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COMMUNICATION

β-Carotene and α-Tocopherol Are Synergistic Antioxidants

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The possibility of a cooperative interaction between fat-soluble antioxidants is examined in a membrane model. A combination of β-carotene and α-tocopherol results in an inhibition of lipid peroxidation significantly greater than the sum of the individual inhibitions. Our data provide the first evidence that β-carotene can act synergistically with α-tocopherol as an effective radical-trapping antioxidant in membranes.

β-Carotene has been postulated to be a dietary anticarcinogen (1) and this function is under active investigation. In addition, there is increasing epidemiological evidence that carotenoids may also play a preventive role in cardiovascular disease (2), and a single, preliminary report of direct supplementation with β-carotene indicates that it may function in preventing the recurrence of coronary and vascular events (3). These exciting developments raise the question as to how this nontoxic compound might function in animal tissues. Most recent observations relating β-carotene to disease prevention suggest that antioxidant properties (4, 5) might explain the observed effects. However, this antioxidant effect has not been clearly demonstrated in humans. α-Tocopherol has been proposed to be the most important lipid-soluble radical scavenging antioxidant in membranes and plasma (6), but other fat-soluble compounds, such as ubiquinones and carotenoids, have also been shown to protect biological systems from oxidative damage (4, 7). In contrast to α-tocopherol, little is known about their precise antioxidant mechanisms. The antioxidant activity of ubiquinones–ubiquinols can be due either to a direct scavenging mechanism (8) or to an indirect mechanism involving recycling of α-tocopherol (9). The latter mechanism has been clearly demonstrated for ascorbic acid (10). Although β-carotene has been demonstrated to possess antioxidant activity in liposomes (11), microsomal membranes (12), lipoproteins such as LDL (13), and corneal endothelial cells (14), no information is available with respect to the *mechanism* of its antioxidant activity. It has been suggested that β-carotene directly traps the chain-propagating peroxyl radical (15). Several reports indicate that the concentration of oxygen may have an important role in the antioxidant activity of this compound (15–18). It has also been hypothesized that α-tocopherol and β-carotene have complementary roles relative to the varying oxygen tensions in biological membranes, since the antioxidant activity of β-carotene improves at physiological oxygen tensions (19).

We have recently demonstrated that α-tocopherol and β-carotene exert an *additive* effect in inhibiting radical-initiated lipid peroxidation in hexane solutions of lipid extracts from rat liver microsomes (18). The present work investigates possible interactions between β-carotene and α-tocopherol in a membrane system and attempts to elucidate the mechanism of antioxidant activity of β-carotene.

EXPERIMENTAL PROCEDURES

Microsomal preparation. Liver microsomes were prepared from Sprague–Dawley rats (20) and suspended in 0.1 M potassium phosphate buffer, pH 7.5 (12). Microsomal proteins were determined by the Bio-Rad method (21).

Incorporation of β-carotene and α-tocopherol into microsomal membranes. An aliquot of β-carotene in nitrogen-saturated $CHCl_3/CH_3OH$ (2/1, v/v) solution was evaporated to dryness in a Potter–Elvehjem homogenizer under a stream of nitrogen. Microsomes in phosphate buffer were added and gently homogenized at 0°C until all the β-carotene was dispersed in the microsomes, as described earlier (12). When the microsomes were resedimented, all of the added β-carotene was present in the pellet, indicating that the β-carotene was incorporated or associated with the microsomes. Comparable effects were found adding α-tocopherol, either in $CHCl_3/CH_3OH$ (2/1, v/v) solution and drying, as above, or in an ethanol solution (0.5 vol% or less). In the latter case, the added ethanol did not inhibit lipid peroxidation (data not shown).

Lipid peroxidation. Lipid peroxidation was initiated by the addition of either NADPH/ADP/Fe^{3+} or 2,2′-azobis(2-amidinopropane) (AAPH)[2] to the microsomal suspension (12). The reaction mixtures were shaken in air at 37°C. Lipid peroxidation products were determined as malondialdehyde (MDA) formation at 535 nm (22). In the AAPH experiments, the stock reagent for MDA measurements contained 0.02% (w/v) butylated hydroxytoluene (BHT). This amount of BHT completely prevents the formation of any nonspecific thiobarbituric acid reactive

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[2] Abbreviations used: AAPH, 2,2′-azobis(2-amidinopropane); MDA, malondialdehyde; BHT, butylated hydroxytoluene.

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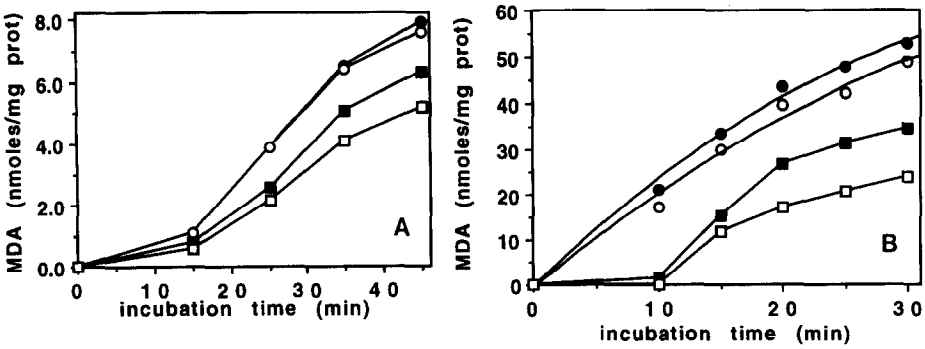

FIG. 1. The induction of lipid peroxidation in rat liver microsomes by the addition of (A) AAPH (●) or (B) NADPH/ADP/Fe^{3+} (●), as monitored by MDA formation at 535 nm, in the presence of β-carotene (○), α-tocopherol (■), or a combination of both antioxidants (□) under air at 37°C. (A) 25 mM AAPH in 0.1 M phosphate buffer, pH 7.5, 0.5 mg/ml protein, β-carotene at 10 nmol/mg protein, α-tocopherol at 6.5 nmol/mg protein. (B) 0.4 mM NADPH in 0.15 M KCl, 50 mM Hepes, pH 7.5, 1 mM ADP, 50 mM $FeCl_3$, 0.33 mg/ml protein, β-carotene at 10 nmol/mg protein, α-tocopherol at 6.0 nmol/mg protein. The points represent the means of three experiments.

substances, as well as preventing decomposition of AAPH during the boiling.

Assay of β-carotene and α-tocopherol. The antioxidants were extracted and analyzed by HPLC as described earlier (12, 18). In these experiments, β-carotene was present at 10 nmol/mg protein, and α-tocopherol was present at 6.0–6.5 nmol/mg protein.

Reagents. α-Tocopherol and β-carotene were obtained from Fluka Chemica-Biochemica (Buchs, Switzerland); AAPH was from Polysciences, Inc. (Warrington, PA); β-nicotinamide adenine dinucleotide phosphate (β-NADPH), adenosine 5'-diphosphate (ADP), thiobarbituric acid, and BHT were from Sigma Chemical Co. (St. Louis, MO); and ferric chloride, trichloroacetic acid, and hydrochloric acid were from Fisher Scientific Co. (Fair Lawn, NJ). The solvents were of HPLC grade and used without purification.

RESULTS

Effects of a combination of α-tocopherol and β-carotene on lipid peroxidation. Lipid peroxidation was initiated in rat liver microsomal membranes by the addition of either AAPH, which produces peroxyl radicals by thermal decomposition (23), or by NADPH/ADP/Fe^{3+}, which produces initiators of still uncertain origin, including perferryl and hydroxyl radicals (24). The effects in this system of β-carotene and α-tocopherol, added either individually or in combination, are shown in Fig. 1. It should be noted that a lag period, due to endogenous antioxidants, is clearly seen when azo-initiators such as AAPH are used (12), but is not demonstrable when NADPH/ADP/Fe^{3+} is used to initiate lipid peroxidation (12, 20). When β-carotene (10 nmol/mg protein) was added alone, no significant effects were observed following AAPH treatment, whereas α-tocopherol (6.5 nmol/mg protein) was protective (Fig. 1A). When NADPH/ADP/Fe^{3+} was the initiator, β-carotene was marginally protective, whereas α-tocopherol was very protective (Fig. 1B). The protection by β-carotene is a function of concentration, since we previously observed a significant inhibition of AAPH-induced MDA formation in microsomal membranes at 50 nmol β-carotene/mg protein (12). On the other hand, 10–100 nmol β-carotene/mg protein has been reported to inhibit MDA formation in microsomal membranes treated with adriamycin/NADPH/Fe^{3+} (16). In addition, lowering the oxygen tension increased the antioxidant effects of β-carotene (16).

The addition of both antioxidants markedly decreases MDA formation induced either by AAPH (Fig. 1A) or by NADPH (Fig. 1B). This inhibition is greater than the sum of the inhibitions observed individually. A statistical analysis of the values from Figs. 1 and 2 is presented in Table I for the last time of incubation for each experiment, showing clearly that a *synergistic* interaction occurs between α-tocopherol and β-carotene in this membrane system. On the other hand, no evident increase in lag time was found when both antioxidants were present. This observation is interesting because of previous results obtained in solution (18, 25), or in membranes (12), showing that the inhibition of azo-initiated lipid peroxidation by β-carotene does not produce a clearly defined lag phase as seen with α-tocopherol.

Effects of a combination of α-tocopherol and β-carotene on AAPH-induced loss of the antioxidants. To examine further the interactions between the two antioxidants, we have evaluated their consumption, individually or in combination, during the phases of lipid peroxidation. Figure 2 shows that added β-carotene (10 nmol/mg protein) is consumed very slowly during AAPH-induced lipid peroxidation in the presence or absence of added α-tocopherol (6.5 nmol/mg protein). On the other hand,

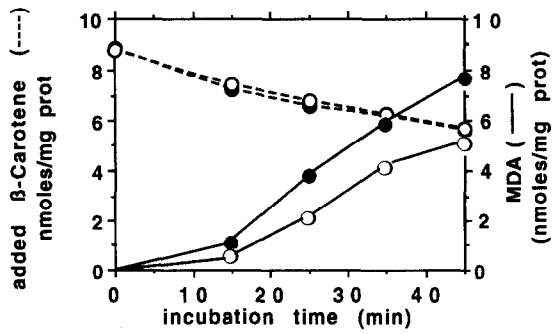

FIG. 2. AAPH-induced loss of added β-carotene in relation to MDA formation in the absence (●) or in the presence (○) of added α-tocopherol (6.5 nmol/mg protein) in rat liver microsomes. β-Carotene was extracted with 1 vol of methanol and 3 vol of hexane:diethyl ether (1:1) and the concentration was calculated spectrophotometrically at 450 nm (35). The points represent the means of three experiments.

TABLE I

The Effect of a Combination of β-Carotene and α-Tocopherol on the Inhibition of Radical-Induced Lipid Peroxidation in Rat Liver Microsomes

Antioxidant	nmol/mg protein	Inhibition of MDA formation[1] (% of control) Radical initiator: AAPH (45 min)	Radical initiator: NADPH/ADP/Fe^{3+} (30 min)
α-Tocopherol	6.5	20.0 ± 2.0	
	6.0		34.0 ± 2.8
β-Carotene	10.0	4.0 ± 0.2	7.0 ± 0.7
α-Tocopherol + β-carotene			
Expected[2]		24.0 ± 2.2[a]	41.0 ± 3.5[a]
Observed		34.0 ± 3.0[b]	54.0 ± 5.0[b]

Note. Values in the same column with different superscript letters are significantly different ($P \leq 0.05$) from each other.

[1]Values obtained from Figs. 1 and 2.

[2]If antioxidants act in an *additive* fashion.

lipid peroxidation can be observed even when high levels of β-carotene are still present. The addition of α-tocopherol has no effect on the AAPH-induced loss of β-carotene, although it slows down MDA formation.

The addition of AAPH to initiate lipid peroxidation in microsomal membranes also results in the loss of added α-tocopherol (Fig. 3). We observed earlier that the AAPH-induced propagation phase only begins when a substantial depletion of endogenous α-tocopherol occurs (12), but, as seen in Fig. 3, the propagation phase can begin when high levels of exogenous α-tocopherol (about 4 nmol/mg protein) remain. These observations suggest a difference in the effects of exogenous and endogenous α-tocopherol, presumably due to some difference in incorporation in the membrane system (26, 27). In addition, the exogenous β-carotene actually increases the loss of α-tocopherol (Fig. 3).

DISCUSSION

Although some evidence has appeared that β-carotene and α-tocopherol act synergistically *in vivo* (28), our results offer the first attempt at understanding the mechanisms of this phenomenon. The synergistic interaction between these antioxidants that results in both an increase of membrane resistance to oxidative stress and an increase of the loss of α-tocopherol could be explained as follows. In the absence of added α-tocopherol, β-carotene could react with peroxyl or alkoxyl radicals and form a resonance-stabilized carbon-centered radical (β-C•), as proposed earlier (15, 19). At the atmospheric oxygen concentration (150 Torr) of our system, a new chain-carrying β-carotene peroxyl radical (β-COO•) could be formed (15, 19). The relative amount and reactivity of β-COO• could represent the balance between the antioxidant activity of β-carotene and the prooxidant character of β-COO•, resulting in marginal (NADPH) or no (AAPH) inhibition of lipid peroxidation (Fig. 1). Our data and evidence in the literature support this hypothesis. β-Carotene is directly consumed during AAPH-induced lipid peroxidation, suggesting its reaction with the radicals generated by AAPH. In parallel, several observations show the ability of β-carotene to react directly with oxygen radicals and peroxyl radicals (4) or alkoxyl radicals (29) generated during lipid peroxidation. β-Carotene also competes with fatty acids in reacting with peroxyl radicals (30). In addition, increasing evidence indicates that the antioxidant activity of β-carotene is markedly potentiated at low oxygen tensions (15–19, 31), where the accumulation of β-COO• is less likely to occur.

The addition of both antioxidants to our system substantially facilitates the antioxidant character of β-carotene by limiting the production or reactivity of β-COO•. Although at present no evidence is available indicating the formation of β-COO• or a reaction between α-tocopherol and β-carotene, the following observations encourage this hypothesis. Tocopherols prolong the inhibitory effect of β-carotene on singlet oxygen-initiated photo-oxidation of methyl linoleate and soybean oil, by a mechanism involving the prevention of β-carotene decomposition (32). Also, α-tocopherol protects β-carotene from autoxidation (33). This protection is dose-dependent and occurs only at sufficiently high concentrations of α-tocopherol. This could explain the lack of synergism between the two antioxidants that we observed both in membranes and in homogeneous solution (18), when the levels of α-tocopherol were much lower than the levels of β-carotene. In addition, the products formed from β-carotene by autoxidation or by initiation with an azocompound are similar, suggesting the presence of a similar peroxyl radical intermediate (33).

The hypothesis of α-tocopherol protection of β-carotene is in agreement with our data (Fig. 3) showing an increase of α-tocopherol consumption induced by AAPH in the presence of a combination of the two antioxidants. Such protection could explain the results in lipoproteins (26), where during copper-induced lipid peroxidation there is a more rapid consumption of α-tocopherol than β-carotene.

The opposite hypothesis, that β-carotene protects α-tocopherol by a mechanism involving recycling of α-tocopheroxyl radicals seems improbable on the basis of pulse radiolysis studies, which indicate a lower ability of β-carotene to act as hydrogen or electron donor than α-tocopherol (34). If β-carotene were able to recycle α-tocopherol, the combination of the two an-

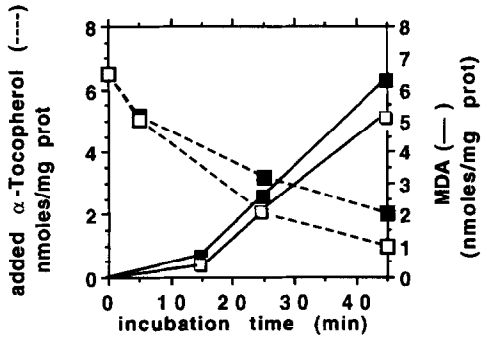

FIG. 3. AAPH-induced loss of added α-tocopherol in relation to MDA formation in rat liver microsomes in the absence (■) or in the presence (□) of added β-carotene (10 nmol/mg protein) in rat liver microsomes. α-Tocopherol was extracted and analyzed by HPLC (12). The points represent the means of three experiments.

tioxidants should produce, in our system, a clear increase of lag time and more rapid consumption of the β-carotene than α-tocopherol.

Further studies are required to elucidate the mechanism of the synergistic interactions between β-carotene and α-tocopherol in protecting microsomal membranes from oxidative damage and to establish the importance of this finding at physiological conditions. However, our data clearly show potential antioxidant benefits of an association of β-carotene and α-tocopherol in biological membranes. These observations, which are in contrast with our findings of additive effects in solution (18), may be a function of different localization of these two lipid-soluble antioxidants in biological membranes.

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