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Carotenoid-Containing Unilamellar Liposomes Loaded with Glutathione: a Model to Study Hydrophobic-Hydrophilic Antioxidant Interaction

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Unilamellar liposomes are used as a simple two-compartment model to study the interaction of antioxidants. The vesicle membrane can be loaded with lipophilic compounds such as carotenoids or tocopherols, and the aqueous core space with hydrophilic substances like glutathione (GSH) or ascorbate, mimicking the interphase between an aqueous compartment of a cell and its surrounding membrane.

Unilamellar liposomes were used to investigate the interaction of GSH with the carotenoids lutein, β-carotene and lycopene in preventing lipid peroxidation. Lipid peroxidation was initiated with 2,2'-azo-bis-[2,4-dimethylvaleronitrile] (AMVN). Malondialdehyde (MDA) formation was measured as an indicator of oxidation; additionally, the loss of GSH was followed. In liposomes without added antioxidant, MDA levels of 119 ± 6 nmol/mg phospholipid were detected after incubation with AMVN for 2 h at 37°C. Considerably lower levels of 57 ± 8 nmol MDA/mg phospholipid were found when the liposomal vesicles had been loaded with GSH. Upon incorporation of β-carotene, lycopene or lutein, the resistance of unilamellar liposomes towards lipid peroxidation was further modified. An optimal further protection was observed with 0.02 nmol β-carotene/mg phospholipid or 0.06 nmol lycopene/mg phospholipid. At higher levels both these carotenoids exhibited prooxidant effects. Lutein inhibited lipid peroxidation in a dose-dependent manner between 0.02 and 2.6 nmol/mg phospholipid. With increasing levels of lycopene and lutein the consumption of encapsulated GSH decreased moderately, and high levels of β-carotene led to a more pronounced loss of GSH.

The data demonstrate that interactions between GSH and carotenoids may improve resistance of biological membranes towards lipid peroxidation. Different carotenoids exhibit specific properties, and the level for optimal protection varies between the carotenoids.

Keywords: carotenoids, glutathione, lipid peroxidation, peroxyl radicals, AMVM

INTRODUCTION

Lipophilic compartments are protected against lipid peroxidation by lipophilic antioxidants such as carotenoids and tocopherols [1,2], but hydrophilic compounds are also involved in cellular defense against oxidative deterioration of

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lipids. Vitamin C and GSH interact with tocopherol, thereby regenerating it from the tocopheroxyl radical which is formed upon the initial reduction of a lipid peroxyl radical by vitamin E[3–5]. This interplay allows for the transfer of a radical load from a lipophilic to an aqueous compartment where antioxidant enzymes are operative, constituting a major strategy of antioxidant defense (see Ref. [6] for review).

Cooperative interactions have also been found among lipophilic antioxidants. There is evidence that β-carotene and α-tocopherol act synergistically to inhibit lipid peroxidation challenged by peroxyl radicals [7]. Mixtures of carotenoids are superior to single compounds in preventing the oxidation of lipids in multilamellar liposomes[8]. A synergistic effect has been ascribed to lycopene and lutein, which are among the major carotenoids in human blood and tissues. [8]

Interactions of carotenoids with hydrophilic antioxidants have not yet been characterized in much detail, and we therefore studied a model system based on unilamellar liposomes, which is presented here. Such model studies may have a bearing on *in vivo* studies. For example, the interaction of β-carotene with ascorbate was not addressed in human intervention studies in risk populations such as smokers, although it is known that smokers have a particularly low ascorbate plasma level.

Specific antioxidant effects of β-carotene and lycopene were shown to be inversely dose-dependent. [9] Only low concentrations of these unpolar carotenoids protect HT29 cells against oxidative damage to DNA and membranes, and at higher levels the protection is less pronounced. Furthermore, even a prooxidative effect of β-carotene was reported in a study with human skin fibroblasts exposed to ultraviolet-A light. [10]

The present study investigates the effect of different carotenoid levels on lipid peroxidation in unilamellar liposomes loaded with GSH which plays an important role as hydrophilic antioxidant[11].

MATERIALS AND METHODS

Chemicals

β-Carotene and lutein were a kind gift from Hoffmann-La Roche (Basel, Switzerland). Lycopene, glutathione, and butylated hydroxytoluene (BHT) were purchased from Sigma (Deisenhofen, Germany). 3 -sn-Phosphatidylcholine (egg yolk), fluorescamin and β-apo-8'-carotenal were supplied by Fluka (Buchs, Switzerland). 2,2' -Azobis-[2,4-dimethylvaleronitrile] (AMVN) was from Wako (Osaka, Japan). All other chemicals and solvents were obtained from Merck (Darmstadt, Germany).

Preparation of Liposomes and Incubation

Appropriate amounts of phosphatidylcholine and carotenoids were dissolved in chloroform in a round-bottomed flask. The solvent was evaporated by rotary evaporation so that a thin film of lipids remained at the wall. Sodium phosphate buffer (100 mM; pH 7.4) containing 100 mM glutathione was added to obtain a final concentration of 5 mg phospholipid/ml and the suspension was sonicated for 20 min. For the preparation of unilamellar liposomes of defined size the mixture was extruded through a polycarbonate filter (Avestin; 0,4 μm diameter; Milsch Equipment, Laudenbach, Germany)[12]. Control liposomes were prepared without carotenoids or GSH.

Untrapped glutathione was removed by centrifugation at 47,800 × g for 10 min at 10°C with a Sorvall RC-5B centrifuge (Sorvall; DuPont Instruments; Newtown, CT). The resulting pellet was resuspended in 100 mM sodium phosphate buffer (pH 7.4) and centrifuged again at 47,800 × g for 10 min at 10°C. The liposomes in the sediment were suspended in 100 mM sodium phosphate buffer (pH 7.4).

The size of the liposomes was checked by electron microscopy.

Lipid Peroxidation

Lipid peroxidation was initiated by 10 mM AMVN dissolved in tetrahydrofuran; final concentration of tetrahydrofuran in the suspension was 1 %. Reactions were carried out in the dark at 37°C for 2 h at ambient oxygen tension[13]. Aliquots were taken for analysis, and the reaction was stopped by freezing the sample in liquid nitrogen.

Analysis of Glutathione

An equal volume of 1-butanol was added to a liposomal suspension; the mixture was centrifuged for 30 sec at 4,000 rpm. The aqueous phase (50 µl) was transferred into 0.75 ml 100 mM borate buffer (pH 8.0). After derivatisation with 0.2 ml fluorescamin (0.5 mg/ml acetone) and separation by HPLC on a 5 × 250-mm Lichrospher 100 RP 18-e column (Merck, Darmstadt, Germany), fluorescence was monitored at excitation and emission wavelengths of 390 and 475 nm, respectively. The mobile phase consisted of 70% 0.1 M citrate buffer (pH 3.0), and 30% acetonitrile. The flow rate was 0.8 ml/min[14].

Measurement of Malondialdehyde (MDA)

MDA was measured following a published method [15] with minor modifications. BHT, final concentration 0.1%, was added to the samples to prevent formation of further MDA. After the reaction with thiobarbituric acid the samples were separated by HPLC on a 5 × 250-mm Lichrospher 100 RP 18-e column (Merck, Darmstadt, Germany); fluorescence was monitored at excitation and emission wavelengths of 513 and 550 nm, respectively. The mobile phase consisted of 55% 50 mM potassium phosphate buffer (pH 5.5), and 45% methanol; flow rate was 0.75 ml/min.

The oxidation of carotenoids with AMVN in the absence of lipids did not lead to signals interfering with the determination of MDA.

Determination of Carotenoids

Carotenoid levels were determined using a previously described method [16]with minor modifications. Samples were mixed with buffer (2 mM potassium phosphate, pH 7.2; 250 mg/ml EDTA), internal standard (β-apo-8'-carotenal) and ethanol. After addition of n-hexane/dichloromethane(5:1, v:v) and BHT (final concentration 0.01 %), the samples were vortexed for 1 min, sonicated for 5 min, vortexed for 30 sec again and centrifuged at 4,500 rpm for 10 min. The supernatant was collected, dried in the dark under a gentle stream of nitrogen, resuspended with diethylether, vortexed briefly, and dried again. The remaining carotenoids were dissolved in 20 µl dichloromethane and 180 µl of mobile phase (methanol/acetonitrile/2-propanol, 54:44:2). After separation by HPLC (Suplex pKb-100; 5 µm; Supelco, Bellefonte, PA) carotenoids were detected at 450 nm (β-carotene, lutein), or 472 nm (lycopene).

Statistics

Two-sided unpaired Student's t-test was applied for statistical analyses.

RESULTS

Characterization of Liposomes

Unilamellar liposomes were characterized with respect to size and structure by means of electron microscopy. Loaded and unloaded vesicles were unilamellar with a mean size of 214 ± 20 nm (Figures 1 and 2). The liposomal core space was loaded with GSH, and non-incorporated antioxidant was eliminated by centrifugation.

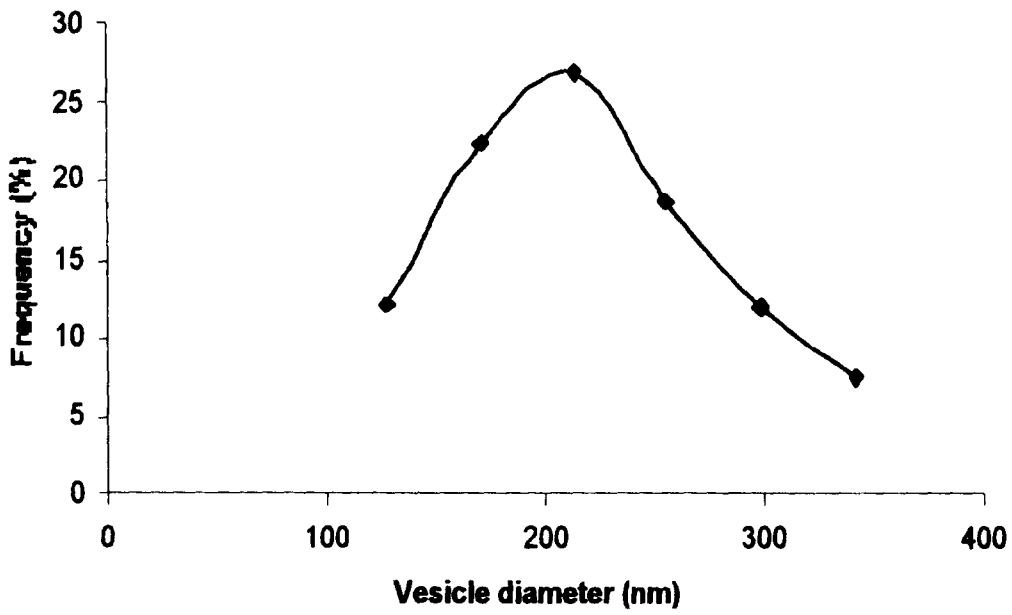

FIGURE 1 Size distribution of 3-sn-phosphatidylcholine (egg yolk) liposomes obtained after extrusion through a 400 nm pore polycarbonate membrane measured by electron microscopy

FIGURE 2 Electron micrograph of 3-sn-phosphatidylcholine (egg yolk) liposomes obtained after extrusion through a 400 nm pore polycarbonate membrane

The amount of non-encapsulated GSH was 6.7 ± 0.9% of total GSH in suspensions of control vesicles and 6.4 ± 0.9 % in suspensions of carotenoid-loaded liposomes. In the present system GSH is not being regenerated subsequent to oxidation. Thus, a relatively high initial GSH level within the liposomes (20 ± 2 μmol/mg phospholipid) was used. The efficacy of carotenoid incorporation into the membrane varied between carotenoids. High amounts of lutein (2.6 nmol/mg phospholipid) and β-carotene (2.0 nmol/mg phospholipid) were incorporated, whereas only a level of 0.12 nmol/mg phospholipid was achieved with lycopene. No leakage of GSH into the incubation medium was observed when the liposomes were incubated with AMVN.

The data demonstrate that the extrusion method provides a simple way to prepare unilamellar liposomes within a defined range of size. The purification from non-incorporated GSH is efficient, and losses of this antioxidant can be attributed to reactions occurring within the core space of the vesicles.

Antioxidant and Prooxidant Effects

When GSH was incorporated into the hydrophilic compartment of unilamellar liposomes the resistance of the particles towards lipid peroxidation increased. Without GSH, 119 ± 6 nmol MDA/mg phospholipid was formed after incubation with AMVN for 2 h at 37°C. Considerably lower levels of 57 ± 8 nmol MDA/mg phospholipid were detected when the liposomes had been loaded with GSH.

The protection against oxidation was modified when carotenoids were additionally incorporated into the membrane (Table I). The incorporation of lutein into membranes of GSH-loaded liposomes further diminished MDA formation only at the highest lutein level of 2.6 nmol/mg phospholipid; MDA levels significantly decreased to 44 nmol MDA/mg phospholipid. At lower lutein levels, even a prooxidant effect was observed: MDA formation in GSH-loaded liposomes containing lutein at 0.02 nmol/mg phospholipid was significantly higher than in liposomes only containing GSH, 76 and 57 nmol MDA/mg phospholipid, respectively. This was also reflected in the loss of GSH, measured as an indicator of oxidation. At high levels of lutein (2.6 nmol/mg phospholipid), loss of GSH was only 3.0 μmol/mg phospholipid as compared to a loss of 5.0 μmol/mg phospholipid in liposomes without the carotenoid. In accordance with the prooxidant effect at 0.02 nmol lutein/mg phospholipid observed with MDA formation, the loss of GSH was higher in vesicles loaded with lutein and GSH (7.4 μmol/mg phospholipid) compared to liposomes containing only GSH (5.0 μmol/mg phospholipid).

In contrast to lutein, a small but statistically significant effect on MDA formation was observed with β-carotene only at the lowest level of 0.02 nmol carotenoid/mg phospholipid. MDA formation was diminished from 57 to 48 nmol MDA/mg phospholipid. The incorporation of higher amounts of β-carotene into the membrane did not improve antioxidant protection (Figure 3). In contrast, there is a trend towards a prooxidative effect with increasing amounts of β-carotene, which is statistically significant at 2.0 nmol/mg phospholipid of the carotenoid (64 versus 57 nmol MDA/mg phospholipid). β-Carotene was more effective in preventing the loss of GSH than lutein. Again, lower amounts were more protective than higher levels of β-carotene. At concentrations of 0.02–0.52 nmol/mg phospholipid, the loss of GSH was diminished from 5.0 μmol/mg phospholipid in GSH-loaded liposomes to about 2.0 μmol/mg phospholipid in liposomes with GSH and β-carotene.

TABLE I MDA formation and loss of antioxidants in unilamellar glutathione-loaded liposomes. MDA formation was initiated by 10 mM AMVN; values at t=0 (about 30 nmol/mg phospholipid) were subtracted from each corresponding sample (n = 6–8 for each concentration); start content of the GSH-loaded liposomes was 20 ± 2 μmol GSH/mg phospholipid

	Carotenoid (nmol/mg phospholipid)	*Carotenoid consumed (mnol/mg phospholipid)*	*MDA (nmol/mg phospholipid)*	*Loss of GSH (μmol/mg phospholipid)*
Empty	–	–	119 ± 6	–
GSH	–	–	57 ± 8[*a]	5.0 ± 0.8
+ lutein	0.02	0.012 ± 0.002	76 ± 9[*b]	7.4 ± 0.2
	0.16	0.116 ± 0.006	69 ± 3[*c]	4.2 ± 0.2
	0.52	0.328 ± 0.010	61 ± 5	4.2 ± 2.2
	2.60	1.334 ± 0.064	44 ± 2[*c]	3.0 ± 2.6
+ β-carotene	0.02	0.018 ± 0.001	48 ± 8[*d]	1.8 ± 1.6
	0.16	0.116 ± 0.008	57 ± 1	2.2 ± 0.0
	0.52	0.484 ± 0.002	62 ± 4	2.0 ± 0.4
	2.00	1.700 ± 0.018	64 ± 2[*e]	5.6 ± 1.4
+ lycopene	0.02	0.010 ± 0.002	41 ± 4[*a]	5.4 ± 3.2
	0.06	0.012 ± 0.001	25 ± 7[*a]	4.2 ± 2.6
	0.10	0.014 ± 0.030	55 ± 3	3.0 ± 0.8
	0.12	0.012 ± 0.008	71 ± 5[*c]	2.6 ± 2.0

GSH vs. Empty: [*a]: $P < 0.0005$.
Carotenoid liposomes vs. GSH: [*a]: $P < 0.0005$, [*b]: $P < 0.001$, [*c]: $P < 0.005$, [*d]: $P < 0.025$, [*e]: $P < 0.05$.

In comparison to lutein and β-carotene, incorporation of lycopene into the membrane of GSH-loaded liposomes was relatively low, the range being between 0.02 and 0.12 nmol lycopene/mg phospholipid (Figure 3). Lycopene was most protective at 0.06 nmol/mg phospholipid, diminishing the amount of MDA from 57 to 25 nmol/mg phospholipid, which was the highest protection observed in this study. At lower levels, this effect was less pronounced and at the higher level a prooxidant effect of lycopene was detected (Figure 3), with MDA formation increasing from 57 to 71 nmol MDA/mg phospholipid. Lycopene protected GSH from oxidation in a dose-dependent manner.

Loss of Carotenoid

The loss of carotenoids in GSH-loaded liposomes during incubation was followed by HPLC (Table I). In vesicles containing lutein and β-carotene the carotenoid content decreased upon incubation with AMVN. With elevated levels, increasing amounts of the carotenoids were consumed. Opposing results were observed with lycopene. In all samples only a minor loss of about 0.012 nmol/mg phospholipid was found, which might be related more to sample preparation than to a chemical reaction.

DISCUSSION

Unilamellar liposomes are a suitable system to study interactions between lipophilic and hydrophilic antioxidants. Liposomes which contain GSH in the core space are more resistant towards lipid peroxidation than unloaded vesicles. This is remarkable since the oxidation process likely proceeds in the lipid compartment.

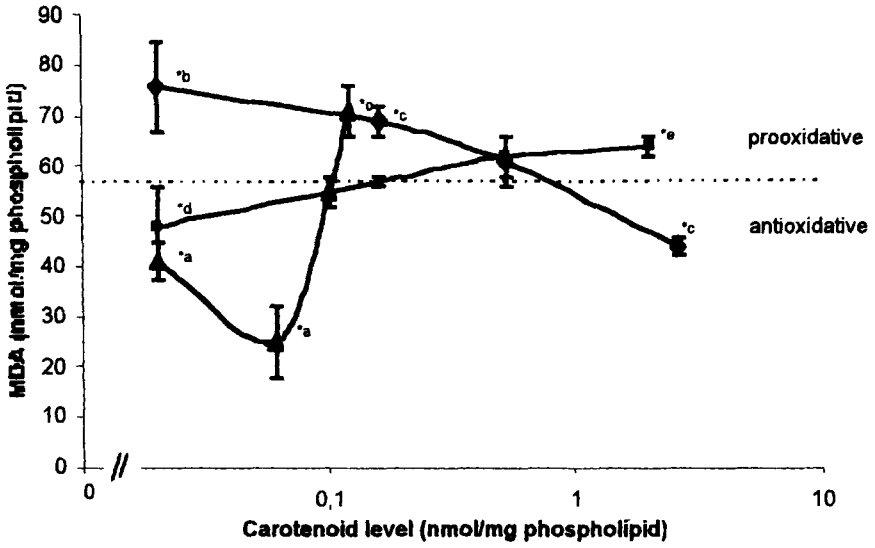

FIGURE 3 Lipid peroxidation in unilamellar carotenoid liposomes loaded with glutathione. Oxidation was initiated with 10 mM AMVN. Reactions were carried out for 2 h at 37°C. The graphs show the different dose-dependences of the lipid protective effect of lutein (♦), β-carotene (■) and lycopene (▲). Data points with different letters are significantly different from control without carotenoid (dashed line). Above dashed line, prooxidant; below dashed line, antioxidant effects. ^{*a}: $P < 0.0005$, ^{*b}: $P < 0.001$, ^{*c}: $P < 0.005$, ^{*d}: $P < 0.025$, ^{*e}: $P < 0.05$

Due to its hydrophilic character, GSH might exert its protective effect at the interface of the hydrophilic and lipophilic compartments, scavenging more polar reactive intermediates formed during lipid peroxidation or AMVN decomposition. Because no leakage of GSH from the core space of the liposomes was observed during incubation, its loss is likely due to interactions with reactive oxygen species with a considerable mobility within or even through the membrane.

Upon incorporation of carotenoids into the membrane of GSH-loaded liposomes, protection against oxidation was modified. The most polar carotenoid lutein provided additional protection, increasing with the amount incorporated into the membrane which suggests a cooperative interaction between both antioxidants. In the case of lycopene and β-carotene the situation is more complicated. Optimal protection against lipid peroxidation was achieved at specific carotenoid levels. It appears that the ratio between membrane lipids and incorporated carotenoids is important for optimal protection. The amount of MDA formed at the optimal level of 0.06 nmol lycopene/mg phospholipid is about only half of that produced in liposomes loaded only with GSH, and lycopene at the optimum level was found to be the most efficient carotenoid (Figure 3).

The different antioxidant activities of the investigated carotenoids in GSH-loaded liposomes might be related to their different polarity and structure, which govern orientation of the carotenoid in the membrane. It has been shown that xanthophylls such as zeaxanthin or violaxanthin span the membrane, with the polar end-groups anchored at the polar sites [17,18]. β-Carotene and lycopene, lacking hydrophilic substituents, remain entirely within the hydrophobic core of the membrane and retain a substantial degree of mobility [19]. Compared to β-carotene, the incorporation of lycopene into the membrane was low. High lycopene levels

might disturb the bilayer structure, and as a consequence there would be a loss of the membrane integrity, as shown by Lowe et al. [9] in the case of the HT29 cells. Only low concentrations of β-carotene and lycopene had an antioxidant effect on the lipid membrane.

The present study demonstrates that the lipid protection decreases when the concentration of unpolar carotenoids is augmented; elevated levels are even prooxidative. It also suggests that there is an optimal ratio of lipid to carotenoids in membrane systems providing optimal protection against lipid peroxidation. This study adds an example that combinations of hydrophilic and hydrophobic antioxidants generate synergism.

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