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β-Carotene: interactions with α-tocopherol and ascorbic acid in microsomal lipid peroxidation

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Abstract

β-Carotene, α-tocopherol, and ascorbic acid were tested for their ability to inhibit, enhance, or react synergistically with O_2 (15, 150, 760 torr) and, 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH) or 1,1′-azobis (cyclohexane-carbonitrile) (ACCN) in isolated rat liver microsomes. β-Carotene did not protect against lipid peroxidation, i.e., malondialdehyde (MDA) formation, in microsomal samples incubated at 37°C with aqueous soluble AAPH at all added β-carotene concentrations and oxygen tensions. More MDA (16%, $p < 0.001$) was produced at 15 torr of O_2, and 160 nmol/mg protein of β-carotene compared to respective vehicle control. Individually, α-tocopherol and ascorbic acid exhibited antioxidant protection (ascorbic acid ≫ α-tocopherol); however, a mixture of both compounds was no more protective than ascorbic acid alone. β-Carotene demonstrated a concentration-dependent antioxidant affect at 15 torr O_2 ($p < 0.01$); but a prooxidant effect at higher O_2 at 150 and 760 torr (>57%, $p < 0.001$) by lipid-soluble ACCN. α-Tocopherol exhibited concentration-dependent inhibitory effects on microsomal MDA formation at all oxygen tensions, but was most effective under 150 torr. Ascorbic acid demonstrated a concentration-dependent antioxidant effect only at 150 torr. ACCN-induced lipid peroxidation was no greater for the combination of the three compounds than ascorbic acid added alone. Thus, antioxidant or prooxidant activities for β-carotene, α-tocopherol, and ascorbic acid in microsomal suspensions are related to O_2 tension, solubility, antioxidant concentrations and are governed by complex interactions. Differences between AAPH- and ACCN-induced lipid peroxidation are related to differences in lipid solubility.

Keywords: Microsomes; Lipid peroxidation; β-carotene; α-tocopherol; Ascorbic acid; 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH); 1,1′-azobis (cyclohexane-carbonitrile) (ACCN)

1. Introduction

β-Carotene, α-tocopherol, and ascorbic acid act as potent antioxidants in protecting biological membranes or lipids against free radical damage. A direct scavenging activity of β-carotene with chemical species, such as singlet oxygen, triplet photochemical sensitizers, and free radicals, has been demonstrated both in vivo and in vitro [1–3]. However, the antioxidant affect of β-carotene is conditional. β-Carotene is effective as an antioxidant only at low oxygen partial pressure, which is often found in tissues under normal physiological conditions [4–6]. Some epidemiological studies have shown that β-carotene not only had little affect in preventing lung cancers, but may have increased the incidence of lung cancer [7–9] which may have been related to prooxidant action.

Unlike β-carotene, the antioxidant activity of α-tocopherol is decreased at low pO_2 [10,11]. α-Tocopheroxyl radicals, generated when α-tocopherol scavenges a free radical, react efficiently with vitamin C near the membrane surface to regenerate α-tocopherol for further scavenging [12,13]. Although such a mechanism has been reported by several investigators involving homogeneous solution [14], and in micellar [15] and liposomal suspensions [16,17], it is still unclear whether this mechanism operates in vivo because we know little about the precise location of α-tocopherol and ascorbic acid in cellular membranes. Therefore, antioxidant affects of β-carotene and α-tocopherol are affected by oxygen partial pressure and interaction among the three antioxidant compounds.

Our objective was to study the antioxidant efficiency of β-carotene, α-tocopherol, and ascorbic acid in preventing lipid peroxidation induced by various sources of radicals in rat liver microsomes and examine the relationships between oxygen tension and concentrations of these three compounds. The prooxidants used in this study were azocom-

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pounds: water-soluble 2,2′-azobis (2-amidinopropane) (AAPH) and lipid-soluble 1,1′-azobis (cyclohexane-carbonitrile) (ACCN), both capable of generating peroxyl radicals at constant and measurable rates [14]. However, because of the different lipid solubility characteristics of both compounds, will promote different lipid peroxidation outcomes.

2. Materials and methods

2.1. Chemicals

β-Carotene, α-tocopherol (vitamin E), ascorbic acid (vitamin C), thiobarbituric acid (TBA) and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Company (St. Louis, MO). 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) and 1,1′-azobis (cyclohexane-carbonitrile) (ACCN) were purchased from Wako Chemicals (Richmond, VA). Tetrahydrofuran (THF) and trichloroacetic acid (TCA) were purchased from Fisher Scientific Co. (Fairlawn, NJ). Stock solutions of β-carotene, α-tocopherol, and ascorbic acid were freshly prepared as needed.

2.2. Microsome preparations

Liver microsomes were prepared from Sprague-Dawley rats by tissue homogenization with 5 volumes of ice-cold 0.25 M sucrose containing 5 mM Hepes, 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5, in a Potter-Elvehjem homogenizer. Microsomal vesicles were isolated by removal of the nuclear fraction at 800 *g* for 10 min and removal of the mitochondrial fraction at 18,000 *g* for 10 min, washed once in 0.15 M KCl, and centrifuged again at 105,000 *g* for 30 min (Beckman-Spinco untracentrifuge, using type 50 Ti rotor). The membranes, suspended in 0.1 M potassium phosphate buffer, pH 7.5, were stored at –70°C until use. Microsomal proteins were determined by BCA Protein Assay adapted for microtiter plates using bovine serum albumin as standards (Pierce, Rockfor, IL).

2.3. Incorporation of β-carotene, α-tocopherol and ascorbic acid into microsomal solution

β-Carotene and α-tocopherol were dissolved in THF containing 0.125% BHT. Ascorbic acid was dissolved in distilled water. Compounds were added to microsomal suspensions and gently homogenized at 0°C until all the compounds were homogeneously dispersed. The final concentration of β-carotene, α-tocopherol and ascorbic acid were determined by HPLC and spectrophotometrically [18–20]. The final concentration of THF in the solution was ≤0.5%, which had no effect on lipid peroxidation.

Rat liver microsomes were mixed with β-carotene, α-tocopherol, ascorbic acid, and mixture of these three compounds. β-Carotene concentrations were: 10, 20, 40, 80, and 160 nmol/mg protein, respectively. α-Tocopherol concentrations were: 2.5, 5, 10, 20, and 40 nmol/mg protein. Ascorbic acid concentrations were: 20, 40, 80, 160, and 320 nmol/mg protein. Concentrations of mixtures (Table 1) varied from: 1) 10, 2.5 and 20 nmol/mg protein of β-carotene, α-tocopherol, and ascorbic acid, respectively; 2) 20, 5 and 40 nmol/mg protein of β-carotene, α-tocopherol, and ascorbic acid, respectively; 3) 40, 10 and 80 nmol/mg protein of β-carotene, α-tocopherol, and ascorbic acid, respectively; 4) 80, 20, and 160 nmol/mg protein of β-carotene, α-tocopherol, and ascorbic acid, respectively; to 5) 160, 40, and 320 nmol/mg protein of β-carotene, α-tocopherol, and ascorbic acid, respectively. Controls were mixed with solvents only. Oxidation was induced by incubation with 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) or 1,1′azobis (cyclohexane-carbonitrile) (ACCN) at 37°C in the dark for 1 hr.

Table 1
Concentrations of β-carotene, α-tocopherol, and ascorbic acid in each antioxidant mixture

Mixture no.	β-Carotene (nmol/mg protein)	α-Tocopherol (nmol/mg protein)	Ascorbic acid (nmol/mg protein)
1	10	2.5	20
2	20	5	40
3	40	10	80
4	80	20	160
5	160	40	320

2.4. Oxygen tensions

To mimic 15 torr O_2 tension, microsomal suspensions were injected and maintained in sealed Vacutainer tubes [21]. To mimic 150 torr O_2 tension, micrsomal suspensions were maintained at atmospheric conditions. To mimic 760 torr O_2 tension, microsomal suspensions were maintained with 100% O_2 gases and sealed in Fisherbrand centrifuge tubes with Parafilm M film and plug seal caps [21].

2.5. Lipid peroxidation:

Lipid peroxidation was induced by the addition of 25 mM (final concentration) 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) or 1,1′-azobis (cyclohexane-carbonitrile) (ACCN) to microsomal suspensions. The reaction mixtures were incubated in the dark at 37°C for 60 min shaking every 10 min. The samples were mixed with 1 ml of stock reagent for malondialdehyde (MDA) measurement and heated for 15 min in a boiling water bath. Stock reagent was prepared by mixing 15% w/v TCA, 0.375% w/v 2-thiobarbituric acid, 0.25 N HCl, and 0.02% w/v butylated hydroxytoluene, BHT. After cooling, the precipitate was removed by centrifugation at 600 *g* for 10 min. The amount of BHT used should completely prevent any TBARS formation due to nonspecific chromophore compounds and to the decomposition of AAPH and ACCN during the boiling. Blanks contained all components of MDA reagents except lipid samples. The MDA formation of the samples

Table 2
Effects of antioxidants on 2,2′-azobis (2-amidinopropane) dihydrochloride induced microsomal lipid oxidation

β-Carotene (nmol/mg protein)	MDA (μM)‡	α-Tocopherol (nmol/mg protein)	MDA (μM)‡	Ascorbic acid (nmol/mg protein)	MDA (μM)‡	Mixture no. (nmol/mg protein)	MDA (μM)‡
			15 torr oxygen tension				
0	1.38 ± 0.04	0	1.38 ± 0.17	0	1.52 ± 0.16	0	1.49 ± 0.20
0 (THF)	1.62 ± 0.04	0 (THF)	1.62 ± 0.02	0 (THF)	1.40 ± 0.03	0 (THF)	1.74 ± 0.04
10	1.75 ± 0.03***	2.5	1.64 ± 0.03	20	1.31 ± 0.12	1	1.44 ± 0.03***
20	1.74 ± 0.09*	5	1.49 ± 0.08**	40	1.29 ± 0.01***	2	1.25 ± 0.04***
40	1.56 ± 0.13	10	1.31 ± 0.15**	80	1.20 ± 0.04***	3	0.99 ± 0.04***
80	1.70 ± 0.22	20	1.35 ± 0.02***	160	0.67 ± 0.15***	4	0.21 ± 0.02***
160	1.88 ± 0.04***	40	1.29 ± 0.03***	320	0.01 ± 0.03***	5	0.04 ± 0.01***
			150 torr oxygen tension				
0	1.64 ± 0.08	0	1.66 ± 0.12	0	1.65 ± 0.06	0	1.56 ± 0.13
0 (THF)	2.65 ± 0.13	0 (THF)	2.16 ± 0.05	0 (THF)	1.64 ± 0.08	0 (THF)	2.62 ± 0.04
10	2.67 ± 0.05	2.5	2.06 ± 0.11	20	1.53 ± 0.04*	1	2.28 ± 0.09***
20	2.78 ± 0.15	5	1.97 ± 0.06***	40	1.39 ± 0.02***	2	1.95 ± 0.08***
40	2.78 ± 0.02	10	1.90 ± 0.04***	80	0.75 ± 0.06***	3	0.85 ± 0.08***
80	2.72 ± 0.12	20	1.79 ± 0.12***	160	0.0 ± 0.0***	4	0.0 ± 0.0***
160	2.71 ± 0.15	40	1.71 ± 0.08***	320	0.0 ± 0.0***	5	0.0 ± 0.0***
			760 torr oxygen tension				
0	5.17 ± 0.13	0	4.94 ± 0.17	0	5.05 ± 0.35	0	5.15 ± 0.22
0 (THF)	6.34 ± 0.37	0 (THF)	5.61 ± 0.37	0 (THF)	5.05 ± 0.21	0 (THF)	5.83 ± 0.13
10	6.55 ± 0.42	2.5	5.51 ± 0.51	20	4.37 ± 0.08***	1	5.58 ± 0.15*
20	6.47 ± 0.13	5	5.51 ± 0.22	40	3.85 ± 0.18***	2	5.32 ± 0.29**
40	7.05 ± 0.33	10	5.19 ± 0.10	80	3.82 ± 0.39***	3	5.13 ± 0.33**
80	6.67 ± 0.23	20	5.41 ± 0.27	160	2.23 ± 0.16***	4	2.88 ± 0.25***
160	6.24 ± 0.29	40	4.42 ± 0.22***	320	0.0 ± 0.0***	5	0.0 ± 0.0***

† Concentrations of antioxidants in the mixtures are described in Table 1.
‡ Malondialdehyde (MA); tetrahydrofuran (THF). Data represents mean ± S.D. (triplicate). Values significantly different from vehicle are labeled with * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

was quantitated by using $\epsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and absorbency value of 533 nm.

2.6. Data analysis and statistics

Values were expressed as mean ± SD. Statistical comparisons between different treatment groups were made by two-way analysis of variance (ANOVA) with significance set at $p < 0.05$.

3. Results

3.1. AAPH-induced lipid peroxidation

3.1.1. 15 torr of oxygen tension

Table 2 documents the effect of β-carotene, added to rat liver microsomal membranes treated with 25 mM AAPH, on lipid peroxidation, i.e., MDA formation. Addition of 10 nmol/mg protein β-carotene to rat liver microsomes treated with AAPH did not provide protection but increased MDA formation by 8% ($p < 0.001$). Addition of 160 nmol/mg protein β-carotene resulted in 16% increased MDA compared to respective solvent control.

However, α-tocopherol provided significant protection against AAPH-induced MDA formation at 15 torr. Addition of 5 nmol/mg protein α-tocopherol resulted in 8% decreased MDA formation ($p < 0.01$). Adding higher concentrations of 10, 20, and 40 nmol/mg protein, α-tocopherol provided 19.1, 16.7, and 20.3% protection, respectively.

Ascorbic acid exhibited a very strong concentration-response effect in protecting against AAPH-induced MDA formation. Ascorbic acid reduced MDA formation by 7.9, 14.3, 52.1 and 99.3% at concentrations of 40, 80, 160, and 320 nmol/mg protein respectively ($p < 0.001$).

We also investigated the affects of the mixture of these three compounds on AAPH-induced MDA formation at 15 torr of oxygen tension. The concentrations for each compound were as mentioned above for the mixture. Protection against MDA production was significant even with mixture group 1 (17.2%, $p < 0.001$, i.e., the lowest concentration combination of the three compounds. Protection was also concentration-dependent with 97.7% protection for mixture group 5.

3.1.2. 150 torr of oxygen tension

An antioxidant affect for β-carotene was not demonstrated. Higher oxygen tension resulted in increased but not significant MDA formation (Table 2).

Adding α-tocopherol to microsomal suspensions under

Table 3
Effects of antioxidants on 1,1′-azobis (cyclohexane carbonitrile) induced microsomal lipid oxidation

β-Carotene (nmol/mg protein)	MDA (μM)‡	α-Tocopherol (nmol/mg protein)	MDA (μM)‡	Ascorbic acid (nmol/mg protein)	MDA (μM)‡	Mixture no. (nmol/mg protein)	MDA (μM)‡
			15 torr oxygen tension				
0	0.51 ± 0.05	0	0.58 ± 0.03	0	0.54 ± 0.07	0	0.55 ± 0.04
0 (THF)	0.46 ± 0.02	0 (THF)	0.57 ± 0.03	0 (THF)	0.54 ± 0.06	0 (THF)	0.60 ± 0.04
10	0.39 ± 0.02***	2.5	0.58 ± 0.02	20	0.56 ± 0.04	1	0.47 ± 0.02***
20	0.38 ± 0.03**	5	0.52 ± 0.03*	40	0.49 ± 0.08	2	0.53 ± 0.07
40	0.37 ± 0.02***	10	0.45 ± 0.05**	80	0.53 ± 0.05	3	0.46 ± 0.05**
80	0.33 ± 0.07**	20	0.37 ± 0.06***	160	0.53 ± 0.07	4	0.42 ± 0.04***
160	0.22 ± 0.05***	40	0.38 ± 0.04***	320	0.51 ± 0.04	5	0.54 ± 0.03*
			150 torr oxygen tension				
0	0.42 ± 0.02	0	0.45 ± 0.05	0	0.40 ± 0.04	0	0.37 ± 0.05
0 (THF)	0.43 ± 0.02	0 (THF)	0.38 ± 0.03	0 (THF)	0.36 ± 0.08	0 (THF)	0.49 ± 0.04
10	0.53 ± 0.06**	2.5	0.24 ± 0.01***	20	0.07 ± 0.03***	1	0.03 ± 0.02***
20	0.53 ± 0.03***	5	0.17 ± 0.03***	40	0.0 ± 0.0***	2	0.0 ± 0.0***
40	0.59 ± 0.02***	10	0.15 ± 0.02***	80	0.0 ± 0.0***	3	0.0 ± 0.0***
80	0.58 ± 0.06***	20	0.13 ± 0.03***	160	0.0 ± 0.0***	4	0.0 ± 0.0***
160	0.68 ± 0.02***	40	0.06 ± 0.01***	320	0.0 ± 0.0***	5	0.0 ± 0.0***
			760 torr oxygen tension				
0	0.87 ± 0.14	0	0.83 ± 0.06	0	0.85 ± 0.06	0	0.81 ± 0.05
0 (THF)	0.85 ± 0.04	0 (THF)	0.77 ± 0.04	0 (THF)	0.97 ± 0.12	0 (THF)	1.01 ± 0.04
10	0.78 ± 0.06*	2.5	0.75 ± 0.09	20	0.62 ± 0.03***	1	0.58 ± 0.06***
20	0.80 ± 0.05	5	0.68 ± 0.02**	40	0.69 ± 0.03***	2	0.63 ± 0.05***
40	0.97 ± 0.03***	10	0.73 ± 0.01	80	0.59 ± 0.04***	3	0.59 ± 0.10***
80	1.08 ± 0.19***	20	0.78 ± 0.08	160	0.63 ± 0.06***	4	0.59 ± 0.02***
160	1.34 ± 0.06***	40	0.56 ± 0.06***	320	0.71 ± 0.06**	5	0.62 ± 0.07***

† Concentrations of antioxidants in the mixtures are described in Table 1.
‡ Malondialdehyde (MA); tetrahydrofuran (THF). Data represents mean ± S.D. (triplicate). Values significantly different from vehicle are labeled with * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

150 torr resulted in concentration-dependent antioxidant effects, reducing MDA formation by 8.8, 12.0, 17.1 and 20.8% at concentrations of 5, 10, 20 and 40 nmol/mg protein, respectively.

Ascorbic acid also protected microsomes from oxidation at 20 nmol/mg protein. At concentrations of 160 nmol/mg protein and higher, ascorbic acid completely eliminated MDA formation.

The mixture of these three compounds showed an enhanced protection of microsomes from lipid peroxidation. MDA was reduced, 13.0, 25.6, 67.6% at mixture groups 1, 2, and 3, respectively ($p < 0.001$). Adding mixture groups 4 and 5 to the microsomal suspension completely inhibited MDA formation.

3.1.3. 760 torr of oxygen tension

Table 2 also documents the effect of adding β-carotene to the microsomal suspension. We observed increased MDA formation at most of the concentrations tested (exception was β-carotene added at 160 nmol/mg protein). However, the increase of MDA formation was only significant with the addition of 40 nmol/mg protein of β-carotene.

The antioxidant effect was attenuated when α-tocopherol was added alone to the microsomes at 760 torr of oxygen tension. Adding α-tocopherol resulted in protecting microsomal suspensions from oxidation at 40 nmol/mg protein by reducing MDA formation 21.2% ($p < 0.001$).

The protective effect of ascorbic acid on microsomal suspension lipid peroxidation was not affected by the high oxygen tension. Ascorbic acid provided concentration-dependent antioxidant protection against AAPH-induced lipid peroxidation. At concentrations as low as 20 nmol/mg protein, ascorbic acid reduced MDA formation by 13.5% ($p < 0.001$). At the highest concentration of 320 nmol/mg protein, ascorbic acid inhibited MDA formation by 100% ($p < 0.001$).

Adding a mixture of these three compounds results in a concentration-dependent antioxidant effect. The mixture was no more protective than that found for ascorbic acid; however, was more protective than that found for either β-carotene or α-tocopherol.

3.2. ACCN-induced lipid peroxidation

3.2.1. 15 torr of oxygen tension

Lipid soluble prooxidant ACCN and β-carotene added to microsomal suspensions resulted in a concentration-dependent antioxidant effect. β-Carotene reduced MDA formation by 14, 16.9, 18.3, 26.8, and 50.7% at concentrations of

10, 20, 40, 80, and 160 nmol/mg protein, respectively (Table 3).

α-Tocopherol exhibited an antioxidant effect, but this effect did not appear to be concentration-dependent. Reductions of MDA formation of 9, 21.1, 35.1, and 33.3% was observed at concentrations of 5, 10, 20, and 40 nmol/mg protein of α-tocopherol respectively.

Unlike what we observed for AAPH-induced lipid peroxidation, ascorbic acid did not provide any antioxidant protection, or promotion of lipid peroxidation at this oxygen tension.

For the mixture of these three compounds, the best protection was observed with mixture 4, 30% reduction of MDA formation ($p < 0.001$). Other mixture groups provided various levels protection and were not concentration-dependent.

3.2.2. 150 torr of oxygen tension

Adding β-carotene to microsomal suspensions resulted in a prooxidant effect at 150 torr. Adding β-carotene increased MDA formation by 23.3, 23.3, 37.2, 34.9, and 58.1% at concentrations of 10, 20, 40, 80, and 160 nmol/mg protein of β-carotene respectively.

In contrast, α-tocopherol exhibited concentration-dependent antioxidant protection against ACCN-induced lipid peroxidation. Maximum protection was observed at 40 nmol/mg protein of α-tocopherol with 84.2% reduction of MDA formation.

Adding ascorbic acid to microsomal suspension resulted in better protection compared to adding α-tocopherol. At the lowest concentration of added ascorbic acid, 20 nmol/mg protein, MDA was reduced by 80.6%. Furthermore protection was observed beyond 20 nmol/mg protein with completely diminished MDA.

Adding the mixture to microsomal suspension resulted a similar pattern of protection to that observed for ascorbic acid. Adding mixture group 1 resulted in a 93.9% decrease of MDA compared to respective solvent control ($p < 0.001$). Other mixture groups provided 100% protection.

3.2.4. 760 torr of oxygen tension

Table 3 also documents the effect of adding β-carotene to microsomal suspension with ACCN-induced lipid peroxidation. At low β-carotene concentrations (<40 nmol/mg protein), β-carotene exhibited antioxidant effects with a 6% reduction of MDA at 20 nmol/mg protein (non significant, $p = 0.09$). This antioxidant effect was replaced with a prooxidant effect at 40 nmol/mg protein, with 13.5% more MDA production. Maximum MDA promotion was observed at 160 nmol/mg protein β-carotene with 57% increase of MDA formation.

High oxygen tension resulted in a significant impact regarding the effect of α-tocopherol on lipid peroxidation. An antioxidant effect was only evident at a concentration of 40 nmol/mg protein with 27.3% reduction of MDA formation. Other concentrations of α-tocopherol demonstrated neither significant protection nor promotion of lipid peroxidation.

Ascorbic acid exhibited an antioxidant effect at all concentrations. However, this effect was not concentration-dependent. The largest antioxidant effect was found at concentration of 80 nmol/mg protein with 39.2% reduction of MDA formation, while the least protection was observed at the highest concentration, 320 nmol/mg protein, with 26.8% protection.

Adding a mixture of these three compounds demonstrated more protection than any single compound. However, such effects were not concentration-dependent within the concentration tested. MDA formation was decreased 42.4, 37.6, 41.6, 41.6, and 38.6% for mixture groups 1, 2, 3, 4, and 5, respectively.

4. Discussion

To discriminate between the effectiveness of β-carotene, α-tocopherol and ascorbic acid in protecting membranes from oxidative damage relative to different oxygen tensions, we initiated lipid peroxidation in microsomal membrane using AAPH and ACCN prooxidants. Azocompounds produce peroxyl radicals via an oxygen-dependent, iron-independent mechanism at specific sites and at constant rates, i.e., at either the aqueous, lipid, or aqueous/lipid interphase [14]. The thermal decomposition of either AAPH or ACCN at 37°C induces a free radical chain oxidation [22–25]. The rate of radical production in the microsomes is directly proportional to the concentration of azocompound used. The rate of free radical generation with 25 mM AAPH at 37°C was calculated to be about 0.03×10^{-6} mol/L/s [26].

We found that during AAPH-induced microsomal peroxidation, β-carotene did not inhibit lipid peroxidation consistent with other investigations [26,27], showing that β-carotene is very ineffective in inhibiting AAPH-induced lipid peroxidation. Such effects could be explained by β-carotene being rapidly consumed during AAPH-induced peroxidation. In addition, high oxygen tension and β-carotene concentrations did not change the ineffectiveness of β-carotene in AAPH-induced lipid peroxidation. Thus, other factors, such as the kind and the site of peroxyl radical production, seem to influence significantly the effectiveness of β-carotene in inhibiting or quenching peroxyl radical reactions, e.g., lipid, water, or interface phases. Due to lipid solubility, β-carotene can react with either the primary or secondary radicals generated in the lipid phase of the microsomal membranes by the lipid-soluble azocompound [26]. Also, microsomes are more exposed to β-carotene and lipid prooxidant. β-Carotene protects the microsomes from lipid peroxidation with 14–50% protection at a concentration range of 10–160 nmol/mg protein at 15 torr of oxygen. At higher oxygen tension, however, β-carotene lost antioxidant activity and promoted lipid peroxidation. Lipid peroxidation was most evident at the highest concentration,

160 nmol/mg protein at both 150 torr and 760 torr of oxygen tension. It has been observed that β-carotene reacts with the peroxyl radicals generated in solution by thermolysis of lipid soluble prooxidant [28]. The reaction produces 5,6- and 15,15′-epoxy-β,β-carotene, in addition to several unidentified polar products. Moreover, β-carotene is a more effective antioxidant when AMVN is used as the prooxidant, rather than AAPH [26]. We found that β-carotene is more active as an antioxidant at low oxygen tension in lipid peroxidation induced by lipid soluble prooxidant. However, β-carotene became a prooxidant at high oxygen tension and this effect is enhanced by increasing the concentration of β-carotene. The differences in the production rate of oxidation products may be related to lipid vs. water solubility.

It is well known that peroxyl radicals, because of the selectivity of their reactions and their ability to diffuse in biological systems, are potentially more dangerous than many other radical species [29]. These species may be implicated in the toxic action of many chemicals and environmental agents [30], and may be connected with a variety of pathological events, such as heart disease, cancer, and the process of aging [31]. There is increasing evidence that carotenoids are very effective quenchers of peroxyl radicals [1], but the mechanism of their action has not yet been defined. One might expect that the reaction of β-carotene with a peroxyl radical would form a carotenoid radical species shown in equation 1 [4,5].

$$\beta\text{-carotene} + \text{ROO}^{\cdot} \rightarrow \beta\text{-carotene} + \text{ROOH} \quad (1)$$

β-Carotene may react directly with a peroxyl radical to form a resonance-stabilized carbon-centered radical (equation 2)

$$\beta\text{-carotene} + \text{ROO}^{\cdot} \rightarrow \text{ROO-}\beta\text{-carotene}^{\cdot} \quad (2)$$

thus, providing an explanation of the antioxidant effect of β-carotene on lipid peroxidation induced by peroxyl radicals, i.e., β-carotene reacting with peroxyl radicals.

In the presence of oxygen, the β-carotene radical in the equation (1) would combine with oxygen to form a carotenoid-peroxyl radical (equation 3).

$$\beta\text{-carotene}^{\cdot} + \text{O}_2 \leftrightarrow \beta\text{-carotene-OO}^{\cdot} \quad (3)$$

This reaction would be dependent on the oxygen tension in the system. If the oxygen tension is sufficiently low, the equilibrium of reaction (3) shifts to the left, reducing the amount of chain-carrying peroxyl radical. In addition, the β-carotene-peroxyl complex could react with another peroxyl radical, leading to a termination reaction, as shown in reaction (4).

$$\beta\text{-carotene-OO}^{\cdot} + \text{ROO}^{\cdot} \rightarrow \text{inactive products} \quad (4)$$

On the other hand, if the oxygen tension is high, the equilibrium of reaction (3) would shift to the right and form a peroxyl radical capable of acting as a prooxidant. The last reaction is also referred to as autoxidation of β-carotene [4]. Although none of the potential intermediate forms proposed by Burton and Ingold [4] have been isolated, a variety of products arising from the reactions of radicals with β-carotene have been described in some studies [28,32]. Our results are consistent with these suggestions.

Beside the effects of high oxygen tension, we also found that high concentration of β-carotene is another factor influencing the antioxidant property of β-carotene. We found that high concentrations of β-carotene caused more MDA formation (Table 3). In equation 3, β-carotene radical (β-carotene$^{\cdot}$) is another component able to shift the equilibrium of the reaction to the right though O_2 tension is low (4). If the β-carotene concentration is high, more β-carotene$^{\cdot}$ could be produced, providing more resources for making the chain-carrying peroxyl radical (equation 3).

Several forms of reactive oxygen are generated in the cells as a result of various metabolic processes or following exposure to xenobiotics. The possible species are molecular oxygen (O_2), superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$). Many studies have indicated that carotenoids are able to limit the oxidative damage induced by oxy radical-generating systems. This protection involves both nuclear and lipid molecules. β-Carotene is able to reduce the extent of nuclear damage induced by xanthine oxidase/hypoxanthine or by activated polymorphonuclear leukocytes [33]. β-Carotene inhibits lipid peroxidation induced either by enzymatic sources of oxy radicals, e.g., xanthine oxidase system [34], and NADPH/cytochrome P-450 reductase [35], or by nonenzymatic sources, such as transition metal salts [36]. In addition, there is experimental evidence indicating the effectiveness of carotenoids in inhibiting lipid peroxidation induced by xenobiotics [10,37] well-known to be implicated in the production of oxy radicals.

Although it is clear that there is an involvement of carotenoids in reactions with oxygen radicals, it is not clear which radical species is primarily involved. In addition to peroxyl radicals, reaction of β-carotene with oxygen radicals would further consume more β-carotene and thus attenuate its antioxidant capability. Our results are consistent with such assumptions (Table 2) because we found that oxidation paralleled the concentration of oxygen.

We found that α-tocopherol effectively suppressed the lipid peroxidation in a concentration-dependent manner, either in the presence AAPH or ACCN. α-Tocopherol acts as a chain-breaking antioxidant by donating its phenolic hydrogen to the chain-propagating lipid peroxyl radical and replacing the latter with the less reactive α-tocopheroxyl radical. α-Tocopheroxyl radical can further react with another lipid peroxyl radical to stop propagation of lipid peroxidation. Each molecule of α-tocopherol has the capacity to scavenge two radicals [38]. The key to α-tocopherol actions under these conditions is that there must be enough antioxidant available to keep trapping free radicals. After complete consumption of the vitamin, the rate of lipid peroxidation increases rapidly. Another consideration to explain the effectiveness of α-tocopherol in both AAPH and

ACCN induced lipid peroxidation is the location of α-tocopherol in membrane. There are two pools of α-tocopherol, one near the surface and the other in the depth of the membrane, with consequently different susceptibility to peroxyl radicals derived from water- or lipid-soluble prooxidants [26].

We found that α-tocopherol exerted a more effective protection against ACCN-induced lipid peroxidation at 150 torr than at 15 torr and 760 torr. This result is consistent with results of others [10,11] suggesting that the antioxidant activity of α-tocopherol is decreased at low oxygen partial pressure. Others have recently suggested that oxidants could interact directly with either surface lipids or α-tocopherol [38]. Although the surface lipids are more abundant, α-tocopherol is approximately five orders of magnitude more reactive toward peroxyl radical $ROO^{\cdot}$ and the redox-active chromanol hydrogen that resides predominantly at, or close to, the surface of the lipid. Therefore, $ROO^{\cdot}$ tends to react preferentially with α-tocopherol. The resulting α-tocopheroxyl radical is trapped within LDL and therefore cannot undergo radical-radical termination unless a second $ROO^{\cdot}$ enters the oxidizing particle. The flux of free radical, i.e., the frequency with which LDL encounters $ROO^{\cdot}$, fundamentally controls the net effect of α-tocopherol. Under relatively high flux conditions, termination reactions between α-tocopheroxyl radical and $ROO^{\cdot}$ are frequent, resulting in both the prevention of lipid peroxidation and the rapid consumption of α-tocopherol. Under low flux conditions, however, termination reactions are infrequent. The relatively less polar α-tocopheroxyl radical is trapped within LDL and, therefore, cannot undergo radical-radical termination. Under extremely high flux conditions, $ROO^{\cdot}$ or more reactive $OH^{\cdot}$ can directly attack lipid molecules and the termination reaction cannot protect lipid from peroxidation [38]. We found that 760 torr oxygen could produce a variety of reactive oxygen species including $OH^{\cdot}$, and thus attenuate the antioxidant effect of α-tocopherol.

We found that ascorbic acid exhibited the most effective antioxidant protection against AAPH-induced lipid peroxidation. Ascorbic acid is an effective antioxidant for several reasons. First, both ascorbate and the ascorbyl radical, the latter formed by one electron oxidation of ascorbate, have low reduction potentials [39] and can react with most other biologically relevant radicals and oxidants. Second, the ascorbyl radical has a low reactivity due to resonance stabilization of the unpaired electron and readily dismutates ($k_2 = 2 \times 10^5\ M^{-1}\ s^{-1}$) to ascorbate and dehydroascorbic acid (DHA) [40]. In addition, ascorbate can be regenerated from both the ascorbyl radical and DHA by enzyme-dependent and independent pathways. Ascorbic acid is more effective in an aqueous environment when a water-soluble prooxidant is involved, and its antioxidant effect is not influenced by oxygen tension. What is surprising is the effect of ascorbic acid on ACCN-induced lipid peroxidation at 150 torr of oxygen. Ascorbic acid may be less effective as an antioxidant in ACCN-induced lipid peroxidation. Our findings of 15 torr and 760 torr oxygen tension also were in agreement with this expectation. However, ascorbic acid was found more effective to inhibit ACCN-induced lipid peroxidation. Some studies suggest that endogenous and exogenous vitamin C inhibit the formation of lipid hydroperoxides more effectively, even in iron-overloaded or copper supplemented human plasma [41, 42].

The mixture of β-carotene, α-tocopherol, and ascorbic acid provided slightly better protection in lipids from AAPH-induced peroxidation than each compound alone. The regeneration of α-tocopherol from α-tocopherol radical by ascorbate, with concomitant generation of the ascorbyl radical, is well established [43,44]. Addition of ascorbate to LDL undergoing oxidation induced by aqueous $ROO^{\cdot}$ results in immediate cessation of α-tocopherol consumption and lipid oxidation and formation of the relatively nonreactive ascorbyl radical [45]. Thus, ascorbate is a phase transfer agent that facilitates the export of a radical from within the lipoproteins to the aqueous compartment and thereby prevents lipid peroxidation [38].

Several studies demonstrate that interaction between carotenoid and tocopherols can occur in vitro [6,32,46]. Although the mechanism of the interaction of carotenoid-tocopherol needs further study, our findings suggest that tocopherol may limit the proooxidant effects of carotenoids in biologic systems. However, no significant interaction between these two compounds was observed.

The possibility of interactions in vitro between β-carotene and ascorbic acid was reported by several authors [47,48]. Our results imply that β-carotene and ascorbate may be sparing each other.

In conclusion, our findings illustrate that β-carotene, α-tocopherol, and ascorbic acid can act as antioxidants in microsomal membranes, and that their antioxidant efficacy is influenced by many factors, including: oxygen tension, antioxidant, source of oxidants, and interactions with each other. Many in vitro results have not been substantiated in vivo. Moreover, the products of β-carotene responsible for the prooxidant activity have not been identified yet.

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