Effect of Dietary Protein on Vitamin E Levels in Erythrocytes and Tissues of Rats

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Summary The effect of dietary protein level on the transfer of α -tocopherol in blood to tissues including RBC was studied using rats. The first experiment comprised a 10% casein (low protein), 20% casein (normal) and 20% SPI (normal soybean protein) diet groups supplemented with 71.5 mg of α -Toc/kg diet. In Exp. 2 the relationship of the tissue α -Toc level and protein level in diets, as shown by recovery from vitamin E-deficient status after the administration of α -Toc for 3 days, was checked by adjusting the protein level in diets to 10%, 20% and 40% casein. In Exp. 1 α -Toc in RBC decreased significantly in the 10% casein and 20% SPI groups compared to the 20% casein group. Moreover, α -Toc in kidney, lung and muscle decreased significantly in the 10% casein and 20% SPI groups. α -Toc in liver in the 20% SPI group decreased significantly compared to the 20% casein group. In Exp. 2 similar results were observed (Table 4), but α -Toc in RBC showed only a tendency to decrease with the low protein diet. In Exp. 1 free cholesterol in RBC increased significantly in the 10% casein group compared with the other two groups.

Key Words α -tocopherol concentration in tissues, erythrocyte α -tocopherol level, protein level in diets, transfer of α -tocopherol, vitamin E-deficient

In a previous paper (1), it was shown that the transfer of circulating vitamin E (α -tocopherol, α -Toc) to erythrocytes (RBC) in rats was affected by the quantity and quality of dietary protein, even when the serum α -Toc was kept at exactly the same level in all dietary protein groups. When the dietary casein level was kept at a level lower than 10%, α -Toc in RBC significantly decreased as compared with the control (20% casein) group. This occurred independently of the level of serum

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cholesterol. The effect of soy protein isolate (SPI) was examined and compared to casein in the previous study (1). α -Toc levels in serum and RBC were lower in the SPI group than in the casein group, both at 10% and 20% dietary protein levels. The reason why dietary proteins affect tocopherol concentrations is assumed to be secondary to changes in protein concentrations affiliating to tocopherol, such as lipoproteins in serum and binding proteins in tissue cells, since it has been recognized that tocopherol is localized in lipoproteins in serum (2), while it exists in a form bound with specific binding proteins in RBC membranes (3) and in cytosol in tissue cells of liver, small intestine, lung and kidney (4-7).

Half of present investigation was attempted to further extend and confirm the above results, especially changes in tocopherol concentrations in several tissues besides the liver, by a trial with a similar dietary protein. α -Toc in RBC responded to the quantity and quality of dietary protein, while α -Toc in liver did not. Clinically, RBC or platelet α -Toc was used to evaluate vitamin E status (8). It is important to know whether the RBC α -Toc level interrelates with α -Toc levels in tissues such as kidney, lung, heart and muscle under low-protein nutrition or not. The other half of the present investigation was attempted to determine the effect of dietary protein on α -Toc levels in tissues including RBC after recovery from α -Toc deficiency by refeeding α -Toc.

EXPERIMENTAL

1. Animals and feeding

Male Sprague-Dawley strain rats weighing 120 to 150 g initially were fed the following standard diet for a week: vitamin-free casein, 20.0%; sucrose, 63.8%; vitamin E-free stripped corn oil, 8.0%; mineral mixture, 4.0%; vitamin mixture, 1.0%; choline chloride, 0.2%; and cellulose powder, 3.0%. Compositions of the mineral mixture and vitamins except for vitamin E followed Harper (9).

Experiment 1. Animals were divided into 3 groups (each group containing 10 animals): 20% casein, 10% casein and 20% soybean protein isolate (SPI)-fed groups. In the 10% protein group the protein level in the diet was adjusted by sucrose. For SPI commercially available Fujipro-R was used and kindly donated by Fuji Oil Manufacturing Co., Osaka, Japan. This SPI was prepared from soybean extract after heat treatment to inactivate trypsin inhibitors. Animals in each group were pair-fed with 17 g/day of the diet for 6 weeks. α -Toc levels in each diet were kept at 71.5 mg/kg diet during feeding. Tocopherol contents in SPI were as follows: α -Toc 76, γ -Toc 164 and δ -Toc 94 μ g/kg.

Experiment 2. The rats were prefed a diet containing α -Toc (71.5 mg/kg diet) for 1 week, and then divided into 3 groups (10 animals each): the 40%, 20% and 10% casein groups. After a vitamin E-deficient diet had been administered for 6 weeks by pair-feeding (daily intake 17 g), about half of the animals in each group were sacrificed and the remaining animals were fed a vitamin E-supplemented diet (*dl*- α -Toc 71 mg/kg diet, daily intake 10 g) for 3 days. The daily intake of α -Toc for each animal was *ca.* 2.5 mg/kg body weight.

Before sacrifice the animals were fasted overnight. Blood was drawn from the aorta after ethyl ether anesthesia. Part of the blood was heparinized for separation of RBC while the remaining blood was untreated with heparine to separate serum. Liver, kidney, lung and skeletal muscle (*musculus quadriceps femoris*) were used to analyze the α -Toc level and 2-thiobarbituric acid reactive substances (TBARS).

2. Materials

dl- α -Toc for diets and the authentic specimens of *d*- α -, *d*- β -, *d*- γ - and *d*- δ -tocopherols for high performance liquid chromatography (HPLC) were kindly donated by Eisai Co., Ltd., Tokyo. 2,2,5,7,8-Pentamethyl-6-hydroxy-chroman (PMHC) as the internal standard for HPLC was synthesized in our laboratory using the method of Smith *et al.* (10,11).

3. Analytical methods

Total cholesterol in serum was determined by the enzymatic method using a kit from Kyowa Fermentation Co., Ltd., Tokyo. α -Toc in serum was determined by HPLC following the method of Abe *et al.* (12), and that in tissues and RBC was similarly determined by HPLC after saponification following the method of Abe *et al.* (13,14). PMHC as an internal standard was added to the extract as *n*-hexane solution before HPLC.

Free cholesterol in RBC was determined following Rose's method as modified by Shibata *et al.* (15). Lipid peroxide amounts (TBARS) in RBC were determined by the method of Brownlee *et al.* (16) and those in liver by the method of Uchiyama and Mihara (17).

RESULTS

1. Experiment 1

Table 1 shows α -Toc concentrations in RBC and serum, and total cholesterol level in serum and free cholesterol in RBC in the 10% and 20% casein groups and the 20% SPI group, respectively. α -Toc in RBC was significantly higher only in the 20% casein group as compared with the other groups, while α -Toc and total cholesterol in serum did not differ from each other. α -Toc in sera of the 20% and 10% casein groups was slightly higher than that of the 20% SPI group, but the difference was statistically not significant. In the 10% casein group, free cholesterol in RBC significantly increased compared to that in the 20% protein group, but it did not differ between the 20% protein groups.

In Table 2 the α -Toc concentrations in tissues, liver, kidney, lung and muscle are shown. The α -Toc concentration in every tissue of the 20% casein group was higher than in the other two groups. In the 10% casein and 20% SPI groups there was no difference in the α -Toc level in any of the tissues examined.

2. Experiment 2

Tables 3 and 4 show the results of hemolysis induced by dialuric acid, TBARS in RBC and liver, and α -Toc concentrations in the tissues after feeding a vitamin E-

Table 1. Effect of protein level in diets on α -tocopherol and free cholesterol levels in RBC, and α -tocopherol and total cholesterol in serum of rats (Exp. 1).

Group	RBC		Serum	
	α -Toc (μ g/ml packed cell)	Free cholesterol (mg/dl packed cell)	α -Toc (μ g/ml)	Total cholesterol (mg/dl)
20% Casein	6.1 $\pm$ 1.0*	105.2 $\pm$ 4.2*	6.8 $\pm$ 0.8*	67.5 $\pm$ 7.4
10% Casein	5.0 $\pm$ 0.5 ^a	129.0 $\pm$ 6.3 ^a	6.7 $\pm$ 0.7	68.0 $\pm$ 7.7
20% SPI	4.3 $\pm$ 0.7 ^a	107.6 $\pm$ 10.1	6.1 $\pm$ 1.6	65.3 $\pm$ 7.8

* Mean $\pm$ SD. ^a Significantly different from 20% casein group ($p < 0.05$).

Table 2. The effect of diet protein levels upon α -tocopherol concentration in tissues of rats.

Group	Liver*	Kidney*	Lung*	Muscle*
20% Casein	40.0 $\pm$ 8.5	25.1 $\pm$ 2.9	45.2 $\pm$ 4.8	16.4 $\pm$ 3.0
10% Casein	37.5 $\pm$ 6.0	21.4 $\pm$ 6.0 ^b	35.2 $\pm$ 4.6 ^c	13.5 $\pm$ 0.8 ^b
20% SPI	33.0 $\pm$ 9.0 ^a	19.8 $\pm$ 2.6 ^b	30.7 $\pm$ 5.0 ^c	13.2 $\pm$ 2.2 ^b

* μ g/g fresh tissue. Mean $\pm$ SD. Values within column sharing a superscript letter differ significantly from 20% casein group (^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$).

Table 3. Hemolysis of RBC with dialuric acid and TBARS in erythrocyte and liver from vitamin E-deficient rats (Exp. 2).

Group	Hemolysis	TBARS	
		RBC (MDA nmol/ml packed cell)	Liver (MDA nmol/g)
40% Casein	88.3 $\pm$ 7.5*	2.1 $\pm$ 0.3	105.0 $\pm$ 27
20% Casein	84.2 $\pm$ 6.6	2.6 $\pm$ 0.4	98.9 $\pm$ 27
10% Casein	81.8 $\pm$ 18.8	2.7 $\pm$ 0.4	108.0 $\pm$ 27

* Mean $\pm$ SD.

deficient diet for 6 weeks, respectively. Table 5 shows α -Toc and free cholesterol concentrations in RBC, and α -Toc and total cholesterol in serum in rats fed an α -Toc-supplemented diet for 3 days after 6-week administration of α -Toc-deficient diets. Table 6 shows α -Toc concentrations in the tissues, liver, kidney, lung, heart and muscle of the same rats. In this experiment protein levels in diets were kept at 10, 20 and 40% with casein alone.

Table 4. α -Tocopherol levels in liver, kidney, lung, heart and muscle of vitamin E-deficient rats (Exp. 2).

Group	Liver*	Kidney*	Lung*	Heart*	Muscle*
40% Casein	2.70 $\pm$ 0.67	3.98 $\pm$ 0.17	3.99 $\pm$ 0.98	5.39 $\pm$ 0.35	2.90 $\pm$ 0.55
20% Casein	2.33 $\pm$ 0.13	2.91 $\pm$ 0.02	3.46 $\pm$ 0.31 ^b	4.51 $\pm$ 0.37 ^c	2.50 $\pm$ 0.20
10% Casein	2.08 $\pm$ 0.62	2.44 $\pm$ 0.43 ^a	2.69 $\pm$ 0.63 ^b	4.13 $\pm$ 0.53 ^c	2.39 $\pm$ 0.48

* $\mu\text{g/g}$ fresh tissue. Mean $\pm$ SD. Values within column sharing a superscript letter differ significantly from 40% casein group (^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$).

Table 5. α -Tocopherol levels in liver, kidney, lung, heart and muscle of vitamin E-refed rats (Exp. 2).

Group	Liver	Kidney	Lung	Heart	Muscle
			($\mu\text{g/g}$ fresh weight)		
40% Casein	15.9 $\pm$ 3.6*	7.49 $\pm$ 0.66	14.8 $\pm$ 1.8	11.3 $\pm$ 1.8	3.74 $\pm$ 0.20
20% Casein	17.7 $\pm$ 3.2	6.18 $\pm$ 0.40 ^a	12.7 $\pm$ 1.3 ^c	10.7 $\pm$ 1.3	3.27 $\pm$ 0.22 ^e
10% Casein	14.8 $\pm$ 2.9	4.20 $\pm$ 0.50 ^b	12.3 $\pm$ 1.3 ^c	8.75 $\pm$ 0.8 ^d	2.91 $\pm$ 0.51 ^e

* Mean $\pm$ SD. ^{a,c} and ^e superscript letters mean a significant difference from 40% casein group (^a $p < 0.001$, ^c $p < 0.05$, ^e $p < 0.01$). ^b and ^d superscript letters mean a significant difference from 20% and 40% casein groups (^b $p < 0.001$, ^d $p < 0.05$).

Table 6. α -Tocopherol and free cholesterol levels in erythrocyte and α -tocopherol and total lipids in serum of vitamin E-refed rats (Exp. 2).

Group	Erythrocyte		Serum	
	α -Toc ($\mu\text{g/ml}$ packed cell)	Free cholesterol (mg/dl packed cell)	α -Toc ($\mu\text{g/ml}$)	Total lipids (mg/dl)
40% Casein	2.99 $\pm$ 0.46*	122 $\pm$ 10	3.65 $\pm$ 0.25	135 $\pm$ 1.5
20% Casein	3.15 $\pm$ 0.24	117 $\pm$ 7.2	4.17 $\pm$ 0.97	131 $\pm$ 22.0
10% Casein	2.83 $\pm$ 0.40	125 $\pm$ 12	4.50 $\pm$ 0.59 ^a	158 $\pm$ 22.0 ^b

* Mean $\pm$ SD. ^a Significantly different from 40% casein group ($p < 0.05$). ^b Significantly different from 40% and 20% casein groups ($p < 0.05$).

Hemolysis induced by dialuric acid and TBARS of RBC and liver did not differ among the groups (Table 3). α -Toc in RBC in each group was not detectable by HPLC. The α -Toc concentration in each tissue of vitamin E-deficient rats (Table 4) was low, compared with the vitamin E-supplemented groups as shown in Table 5. It is noteworthy that a higher protein diet with 40% casein prevents the decrease of

α -Toc levels in tissues, especially in lung and heart, as shown in Table 4. No difference in free cholesterol in RBC was found among the groups (Table 6).

Vitamin E supplementation for 3 days after administration of a vitamin E-deficient diet for 6 weeks increased the levels of α -Toc in every tissue and in serum and RBC (Tables 5 and 6). The 40% casein group showed the highest α -Toc concentrations in kidney, lung and muscle. In heart, the 40% and 20% casein groups had significantly higher α -Toc levels than the 10% casein group, but α -Toc in RBC and liver was not significantly different among the groups (Tables 5 and 6). Serum total lipids was significantly higher in the 10% casein group than in the other two groups.

DISCUSSION

1. Experiment 1. In a previous paper, it was shown that a low protein diet or a diet with poorer proteins (SPI) caused tocopherol concentrations in RBC to decrease as compared with the control diet group. The present data are consistent with the previous results, because in the rats on a low (10%) casein diets and a diet consisting of poor quality proteins (SPI), tocopherol concentrations in RBCs were low, as compared with the control group (20% casein). Although it is well known that tocopherol concentrations in serum (18) and RBC are affected by lipid concentrations in serum, so that a higher tocopherol concentration in hyperlipemic serum is accompanied by a lower RBC tocopherol concentration, our previous and present data show that there are no changes in serum lipids, RBC tocopherol concentrations decreased by poor quality and a small quantity of dietary proteins. In addition, it was confirmed in this study that in spite of the intake of the same amount of vitamin E, a decrease in tocopherol concentrations in tissues (except for liver) was also documented when dietary proteins were low, and there was a decrease in the RBC tocopherol concentration as well. From these findings, we assume that intake of low-protein diets may decrease the efficacy of tocopherol transport from blood to tissue cells, and furthermore, it may be difficult to maintain adequate tocopherol into the cells, secondary to a decrease in circulating proteins containing tocopherol (lipoproteins) in blood and a decrease in cytosol binding proteins to tocopherol in the cells, respectively. However, in the liver, no significant changes were observed in the low-protein diet group. Since liver may be a depositary organ for tocopherol, thus differing from other tissues, a decrease in tocopherol in liver occur last among tissues as tocopherol deficiency occurs. On the other hand, changes in tocopherol concentrations in RBCs were found to reflect those in tissues examined, except for liver. Mino *et al.* (8) proposed that RBC tocopherol is an expression of the nutritional vitamin E status, apart from the Horwitt's index of plasma tocopherol expressed in terms of total lipids (the tocopherol/lipids ratio) which was previously thought to be a very reliable index of vitamin E status (19). Our results agree with the view that RBC tocopherol is an extremely good index for the assessment of bioavailable vitamin E.

From the results on the effect of SPI, the quality of protein is also known to influence the vitamin E nutritional status. Two hypotheses may be suggested to account for this: 1) the lower protein score of SPI than that of casein, and 2) secondary to cholesterol changes as indicated in previous reports, in which SPI was shown to reduce serum cholesterol (20-23). The mechanism however remains to be solved. In our present study, the low-protein diet (10% casein) induced an elevation of cholesterol in RBC, indicating that membrane cholesterol may be affected by protein content in diet. Nakagawa (24) reported that a negative correlation was shown in RBC in normal children between membrane cholesterol and tocopherol. Our result thus agreed with Nakagawa's observation. However, when comparing the 20% casein group and the 20% SPI group, there was no difference in RBC cholesterol concentrations, while tocopherol concentrations were lower in the SPI group than in the casein group. This difference is yet unexplainable and remains to be studied further.

2. *Experiment 2.* Experiment 2 was undertaken to investigate the effect of dietary protein intake on vitamin E-deficient RBC and tissues, and additionally, the effect on the tocopherol levels after refeeding with a vitamin E-supplemented diet in vitamin E-deficient rats. After consuming deficient diets for 6 weeks, almost complete hemolysis induced by dialuric acid was documented in all the groups receiving the three amounts of dietary protein, indicating development of vitamin E deficiency. Differences in the amounts of protein intake did not affect lipid peroxidation in the liver and RBC, as shown in the TBARS levels. This may possibly be due to similar tocopherol concentrations in the RBC and liver among the three groups (Table 4). In addition to liver and muscle, a significant reduction of tocopherol concentrations occurred in the lowest protein group as compared with the highest one. In the 20% group tocopherol concentrations in tissues showed moderate elevation from the lowest group, but the difference was not significant. For long-term deficiency of vitamin E, a higher intake of protein than the regular level (20%) would be needed to maintain a higher concentration of tocopherol in tissues. This finding suggests that either consumption of tocopherol in tissues becomes greater or the retention of tocopherol in tissues becomes smaller in low-protein nutrition. This tendency was more clear in the increment of tocopherol concentration in rats which recovered from vitamin E deficiency after administration of a vitamin E-supplemented diet for 3 days. These results in Exp. 2 were slightly different from those in Exp. 1, because in the former a significant difference in tocopherol concentrations in tissues in vitamin E-deficient rats were observed only in kidney, lung and heart between the 40% casein and the other groups, while in the latter it was additionally found between the 20% and 10% casein groups. We think that this difference comes from a decrease in the retention of tocopherol in the body after a 6-week deficiency. Since after long-term deficiency the concentrations of tocopherol in tissues became quite small, we could not observe a statistically significant difference in tissues between the 20% and 10% casein groups, but a tendency of decrease was shown. Although after refeeding vitamin E for 3 days

tocopherol was transported into every tissue cell, a difference might occur in the rate of incorporation of tocopherol among tissue cells. Thus, recovery from vitamin E deficiency may differ among tissues. This also indicates that protein nutrition may affect the tocopherol nutritional status through changes in the transporting proteins in serum and/or binding proteins in cells. However, RBC and liver tocopherol concentrations were not different among the three groups, in which serum cholesterol levels were twice as high as those in the rats sufficient in vitamin E (Exp. 1). A high concentration of cholesterol may suppress the transportation of tocopherol to RBC. There are many problems remaining to be solved. Further study should lead to an explanation of the respective problems.

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