Effects of α -Tocopherol-Nicotinate Administration on the Microdynamics of Phospholipids of Erythrocyte Membranes in Human Subjects

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(Received June 2, 1988)

Summary The effects of α -tocopherol-nicotinate on the microdynamic parameters, membrane viscosity, and wobbling angle of phospholipid molecules of healthy human erythrocyte membranes were studied with a nanosecond time-resolved fluorometer. The incorporation of α -tocopherol was achieved by oral administration of α -tocopherol-nicotinate for one month with dosage of 400 mg/day. The increase in α -tocopherol concentration in erythrocytes was confirmed in two subjects. The membrane viscosity in seven healthy subjects decreased significantly from 1.015 ± 0.137 to 0.888 ± 0.133 mPa·s. But no changes in the wobbling angle of phospholipid molecules were observed.

Key Words tocopherol-nicotinate, erythrocyte membrane, fluorescence anisotropy decay, microdynamics, membrane viscosity, wobbling angle

The stabilization effect of α -tocopherol on phospholipid layers has been studied in liposomes by many authors using different methods. Incorporation of α -tocopherol and the phytol chain into liposome membranes causes a reduction in glucose release from liposomes at temperatures higher than the phase transition (1, 2). This decrease in glucose permeation is attributed to a decrease in the mobility of the acyl chain of phospholipid molecules, since α -tocopherol is aligned with the prevailing direction of the phospholipid acyl chains and perturbs the lipid packing (3). In a concentration of 28 mol%, tocopherol resulted in a decrease in the lateral diffusion coefficient for pyrene and an increased order of acyl chains in dipalmitoyl lecithin liposomes above phase transition temperatures (4). Fukuzawa *et al.* (5) studied the effect of α -tocopherol on membrane fluidity by measuring the polarity (P) of the steady state fluorescence from diphenyl hexatriene (DPH) entrapped in the phospholipid layer. They found that the incorporation of α -tocopherol caused different effects on the membrane fluidity of liposomes of dipalmitoyl-phosphatidylcholine (DPPC) at different temperatures. DPPC showed a phase

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transition at 42°C and tocopherol caused an increase in membrane fluidity at 37°C and a decrease at 43°C. They found that with egg lecithin liposomes, which consisted of several species of phospholipid and showed no phase transition, the same treatment decreased the fluidity at all temperatures. These effects of α -tocopherol on the membrane fluidity of liposomes were similar to those of cholesterol(5). Both α -tocopherol and cholesterol increased the mobility of the phospholipid molecules in liquid-crystalline phase at temperatures lower than the phase transition and rigidified them in the liquid phase at temperatures higher than phase transition. They decreased the fluidity of liposomes which showed no phase transitions.

Biomembranes contain several different species of phospholipids and show no clear phase transition. If these are randomly distributed then, on the basis of the findings with liposomes, the most likely effect of α -tocopherol and cholesterol would be a decrease in membrane fluidity. The *in vitro* addition of α -tocopherol to biomembranes such as those of platelets(6) and intestinal brush-border(7) caused a decrease in membrane fluidity. The *in vivo* administration of α -tocopherol, however, failed to cause any significant increase in the order parameter of acyl chains of phospholipid molecules in the measurement of electron spin resonance in rats(8). The order parameter of acyl chains provides a measure of the localized oscillation of the spin label bound on the acyl chain. Nevertheless the order parameter given by the spin label provides information on the fluidity and membrane functions(9, 10). A further analysis of the effects of α -tocopherol on membrane viscosity and wobbling angle of phospholipid molecules by the time-resolved depolarization method will be useful to obtain more information on the tocopherol effects on erythrocyte membranes.

Numerous studies have confirmed the membrane stabilization effects of tocopherol on premature infant blood. In premature infants the low content of α -tocopherol in erythrocytes is related with hemolytic anemia(11). Supplementation with α -tocopherol improves their hematocrit and other hematological parameters(12-14). In healthy human subjects, however, its effects on microdynamic properties of erythrocyte membranes remain unstudied.

The fluidity which is often used to express the microdynamic properties of cellular membranes is a combined term for the membrane viscosity and the wobbling angle of phospholipid molecules(15). In the present study these two microdynamic parameters were measured in erythrocyte membranes by a nano-second time-resolved fluorescence anisotropy depolarization method. Erythrocytes were obtained from healthy human subjects before and after one-month oral administration of α -tocopherol-nicotinate. Additionally the concentrations of α -tocopherol in erythrocyte membranes and blood plasma, and the osmotic fragility of erythrocytes were measured in a limited number of human subjects, to confirm the incorporation of α -tocopherol in the human erythrocyte membranes by oral administration of α -tocopherol-nicotinate.

METHODS

Seven healthy subjects (6 male and 1 female, 30 to 52 years old) took, orally, one capsule containing 200 mg of α -tocopherol-nicotinate (kindly supplied by Eisai Co., Japan) after breakfast and supper every day for one month. Venous blood was sampled from the antecubital vein into a heparinized plastic syringe before and after the one-month α -tocopherol-nicotinate administration (16).

The osmotic fragility of erythrocytes was measured by the coil planet method (17) in two subjects and expressed in terms of hemolysis starting point (HSP), hemolysis middle point (HMP), and hemolysis end point (HEP). The concentrations of α -tocopherol in erythrocyte and blood plasma were measured with high-speed liquid chromatography using microparticle columns (18, 19). Plasma cholesterol, high-density lipoprotein cholesterol (HDL), and beta-lipoprotein were measured by routine methods.

For the time-resolved fluorescence depolarization measurement, erythrocyte membrane ghosts were prepared as follows. After removal of plasma and the buffy coat, the erythrocytes were washed three times in isotonic salt solution with centrifugation at 4,000 rpm for 5 min at 4°C, then lysed with a hypotonic solution (30 mOsm/liter). The lysed erythrocytes were washed twice in the hypotonic solution with centrifugation at 16,500 rpm for 20 min at 4°C and finally washed in normotonic saline. The resultant erythrocyte ghost membranes were incubated with the rod-shaped fluorescence molecular probe, diphenyl hexatriene (DPH) emulsified in tetrahydrofuran (20).

The principle and the procedure for the measurements of the microdynamic parameters, the time-resolved fluorescence depolarization has been described previously (15, 21, 22). Briefly, membrane ghosts containing DPH are illuminated with vertically polarized pulsed light. DPH molecules with molecular axes parallel to the direction of polarization of the excitatory light can absorb energy and emit fluorescent light. If the thermal motion of DPH is strictly limited by the arrangement of the lipid bilayer, the emitted fluorescent light would be vertically polarized in the direction parallel to the polarization of the exciting light. In cell membranes the phospholipid molecules oscillate with a definite wobbling angle. DPH molecules oscillate together with surrounding phospholipid molecules. Their oscillation causes a decrease in the polarization of the fluorescent light and an increase in the horizontal component of the fluorescent light. Phospholipid molecules laterally collide with one another when oscillating and are subjected to a frictional resistance caused by the collision. The polarization of DPH fluorescence, therefore, decreases after pulsed excitation and reaches a constant value with a characteristic time constant. The time constant is related to the frictional resistance and represents the membrane viscosity. The final value of the polarization of fluorescence relates to the wobbling angle of phospholipid molecules.

The parallel and perpendicular components of the fluorescent light, $I_{\parallel}(t)$ and $I_{\perp}(t)$, were measured by the single photon counting method controlled with a personal computer. The time course of anisotropy decay to express the process of the fluorescence depolarization was obtained from the equation: $r(t) = [I_{\parallel}(t) - I_{\perp}(t)]/[I_{\parallel}(t) + 2I_{\perp}(t)]$. From deconvolution analysis, the anisotropy decay time (ϕ) and the anisotropy value at infinite time (r_{∞}) were calculated according to the equation: $r(t) = (r_0 - r_{\infty}) \exp(-t/\phi) + r_{\infty}$. The ϕ value relates to the membrane viscosity and the r_{∞} provides the wobbling angle of the phospholipid in membranes. The decay curve of the total fluorescence is given by $S(t) = I_{\parallel}(t) + 2I_{\perp}(t) = A \exp(-t/\tau)$, where τ is the lifetime of the total fluorescence.

The fluorescence anisotropy yielded by steady state excitation (r_s) was measured with a Shimadzu spectrofluorometer model RF-540 equipped with a thermostatically controlled cell compartment.

The fluorescence measurements were made at 37°C. The significance of changes in the relevant parameters was checked by Student's paired *t*-test.

RESULTS AND DISCUSSION

The results of the osmotic fragility test and lipid analysis are listed in Table 1. The administration of α -tocopherol-nicotinate caused a slight decrease in HMP and

Table 1. Effects of one month's administration of α -tocopherol-nicotinate on osmotic fragility of erythrocytes and tocopherol (α , β , and γ) concentration in erythrocytes and plasma from two subjects.

	Subj. 1 (ET)		Subj. 2 (TK)	
	Control	α -Tocopherol-nicotinate 1 month	Control	α -Tocopherol-nicotinate 1 month
RBC tocopherol α	2.03	2.91	1.79	2.85
($\mu\text{g/ml}$) β	0.08	0.07	0.06	—
γ	0.29	0.05	0.05	0.23
Plasma tocopherol α	10.48	21.60	7.28	11.21
($\mu\text{g/ml}$ plasma) β	0.14	—	0.14	0.10
γ	0.53	0.30	0.85	0.33
Osmotic fragility (mmHg)				
HSP	120	126	120	110
HMP	114	100	106	96
HEP	80	76	82	77
Blood plasma lipids (mg/dl)				
Total cholesterol	164	160	120	110
β -Lipoprotein	403	330	262	247
Triglyceride	228	227	84	64

Fig. 1. Decay curves of polarized DPH fluorescence, $I_{\parallel}(t)$ and $I_{\perp}(t)$, and emission anisotropy, $r(t)$, in erythrocyte membranes. Erythrocyte membranes were obtained in the control state (solid lines). The emission anisotropy shown with a dashed line was obtained after one month's administration of α -tocopherol-nicotinate. The ordinate shows the normalized number of counted photons and the abscissa, the lapse of time in nanoseconds.

HEP in both subjects. The total cholesterol and triglyceride concentrations tended to decrease in plasma. The concentration of α -tocopherol in both blood plasma and erythrocyte membranes was higher after one month of α -tocopherol-nicotinate administration than before. These changes are consistent with the generally accepted effects of α -tocopherol administration on rat and human blood (23, 24).

The anisotropy decay curves of DPH fluorescence in erythrocyte membranes can be reproduced on an X-Y plotter by the personal computer. An example in the control state is shown in Fig. 1, where a parallel and a perpendicular fluorescence decay curve are depicted with an anisotropy decay curve. The anisotropy decay curve obtained in erythrocyte membranes from the same human subject after one-month administration of α -tocopherol-nicotinate is superimposed with a dashed line for comparison. The faster decay of the dashed line indicates their lower viscosity. The values for ϕ calculated from these curves were 3.61 and 2.72 ns for the control and α -tocopherol-nicotinate-treated state, respectively. The similar r_{∞} -value in the two states means there was no change in the wobbling angle. The viscosity was reduced from the control value of 1.173 to 0.940 mPa·s by the α -tocopherol-nicotinate treatment.

The membrane viscosity, the wobbling angle of phospholipids, and anisotropy of DPH fluorescence with steady state illumination in seven subjects are sum-

Fig. 2. Effects of one month's administration of α -tocopherol-nicotinate on membrane viscosity, wobbling angle of acyl chains of phospholipids of erythrocyte membranes, and steady state anisotropy of DPH fluorescence in the erythrocyte membranes. Horizontal and vertical bars represent mean values and $\pm$ SD, respectively.

marized in Fig. 2. The membrane viscosity decreased in seven subjects after α -tocopherol-nicotinate treatment. The decrease from 1.015 ± 0.137 to 0.888 ± 0.133 mPa·s on an average was significant ($p < 0.01$). But the wobbling angle showed no significant difference. The anisotropy value of DPH fluorescence obtained with steady illumination (r_s) was not changed by α -tocopherol-nicotinate administration (0.188 ± 0.003 for the control state and 0.184 ± 0.010 for the α -tocopherol-nicotinate-treated state).

The anisotropy decay time, ϕ , decreased from the initial value (mean $\pm$ SD) of 3.04 ± 0.54 to 2.69 ± 0.60 ns after one-month treatment with α -tocopherol-nicotinate on an average ($p < 0.05$). The lifetime of the total fluorescent light decreased from the initial value (mean $\pm$ SD) of 10.39 ± 0.24 to 10.21 ± 0.69 ns after α -tocopherol-nicotinate administration but this was statistically nonsignificant.

The tendency of the lifetime of DPH fluorescence to decrease with tocopherol incorporation was consistent with the previously reported observation that the lifetime of the fluorescence of pyrene was reduced by tocopherol (4). The term r_s is related to the parameters obtained with the time-resolved depolarization by the equation: $r_s = (r_0 - r_\infty) / [1 + \tau / \phi] + r_\infty$ (11). It should be mentioned that r_s is a complex term for viscosity, wobbling angle, and fluorescence lifetime. When τ decreases and other terms remain constant, r_s increases. This change may be

interpreted as a decrease in fluidity. In the present study the membrane viscosity was calculated from the value of ϕ and found to decrease with the increased content of α -tocopherol in erythrocyte membranes.

The chroman ring of tocopherol attracts the phosphorus head of the phospholipid molecules (5) and reduces the membrane fluidity. The long phytol chain of α -tocopherol acts so as to incorporate and retain tocopherol in membranes (25). At least two isoprene units are necessary as an anchor of tocopherol in liposomes (26). The phytol chain inevitably restricts the molecular oscillation of surrounding phospholipid molecules. The application of tocopherol in a high dose to erythrocytes *in vitro* results in a decrease in the membrane fluidity (4, 6, and 7). The present *in vivo* application caused a small increase in α -tocopherol concentration in erythrocyte membranes. The small increase in tocopherol should have caused an increase in the tocopherol-phospholipid interaction. This effect, however, was probably overshadowed by other changes. The slight decrease in the membrane viscosity of erythrocytes from tocopherol-administered human subjects may be attributed to small changes in constituents of phospholipids. In support for this consideration there are some reported observations. Kon *et al.* (8) observed a small increase in linoleic acids in erythrocytes from tocopherol-fed rats. Underwoods *et al.* (27) reported an increase in the polyunsaturated fatty acids in blood plasma of tocopherol-treated human patients. Imamura *et al.* (28) found an increase in polyunsaturated phospholipids in erythrocyte membranes of cholesterol-fed rabbits, following a tocopherol administration. An experiment of the inverse direction was made in rats fed with tocopherol-free diet (29). Phosphatidyl ethanolamine and sphingomyelin decreased markedly as tocopherol content became negligible in erythrocytes. Thus a small change in phospholipid constituents seems possible also in the present human erythrocytes. Analysis of phospholipids, however, could not be made in the present study.

It was expected that the incorporation of the long phytol chain to the microstructure of erythrocyte membranes would limit the molecular motion of phospholipid chains and, therefore, would result in a decrease in wobbling angle. Contrary to this expectation, no consistent changes were found in the wobbling angle. This result too suggests that the direct effect of α -tocopherol on the microdynamics of erythrocyte membranes is overshadowed by such changes as mentioned above.

In conclusion, administration of α -tocopherol-nicotinate for one month caused a small reduction in the viscosity of the phospholipid bilayer in human erythrocyte membranes, in sharp contrast to the rigidifying effect of tocopherol on biomembranes established in *in vitro* tests.

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