Carotenoid mixtures protect multilamellar liposomes against oxidative damage: synergistic effects of lycopene and lutein

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Abstract Antioxidant activity of carotenoids in multilamellar liposomes assayed by inhibition of formation of thiobarbituric acid-reactive substances was in the ranking: lycopene > α-tocopherol > α-carotene > β-cryptoxanthin > zeaxanthin = β-carotene > lutein. Mixtures of carotenoids were more effective than the single compounds. This synergistic effect was most pronounced when lycopene or lutein was present. The superior protection of mixtures may be related to specific positioning of different carotenoids in membranes.

Key words: α-Tocopherol; Lipid peroxidation; Peroxyl radical; 2,2′-Azo-bis(2,4-dimethylvaleronitrile); Antioxidant activity

1. Introduction

Several lines of evidence indicate that carotenoids and tocopherols contribute to the beneficial health effects associated with an increased consumption of fruits and vegetables. It has been suggested that these effects are at least in part related to their antioxidant activity [1–4]. Carotenoids and α-tocopherol trap various types of organic radicals. An interaction between β-carotene and the trichloromethylperoxyl radical was described [5], producing the carotenoid radical cation and a radical adduct [6]. The radical adduct is less stable than the radical cation and decays to the cation with a rate constant of 1.8×10^4 s^{-1} [6]. Other free radicals such as nitrogen dioxide, thiyl, and sulfonyl radicals are also rapidly scavenged by β-carotene [7]. Carotenoids react with the radical cation $ABTS^{\bullet+}$ derived from ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) [8]. This scavenging ability becomes more pronounced with increasing polarity and number of conjugated double bonds [9].

Carotenoids efficiently inhibit lipid peroxidation at low oxygen tension, and an interaction of β-carotene with the lipid peroxyl radical leading to the formation of a radical adduct was postulated [10]. It has been demonstrated that β-carotene incorporated into liposomes prevents them from being oxidized [11].

The reaction of α-tocopherol with a peroxyl radical leads to a relatively stable α-tocopheroxyl radical [12] which is less effective in chain initiation than primary radicals [13]. The α-tocopheroxyl radical can be reduced to α-tocopherol by vitamin C, bile pigments or thiols [13–15]. The protective effect of exogenous α-tocopherol is considerably less than endogenous α-tocopherol in biological membranes due to cooperative interaction with other endogenous antioxidants [9,16].

Little is known so far about the antioxidant efficiency and cooperative interactions of different carotenoids and mixtures of carotenoids with vitamin E. The present study was carried out in a simple model system to compare carotenoids of different chemical structure, α-tocopherol, and their mixtures regarding their capacity to inhibit lipid peroxidation.

2. Materials and methods

2.1. Chemicals

Carotenoids were a kind gift from Hoffmann-La Roche (Basel, Switzerland); purity was >98% except for zeaxanthin (90%). α-Tocopherol, phosphatidylcholine from egg yolk, 2-thiobarbituric acid (TBA), and butylated hydroxytoluene (BHT) were obtained from Sigma (Deisenhofen, Germany). 2,2′-Azo-bis(2,4-dimethylvaleronitrile) (AMVN) was from Wako (Osaka, Japan). All other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Mixtures

Table 1 shows the composition of the mixtures examined. Mixture M1 contains the major carotenoids found in human blood and tissues. All other mixtures are either modifications of M1 or two-component mixtures to test for the activity of specific compounds. M6 was used to investigate the interaction between carotenoid mixture M1 and α-tocopherol.

2.3. Preparation of liposomes

Carotenoids, α-tocopherol or mixtures were dissolved together with phosphatidylcholine in chloroform. The solvent was evaporated under a stream of nitrogen. Multilamellar liposomes containing the respective compounds were prepared by addition of 100 mM phosphate buffer (pH 7.4) to the lipid film followed by sonication for 5 min. The final concentration of liposomes was 1 mg/ml. Lipid peroxidation was initiated by 10 mM AMVN dissolved in tetrahydrofuran; final concentration of tetrahydrofuran in the suspension was 2%. Reactions were carried out in the dark at 37°C for 2 h at constant rate of thermodecomposition of the azo-initiator at ambient oxygen tension [17]. There was a loss of carotenoids during the incubation, but no complete decoloration was observed within the 2 h experimental period.

2.4. Measurement of thiobarbituric acid-reactive substances (TBARS)

After 2 h of incubation, TBARS were measured following a published method [18] with minor modifications. 0.1% BHT was added to the samples before boiling to prevent formation of further TBA-reactive products. Malondialdehyde equivalents were calculated using the extinction coefficient $\varepsilon_{532}=1.56\times10^5$ M^{-1} cm^{-1}. Absorbance at 532 nm was calculated from spectra using the formula: $\Delta A_{532}=A_{532}-1/2(A_{515}+A_{550})$. TBARS formation without addition of compounds was 2.1 nmol MDA equivalents per mg of phospholipid with 10 mM of AMVN. The presence of carotenoids did not affect the assay itself as demonstrated by control experiments including a reagent blank together with the carotenoids.

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Table 1
Contribution of single compounds to carotenoid mixtures (μM)

Code	Lycopene	α-Carotene	β-Carotene	β-Cryptoxanthin	Zeaxanthin	Lutein	α-Tocopherol
M1	0.66	0.33	0.66	0.33	0.33	0.66	–
M2	–	0.43	0.86	0.43	0.43	0.86	–
M3	0.86	0.43	0.86	0.43	0.43	–	–
M4	–	0.60	1.20	0.60	0.60	–	–
M5	1.20	0.60	–	0.60	0.60	–	–
M6	0.07	0.03	0.07	0.03	0.03	0.07	2.70
M7	1.50	–	1.50	–	–	–	–
M8	2.00	1.00	–	–	–	–	–
M9	2.00	–	–	1.00	–	–	–
M10	1.50	–	–	–	–	1.50	–
M11	2.00	–	–	–	1.00	–	–
M12	–	–	1.00	–	–	2.00	–
M13	–	1.00	2.00	–	–	–	–

Final concentration in each mixture is 3 μM.

2.5. Calculated values

For the mixtures M1–M13 a theoretical additive value was calculated. The measured TBARS values obtained with the single components (see Table 2) were normalized for their molar contribution, summed up and divided by the total molarity of the mixture:

$$\text{Calculated value} = \frac{\Sigma\ (\text{exp. value}[\%] \times \text{molarity}\ [\mu\text{M}])}{3\ [\mu\text{M}]}$$

Thus, the 'calculated value' for TBARS in Tables 3 and 4 gives the TBARS level expected for simply additive effects of the components. A synergistic effect is shown by a positive value in the column 'difference', obtained by comparison with the measured value.

3. Results and discussion

The composition of the mixtures and the concentration of single components in the mixtures are shown in Table 1. The molarity of each mixture was 3 μM total. Mixture M1 contains the major carotenoids detectable in human serum and tissues; the concentrations of carotenoids are in the upper range of the levels found in human plasma.

3.1. Single compounds

The effects of single carotenoids and α-tocopherol on lipid peroxidation induced by AMVN (10 mM) are summarized in Table 2. Lycopene was the most efficient carotenoid, diminishing TBARS formation to about 25% of control; other carotenoids such as β-carotene, zeaxanthin or lutein were less active. Under the present conditions inhibitory effects were observed in the order: lycopene > α-tocopherol > α-carotene > β-cryptoxanthin > zeaxanthin = β-carotene > lutein. The lipid-soluble antioxidant α-tocopherol is among the more efficient inhibitors of AMVN-induced lipid peroxidation. In a similar liposomal system, several carotenoids and α-tocopherol have been used to prevent the formation of phospholipid hydroperoxides upon incubation with AMVN [19]; there α-tocopherol was found to be superior to all carotenoids investigated. In contrast to the present study, a different ranking of carotenoids was found with zeaxanthin > cryptoxanthin > β-carotene > lycopene. However, the endpoints investigated in both studies were different, and the carotenoid levels were higher (50 μM in [19]) than in the present study (3 μM).

The interaction of carotenoids with the stable radical cation of $ABTS^{\bullet+}$ has been studied to investigate the antioxidant effects of carotenoids [8,20]. Here also, lycopene was shown to be the most powerful antioxidant followed by cryptoxanthin and β-carotene; zeaxanthin, lutein and α-carotene were less efficient but still superior to α-tocopherol. Lycopene is more active than β-carotene in protecting cellular membranes from damage induced by the nitrogen dioxide radical [21]. No significant differences were found in the reaction of β-carotene, zeaxanthin, and lutein with the trichloromethyl peroxyl radical. Studies on the interaction of carotenoids with nitrogen dioxide, thiyl and sulfonyl radicals suggest that the rate of scavenging as well as the scavenging mechanism strongly depends on the nature of the oxidizing species [22]. Several radical species including AMVN-derived peroxyl radicals or lipid peroxyl radicals may be formed under the conditions of the present study. Thus, scavenging of specific radicals by carotenoids can not be assigned.

3.2. Mixtures

The data obtained for mixtures of carotenoids are shown in Tables 3 and 4. M1 contains all the carotenoids tested as single components at a final concentration of 3 μM (sum of all carotenoids). Lipid peroxidation in this mixture was 37% that of control. Based on the data obtained for the single components as shown in Table 2 and normalized for their molar contribution to M1, the theoretical value was calculated to be 59%. Thus, the expected additive inhibition of lipid peroxidation was exceeded by 22%, i.e. the mixture was synergistic with respect to the inhibition of TBARS formation. When lycopene was omitted from the mixture (M2) and other carotenoids were increased to yield a final concentration of 3 μM, the difference between the calculated additive value (68%) and the measured value (34%) was 34%. Such a synergistic effect was also observed when lutein was withdrawn from M1

Table 2
TBARS formation in multilamellar liposomes in the presence of single antioxidants (3 μM)

Compound	Measured TBARS (%)	*n*
Control[a]	100 ± 9	35
Lycopene	25 ± 10	17
α-Carotene	51 ± 8	33
β-Carotene	73 ± 9	30
β-Cryptoxanthin	55 ± 8	30
Zeaxanthin	73 ± 9	23
Lutein	77 ± 9	14
α-Tocopherol	43 ± 7	5

Lipid peroxidation was induced by 10 mM AMVN. Data are given as % of control.
[a]Control: 100% refers to 2.1 ± 0.2 nmol TBARS/mg phospholipid.

Table 3
TBARS formation in multilamellar liposomes in the presence of antioxidant mixtures (3 μM)

Code	Mixture	Measured TBARS (%)	Calculated TBARS (%)[a]	Difference[b]	*n*
M1	Complete carotenoid mixture	37 ± 7	59	22	8
M2	M1 minus lycopene	34 ± 14	68	34	6
M3	M1 minus lutein	43 ± 11	54	11	5
M4	M1 minus lycopene minuslLutein	58 ± 10	65	7	7
M5	M1 minus lutein minus β-carotene	27 ± 18	46	19	5
M6	M1 plus α-tocopherol	24 ± 9	45	21	6

Lipid peroxidation was induced by 10 mM AMVN. Data are given as % of control (see Table 2).
[a]Calculated from data for single components; see Table 2.
[b]Calculated minus measured value.

to yield M3. The additive value in M3 was calculated with 54% while 43% were measured.

When both lycopene and lutein were omitted from the mixture (M4) the inhibitory effect of the carotenoid mixture on the inhibition of lipid peroxidation was just additive, 58 ± 10% inhibition vs. 65% calculated. The data demonstrate that lutein and lycopene are responsible for synergistic antioxidant effects observed with a mixture of carotenoids. This is also obvious from the data obtained for M5. β-Carotene and the other carotenoids tested here have only limited synergistic effects. For the mixture of β-carotene and α-carotene (M13) an additive value of 66% was calculated which is close to the measured inhibition of 60%.

Synergism for lycopene and lutein was also found in two-component mixtures containing one of these compounds together with only one other carotenoid (M7, M8, M9, M11, M12). The most efficient mixture used in the present study consisted of lycopene and lutein (M10). For additive effects, a value of 51% was calculated which is significantly different from the measured value of 16 ± 8%.

The data obtained in this simple in vitro system show that combinations of carotenoids are more effective than single compounds in preventing oxidative damage, i.e. they are synergistic. These effects might be related to different physicochemical properties and/or the location in the biomembranes. Xanthophylls such as zeaxanthin or violaxanthin span the membrane, with the polar endgroups anchored at polar sites of the membrane [23,24]. β-Carotene and lycopene, lacking hydrophilic substituents, remain entirely within the inner part of the membrane and retain a substantial degree of mobility [25]. Therefore, specific orientations of carotenoids might provide shielding effects that account for synergistic properties.

Synergistic antioxidant efficacy was also found when M1 was modified with α-tocopherol. In comparison to the calculated value of 45% an inhibition of 24 ± 9% was found experimentally (M6). Several studies revealed interactions between β-carotene and α-tocopherol in preventing radical initiated reactions [15,26]. These data support the results obtained in the present study on interactions of carotenoid mixtures and vitamin E. As in the case of carotenoid mixtures, suppression of lipid peroxidation by various carotenoids and α-tocopherol may be influenced by the site and the rate of radical production [9,16]. Thus a different compartmentation of the lipophilic antioxidants may be important.

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Table 4
TBARS formation in multilamellar liposomes in the presence of two-component carotenoid mixtures (3 μM)

Code	Mixture	Measured TBARS (%)	Calculated TBARS (%)[a]	Difference[b]	*n*
M7	Lycopene plus β-carotene	31 ± 10	49	18	5
M8	Lycopene plus α-carotene	21 ± 4	34	13	5
M9	Lycopene plus β-cryptoxanthin	25 ± 8	35	10	3
M10	Lycopene plus lutein	16 ± 8	51	35	4
M11	Lycopene plus zeaxanthin	22 ± 7	41	19	4
M12	Lutein plus β-carotene	44 ± 8	76	32	9
M13	β-Carotene plus α-carotene	60 ± 11	66	6	6

Lipid peroxidation was induced by 10 mM AMVN. Data are given as % of control (see Table 2).
[a]Calculated from data for single components; see Table 2.
[b]Calculated minus measured value.

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