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Liposomes Containing α-Tocopherol and Ascorbate are Protected from an External Oxidant Stress

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The interaction between α-tocopherol and ascorbate in protecting membrane lipids from peroxidation was studied in unilamellar liposomes in which α-tocopherol was incorporated into the liposomal membrane, and ascorbate was trapped within the vesicles. Extravesicular ferricyanide was reduced by ascorbate-derived electrons, and this was enhanced by the presence of α-tocopherol in the lipid bilayer. When a water-soluble free radical initiator was added to the outside of liposomes, intravesicular ascorbate prevented oxidation of α-tocopherol, and this effect was associated with complete protection against peroxidation of membrane lipids. These results suggest that ascorbate-dependent recycling of α-tocopherol can protect biological membranes from peroxidation by oxidants originating across the membrane bilayer from ascorbate.

Keywords: Oxidant stress, liposomes, α-tocopherol, ascorbic acid, ferricyanide, lipid peroxidation

INTRODUCTION

Ascorbic acid and α-tocopherol have been shown to work together to protect unsaturated fatty acids from lipid peroxidation in liposomes[1–4] and in LDL.[5,6] In these systems, α-tocopherol serves as the primary antioxidant in the lipids, whereas the action of ascorbate is restricted to the aqueous space.[1,4] Synergism between the two vitamins is thought to involve a chain-breaking reduction by α-tocopherol of lipid-based peroxyl radicals within the bilayer, migration of the resulting α-tocopheroxyl free radical to the interfacial region of one bilayer leaflet, and reduction or recycling of the α-tocopheroxyl free radical by ascorbate present in the aqueous space.[7] In studies with liposomes, oxidant stress has been generated by lipid-soluble free radical initiators within the membrane bilayer,[1,4] or by water-soluble oxidants on the same side of the lipid bilayer as ascorbate.[2–4] Whether similar protection is afforded against an oxidant stress coming from the opposite or *trans* side of the membrane bilayer from ascorbate is unknown. Therefore, in this work we have investigated the vectorial nature and synergism between ascorbate and α-tocopherol by separating ascorbate from an oxidant across the lipid bilayer of liposomes.

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MATERIALS AND METHODS

Liposome Preparation

Several types of unilamellar liposomes were tested in these studies. Large unilamellar liposomes were prepared by a freeze-thaw procedure as described by Pick.[8] Soybean lecithin (1.4 μmol of soybean lecithin, Sigma Type II-S) was dissolved in chloroform and α-tocopherol was added where indicated to give 70 nmols of α-tocopherol per μmol of α-lecithin. The solution was dried under a stream of nitrogen gas, and placed under vacuum for 2–3 hours. The thin film of lipid was resuspended in 0.5 ml of 50 mM Tris-HCl buffer, pH 7.4, containing either 100 mM NaCl, or 100 mM ascorbate, and clarified by sonication for 1 min using the full microtip power of an Ultrasonics Model W-220F sonicator. The resulting suspension was frozen in dry ice-acetone, and allowed to thaw slowly at room temperature. Liposomes were sonicated for an additional 30 seconds in a bath sonicator to decrease bilayer permeability.[8] Aliquots of these liposomes (100 μl) were separated from the preparation buffer using Bio-Spin 6 columns as described by the manufacturer (Bio-Rad, Hercules, CA). The column step was repeated and the liposomes were placed on ice and used immediately. Small unilamellar liposomes were prepared and purified as described by Futami, *et al.*[9] using reagents and conditions noted above.

Liposomes containing endogenous erythrocyte lipids were prepared from reconstituted erythrocyte band 4.5.[10] Approximately 1 ml of reconstituted band 4.5 (≈1 mg of protein) was extracted by the method of Folch, *et al.*.[11] The resulting lipid-containing chloroform fraction was mixed with 25 mg of soybean lecithin and this solution was dried under nitrogen. The lipids were reconstituted by adding 0.5–0.7 mL of 50 mM Tris buffer, pH 7.4, that contained ascorbate or ferricyanide as required. The mixture was warmed to 37°C, sonicated for 3–5 min, and chromatographed on Sephadex G-50 to remove unincorporated agents.[9]

Assay of Liposomal Membrane Tocopherol Content

A 0.1 mL aliquot of liposome suspension was dissolved in 0.4 ml of ice-cold methanol and 100–200 μL was sampled for assay of α-tocopherol by HPLC.[12]

Other Assays

Reduction of ferricyanide was assessed either by following the decrease in absorbance at 420 nm with time on a Gilford 250 spectrophotometer, or by measuring the appearance of ferrocyanide with the assay of Avron and Shavit,[13] as described previously.[14] Lipid hydroperoxides in liposomes and erythrocyte ghosts were measured using the FOX2 assay with *tert*-butyl hydroperoxide as a standard.[15] The presence of a small amount of ascorbate in the vesicles was not found to interfere with the assay of lipid hydroperoxides. The phospholipid content of the liposomes was measured using the method of Ames.[16] Data are expressed as mean ± standard error. Statistical significance was assessed by one- or two-way analysis of variance using the statistical software package SigmaStat (Jandel Scientific, St. Louis, MO).

RESULTS

When ascorbate was sealed inside phospholipid liposomes containing α-tocopherol, extravesicular ferricyanide was reduced in a time-dependent manner at a rate several-fold greater than that observed in liposomes containing α-tocopherol, but not ascorbate (Fig. 1). In the absence of α-tocopherol, ascorbate-containing vesicles showed little reduction of ferricyanide, indicating low permeability of the liposomal membrane to ascorbate (Fig. 1). The possibility that α-tocopherol in the liposomes had made them more permeable to ascorbate was examined by including 0.1 μCi of [1-^{14}C]ascorbate (New England Nuclear, 4.7 Ci/mol) along with 100 mM unlabeled ascorbate during

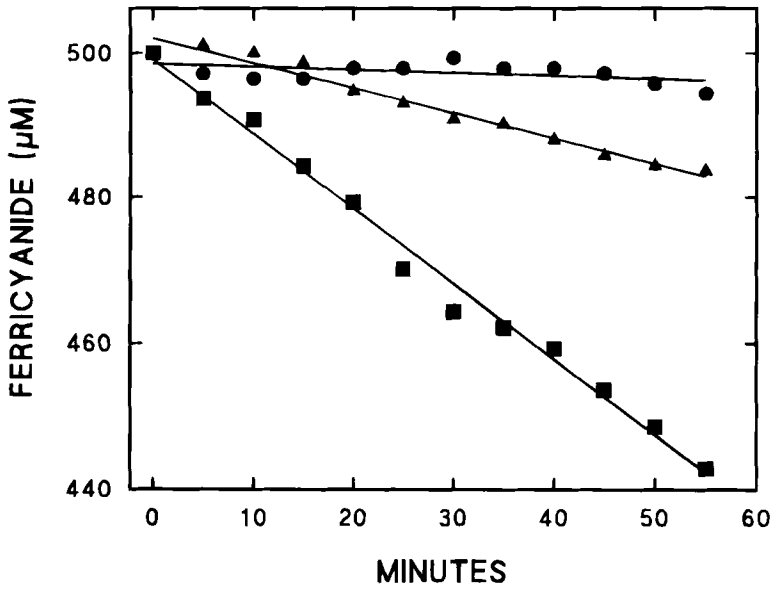

FIGURE 1 *Time course of ferricyanide reduction in liposomes.* Large unilamellar liposomes were prepared to contain initially either 100 mM ascorbate (circles), 70 nmol of α-tocopherol per μmol of lecithin (triangles), or both (squares). An aliquot of liposomes (70 nmol lecithin) was placed in a cuvette containing Tris buffer, ferricyanide was added to a concentration of 500 μM and a total volume of 0.5 ml, and the change in absorbance at 420 nm at room temperature was recorded. The results of two experiments are averaged and shown as a decline in the ferricyanide concentration.

liposome formation. Following two centrifugations on spin-columns, 2–3% of the [^{14}C]ascorbate in the original solution was incorporated into the liposomes. Over an hour of incubation under the conditions of Fig. 1, loss of [^{14}C]ascorbate was slow in α-tocopherol-containing liposomes (≈5%) and no different from control (results not shown). It was necessary for the α-tocopherol to be incorporated into the liposomal membrane at the time of formation. Addition of α-tocopherol to ascorbate-containing liposomes just before the chromatographic step resulted in little reduction of extravesicular ferricyanide (results not shown).

In the presence of intravesicular ascorbate, increases in ferricyanide reduction occurred in liposomes that contained added α-tocopherol as well as in liposomes prepared with erythrocyte phospholipids that contained *endogenous* α-tocopherol. In the latter, the content of α-tocopherol (1.3 ± 0.4 nmol/μmol phospholipid, N = 4) was manyfold lower than that obtained when exogenous α-tocopherol was added to liposomes (Table I). Nonetheless, ferricyanide reduction was increased by the presence of 100 mM ascorbate in liposomes prepared with endogenous erythrocyte lipids compared to similar liposomes lacking ascorbate (1.7 ± 0.4 *vs.* 0.5 ± 0.1 nmol/nmol phospholipid, respectively, $p < 0.02$, N = 6). In liposomes prepared with exogenous α-tocopherol, ascorbate was necessary for a significant increase in ferricyanide reduction (Table I). The reduction of ferricyanide by liposomes containing α-tocopherol alone is unexplained, but could be due to a catalytic effect of α-tocopherol to facilitate oxidation of unsaturated fatty acids by ferricyanide. Reduction of ferricyanide in ascorbate-containing liposomes is likely due to some leakage of ascorbate across the membrane of these small unilamellar liposomes. Such leakage was less in the large unilamellar liposomes (Fig. 1). In liposomes loaded with the same amount of ascorbate (100 mM), the extent of ferricyanide reduction correlated with the amount of α-tocopherol present in the liposomal membrane (Fig. 2). This suggests that α-tocopherol-dependent elec-

TABLE I Liposomal content of α-tocopherol and capacity for ferricyanide reduction[a]

Content	α-Tocopherol	N[c]	Ferrocyanide	N[c]
	(nmol/μmol PL)		(nmol/nmol PL)	
α-Tocopherol	48 ± 11	9	1.8 ± 0.9	6
Ascorbate	N.D.[b]	—	3.9 ± 1.9	9
α-Tocopherol + Ascorbate	49 ± 10	9	21 ± 4[d]	6

[a]Small unilamellar liposomes (80 nmol phospholipid/0.5 ml incubation) were prepared to contain α-tocopherol (70 nmol/μmol *lecithin*) and/or ascorbate (100 mM). The content of α-tocopherol was assayed in paired samples not treated with ferricyanide. Ferrocyanide appearance was measured at the end of a 30 min incubation at 37 °C with 1 mM ferricyanide, with correction in each experiment for background observed in the presence of liposomes alone (0.91 ± 0.15 nmol/nmol phospholipid (PL)).
[b]Not determined.
[c]Number of experiments.
[d]$p < 0.05$ compared to liposomes containing either ascorbate or α-tocopherol alone.

tron transfer from intracellular ascorbate to extracellular ferricyanide was rate-limiting.

To determine whether intravesicular ascorbate can interact with α-tocopherol to prevent lipid peroxidation by an extravesicular agent, the water-soluble free radical initiator 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) (Wako Chemical Co., Richmond, VA) was added to liposomes containing various combinations of ascorbate and α-tocopherol. Over a 4 hour incubation at 37 °C, the α-tocopherol content of liposomes without ascorbate progressively decreased, with nearly complete disappearance by the end of the incubation with AAPH (Fig. 3A). When ascorbate was trapped within the vesicles, loss of α-tocopherol was prevented (Fig. 3B). This was

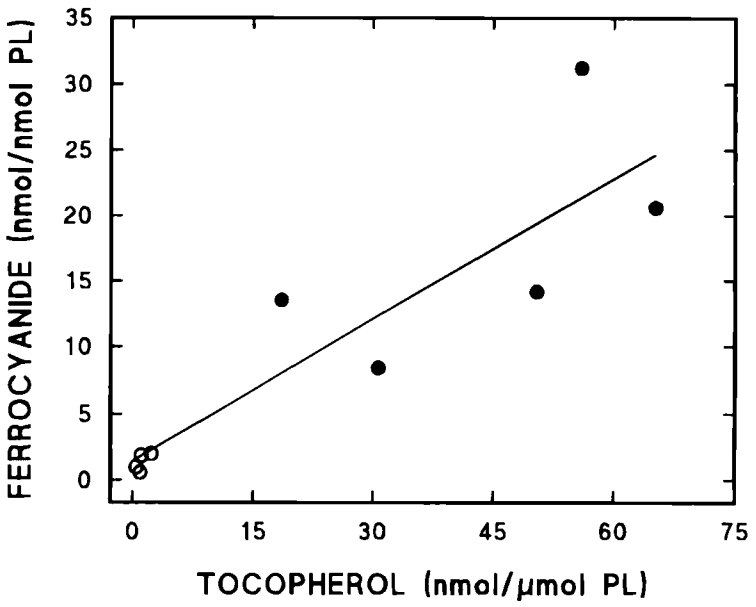

FIGURE 2 *Reduction of ferricyanide as a function of liposomal α-tocopherol content.* Small unilamellar liposomes (filled circles) were loaded with the indicated α-tocopherol concentration and with 100 mM ascorbate. An aliquot (63 nmol phospholipid/0.5 ml Tris buffer) was incubated as described in the legend to Table I. Liposomes containing endogenous erythrocyte lipids (open circles) were prepared to contain 100 mM ascorbate and incubated with 1 mM ferricyanide at a final phospholipid concentration of 176 nmol phospholipid/0.5 ml Tris buffer, also under the conditions noted in the legend to Table I. Paired data are expressed relative to the liposome phospholipid content (PL). The solid line represents a linear fit ($r = 0.89$, $p < 0.01$).

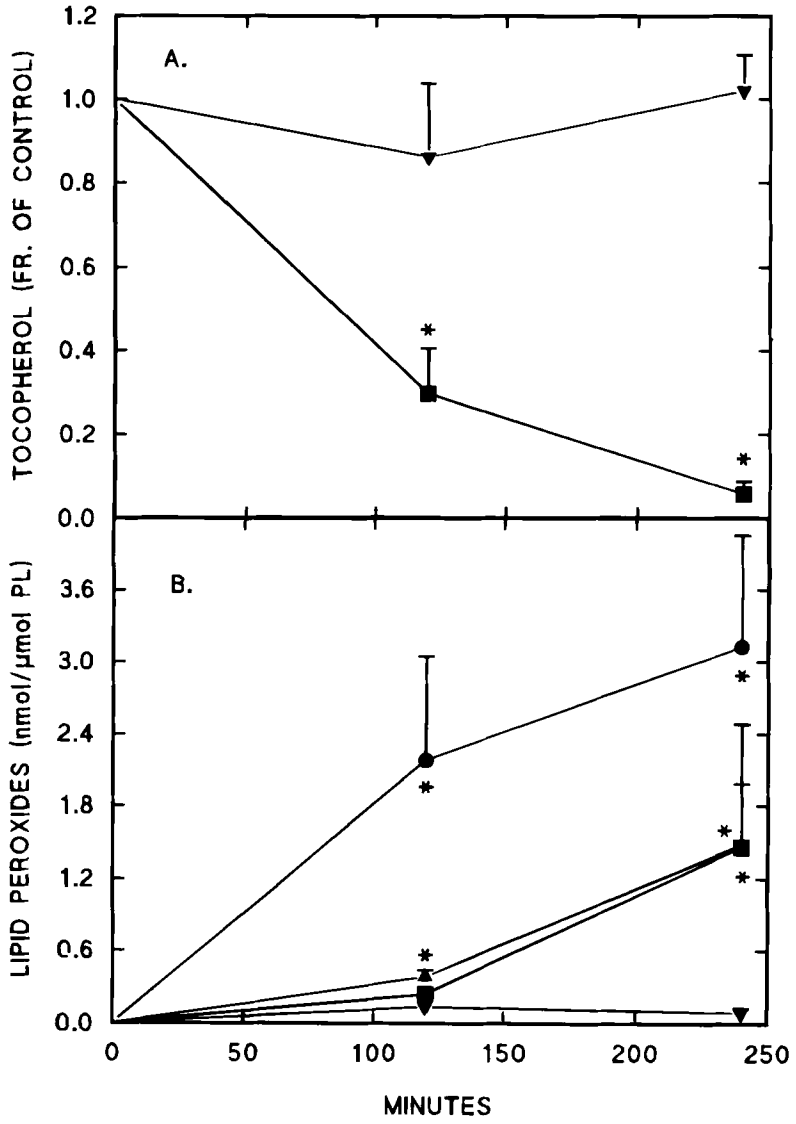

FIGURE 3 *Oxidation of liposomes by AAPH.* Small unilamellar liposomes were prepared in the absence of additives (circles), or to contain 100 mM ascorbate (triangles), α-tocopherol at the concentration noted in Table I (squares), or both ascorbate and α-tocopherol (inverted triangles). Liposomes (80 nmol phospholipid/0.5 ml Tris buffer) were incubated in the presence or absence of 10 mM AAPH at 37 °C in 50 mM Tris-HCl buffer, pH 7.4. At the indicated times, aliquots were removed for assay the liposomal content of α-tocopherol (Panel A) or of lipid hydroperoxides (Panel B). Values for α-tocopherol are expressed as a fraction of control incubations not treated with AAPH. Lipid hydroperoxides are expressed relative to the phospholipid content (PL) of the liposomes, and are corrected at each time point for hydroperoxide formation found in the samples not treated with AAPH. A single asterisk (*) indicates $p < 0.05$ compared to liposomes containing both ascorbate and α-tocopherol. Data are from 6 experiments.

associated with complete protection against lipid peroxidation by AAPH (Fig. 3B). Either ascorbate or α-tocopherol alone afforded protection for 2 hours against oxidation of liposomal fatty acids by AAPH, but not for 4 hours.

DISCUSSION

The results of this and earlier studies indicate that α-tocopherol can mediate the transbilayer reduction of ferricyanide by ascorbate, a process also associated in this work with protection of the membrane from external oxidant stress. Transbilayer electron transfer by α-tocopherol is slow compared to that mediated by less hydrophobic quinones or chromanols.[17,18] Kagan, et al.[17] showed that α-tocopherol fluorescence in liposomes was quenched by ferricyanide in two phases. A rapid phase, not detected in this and another study,[18] corresponded to oxidation of α-tocopherol molecules exposed to the external aqueous solution. A much slower phase, also observed in this study (Fig. 1), was thought to be due to diffusion of α-tocopherol from within or across the liposomal membrane to the outer face where it could be oxidized.[17] Short chain 6-hydroxy chromanes showed only a rapid phase under such conditions.[17] Ilani and Krakover reported a slow diffusion rate of α-tocopherol across dipalmitoyl phosphatidylcholine liposomal bilayers compared to 2,6-dichlorobenzoquinone,[18] and they also found that the rate of reaction of α-tocopherol at the lipid-water interface was much slower than its reaction in solution.[18] Thus transmembrane electron transfer by α-tocopherol appears to be limited both by its shuttle frequency across the bilayer and by the rates of redox reactions at the lipid-water interface on either side of the bilayer. In a recent study from this laboratory, the rate of external ferricyanide reduction by ascorbate resealed within human erythrocyte ghosts also depended on the membrane content of α-tocopherol.[12] Ferricyanide reduction by resealed ghosts was increased by addition of exogenous α-tocopherol during ghost preparation, but was decreased by removal of endogenous α-tocopherol. This may indicate that α-tocopherol can contribute to the transfer of reducing equivalents across cell membranes as well as across artificial liposomal membranes.

The present results suggest that trans- or intramembrane transfer of ascorbate-derived reducing equivalents by α-tocopherol can protect liposomes from extravesicular oxidant stress. This conclusion derives from use of the water soluble free radical initiator AAPH added to the outside of ascorbate-containing liposomes. At physiological pH, both AAPH and its free radical fission products will carry a positive charge[19] and should only slowly cross the sealed liposomal membrane. When AAPH is added outside vesicles, the resulting free radicals are likely to be intercepted by α-tocopherol in the membrane bilayer. One-electron oxidation of α-tocopherol produces tocopheroxyl radicals, which then migrate to the inner bilayer surface where they are reduced by ascorbate within the liposome. This mechanism seems more plausible than direct reduction of the AAPH radical or its derivatives by ascorbate, given the initial separation of reagents across the membrane, and since intravesicular ascorbate completely prevented both the oxidation of α-tocopherol and lipid peroxidation in the liposomal bilayer (Fig. 3). These results in a simple liposomal system support the possibility that a similar synergism exists for ascorbate and α-tocopherol in erythrocyte ghosts exposed to an external oxidant stress.[12]

Acknowledgments

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