Interaction among vitamin C, vitamin E, and β-carotene[1–3]

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ABSTRACT The effects of vitamin C (ascorbic acid), vitamin E (α-tocopherol), and β-carotene as antioxidants and their cooperative action against the oxidation of lipid in solution, membranes, and lipoproteins have been studied and reviewed. Ascorbic acid and α-tocopherol act as potent, and probably the most important, hydrophilic and lipophilic antioxidants, respectively. They function at their own site individually and furthermore act synergistically. β-Carotene has lower reactivity toward radicals than does α-tocopherol and acts as a weak antioxidant in solution. It is more lipophilic than α-tocopherol and is assumed to be present at the interior of membranes or lipoproteins, which enables it to scavenge radicals within the lipophilic compartment more efficiently than does α-tocopherol. The cooperative interaction between vitamin C and vitamin E may be quite probable, that of vitamin C and β-carotene is improbable, whereas that between vitamin E and β-carotene may be possible. *Am J Clin Nutr* 1995;62(suppl):1322S–6S.

KEY WORDS Ascorbic acid, α-tocopherol, β-carotene, free radicals, membrane, lipoprotein

INTRODUCTION

The radical-scavenging antioxidants play a vital role in the defense systems in vivo against oxidative stress induced by free radicals and active oxygen species (**Table 1**) (1). Vitamin C, vitamin E, and β-carotene are known to be particularly important and their role in maintaining health and preventing disease has received much attention (2, 3). Vitamin C is the major water-soluble antioxidant and acts as the first defense against free radicals in whole blood (4) and plasma (5). Vitamin E is composed of eight different stereoisomers in the side chain and four homologs on the chroman ring; *RRR*-α-tocopherol is known to have the highest biological activity (6). α-Tocopherol is the most abundant lipophilic antioxidant in vivo (7).

β-Carotene has also received much attention in, for example, the suppression of lung cancer (8), although the recent results of an intervention trial conducted in Finland to evaluate the effects of β-carotene and vitamin E supplementation on the incidence of lung cancer in male smokers did not show a positive effect (9). It is known that β-carotene quenches singlet oxygen rapidly (10) but its role as a radical-scavenging antioxidant and the mechanism of inhibition of oxidation are not yet fully established. These antioxidants act not only individually but also cooperatively and in some cases even synergistically. We examine the interaction of these three antioxidants.

MATERIALS AND METHODS

Methyl linoleate and soybean phosphatidylcholine were purchased from Sigma Chemical Company (St Louis) and purified before use by column chromatography as described previously (11). Dimyristoyl phosphatidylcholine (14:0 phosphatidylcholine) obtained from Sigma was used as received. Soybean phosphatidylcholine liposomes were prepared as described previously (11). Low-density lipoprotein (LDL) was prepared from human plasma of healthy donors by ultracentrifugation within a density cutoff of 1019–1063 g/L and dialyzed with phosphate-buffered saline (PBS, pH 7.4) (12). Water-soluble and lipid-soluble azo compounds, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis(2, 4-dimethylvaleronitrile) (AMVN), respectively, were used as radical initiators to generate free radicals at a known and constant rate and at different sites (13).

Commercial vitamin C (ascorbic acid) and 6-*O*-palmitoylascorbic acid were used as received. *RRR*-α-Tocopherol and β-carotene were kindly supplied by Eisai Company Ltd (Tokyo) and Hoffmann-La Roche (Basel, Switzerland), respectively.

The oxidations were carried out at 37 °C in air, unless otherwise specified. The oxidations of phosphatidylcholine liposomes and LDL induced by either AMVN or AAPH were performed in PBS, pH 7.4, in the presence of 0.1 mol EDTA/L, whereas the oxidation induced by metal ion was carried out in the absence of EDTA.

The hydroperoxides of methyl linoleate, phosphatidylcholine, and cholesteryl ester were measured with HPLC as described elsewhere (11, 12, 14). The consumption of antioxidants was also measured by HPLC (12, 14, 15).

RESULTS

The individual antioxidant activity of ascorbic acid, α-tocopherol, and β-carotene was first measured in the oxidation of

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TABLE 1
Defense system in vivo against oxidative damage[1]

Active agent	Action
1) Preventive antioxidants: suppress the formation of free radicals	
Nonradical decomposition of hydroperoxides and hydrogen peroxide	
Catalase	Decomposition of hydrogen peroxide $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$
Glutathione peroxidase (cellular)	Decomposition of hydrogen peroxide and free fatty acid hydroperoxides $H_2O_2 + 2\ GSH \rightarrow 2\ H_2O + GSSG$ $LOOH + 2\ GSH \rightarrow LOH + H_2O + GSSG$
Gluthathione peroxidase (plasma)	Decomposition of hydrogen peroxide and phospholipid hydroperoxides $PLOOH + 2\ GSH \rightarrow PLOH + H_2O + GSSG$
Phospholipid hydroperoxide glutathione peroxidase	Decomposition of phospholipid hydroperoxides
Peroxidase	Decomposition of hydrogen peroxide and lipid hydroperoxides $LOOH + AH_2 \rightarrow LOH + H_2O + A$ $H_2O_2 + AH_2 \rightarrow 2\ H_2O + A$
Glutathione-*S*-transferase	Decomposition of lipid hydroperoxides
Sequestration of metal by chelation	
Transferrin, lactoferrin	Sequestration of iron
Haptoglobin	Sequestration of hemoglobin
Hemopexin	Stabilization of heme
Ceruloplasmin, albumin	Sequestration of copper
Quenching of active oxygen species	
Superoxide dismutase	Disproportionation of superoxide $2\ O_2^{\cdot-} + 2\ H^+ \rightarrow H_2O_2 + O_2$
Carotenoids, vitamin E	Quenching of singlet oxygen
2) Radical-scavenging antioxidants: scavenge radicals to inhibit chain initiation and break chain propagation	
Hydrophilic: vitamin C, uric acid, bilirubin, albumin	
Lipophilic: vitamin E, ubiquinol, carotenoids, flavonoids	
3) Repair and de novo enzymes: repair the damage and reconstitute membranes	
Lipase, protease, DNA repair enzymes, transferase	
4) Adaptation: generate appropriate antioxidant enzymes and transfer them to the right site at the right time and in the right concentration	

[1] GSH, reduced glutathione; GSSG, oxidized glutathione; LOOH, lipid hydroperoxide; LOH, lipid alcohol; PLOOH, phospholipid hydroperoxide; PLOH, phospholipid alcohol; AH_2, reduced substrate; A, oxidized substrate.

soybean phosphatidylcholine liposomes induced by either AAPH or AMVN. As shown in **Figure 1**, ascorbic acid suppressed the oxidation induced by AAPH efficiently, but it only retarded the oxidation initiated by AMVN. α-Tocopherol inhibited the oxidations of phosphatidylcholine liposomes effectively induced by either AAPH or AMVN. β-Carotene was much less effective than α-tocopherol in both oxidations.

It has been shown previously that α-tocopherol and ascorbic acid inhibit the oxidations of liposomal membranes (16, 17) and LDL (15) synergistically. Ascorbic acid scavenges aqueous radicals and also acts as a synergist to regenerate α-tocopherol by reducing the α-tocopheroxyl radical formed from α-tocopherol when it reacts with a radical. Such synergistic interaction between α-tocopherol and ascorbic acid was confirmed in the present study in the oxidation of LDL (data not shown).

The effects of α-tocopherol, β-carotene, and their combination on the oxidations of methyl linoleate in acetonitrile solution and soybean phosphatidylcholine liposomes are shown in **Figure 2**. In the oxidation of methyl linoleate in acetonitrile solution in the presence of both α-tocopherol and β-carotene, α-tocopherol was consumed predominantly at first and β-carotene was spared and then consumed rapidly after most of the α-tocopherol disappeared. When 6-*O*-palmitoylascorbic acid was also present, both α-tocopherol and β-carotene were spared at first and then α-tocopherol began to be consumed, followed by β-carotene. On the other hand, in the oxidation of soybean phosphatidylcholine liposomes induced by AMVN and inhibited by a combination of α-tocopherol and β-carotene, β-carotene was consumed faster than α-tocopherol. The oxidation was suppressed slightly more efficiently by their combination than the sum of individual inhibition. In the presence of 6-*O*-palmitoylascorbic acid in the liposomal membrane, the consumption of β-carotene was delayed whereas that of α-tocopherol was suppressed markedly. The combination of these three antioxidants inhibited the oxidation of soybean phosphatidylcholine almost completely.

The rate of consumption of α-tocopherol and β-carotene incorporated simultaneously into the same dimyristoyl phosphatidylcholine liposomal membrane was dependent on the site of radical generation. When the radicals were generated initially in the aqueous phase from AAPH, α-tocopherol was

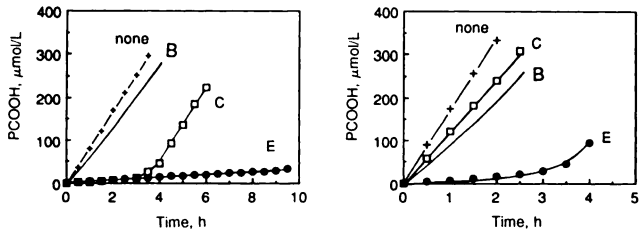

FIGURE 1. Inhibition of oxidation of soybean phosphatidylcholine liposomes by ascorbic acid, α-tocopherol, and β-carotene. The oxidation of soybean phosphatidylcholine (2.80 mmol/L) liposomes was induced either by 1.0 mmol 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)/L (left) or 0.50 mmol 2,2′-azobis(2, 4-dimethylvaleronitrile) (AMVN)/L (right) at 37 °C in air in the absence (+) or presence of either ascorbic acid (C), α-tocopherol (E), or β-carotene (B) and the accumulation of soybean phosphatidylcholine hydroperoxide (PCOOH) was followed. The concentrations of the antioxidants were 10 μmol/L each.

consumed faster than β-carotene, whereas when AMVN was incorporated into the membrane as a radical source and the radicals were generated within the membrane, β-carotene was consumed faster than α-tocopherol (data not shown).

The oxidation of LDL gave cholesteryl ester hydroperoxide and phosphatidylcholine hydroperoxide as major primary products (12, 15). Both α-tocopherol and β-carotene were consumed simultaneously as observed in the liposomal membrane system; ascorbic acid spared α-tocopherol but it only retarded the consumption of β-carotene. Note that the addition of ascorbic acid suppressed the oxidation of LDL completely.

DISCUSSION

The above results show that ascorbic acid, α-tocopherol, and β-carotene act as radical-scavenging antioxidants and suppress lipid peroxidation. It should be appreciated that the antioxidant activities are determined not only by chemical reactivity toward free radicals but also by several other factors, as listed in **Table 2**.

As shown in Figure 1 and as described above, the site of initial radical generation and the location of the antioxidant are important factors. Ascorbic acid and other hydrophilic antioxidants scavenge aqueous radicals efficiently but they cannot scavenge lipophilic radicals in the membranes and LDL as efficiently. Lipophilic antioxidants such as α-tocopherol and β-carotene can suppress the oxidation induced by either lipophilic or aqueous radicals.

The reactivity of an attacking radical is also an important factor in determining antioxidant potency, primarily by a competition between the following two reactions: chain inhibition (Eq 1) and chain propagation (Eq 2),

$$X^{\cdot} + IH \xrightarrow{k_{inh}} XH + I^{\cdot} \qquad (1)$$

$$X^{\cdot} + LH \xrightarrow{k_{p}} XH + L^{\cdot} \qquad (2)$$

where X ·, IH, and LH are an attacking radical, antioxidant, and substrate, and k_{inh} and k_p are the rate constants for equations *1* and *2*, respectively. Antioxidant activity is determined by a ratio of equations *1* and *2*, that is, $k_{inh}(IH)/k_p(LH)$. This means that the antioxidant potency is dependent on k_p, that is, the

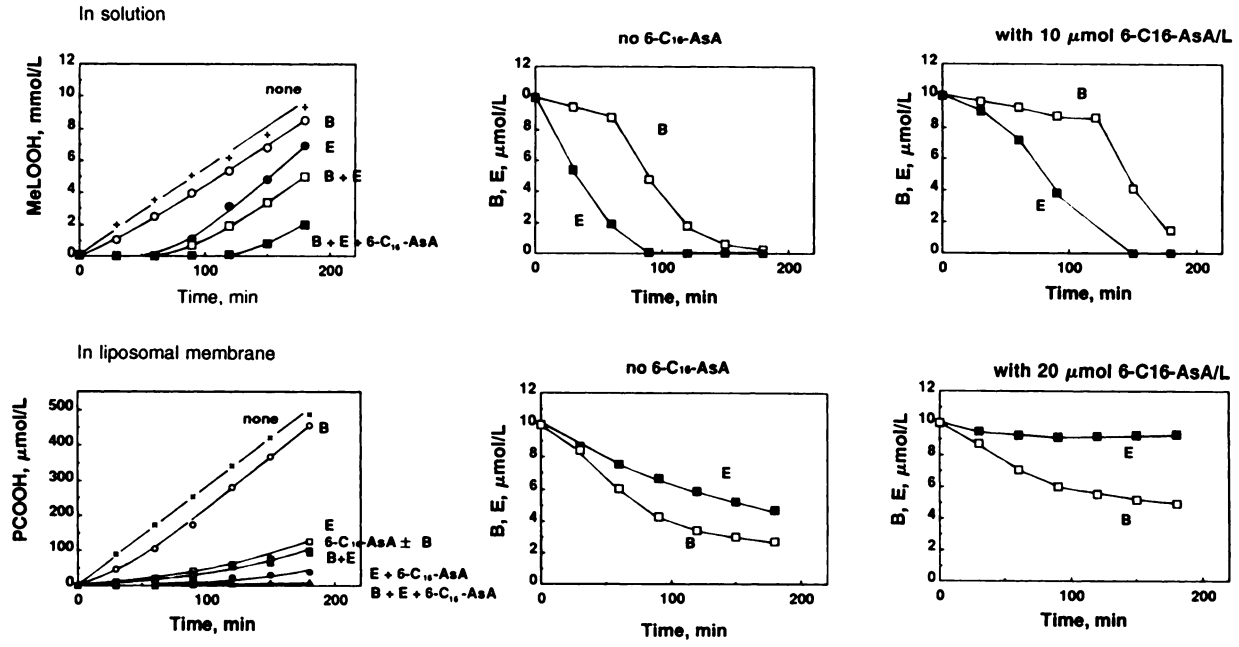

FIGURE 2. Oxidation of methyl linoleate (MeLH) in acetonitrile solution (top row) and soybean phosphatidylcholine liposomes (bottom row) induced by 2,2′-azobis(2, 4-dimethylvaleronitrile) (AMVN) at 37 °C in air in the presence of α-tocopherol (E), β-carotene (B), and 6-*O*-palmitoylascorbic acid (6-C_{16}-AsA). The accumulation of methyl linoleate hydroperoxide (MeLOOH) and soybean phosphatidylcholine hydroperoxide (PCOOH) was followed. Top row: MeLH = 200 mmol/L, AMVN = 1.0 mmol/L, and E and B = 10 μmol/L each; bottom row: soybean phosphatidylcholine = 5.1 mmol/L, AMVN = 1.5 mmol/L, E and B = 10 μmol/L, and 6-C_{16}-AsA = 20 μmol/L.

TABLE 2
Factors that determine the potency of radical-scavenging antioxidants

1) Intrinsic chemical reactivity toward radical, k_{inh}
2) Site of generation and reactivity of attacking radical X$^{\cdot}$
3) Site of antioxidant
4) Concentration and mobility at the microenvironment
5) Fate of antioxidant-derived radical I$^{\cdot}$
6) Interaction with other antioxidants
7) Absorption, distribution, retention, metabolism, and safety

reactivities of attacking radical and substrate, as well as on the concentrations of antioxidant and substrate and k_{inh}. **Table 3** compares the effect of attacking hydroxyl (HO$^{\cdot}$), alkoxyl (LO$^{\cdot}$), and peroxyl ($LO_2^{\cdot}$ and $HO_2^{\cdot}$) radicals on the competition between α-tocopherol and polyunsaturated lipids. The rate constants were estimated from the literature (18). As shown, hydroxyl and alkoxyl radicals are so reactive and so unselective that the difference in the reactivities between α-tocopherol and unsaturated lipids toward these radicals is not large enough for α-tocopherol to scavenge these radicals preferentially before they attack a substrate. In other words, α-tocopherol is a good scavenger only for peroxyl radicals but not for hydroxyl or alkoxyl radicals. The hydroxyl radical is so reactive that there should be no specific scavenger for it. Ascorbic acid acts as the first defense in the blood against aqueous peroxyl radicals (4, 5), but other antioxidants such as uric acid, the concentration of which is in general a few times higher than that of ascorbic acid, might well play more important roles against hydroxyl radicals.

Furthermore, the location of the antioxidant in the membranes and lipoproteins and its mobility in the microenvironment are also important. As shown above, when α-tocopherol and β-carotene were incorporated simultaneously in dimyristoyl phosphatidylcholine liposomal membranes and peroxyl radicals were formed in the aqueous phase, α-tocopherol was consumed faster than β-carotene. However, β-carotene was consumed faster than α-tocopherol when the radicals were generated initially within the liposomal membranes. Figure 2 shows similar results obtained during the oxidation of soybean phosphatidylcholine liposomes. These results suggest that β-carotene is located mainly in the interior of the lipophilic domain of the membrane, which makes it easier for β-carotene to scavenge lipophilic radicals compared with α-tocopherol.

α-Tocopherol is known to be located at or near the membrane surface (19) and it has been shown experimentally that the efficiency of radical scavenging by α-tocopherol decreases as the radical goes deeper into the interior of the membranes (20). Figure 2 also shows that α-tocopherol was spared quite efficiently by 6-*O*-palmitoylascorbic acid, whereas β-carotene was not. Substantially the same results were observed in the oxidation of LDL: vitamin E was spared by aqueous ascorbic acid but β-carotene was not. Furthermore, little oxidation proceeded by a combination of α-tocopherol and ascorbic acid, suggesting that the tocopherol-mediated propagation observed in the in vitro experiment (21–23) should not be important in the presence of ascorbic acid.

Interaction among antioxidants is another important factor. For the combination of two antioxidants to be synergistic, either the induction period, or lag phase, should be longer than the sum of induction periods produced by the individual antioxidant, or the rate of oxidation inhibited by the two antioxidants should be the same or smaller than that inhibited by either antioxidant. The combination of ascorbate and α-tocopherol is effective in inhibiting oxidation. Ascorbate and α-tocopherol are located in different domains and interact at the interface between membrane or lipoprotein and water. Ascorbic acid reduces α-tocopheroxyl radicals rapidly (24, 25) in membranes (16, 17) and LDL (26, 27) to regenerate α-tocopherol and possibly inhibits α-tocopheroxyl radical–mediated propagation. It was found through use of a spin-label technique that the reduction of stable oxygen radicals by ascorbate became slower as the radical went deeper into the interior of the liposomal membrane (28); similar results were observed for LDL (N Gotoh, N Noguchi, J Tsuchiya, H Morita, H Sakai, H Shimasaki, and E Niki, unpublished observations, 1995). Apparently, the relative importance of ascorbic acid as a synergist should decrease as the phenoxyl radical goes deeper into the membrane or lipoprotein.

The interaction between α-tocopherol and β-carotene is not as evident as that between α-tocopherol and ascorbic acid. Palozza and Krinsky (29) reported that the antioxidant effect of the combination of α-tocopherol and β-carotene was additive in the oxidation of lipid extracts from rat liver microsomes in hexane solutions but was synergistic in the oxidation of microsomal membranes (30). The results of the present study also show that α-tocopherol and β-carotene exert an additive effect in oxidation in solution but a slightly greater effect than the sum of their individual inhibitions of oxidation in membranes. This cooperative interaction between α-tocopherol and β-carotene, if any, is not as significant as that between α-tocopherol and ascorbic acid. β-Carotene acts as an antioxidant by scavenging radicals not by hydrogen atom donation but by addition to the double bond to give a conjugated polyene, carbon-centered radical (31). This carbon-centered radical is resonance-stabilized but the peroxyl radical formed from it by interaction with the oxygen molecule is not stable but capable of inducing oxidation. Therefore, the antioxidant activity of β-carotene is higher at lower oxygen pressures (31). β-Carotene and α-tocopherol may exert a synergistic effect by acting at different portions of the membrane and LDL—α-tocopherol at the surface and β-carotene in the interior—and by the scavenging of β-carotene-derived peroxyl radicals by α-tocopherol, thus inhibiting the above-mentioned oxidation induced by the β-carotene-derived peroxyl radical.

The scheme for the possible mechanism of inhibition of oxidation by α-tocopherol, β-carotene, and ascorbic acid is

TABLE 3
Effects of radicals on antioxidant efficacy of α-tocopherol[1]

Radical X$^{\cdot}$	Rate constant k X$^{\cdot}$ + E	Rate constant k X$^{\cdot}$ + lipid	Ratio of (X$^{\cdot}$ + E) to (X$^{\cdot}$ + lipid)[2]
	$mol \cdot L^{-1} \cdot s^{-1}$		
HO$^{\cdot}$	1×10^{10}	1×10^{9}	1:100
LO$^{\cdot}$	1×10^{8}	1×10^{6}	1:10
$LO_2^{\cdot}$, $HO_2^{\cdot}$	1×10^{6}	1×10^{2}	10:1

[1] X$^{\cdot}$, attacking radical; E, α-tocopherol; HO$^{\cdot}$, hydroxyl radical; LO$^{\cdot}$, alkoxyl radical; $LO_2^{\cdot}$, $HO_2^{\cdot}$, peroxyl radicals.

[2] The ratio of the rate of inhibition reaction 1 to that of propagation reaction 2, assuming the ratio of the concentration of α-tocopherol to that of polyunsaturated lipids is 1:1000.

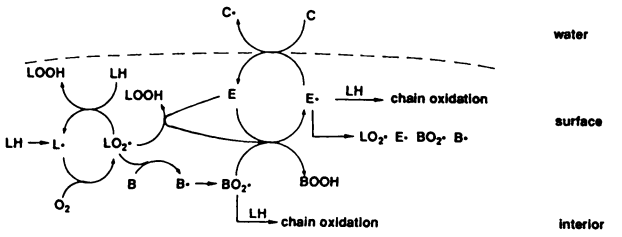

FIGURE 3. Possible scheme for the inhibition of oxidation of the membranes and LDL by a combination of β-carotene (B), vitamin C (C), and vitamin E (E). LH, lipid; L·, lipid radical (carbon-centered); LO_2·, lipid peroxyl radical; LOOH, lipid hydroperoxide; B·, β-carotene-derived conjugated polyene radical; BO_2·, β-carotene peroxyl radical; BOOH, β-carotene hydroperoxide; E·, vitamin E radical; and C·, vitamin C radical.

summarized in **Figure 3**. Ascorbic acid acts in an aqueous phase, whereas α-tocopherol and β-carotene act in the lipophilic compartments. Chemically, β-carotene is less reactive toward radicals than are α-tocopherol and ascorbic acid. Ascorbic acid and α-tocopherol inhibit oxidation synergistically as discussed above. α-Tocopherol is more effective than ascorbic acid in scavenging radicals in membranes and lipoproteins. Ascorbic acid reduces the resulting α-tocopheroxyl radical efficiently to regenerate α-tocopherol and possibly to inhibit oxidation induced by α-tocopheroxyl radicals. α-Tocopherol and β-carotene may exert a cooperative effect by residing and scavenging radicals at different positions in the lipophilic compartment. The direct interaction between β-carotene and ascorbic acid should not be important. In conclusion, the combination of α-tocopherol, β-carotene, and ascorbic acid may be effective in inhibiting oxidative damage, especially in vivo where oxygen concentration is low.

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