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VITAMIN E CHANGES THE MEMBRANE FLUIDITY OF HUMAN PLATELETS

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Summary

The in vitro addition of α-tocopherol to human platelets was evaluated by measuring fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene-labeled platelets and platelet membranes. The fluorescence anisotropy was decreased compared to non-vitamin E-loaded control platelets at temperatures less than 24—27°C but was increased above 27°C. The curve relating temperature to this parameter remained monophasic in α-tocopherol-enriched platelets but exhibited a steeper slope, indicative of increased flow activation energy. The latter was linearly related to platelet α-tocopherol levels. A possible role of membrane proteins in the mechanism of this change was suggested by the increased anisotropy which liposomes prepared from platelet membrane lipids displayed when they were loaded with α-tocopherol. This modulation of 'apparent membrane microviscosity' by α-tocopherol may play a role in the normal effect of this vitamin on human cells.

Introduction

α-Tocopherol has been shown to inhibit platelet aggregation in vitro [1—5]. Initially, this effect was thought to be a manifestation of its antioxidant action as the aggregation-associated lipid peroxide formation was effectively reduced by vitamin E [3]. Further studies, however, made this hypothesis untenable when it was shown that the oxidized metabolite, tocopherylquinone, had antiaggregating properties similar to those of fully reduced vitamin E [6]. A close association of this substance with membranous structures in the platelet can be assumed on the basis of its hydrophobic nature. It therefore

seemed possible that α-tocopherol exerted its antiaggregating action through an effect on the physical state of the platelet membrane, in particular its lipid-lipid and/or lipid-protein interactions. Changes in the composition of membrane lipids readily influence such interactions. One of the best studied examples in this field is cholesterol [7–13]. Its insertion into membranes decreases the random rotational motion of the adjacent phospholipid hydrocarbon chains and increases the degree of order by preventing the dissociation and fusion of hydrocarbon chains as random motion changes with temperature. The increased sensitivity of cholesterol-rich platelets to aggregating agents [14] has been related to a reduction of membrane fluidity [15]. Utilizing a well characterized efficient fluorophore, 1,6-diphenyl-1,3,5-hexatriene which localizes equally well in the fluid and solid lipid domains of biologic membranes and the fluorescence polarization of which reflects almost exclusively the angular displacement of its long axis [16], I investigated the in vitro effect of α-tocopherol on the fluorescence polarization of human platelets. The monophasic temperature dependence of diphenylhexatriene fluorescence polarization observed in normal platelets was pivoted about a point in the 24–27°C temperature range when platelets were enriched with α-tocopherol. The vitamin E-induced change in membrane anisotropy was interpreted as indicative of an increase in the flow activation energy for viscosity and an enhanced degree of fluidity at temperatures above 27°C.

Methods and Materials

Human platelets were obtained from venous blood of normal, healthy volunteers who had abstained from any medication for at least 10 days. Platelets were isolated from acid/citrate/dextrose-anticoagulated blood as previously described [17]. For analysis of platelet anisotropy, platelets were washed three times with 0.145 M NaCl buffered with 0.05 M sodium phosphate, pH 7.3, containing 5% acid/citrate/dextrose and were then resuspended in this buffer at a concentration of $5 \cdot 10^7$ cells/ml. After addition of an equal volume of $2 \cdot 10^{-6}$ M diphenylhexatriene which was prepared according to the method of Shinitzky and Inbar [18], platelets were incubated for 2 h at 37°C. The platelets were then separated by centrifugation, resuspended in phosphate-buffered saline and fluorescence polarization measured as a function of temperature. For this purpose, a fluorescence spectrophotometer (Perkin-Elmer, MPF 44-A) was used which was fitted with polarizers in the excitation and emission paths and a thermostatically controlled cuvette holder. Polarized light of 365 nm wavelength was used to excite fluorescence while emission was measured at 428 nm with a cut-off filter for wavelengths below 390 nm in the light-path. Fluorescence intensities were measured parallel and perpendicular to the direction of the polarized excitation beam. Control samples of diphenylhexatriene suspensions and of unlabeled platelets were examined in each experiment. These light intensities which amounted to approx. 3% of the total fluorescence were neglected. All polarization measurements were corrected for the inability of the instrument to transmit differently polarized light equally. From these measurements the fluorescence anisotropy, r, was calculated according to the equation: $r = I_{\parallel} - I_{\perp}/I_{\parallel} + 2I_{\perp}$, where $I_{\parallel}$ and $I_{\perp}$

are the fluorescence intensities detected through a polarizer oriented parallel and perpendicular, respectively, to the direction of polarization of the excitation beam. The apparent microviscosity was approximated as described by Shinitzky and Barenholz [19] utilizing a maximal limiting anisotropy value of $r_0 = 0.362$ for diphenylhexatriene. Here, r_0 is the upper limit value for r in a medium of infinite viscosity. Because of technical limitations a determination of the fluorescence lifetime of diphenylhexatriene in platelet membranes could not be carried out. Also, the structural parameter, $C(r)$, of the Perrin equation could not be measured. For these reasons, the results with diphenylhexatriene are expressed in terms of polarization or anisotropy. The calculation of the apparent microviscosity, $\overline{\eta}$, and from it that of the flow activation energy, ΔE, is based on a number of assumptions which are arguable. Therefore, the values given below are for comparative purposes only and do not reflect absolute quantities.

Parallel experiments were conducted with isolated platelet membranes prepared by the glycerol-lysis technique [20]. The washed membrane vesicles were loaded with diphenylhexatriene as described above. After centrifugal separation [20], the membranes were resuspended in phosphate-buffered saline, their concentration adjusted to 10—20 μg protein/ml and fluorescence intensities measured as detailed above.

Intact platelets or platelet membranes suspended in plasma were preincubated with varying concentrations of α-tocopherol which was added in concentrated form dissolved in ethanol. The tocopherol content of control and vitamin E-incubated platelets was determined by a fluorimetric method [21].

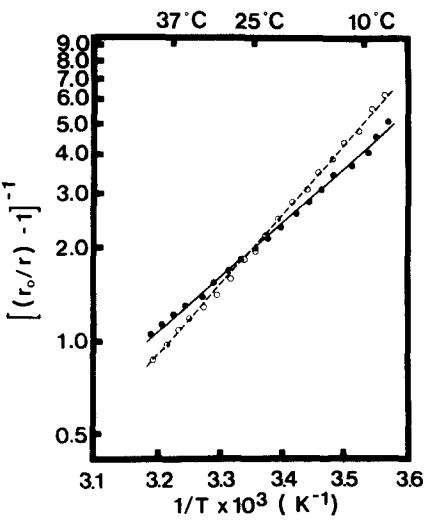

Fig. 1. Temperature dependence of the fluorescence polarization of diphenylhexatriene in normal (●———●) and α-tocopherol-loaded (○- - - - -○) platelets. The ordinate is a fluorescence polarization term and the abscissa is the reciprocal of the absolute temperature. Platelets were incubated for 60 min at 37° C with α-tocopherol which was added in an ethanolic solution. The final concentration of α-tocopherol was 1 mM and of ethanol 0.5%. Normal control platelets were incubated with a corresponding volume of ethanol. After this incubation period the platelets were washed, resuspended in phosphate-buffered saline containing 5% acid/citrate/dextrose and labeled with diphenylhexatriene. This experiment is representative of eight that were performed.

α-Tocopherol at the concentrations used in these experiments exerted no significant quenching of the fluorescence of diphenylhexatriene-labeled platelets or platelet membranes.

Results

Whole platelets loaded with diphenylhexatriene and analyzed over a temperature range from 6—40°C exhibited a monophasic behavior when $[(r_0/r) - 1]^{-1}$ was plotted vs. the reciprocal of the absolute temperature (Fig. 1). In α-tocopherol-preincubated platelets this relationship was characterized by a steeper slope, indicative of a greater flow activation energy, ΔE. In addition, it was interesting to note that in α-tocopherol-loaded platelets anisotropy

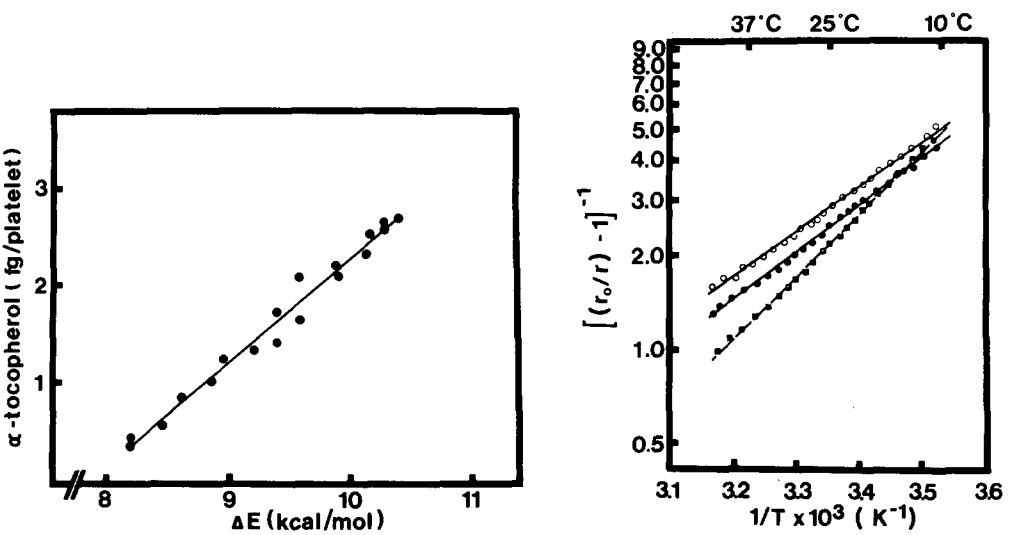

Fig. 2. Relationship between α-tocopherol content of platelets and their flow activation energy for microviscosity. Platelets were incubated with α-tocopherol in concentrations ranging from 0 to 2 mM. The conditions of incubation with vitamin E, the labeling with diphenylhexatriene and the fluorescence measurement are as described in the text and in the legend to Fig. 1. The flow activation energy, ΔE, was calculated from the shape of a plot of log $\overline{\eta}$, the apparent microviscosity of platelets vs. $1/T$, the reciprocal of the absoulute temperature. This relationship, evaluated over the temperature range from 6 to 40°C, was found to be linear. The microviscosity was calculated according to the method of Shinitzky and Barenholz [19] using the approximation, $\overline{\eta} = 2.4\,r/0.362 - r$, suggested by these authors. Each point represents the result of one experiment. The correlation coefficient was +0.98.

Fig. 3. Temperature dependence of fluorescence polarization of diphenylhexatriene in liposomes prepared from platelet membrane lipids. Plasma membranes were isolated from glycerol-lysed platelets [20] and lipids extracted by a modification [22] of the method of Folch et al. [23]. To prepare liposome suspensions, the dried extracted lipid was suspended in phosphate-buffered saline at a final concentration of 0.35 mg/ml. The mixture was sonicated for approx. 10 min under N_2 at 4°C. The resultant suspension was centrifuged at 40 000 × *g* for 10 min at 4°C. The supernatant liposomes were then incubated with α-tocopherol at final concentrations of 2 mM (○———○) and 1 mM (●———●). ■------■, liposomes incubated without additional α-tocopherol. The conditions of incubation were as described for intact platelets or platelet membranes. After this incubation the liposome suspensions were passed through a column of Sephadex G-50 equilibrated with phosphate-buffered saline. The gel-filtered liposomes were then labeled with diphenylhexatriene, were again passed trhough a column of Sephadex G-50 and were concentrated to about 10—15 μg P_i/ml [24] before fluorescence polarization was measured. This experiment is representative of five that were performed.

was decreased at temperatures less than 24—27°C but increased above that of normal control platelets at temperatures above 27°C. Irrespective of the concentration of α-tocopherol in the medium and the amount taken up by the platelets, the crossover region of the $[(r_0/r) - 1]^{-1}$ vs. $1/T$ graph of normal and vitamin E-enriched platelts occurred in the same temperature range, i.e., between 24 and 27°C, The effect of α-tocopherol was dose-dependent up to a concentration of 2 mM in the medium. Virtually the same results were obtained with isolated platelet membranes.

The apparent microviscosity, $\bar{\eta}$, of α-tocopherol-loaded platelets incubated with 1 mM α-tocopherol at 37°C was 2.4 ± 0.05 P (1 S.D.) compared to 2.86 ± 0.12 P for normal control platelets. These changes, as was also the increase in ΔE, were statistically significant ($P < 0.005$) for vitamin E concentrations in excess of 0.2 mM (Fig. 2).

In other experiments, I extracted platelet lipids and prepared liposomes which were then incubated with α-tocopherol. After labeling such liposomes with diphenylhexatriene their fluorescence polarization was measured (Fig. 3). In α-tocopherol-treated liposomes this parameter of fluorescence polarization was increased at temperatures above 15°C.

Discussion

The results obtained with liposomes conform to the expected behavior of a lipid matrix enriched with a substance which aligns itself principally with the prevailing direction of the phospholipid acyl chains. In addition to restricting the motion of these hydrocarbon chains, α-tocopherol may interact specifically with polyunsaturated fatty acids [25] and thereby diminish their effect on membrane microviscosity. The degree of unsaturation of fatty acyl chains is an important determinant of membrane fluidity [11,26]. The postulated 'complex' formation between the phytyl side chain of α-tocopherol and the hydrocarbon skeletons of polyunsaturated fatty acyl residues, especially of arachidonic acid, may result in partial neutralization of the fluidity-enhancing effect of such unsaturated phospholipid acyl chains.

The effect of α-tocopherol on the anisotropy of diphenylhexatriene in intact platelets and isolated membranes stands in sharp contrast to that in liposomes. Although at this time I am unable to offer a precise explanation for this finding, it is possible that membrane proteins are prominently involved in the observed effect. A close association of α-tocopherol with certain proteins of their coordinated lipids may lead to a partitioning of the vitamin into distinct lipid domains. The increased disorder of α-tocopherol-treated platelet membranes revealed by their greater apparent fusion activation energy may thus be a phenomenon which affects primarily the lipid - protein interaction. This could provide the explanation for the effect of α-tocopherol on platelet aggregation. As increased membrane viscosity has been linked to greater exposure of membrane proteins [12,14], so could decreased membrane viscosity be associated with reduced accessibility of membrane proteins. It must be pointed out, however, that factors other than lipid-protein interactions may play a role in the different behavior of vitamin E-loaded platelets as opposed to sonicated membrane extracts. Lipid interactions, the sidedness and asym-

metry of phospholipids and the angle of curvature of the lipids in the bilayer could be affected in the liposomes.

It is obvious that further work is needed to prove the hypothesis relating vitamin E to decreased membrane viscosity. Nonetheless, these findings suggest a new role for α-tocopherol in human platelets. Whether modulation of membrane fluidity by α-tocopherol is a biological effect common to other mammalian cells remains to be established.

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References

1 Kurokawa, I., Ioshiyuki, K., Nagai, T. and Kamimura, M. (1971) J. Vitaminol. 17, 181—184
2 Higashi, O. and Kikuchi, Y. (1974) Tohoku J. Exp. Med. 112, 271—278
3 Steiner, M. and Anastasi, J. (1976) J. Clin. Invest. 57, 732—737
4 Fong, J.S.C. (1976) Experientia 32, 639—641
5 White, J.G., Rao, G.H.R. and Gerrard, J.M. (1977) Am. J. Pathol. 88, 387—402
6 Steiner, M. (1978) in Tocopherol, Oxygen and Biomembranes (de Duve, C. and Hayaishi, O., eds.), pp. 143—163, Elsevier/North-Holland, Amsterdam
7 Chapman, D. and Penkett, S.A. (1966) Nature 211, 1304—1305
8 Ladbrooke, B.D., Williams, R.H. and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333—340
9 Hubbell, W.L. and McConnell, H.M. (1971) J. Am. Chem. Soc. 93, 314—326
10 Papahadjopoulos, D., Cowden, M. and Kimelberg, H. (1973) Biochim. Biophys. Acta 330, 8—26
11 Cogan, U., Shinitzky, M., Weber, G. and Nishida, T. (1973) Biochemistry 12, 521—528
12 Shinitzky, M. and Inbar, M. (1976) Biochim. Biophys. Acta 433, 133—149
13 Demel, R.A. and de Kruijff, B. (1976) Biochim. Biophys. Acta 457, 109—132
14 Shattil, S.J., Anaya-Garlindo, R., Bennett, Y., Colman, R.W. and Cooper, R.A. (1975) J. Clin. Invest. 55, 636—643
15 Shattil, S.J. and Cooper, R.A. (1976) Biochemistry 15, 4832—4837
16 Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652—2657
17 Ando, Y., Steiner, M. and Baldini, M. (1974) Transfusion 14, 453—461
18 Shinitzky, M. and Inbar, M. (1974) J. Mol. Biol. 85, 603—615
19 Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367—394
20 Ando, Y. and Steiner, M. (1973) Biochim. Biophys. Acta 311, 26—37
21 Taylor, S.L., Lamden, M.P. and Tappel, A.L. (1976) Lipids 11, 530—538
22 Deykin, D. and Desser, R.K. (1968) J. Clin. Invest. 47, 1590—1602
23 Folch, Y., Less, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497—509
24 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
25 Lucy, J.A. (1972) Ann. N.Y. Acad. Sci. 203, 4—11
26 Lentz, R.B., Barenholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4521—4528