.61 ± 1.13	15.42 ± 3.54	4.96 ± 1.88
18:2	4.51 ± 1.47	6.29 ± 3.61	3.24 ± 0.58	6.88 ± 0.24	3.04 ± 0.49	6.51 ± 1.40	2.46 ± 0.23
20:4	0.45 ± 0.11	0.50 ± 0.32	0.18 ± 0.18	0.16 ± 0.01	0.21 ± 0.04	0.39 ± 0.07	0.18 ± 0.03

[1] Mean ± S.D.
See text for details.

TABLE IV
Levels of fatty acid residues of hepatic phospholipids, phosphatidylcholine, and phosphatidylethanolamine of rats fed the vitamin E-deficient diet for 9 months

	Major fatty acids mg/g wet weight of liver								
	Total phospholipids			phosphatidylcholine			phosphatidylethanolamine		
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
16:0	2.61 ± 0.13[1]	2.43 ± 0.17	2.61 ± 0.17	1.77 ± 0.11	1.62 ± 0.11	1.74 ± 0.11	0.59 ± 0.04	0.54 ± 0.08	0.52 ± 0.07
16:1	0.16 ± 0.03	0.15 ± 0.04	0.17 ± 0.05	0.06 ± 0.01	0.07 ± 0.02	0.09 ± 0.02	tr	tr	tr
18:0	4.15 ± 0.33	4.74 ± 0.15	4.19 ± 0.51	2.89 ± 0.24	3.11 ± 0.22	2.77 ± 0.31	1.10 ± 0.04	1.07 ± 0.05	0.89 ± 0.06
18:1	1.40 ± 0.04	1.27 ± 0.15	1.46 ± 0.22	0.63 ± 0.03	0.65 ± 0.10	0.71 ± 0.06	0.28 ± 0.01	0.29 ± 0.04	0.27 ± 0.04
18:2	1.71 ± 0.19	1.27 ± 0.15	1.69 ± 0.34	0.79 ± 0.11	0.65 ± 0.08	0.90 ± 0.21	0.33 ± 0.04	0.26 ± 0.06	0.27 ± 0.06
20:4	6.09 ± 0.47	6.23 ± 0.21	5.83 ± 0.58	4.25 ± 0.35	4.46 ± 0.27	4.24 ± 0.46	1.61 ± 0.10	1.52 ± 0.16	1.37 ± 0.27

[1] Mean ± S.D. from analysis of 4 rats.
See text for details.

TABLE V
Levels of fatty acids in plasma and red blood cell total lipids of rats fed the vitamin E-deficient diet for 9 months

	Plasma (mg/ml plasma)			Red blood cells (μg/μmol Phosphorus)		
	Group1	Group 2	Group 3	Group 1	Group 2	Group 3
16:0	0.43 ± 0.11[1]	0.43 ± 0.03	0.41 ± 0.13	25.82 ± 1.04	25.67 ± 1.95	23.81 ± 2.09
16:1	0.06 ± 0.02	0.07 ± 0.01	0.07 ± 0.02	1.44 ± 0.11	1.59 ± 0.15	1.26 ± 0.17
18:0	0.20 ± 0.04	0.19 ± 0.02	0.23 ± 0.05	11.96 ± 0.48	11.56 ± 2.71	10.88 ± 0.47
18:1	0.45 ± 0.14	0.47 ± 0.15	0.53 ± 0.16	8.82 ± 0.29	8.79 ± 0.92	7.97 ± 0.85
18:2	0.33 ± 0.08	0.35 ± 0.05	0.36 ± 0.09	8.65 ± 1.27	8.03 ± 0.99	7.98 ± 0.63
20:4	0.59 ± 0.13	0.51 ± 0.07	0.62 ± 0.14	25.36 ± 2.22	25.58 ± 3.30	22.36 ± 0.89

[1] Mean ± S.D. from 4 rats
See text for details.

Table III shows the fatty acid composition of hepatic TAG-acyl residues in the vitamin E-deficient and control rats. The levels of palmitic acid (16:0) and oleic acid (18:1 n-9) increased particularly in the hepatic lipids of the vitamin E-deficient rats (group 2), but those were normal in group 3 (Table III). It should be noted that linoleic acid also increased in hepatic TAG under the condition of dietary vitamin E deficiency.

The fatty acid compositions of hepatic phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, in vitamin E-deficient rats were essentially the same as those of the control rats (Table IV). No significant differences were noted between group 1 and group 2 in the fatty acid compositions of plasma and red blood cell total lipids (Table V). In preliminary experiments, these groups showed no significant changes in the fatty acid compositions of total lipids from various organs, such as the heart, lung, kidney, spleen, testis, and brain (data not shown).

DISCUSSION

Quantitative analysis of tissue and plasma α-tocopherol in rats fed the vitamin E-deficient diet for 4 months showed the lowest levels of this vitamin in the animals except the brain where it continued to decrease for 9 months. The dietary vitamin E deficiency caused TAG accumulation in the liver of rats, despite a normal level of phospholipids. The TAG level of the liver in the vitamin E-deficient rats, however, decreased to the control level when the antioxidant, α-tocopheryl acetate, was added to the diet. The result indicates that the antioxidant deficiency leads to free radical-induced liver injury, such as fatty liver. The administration of AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) and CCl_4 (carbon tetrachloride), well-known radical initiators *in vivo*, caused TAG accumulation in the liver of experimental animals.[18,22,23] The mechanism of TAG accumulation is not known, but the action of free radicals is resembles that caused by dietary vitamin E deficiency.

In early studies[13,14] the loss of polyunsaturated fatty acids (PUFA) in hepatic phospholipids has been reported in rats fed a vitamin E-deficient diet. Recent studies,[16,17] however, show no significant loss in hepatic PUFA from vitamin E-deficient rats. Change in PUFA as observed in early studies may possibly have been due to inadequate.[16] No significant differences in the PUFA profiles of hepatic phospholipids could be found in this study between the vitamin E-deficient and control rats. In the antioxidant-deficient animals, loss of PUFA may have been due to the small area of tissue lipids *in vivo*.[16] However, it was not detectable in a whole tissue homogenate, such as a liver homogenate under the present conditions. Moreover, the tissue lipids can be protected from free radical mediated lipid peroxidation by *in vivo* homeostatic mechanisms, even in the vitamin E-deficient animals.[12,13] These findings are essentially the same as observed in the rats treated with AAPH and CCl_4.[23]

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