Tocopherol in human platelets

Arne Nordøy and Ellen Strøm

Department of Medicine, Institute of Clinical Medicine, University of Tromsø, Tromsø, Norway

Abstract A spectrophotometric method was used to determine the total tocopherol levels in platelets, plasma, and erythrocytes from human subjects. The platelets contained about three times as much total tocopherol per cell as erythrocytes. This difference was not related to the content of polyunsaturated fatty acids in platelets and erythrocytes. In vitro incubation resulted in significant uptake of tocopherol by plasma and RBC, whereas no uptake was observed into platelets. A 3-month period of tocopherol treatment increased the level of tocopherol in plasma and erythrocytes, whereas the platelet level was unchanged. Tocopherol treatment did not interfere with platelet function or platelet lipid metabolism. The tocopherol fractions of platelets, red cells, and plasma were similar, and α-tocopherol was the main fraction.

Supplementary key words vitamin E · platelet function · vitamin E treatment · tocopherol fractions

Vitamin E is probably one of the most important antioxidants in the body. Lucy (1) has recently suggested that in addition to protecting polyunsaturated fatty acids (PUFA) in phospholipids from oxidation, the vitamin reduces the permeability of membranes that contain highly unsaturated phospholipids and protects the active membrane sites from phospholipases.

Platelets have a high content of PUFA, particularly in the granula and membrane phospholipids (2). Furthermore, a high intake of dietary PUFA will increase the percentage of these fatty acids in the platelet phospholipids and change platelet function as estimated by platelet factor 3 activity (3). This situation could possibly result in adverse effects on platelets as a result of a vitamin E deficiency.

Recent animal studies have shown that, when pigs are fed a PUFA-rich diet with no supplement of vitamin E, they develop vasculitis and thrombosis (4). Ultrastructural studies have confirmed the occurrence of endothelial damage and platelet thrombosis in these animals (5). In vitro studies with human platelets have shown that freshly collected platelets have small quantities of lipid peroxides. Storage of platelets at 4°C and 20°C resulted in rapid and progressive accumulation of lipid peroxide formation. The addition of dl-α-tocopherol prevented lipid peroxide for-

mation (6). These observations indicate that vitamin E may be involved with platelet integrity both in vitro and in vivo. In the present study we have explored the role of vitamin E in human platelets.

MATERIALS AND METHODS

Venous blood was collected from healthy male subjects and from five patients with obliterative atherosclerosis before and after a 3-month period during which they were given a daily dose of 300 mg of dl-α-tocopheryl acetate (Ido-E; A. B. Ferrosane, Malmø, Sweden).

Preparation of test material

36 ml of venous blood was collected after a 12-hr fast in 4 ml of 0.077 M EDTA, pH 6.4. (7). The blood was then centrifuged at 270 g for 15 min at 4°C. The platelet-rich plasma was removed and recentrifuged at 1200 g for 30 min. The platelet-poor plasma was saved for further use. The platelets were washed twice in a platelet washing solution as described earlier (7). The washed platelet pellet was resuspended in 1 ml of 0.9% saline. Platelets were counted in a Thrombo-Counter (Coulter Electronics Ltd., Dunstable, Beds., England.

The red blood cells were washed three times with 5 vol of an isotonic phosphate-buffered NaCl solution containing EDTA. The buffer consisted of 1.42 g of anhydrous Na_2HPO_4, 7.27 g of NaCl, and 0.1 g of disodium EDTA dissolved in a total volume of 1000 ml; the pH was adjusted to 7.4 with dilute HCl. The final hematocrit of the RBC after the third washing was measured in a standard microhematocrit centrifuge, and the cells were counted in a Celloscope 421, Linson Instrument AB, Stockholm, Sweden.

Platelet, plasma, and red cell tocopherol assay

The method of Kayden, Chow, and Bjørnson (8), with a few modifications, was used to determine tocopherol in plasma, platelets, and red cells. The procedure is outlined

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Abbreviations: PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography.

in **Fig. 1.** 0.9 ml of the platelet pellet suspended in 1.0 ml saline, 1 ml of plasma, or 2.0 ml of washed, packed red cells was pipetted into 35-ml glass-stoppered centrifuge tubes. The following modifications of the original method were made. (*1*) The extraction of the saponified material with hexane was carried out in melting ice, thus giving a higher recovery than extraction at room temperature. (*2*) Elution of test material and tocopherol standards from the silica gel (silica gel G; E. Merck A.G., Darmstadt, Germany) after TLC was with 0.1 ml of distilled water and 1.5 ml of 100% ethanol. (*3*) 1.0- to 5.0-μg amounts of pure *dl*-α-tocopherol (Sigma Chemical Co., St. Louis, Mo.) were used as standards in order to have equivalent tocopherol concentrations in both the standards and test materials. (*4*) In the final step before spectrophotometry, 0.5 ml of 0.2% bathophenanthroline, 0.5 ml of 0.001 M $FeCl_3$, and 0.5 ml of 0.001 M H_3PO_4 were used for plasma, whereas 0.2 ml of each solution was used for platelets and red cells.

Separation of tocopherols

dl-α-Tocopherol standards and tocopherol obtained from plasma, red cells, and platelets in volumes similar to those used for the standard tocopherol assay were spotted on TLC plates coated with silica gel G (0.025 mm). These plates were prepared and developed according to Lovelady (9). The spots were identified by using the R_f values of tocopherols in a similar assay system described by Lovelady (9). The silica gel containing the separated tocopherols and blank areas were scraped from the plate into 10-ml centrifuge tubes for elution and further spectrophotometric analysis. The volumes of reagents used were those indicated above for plasma samples in the standard assay.

In vitro incubation

68 ml of venous blood was collected into 12 ml of ACD solution (citric acid 4.8 g, sodium citrate 13.2 g, and glucose 14.7 g in 1000 ml of distilled water). A platelet pellet, packed red cells, and platelet-poor plasma were prepared as described; however, 1 ml of ACD solution per 6 ml of platelet-rich plasma was added before the final centrifugation in the preparation of the platelet pellet. The platelet pellet was resuspended in 5 ml of platelet-poor plasma, and the platelet number was determined.

1 ml of *dl*-α-tocopherol standard (90 μg/ml in 100% ethanol) was pipetted into 35-ml siliconized test tubes. The solution was evaporated under nitrogen at room temperature. Control tubes were prepared with addition of ethanol only. 2 ml of platelet suspension, 2 ml of platelet-poor plasma, and 10 ml of whole blood were added to the tocopherol-coated and the control tubes. The incubation was carried out for 1 hr at 37°C with gentle agitation, in darkness, and in a nitrogen atmosphere. Platelets were counted in the platelet suspension, and test samples were transferred to
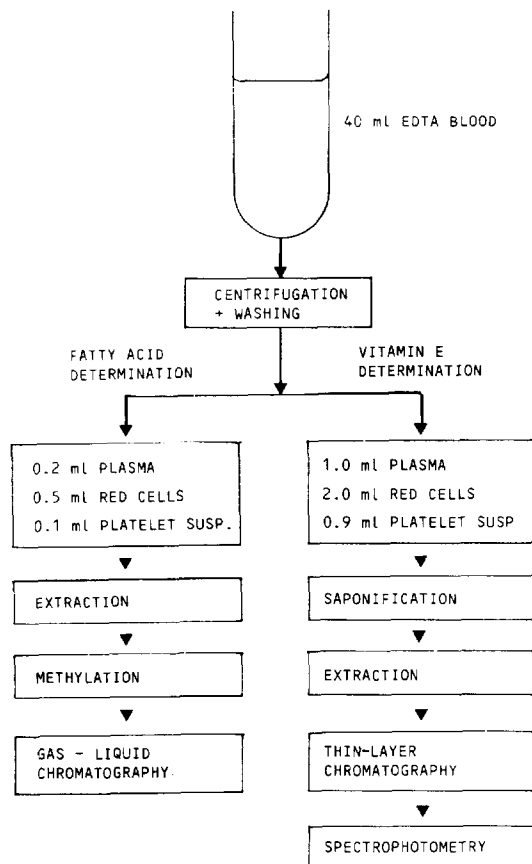

Fig. 1. Outline of the procedure for total tocopherol estimation in plasma, RBC, and platelets.

another test tube and centrifuged at 1200 *g* for 30 min at 4°C. The platelet pellet was washed twice and resuspended in 1 ml of saline. After platelet counting, tocopherol was measured according to the standard assay. The whole blood samples were centrifuged for preparation of packed red cells, washed twice, and processed for tocopherol estimation after the hematocrit was measured. 1-ml samples of plasma were used for the tocopherol assay.

Lipid analyses

Heneicosanoic acid (Nu-Chek-Prep, Elysian, Minn.) was used as an internal standard for fatty acid analyses. 0.06 μmole of 21:0 was added to 0.1 ml of platelet suspension, and 0.6 μmole of 21:0 was added to 0.2 ml of plasma or 0.5 ml of packed red cells. All samples were extracted as described previously (10). The lipids, in chloroform–methanol 2:1, were transferred to 10-ml ampoules, and 2 ml of boron trifluoride in methanol (14% w/v; Applied Science Laboratories, Inc., State College, Pa.) was added. The samples were flushed with nitrogen, sealed, and heated in boiling water for 30 min. The methyl esters were extracted into 4 ml of petroleum ether (Analar; British Drug Houses, Ltd., Poole, England); 2 ml of 5 M sodium hy-

TABLE 1. Tocopherol levels in platelets, RBC, and plasma from 12 healthy male subjects

	Platelets	RBC	Plasma
	$nmoles/10^9$	$nmoles/10^9$	$nmoles/ml$
Mean	1.53 ± 0.676	0.386 ± 0.135	15.9 ± 6.59
Range	$0.91-3.0$	$0.201-0.602$	$9.3-30.7$

droxide was then added dropwise with stirring at 0°C, and the upper phase was collected. The lower phase was extracted with 2 ml of petroleum ether. The methyl esters were analyzed by gas–liquid chromatography performed on an F & M model 402B Hewlett Packard gas chromatograph equipped with paired glass columns, 6 ft × 4 mm ID, packed with 10% EGSS, 100–120 mesh. Programmed runs from 165°C to 195°C were used. Peak areas were calculated by triangulation. The accuracy of the method was estimated by quantification of an NIH fatty acid methyl ester standard mixture for minor and major components as described earlier (7). This standard was analyzed after every tenth test sample. Platelet phospholipids and their fatty acid and aldehyde compositions were measured as described earlier (7).

Standardization of the procedure

Recovery studies using a standard α-tocopherol solution showed a recovery of $78 \pm 12\%$. The standard error of the method was calculated separately for platelets, red blood cells, and plasma. Ten parallel runs for each test agent showed the following standard errors: plasma, 10.5%; platelets, 12%; and red blood cells, 12.8%.

Platelet function tests and coagulation studies

Platelet factor 3 activity and availability after exposure of platelets to ADP and kaolin was measured as described earlier (7). Plasma recalcification time, activated partial thromboplastin time and Normotest were performed ac-

cording to Biggs (11) and Owren (12). The primary bleeding time was estimated by Ivy's technique (11).

RESULTS

Tocopherols in human platelets, red blood cells, and plasma

Blood was collected from 12 healthy male subjects aged 25–50 yr. As shown in **Table 1**, platelets contained about three times as much total tocopherol per cell as RBC. However, whereas there was a significant correlation between the vitamin E content in plasma and RBC, no such correlation could be established between plasma and platelets (**Fig. 2**). Furthermore, no correlation could be established between the vitamin E content in platelets and RBC (**Fig. 3**).

Relationships between tocopherol and total fatty acids and total unsaturated fatty acids

Total fatty acids and total unsaturated fatty acids were estimated in platelets, RBC, and plasma, and the ratios between vitamin E and these fatty acids were calculated. Platelets had a significantly higher content of vitamin E in relation to total fatty acids and total unsaturated fatty acids than RBC and plasma (**Table 2**). However, whereas a significant correlation could be established between the concentration of vitamin E and total fatty acids both in RBC ($r = 0.69$, $P < 0.01$) and in plasma ($r = 0.68$, $P < 0.01$), no such correlation could be established between total fatty acids and vitamin E content in platelets. No significant correlation could be established between the concentration of PUFA and vitamin E content in any of the three compartments.
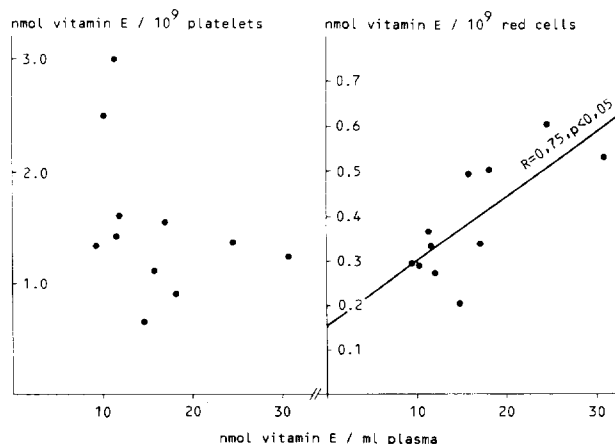

Fig. 2. Correlation between vitamin E content in plasma, platelets, and RBC in 11 healthy male subjects.
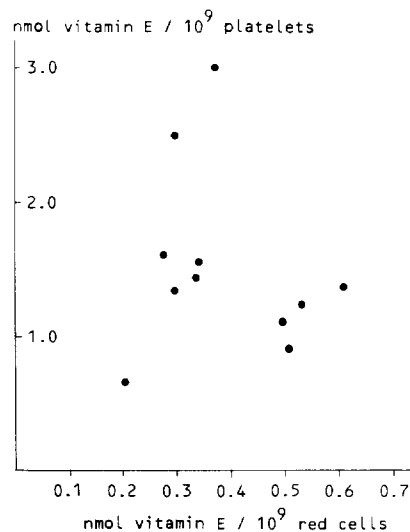

Fig. 3. Correlation between vitamin E content in platelets and RBC in 11 healthy male subjects.
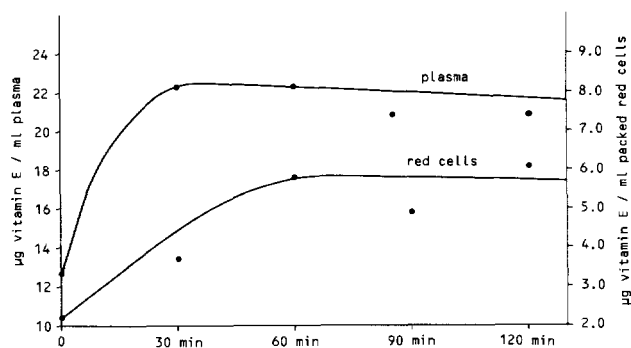
Fig. 4. Uptake of *dl*-α-tocopherol in plasma and RBC during a 2-hr incubation period.

Tocopherol fractions

A good separation of the various tocopherols in plasma, platelets, and red blood cells was obtained by thin-layer chromatography. By quantitation of the various tocopherols it was found that between 70 and 80% of the total tocopherol was accounted for by α-tocopherol in all three compartments. γ-Tocopherol was estimated to be about 20%, and β-tocopherol accounted for only traces of the total tocopherols; δ-tocopherol was not observed. The percentage distributions of the tocopherols in the three compartments were similar (**Table 3**).

Uptake of tocopherol in vitro

When plasma and whole blood, respectively, were incubated with *dl*-α-tocopherol, a continuous increase of the tocopherol concentration was observed during the first 60 min (**Fig. 4**). In plasma, the uptake was maximal at 30 min, whereas equilibrium was established in RBC after 60 min. On the other hand, when platelets were incubated with tocopherol, a fairly constant platelet number was obtained (2.41–2.10 × 10⁹/ml). Thus, a reduction of only 15% in the total platelet number occurred during the incubation period. This reduction in platelet number was similar in the vitamin E-coated tubes and the control tubes. When the tocopherol level was estimated in plasma, platelets, and RBC after incubation, a highly significant increase was observed in both plasma and RBC, whereas no uptake was observed in platelets compared with the controls (**Fig. 5**).

Influence of vitamin E medication

The five subjects with obliterative peripheral atherosclerosis who received tocopherol treatment for a period of 3 months showed no significant improvement in their clinical condition (subjective impression, walking distance, peripheral circulation). They showed a significant increase in the vitamin content of their plasma and RBC, but there were no significant changes of vitamin E levels in the platelets (**Table 4**). The quantity and quality of total fatty acids and total PUFA were unchanged in the three compartments. Platelet number and platelet factor 3 activity in platelet-rich and in platelet-poor plasma as well as platelet factor 3 availability after exposure of platelets to ADP and kaolin and after freezing and thawing three times were essentially unaffected, and bleeding time as well as plasma recalcification time, activated partial thromboplastin time, and Normotest showed no significant changes after the 3-month treatment period. The total platelet phospholipids, the percentage distribution of the phospholipids, and the fatty acid and aldehyde compositions of the platelet phospholipids were estimated, and no significant changes during the treatment period were observed.

DISCUSSION

The present study has shown that platelets have a higher content of vitamin E than red cells on a per cell basis or

TABLE 3. Distribution of tocopherols in plasma, platelets, and RBC

	Plasma	Platelets	RBC
	%	%	%
α-Tocopherol	72	79	80
β-Tocopherol	trace	trace	trace
γ-Tocopherol	17	12	18

TABLE 2. Ratios of tocopherols to total fatty acids and to PUFA in plasma, RBC, and platelets from 12 healthy male subjects

	Plasma	RBC	Platelets	Significance of Difference	
				Platelets/ Plasma	Platelets/ RBC
Tocopherols/ total fatty acids (nmole/nmole)	0.0023 ± 0.0006 (0.0015–0.0042)[a]	0.0011 ± 0.0005 (0.0005–0.0018)	0.0028 ± 0.0016 (0.0010–0.0069)	$P < 0.01$	$P < 0.0001$
Tocopherols/ total PUFA (nmole/nmole)	0.0043 ± 0.0022 (0.0024–0.0167)	0.0025 ± 0.0013 (0.0011–0.0067)	0.0072 ± 0.0072 (0.0020–0.025)	$P < 0.02$	$P < 0.0001$

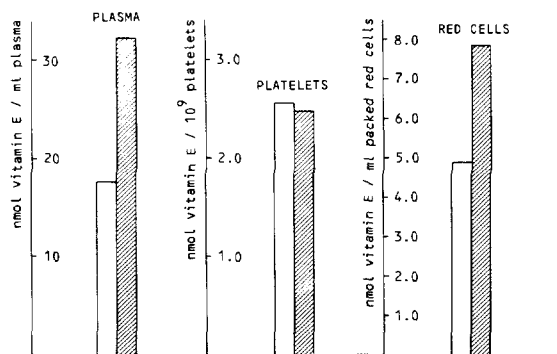[a] Ranges of ratios are in parentheses.

Fig. 5. Tocopherol content in plasma, platelets, and RBC before (*open bars*) and after (*hatched bars*) a 1-hr incubation with *dl*-α-tocopherol.

when the vitamin E content is related to the total fatty acid content in platelets and red cells. These data indicate that the high content of vitamin E in platelets is not a consequence of cell size or total fatty acid content. Furthermore, whereas a significant correlation could be established between the levels of vitamin E in plasma and red cells, no such correlation was observed between plasma and platelets. The high content of PUFA in platelet phospholipids could possibly be the cause of this difference in vitamin E content; however, the high molar ratio of vitamin E to PUFA in platelets excludes this hypothesis (7, 13).

Platelet phospholipids are metabolically very active and have a high turnover rate (14, 15). Recent studies on the role of vitamin E in cellular metabolism indicate that tocopherols have a complex effect on mitochondrial and microsomal membranes by their influence on the stability of phospholipids and by their effects on the respiratory chain and genetic regulation (16–18). This complex nature of the role of vitamin E, which at present is only poorly understood, makes it reasonable to suggest that the variations in vitamin E content in different cells reflect the functions of the cells. This has also been confirmed in animal studies in which it was shown that different tissues show great variations in their vitamin E content (19). The present study thus establishes that the vitamin E content is not only de-

pendent on the content of PUFA but is probably more a reflection of the total function of the cell.

The lack of correlation between the vitamin E levels in plasma and platelets in fasting human subjects probably reflects the fact that platelets, which have their own lipid synthesizing system (14, 20), are influenced by plasma lipids to only a limited degree. Severe lipid abnormalities in plasma have been reflected in red blood cells, whereas platelet lipids have remained unaffected (21). Vitamin E is transported in plasma by the lipoproteins, and the relative ability of lipoproteins to transfer vitamin E to red cells is HDL > LDL > VLDL and chylomicrons (22). In vitro incubation studies have confirmed the differences between platelets and red blood cells. Vitamin E ingestion resulted in increased levels of the vitamin in plasma and red blood cells, whereas the concentration in platelets was unchanged.

Platelets play a fundamental role in the process of thrombosis, and vitamin E has for many years been used in the treatment of a variety of cardiovascular disorders. However, a recent review of the literature (23) could not establish any evidence of its effectiveness during the previous 25 yr. The present study failed to establish any influence of a high intake of vitamin E for 3 months on platelet number or platelet function estimated by platelet factor 3 activity and availability. These findings support the observation of Gomes, Venkatachalapathy, and Haft (24), who reported no effect of vitamin E intake on platelet aggregation. However, Korsan-Bengtsen and Holm (25) observed a prolongation of the plasma recalcification time after a 9-wk period of vitamin E treatment, and it was suggested that this was caused by a reduction in the release of platelet factor 3. The present study failed to confirm these suggestions. Furthermore, no changes in platelet phospholipids or their fatty acid compositions were noted, changes that often are followed by changes in platelet factor 3 activity.

The present study has shown that a high intake of vitamin E for a prolonged period of time does not interfere with platelet lipid metabolism or platelet function. How-

TABLE 4. Plasma, platelet, and RBC tocopherol levels in five subjects with obliterative atherosclerosis before and after a 3-month period of a daily intake of 300 mg of *dl*-α-tocopheryl acetate

Subject	Plasma		Platelets		RBC	
	Before	After	Before	After	Before	After
	nmoles/ml		*nmoles/10⁹*		*nmoles/10⁹*	
J.D.	17.8	37.5		2.73		
D.J.R.	12.4	38.8	2.41	2.47	0.323	0.469
A.G.	22.0	29.0	3.23	1.73		0.619
M.B.	17.0	26.0	2.19	1.39		0.593
M.B.	12.3	29.4	2.78	4.0	0.268	1.010
Mean ± SD	16.2 ± 4.2	32.1 ± 5.66	2.65 ± 0.45	2.46 ± 1.02	0.295[a]	0.739[a]

[a] Mean of two, $t = 4.45$.

ever, in vitamin E deficiency the situation may be the opposite. Animal studies clearly indicate (4, 5) that a thrombotic state is induced by intake of a diet rich in polyunsaturated fats and deficient in vitamin E. In humans, vitamin E deficiency is extremely rare and is related to syndromes with malabsorption and abetalipoproteinemia. This indicates that ordinary mixed foodstuffs contain adequate amounts of vitamin E. Diets rich in PUFA, particularly linoleic acid, have been recommended in the prevention of cardiovascular diseases. However, an increased vitamin E requirement is necessary only if the dietary fats contain more than 25% linoleic acid (26). When it is realized that vegetable oils are the main source of both PUFA and vitamin E, it seems unlikely that vitamin E deficiency could induce a thrombotic state in man. ▮▮

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