Carotenoids are degraded by free radicals but do not affect lipid peroxidation in unilamellar liposomes under different oxygen tensions

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Abstract **It has been questioned whether carotenoids can act as antioxidants in biological membranes. Biological membranes can be modeled for studies of lipid peroxidation using unilamellar liposomes. Both carotenoid depletion and lipid peroxidation were increased with increasing oxygen tension in unilamellar liposomes. Carotenoids in such liposomes were found to be very sensitive to degradation by free radicals generated from iron and 2,2′-azobis(2-amidinopropane) dihydrochloride, but they were not protective against lipid peroxidation. Lycopene and β-carotene were more sensitive to free radical attack than lutein, zeaxanthin, and β-cryptoxanthin.**

Key words: Carotenoid; Liposome; Lipid peroxidation; Free radical

1. Introduction

Epidemiological studies have found that plentiful consumption of fruits and vegetables decreases the risk of various cancers [1–3]. A possible mechanism behind this association may involve the antioxidant effects of carotenoids. Carotenoids are one important class of cancer preventive phytochemicals found in fruits and vegetables. In organic solvent, carotenoids react rapidly with various types of free radicals, including oxygen free radicals, organic radicals and nitrogen oxides [4,5]. When incorporated into liposomes, carotenoids have been shown to protect against lipid damage by agents such UV radiation, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and 2,2′-azobis(2,4-dimethylvaleronitrile) [6,7]. In biological membranes, however, β-carotene appears ineffective as an antioxidant [8,9].

Various types of liposomes can be prepared. For examining the redox properties of carotenoids, unilamellar liposomes may be a better model of biological membranes than multilamellar liposomes. In the latter, lipid peroxidation rates would be governed more by the structural aspects of the liposomes. In this study, conditions were established to completely solubilize carotenoids into unilamellar liposomes, and lipid peroxidation was initiated at physiological pH using 5 μM iron(II) or 1.25 mM AAPH. We found carotenoids to be very sensitive to free radical attacks. If carotenoids were depleted completely in the incubations, it would be difficult to compare their relative antioxidant effects. We therefore selected reaction conditions such that carotenoids were only partially depleted and then the antioxidant effects of lycopene, β-carotene, *trans*-β-apo-8′-carotenal, lutein, β-cryptoxanthin, and zeaxanthin were examined. These compounds represent common carotenoids of varying polarity. We used two well-established lipid oxidation markers as end points, thiobarbituric acid-reactive substances (TBARS) and conjugated dienes (CD), and a relatively newer biomarker for lipid oxidation, 8-isoprostane, which is produced by non-enzymatic peroxidation of arachidonic acid [10].

2. Materials and methods

2.1. Chemicals

β-Arachidonoyl-γ-palmitoyl-L-α-phosphatidylcholine (C20:4, [*cis*]-5,8,11,14/C16:0) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). *trans*-β-Apo-8′-carotenal, *trans*-β-carotene, 2-thiobarbituric acid and butylated hydroxytoluene were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Lycopene, lutein, β-cryptoxanthin, and zeaxanthin were obtained from Indofine Chemical Company (Belle Mead, NJ, USA). The carotenoids were stored as solids until use. Purity was checked by UV spectral and HPLC analyses, and the latter indicated 95–98% purity. The hydroxy-eicosateraenoic acid (HETE) mixture (5-HETE, 8-HETE, 11-HETE, 12-HETE, 15-HETE, catalog number 34002) was obtained from Cayman Chemical (Ann Arbor, MI, USA), and the total 8-isoprostane kit was purchased from Assay Designs (Ann Arbor, MI, USA). The Amicon stirred ultrafiltration cell with polyethersulfone ultrafiltration membranes (NMWL: 50 000) was purchased from Millipore Corporation (Bedford, MA, USA). All other chemicals including AAPH were obtained from Sigma-Aldrich Chemical Co. and solvents were HPLC grade, Burdick&Jackson brand (Muskegon, MI, USA).

2.2. Preparation of liposomes

Synthetic lipid, β-arachidonoyl-γ-palmitoyl-L-α-phosphatidylcholine (C20:4, [*cis*]-5,8,11,14/C16:0) was dissolved with or without carotenoids in chloroform. The lipid was then dried and re-dissolved at 37°C in 1 ml ethanol to give a phospholipid concentration of 7.5 mg/ml. The lipid was injected by syringe into 40 ml of 50 mM Tris buffer (pH 7.4) at 37°C under argon using fast stirring. Using the method of Batzri et al. [11], a stirred ultrafiltration cell (50 ml) with polyethersulfone membranes was used to concentrate the solution to 1–2 ml. The addition of 20 ml buffer was repeated three times to wash out any remaining ethanol. The final concentration of phospholipid in 50 mM Tris pH 7.4 was 2 mM. Carotenoid concentrations in liposome varied somewhat, perhaps due to solubility limits in ethanol and handling factors. The final concentrations for carotenoids in liposomes were 1.85, 4.00, 2.84,2.64, 4.50, 4.19 μM for lycopene, β-car-

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Abbreviations: ANOVA, analysis of variance; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; CD, conjugated dienes; HETE, hydroxy-eicosateraenoic acid; TBARS, thiobarbituric acid-reactive substance

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otene, 8-apo-β-carotenal, lutein, β-cryptoxanthin, and zeaxanthin. The UV spectra of carotenoids in liposomes matched well with published spectra of pure carotenoids, indicating no detectable oxidation [12]. When the carotenoids were extracted from the prepared liposomal suspensions with chloroform, the UV spectra of the extracted carotenoids indicated these same concentrations. This indicates that the carotenoids were not aggregated within the liposomes since aggregation causes a decrease in UV absorbance at the absorption maxima [13]. Literature data also indicate that carotenoid aggregation causes new absorption bands at about 350 and 575 nm [13], which were not found in our liposomal preparations. A final problem to consider is aggregation of the liposomes, but 0.2 mol% carotenoid incorporation was found previously to not have significant effects on liposome structure [6,14].

2.3. Lipid oxidation

Incubations were conducted with 100 μl liposomes suspension under three different oxygen tensions (see tables). The incubations with 0.4% oxygen in the headspace were achieved by evacuating and filling the 10 ml reaction tube four times with argon through. For 2.9% oxygen, the tubes were flushed with argon in the same manner and 1 ml air injected into them afterwards. For 22% oxygen, the incubations were conducted under 100% ambient air. Reactions were started by adding 5 μl of 0.1 mM $FeSO_4$ (freshly made) or 5 μl of 25 mM AAPH solution with needle injection through the septum. Reactions were carried out at 37°C for 30 min.

2.4. Percentage of oxygen in the headspace

The oxygen, nitrogen and argon contents of the headspace were determined by mass spectral analyses. An aliquot of the headspace was sampled with a gas-tight syringe and injected onto a Hewlett-Packard Ultra 2, 25-m (Palo Alto, CA, USA) column at 25°C in a Hewlett-Packard 5890 series II gas chromatograph. The detector was a HP5971A mass selective detector set to scan masses 10–50 *m/e*. The percentage of oxygen was calculated relative to the total of the oxygen, nitrogen and argon content in triplicate. The coefficient of variation in these assays was less than 10%.

2.5. Conjugated diene and carotenoid concentrations

Reactions were stopped by addition of 300 μl of chloroform/methanol (2:1) into the reaction tubes, followed by vortexing and centrifugation at $1000\times g$ for 10 min. The lower layer was removed and dried in a SpeedVac. The extract was dissolved in 400 μl ethanol, and the absorbance was measured at 234 nm to quantify the amount of CD present [15]. Standard curves for quantification of CD were made from a commercial mixture of HETE. The absorbance at the wavelength of maximum absorption of each carotenoid was also recorded to calculate the amount of carotenoid remaining.

2.6. TBARS

TBARS were measured by the method of Burdon et al. [15] with minor modifications. Briefly, 300 μl of reagent (0.1% butylated hydroxytoluene, 0.37% 2-thiobarbituric acid, 15% trichloroacetic acid, 0.25 M HCl) was added into 100 μl of reaction mixture, followed by capping and incubation for 15 min at 100°C. The tubes were then cooled in cold water and centrifuged at $1000\times g$ for 10 min. The absorbance was recorded at 514, 532 and 550 nm. The following formula was used to calculate TBARS: $A = A_{532}-(A_{524}\pm A_{550})/2$, $C = A/0.156$ μM [7].

2.7. Total 8-isoprostane levels

The redissolved extracts from the CD measurements were dried in a SpeedVac. For each dried extract, 200 μl of 2 N NaOH was added, and the mixture was incubated at 45°C for 2 h. This was then neutralized with 200 μl of 2 N HCl and total 8-isoprostanes were quantified by a commercial ELISA kit.

2.8. Statistical methods

For Table 1, one-way analysis of variance (ANOVA) was applied to identify whether Fe(II), AAPH and oxygen tension significantly affected the levels of lipid peroxidation. For Table 2, one-way ANOVA was applied to test if carotenoid depletion depends on the type of carotenoid incorporated into the liposomes. For Tables 3–5, one-way ANOVA was used to test if lipid peroxidation depends on carotenoid incorporation. When significant differences were found in Tables 2–4, Tukey's multiple comparisons test was used to identify those pairs that differed significantly.

3. Results

Liposomes prepared by these methods, either with or without 0.2 mol% carotenoids, contained low levels of pre-existing oxidized lipids. With each of the preparations made over the course of these experiments, CD levels ranged from 15 μM to 22 μM, TBARS ranged from 0.5 μM to 1.3 μM, and 8-isoprostane levels ranged from 1.58 nM to 4.11 nM. There were no significant differences in levels of pre-existing oxidized lipids in liposomes with or without incorporation of carotenoids (not shown).

Levels of lipid peroxidation for liposomes without incorporated carotenoids are shown in Table 1. Liposomes were incubated with 5 μM Fe(II) or 1.25 mM AAPH at 37°C for 30 min under varying oxygen tensions. Under 0.4% oxygen, CD levels were not increased by either 5 μM Fe(II) or 1.25 mM AAPH, but TBARS levels were slightly higher than that of control. Use of 2.9% oxygen for the incubations should be relatively more relevant to physiological conditions than ambient oxygen concentrations since the concentration of oxygen in tissues is thought to be low [16]. Under 2.9% oxygen, lipid peroxidation mediated by Fe(II) and AAPH was significantly induced as determined by all three measurements (Table 1). Using ambient oxygen tensions (22% oxygen), lipid oxidation was much faster than that with 2.9% or with 0.4% oxygen. The levels of 8-isoprostane were determined in incubations with 2.9% oxygen, and levels of 8-isoprostane increased over control by a larger percentage than either TBARS or CD (Table 1).

Carotenoid depletion in liposomes after incubation with Fe(II) or AAPH was substantial (Table 2). Since Fe(II)- and AAPH-mediated CD and TBARS formation were not detected under 0.4% oxygen (Table 1) while carotenoids were depleted (Table 2), carotenoids appeared to be more sensitive

Table 1
Lipid peroxidation in liposomes without added carotenoids[a]

% O_2[b]	Initiator	CD (μM)	TBARS (μM)	8-Isoprostane (nM)
22.0 ± 2.3	None	16.9 ± 1.1	0.81 ± 0.25	2.78 ± 0.54
0.4 ± 0.3	Fe(II)	18.3 ± 2.2	1.51 ± 0.10[c]	–
2.9 ± 0.5	Fe(II)	26.7 ± 3.2[c]	2.56 ± 0.16[c]	11.15 ± 1.20[c]
22.0 ± 2.3	Fe(II)	33.4 ± 5.3[c]	4.35 ± 0.36[c]	–
0.4 ± 0.3	AAPH	17.8 ± 2.1	1.02 ± 0.22	–
2.9 ± 0.5	AAPH	28.1 ± 1.2[c]	1.74 ± 0.16[c]	13.03 ± 0.32[c]
22.0 ± 2.3	AAPH	40.8 ± 5.4[c]	4.56 ± 0.49[c]	–

[a]Values are expressed as mean ± S.D.
[b]Measured percent oxygen from mass spectral analysis of the incubation headspace.
[c]Statistically significant increase ($P < 0.05$, two-tailed test) in comparison with incubations under 22% air without initiator.

Table 2
Percentage of carotenoid depletion

Carotenoid	Initiator	
	Iron	AAPH
	0.4% Oxygen	
β-Carotene	23.6 ± 2.4[a]	30.1 ± 3.2[a]
Lycopene	19.0 ± 5.3[a,b]	20.0 ± 4.2[b]
β-Apo-8′-carotenal	14.3 ± 1.5[b,c]	9.4 ± 3.1[d,c]
Lutein	15.4 ± 1.1[b,c]	1.6 ± 4.0[d,c]
β-Cryptoxanthin	9.8 ± 0.8[c]	5.6 ± 2.7[d,c]
Zeaxanthin	12.2 ± 0.6[b,c]	5.5 ± 2.9[d,c]
	2.9% Oxygen	
β-Carotene	43.9 ± 1.8[a]	49.2 ± 3.5[a]
Lycopene	37.2 ± 4.1[a]	43.0 ± 3.7[a,b]
β-Apo-8′-carotenal	28.4 ± 2.4[b]	39.1 ± 3.6[b]
Lutein	20.1 ± 1.6[c]	24.2 ± 4.1[c]
β-Cryptoxanthin	28.7 ± 2.0[b]	34.5 ± 0.8[b,d]
Zeaxanthin	22.1 ± 3.2[b,c]	27.2 ± 3.5[c,d]
	22% Oxygen	
β-Carotene	58.8 ± 4.0[a]	64.0 ± 0.7[a]
Lycopene	47.2 ± 2.3[a,b]	58.2 ± 1.1[a]
β-Apo-8′-carotenal	31.8 ± 9.2[c,d]	62.3 ± 2.0[a]
Lutein	23.1 ± 4.0[c]	41.1 ± 5.0[b]
β-Cryptoxanthin	39.2 ± 3.4[b,d]	45.2 ± 7.5[b]
Zeaxanthin	31.4 ± 6.4[c,d]	41.2 ± 5.0[b]

Means within a column with different superscripts are significantly different, $P < 0.05$, from ANOVA analyses.

to oxidative stress than formation of those lipid peroxidation products. As oxygen tension was increased, the amount of carotenoid depletion increased also (Table 2). β-Carotene and lycopene appeared to be the two carotenoids most sensitive to oxidation in liposomes under all three oxygen tensions.

To demonstrate the effects of carotenoid incorporation into liposomes on lipid peroxidation, the CD and TBARS levels are presented in Tables 3 and 4 as percentages of that in liposomes without added carotenoids. The percentages of CD and TBARS were all around 100%, indicating that carotenoids did not appreciably affect those indices of lipid peroxidation. This was true under all three oxygen tensions. In the iron-catalyzed reactions, there was one instance of a significant decrease in lipid peroxidation and two instances of a significant increase relative to control. No significant increases or decreases were found in the AAPH-catalyzed reactions. Thus, although substantial percentages of carotenoids were always present in the liposomes during the lipid peroxidation reactions (Table 2), protective effects of carotenoids against lipid peroxidation were largely not observed. Augmentation of lipid peroxidation also was not appreciable.

Table 3
Effects of carotenoid incorporation on CD formation (% of control)

Carotenoid	Initiator	
	Iron	AAPH
	0.4% Oxygen	
β-Carotene	100 ± 7[a]	113 ± 17
Lycopene	133 ± 14[b]*	103 ± 1
β-Apo-8′-carotenal	86 ± 4[a]	90 ± 3
Lutein	103 ± 8[a,b]	96 ± 4
β-Cryptoxanthin	85 ± 7 [a]	111 ± 7
Zeaxanthin	109 ± 21[a,b]	96 ± 4
		2
β-Carotene	107 ± 4	99 ± 9
Lycopene	111 ± 5[a]	96 ± 3
β-Apo-8′-carotenal	100 ± 4	96 ± 6
Lutein	105 ± 8	93 ± 7
β-Cryptoxanthin	90 ± 2[b]	90 ± 6
Zeaxanthin	92 ± 2[b]	89 ± 5
	22% Oxygen	
β-Carotene	116 ± 8[a]	107 ± 1
Lycopene	120 ± 7[a]	101 ± 11
β-Apo-8′-carotenal	95 ± 7	96 ± 14
Lutein	102 ± 6	85 ± 4
β-Cryptoxanthin	79 ± 11[b]	87 ± 7
Zeaxanthin	78 ± 16[b]	89 ± 9

Means within a column with different superscripts are significantly different, $P < 0.05$, from ANOVA analyses. Starred means are significantly different than control (no carotenoid).

Table 4
Effects of carotenoid incorporation on TBARS formation (% of control)

Carotenoid	Initiator	
	Iron	AAPH
	0.4% Oxygen	
β-Carotene	79 ± 15[a,b]	77 ± 16
Lycopene	104 ± 22[a]	86 ± 15
β-Apo-8′-carotenal	76 ± 8[a,b]	96 ± 23
Lutein	90 ± 8[a,b]	100 ± 25
β-Cryptoxanthin	78 ± 0[b]	113 ± 31
Zeaxanthin	72 ± 5[b]	97 ± 18
		2
β-Carotene	95 ± 5[a]	83 ± 14
Lycopene	96 ± 4[a]	94 ± 25
β-Apo-8′-carotenal	123 ± 3[b]*	117 ± 11
Lutein	97 ± 13[a]	90 ± 3
β-Cryptoxanthin	78 ± 5[a]*	83 ± 7
Zeaxanthin	82 ± 7[a]	78 ± 7
	22% Oxygen	
β-Carotene	101 ± 9	86 ± 17
Lycopene	99 ± 12	94 ± 8
β-Apo-8′-carotenal	97 ± 8	93 ± 7
Lutein	123 ± 43	79 ± 4
β-Cryptoxanthin	77 ± 1	83 ± 5
Zeaxanthin	90 ± 7	91 ± 6

Means within a column with different superscripts are significantly different, $P < 0.05$, from ANOVA analyses. Starred means are significantly different than control (no carotenoid).

Table 5 summarizes the effects of carotenoid incorporation into liposomes on 8-isoprostane formation. As shown in Table 1, 8-isoprostane levels are perhaps more sensitive markers for lipid peroxidation under 2.9% oxygen than either CD or TBARS. Neither an antioxidant nor a prooxidant effect of carotenoids on 8-isoprostane levels was observed, however.

4. Discussion

Unilamellar liposomes were chosen for these studies, since

Table 5
Effects of carotenoid incorporation on 8-isoprostane formation under 2.9% oxygen (% of control)[a]

Carotenoid	Initiator	
	Iron	AAPH
β-Carotene	130 ± 41	117 ± 21
Lycopene	124 ± 44	113 ± 11
β-Apo-8′-carotenal	143 ± 70	131 ± 18
Lutein	85 ± 15	97 ± 30
β-Cryptoxanthin	121 ± 38	98 ± 10
Zeaxanthin	94 ± 32	100 ± 1

[a]Iron- and AAPH-mediated 8-isoprostane formation in liposomes without carotenoids was the control. Significant differences were not found between means within a column, $P > 0.05$, from ANOVA analyses.

they mimic biological membranes more closely than multilamellar liposomes. We used the lowest levels of iron and AAPH to oxidize the lipid that were possible while still achieving significant peroxidation. The levels of oxidative stress in vivo would most likely result from low levels of free radicals. It also was important to oxidize the carotenoids partially. If complete depletion occurred, it would be more difficult to ascribe antioxidant or prooxidant effects to the parent carotenoids. These reactions were then examined under two reduced oxygen tensions as well as ambient oxygen tension, since it has been reported that the antioxidant activity of β-carotene varies with oxygen tension [17].

Under all the conditions used in this study, carotenoids were partially depleted, but protection from lipid peroxidation was largely not evident (Tables 3–5). Using higher concentrations of carotenoids was not possible due to solubility problems, especially with lycopene. In studies where protection from lipid peroxidation was shown, multilamellar liposomes made by sonication were used [7,8]. Since carotenoids incorporated into liposomes could change membrane microviscosity, hydrophobicity, permeability to ions and diffusion of oxygen [18], carotenoids in the structure of multilamellar liposomes made by sonication could conceivably decrease accessibility of lipid to free radicals. In unilamellar liposomes, lipid is much more accessible to the aqueous medium. The lack of protection from lipid oxidation by carotenoids while they themselves are being oxidized is consistent with the hypothesis that the anticancer effects of carotenoids may be independent of their antioxidant properties [19,20]. Two other studies have also shown that β-carotene is not a good inhibitor of lipid peroxidation [5,9].

It is also important to determine which of the major carotenoids found in human plasma are most easily oxidized, since these data suggest that oxidized carotenoids may be very sensitive markers of oxidative stress. Many oxidized carotenoids are found in vivo [21]. Our studies allow for the comparison of the relative susceptibility of different carotenoids to oxidation at physiological pH. The data in Table 2 indicate that β-carotene and lycopene are more susceptible to oxidation than the other carotenoids tested. These results in unilamellar liposomes are consistent with the relative ease of oxidation of carotenoids in organic solvent as reported by Siems et al. and Woodall et al. [22,23], as well as with the ease of oxidation predicted by their one-electron reduction potentials [24]. It therefore may be of interest to examine oxidation products of these two carotenoids as sensitive markers of oxidative stress, in addition to their other potential biological properties.

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