α-Tocopherol and Ascorbic Acid Decrease the Production of β-Apo-carotenals and Increase the Formation of Retinoids from β-Carotene in the Lung Tissues of Cigarette Smoke–Exposed Ferrets In Vitro

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ABSTRACT Previously, we found that exposing ferrets to cigarette smoke enhanced oxidative excentric cleavage of β-carotene. In the present study, we examined whether α-tocopherol, ascorbic acid, or the two combined can prevent smoke-altered β-carotene metabolism. In vitro incubation of β-carotene (10 μmol/L) with lung postnuclear fractions from ferrets exposed to cigarette smoke was carried out in the absence or presence of α-tocopherol (50 μmol/L), ascorbic acid (10 or 50 μmol/L), or both vitamins to evaluate their effects on the production of β-apo-carotenals and retinoids from β-carotene. The oxidative cleavage metabolites of β-carotene, β-apo-carotenals (β-apo-14, β-apo-12, β-apo-10, and β-apo-8), retinoic acid (RA), and retinal were analyzed by HPLC. We found that the smoke-enhanced production of individual β-apo-carotenals was significantly decreased by 36–77% when α-tocopherol (50 μmol/L) and ascorbic acid (50 μmol/L) were added together to the incubation mixture. α-Tocopherol alone had a modest effect. Ascorbic acid in the presence of α-tocopherol inhibited the production of β-apo-carotenals in a dose-dependent manner, although ascorbic acid alone had no effect. In contrast, the production of RA and retinal among smoke-exposed ferrets was substantially increased (3–fold, P < 0.05) when both α-tocopherol and ascorbic acid were added to the incubation mixtures. However, when ascorbic acid or α-tocopherol alone was added, the production of RA among smoke-exposed ferrets increased only modestly (80%, P < 0.05) and did not differ from the RA levels in control ferrets. In conclusion, these data indicate that α-tocopherol and ascorbic acid may act synergistically in preventing the enhanced oxidative excentric cleavage of β-carotene induced by smoking exposure, thereby facilitating the conversion of β-carotene into RA and retinal.


KEY WORDS: • carotenoids • retinoids • α-tocopherol • ascorbic acid • cigarette smoke

It has been demonstrated that cigarette smoking substantially decreases plasma levels of β-carotene (1–4) and vitamin C (4–7). Cigarette smoking also slightly reduces plasma levels of vitamin E (8–10) or α-tocopherol (1,11,12).

In our previous study, we found that exposing ferrets to cigarette smoke enhanced in vitro oxidative excentric cleavage of β-carotene (13), which suggests that β-carotene is unstable in the free radical–rich environment of the lungs in cigarette smokers, and that such an environment alters β-carotene metabolism and produces oxidative excentric cleavage metabolites. Several studies including ours showed that these oxidative excentric cleavage products of β-carotene are involved in the carcinogenic process and interfere with retinoic acid (RA)3 metabolism (14–19). A possible explanation for the instability of β-carotene in the lung is that exposure of lung cells to smoke results in increased lung cell oxidative stress, thereby decreasing other antioxidants, such as ascorbic acid and α-tocopherol, which normally have a stabilizing effect on the reduced form of β-carotene.

Ascorbic acid, α-tocopherol, and β-carotene are potent reducing agents that act as antioxidants in vitro and in vivo (20,21). Experimental data suggest that there are interactions among these individual antioxidants with beneficial mutual protection and regeneration. α-Tocopherol is an effective chain-breaking antioxidant, thus inhibiting lipid peroxidation (21). Ascorbic acid can regenerate α-tocopherol from the α-tocopherol radical during lipid peroxidation (21) and convert the β-carotene radical back to its reduced form (22–24). α-Tocopherol also can protect β-carotene from autoxidation (25,26). Conversely, β-carotene can recycle the oxidized form of α-tocopherol into its reduced form (22–24,27). Therefore, using a combination of these antioxidants could be more advantageous in antioxidant protection than using a single antioxidant alone. However, it is unclear whether ascorbic acid, α-tocopherol, or both could prevent the smoke-altered β-carotene metabolism (e.g., enhanced excentric cleavage products) in lung tissue. We thus conducted in vitro incubations of β-carotene with lung postnuclear fractions from ferrets.
exposed to cigarette smoke in the presence and absence of α-tocopherol, ascorbic acid, or both vitamins.

MATERIALS AND METHODS

Chemicals. All-trans-β-carotene, all-trans-RA, all-trans-retinal, retinyl acetate echinenone, α-tocopherol, ascorbic acid, dithiothreitol (DTT), Hepes, NAD⁺, EDTA, potassium chloride, Tween 40, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical. β-Apo-8′-carotenal, apo-10′-carotenal, apo-12′-carotenal, apo-14′-carotenal were gifts from Hoffmann-La Roche. All HPLC solvents were obtained from J. T. Baker Chemical and were filtered through a 0.45-μm membrane filter before use.

Animals, diet and study groups. The maintenance and husbandry of ferrets as well as the design of the in vivo study were described in detail elsewhere (13,28). Male ferrets were from Marshall Farms. Ferrets were fed dry ferret food (Purina Ferret Chow) and were watered ad libitum.

Preparation of postnuclear fractions from lung tissue. We used the postnuclear fraction of lung tissue in the in vitro incubations because this fraction contains excentric cleavage activity (29). The amount of smoke exposure in the ferret is approximately equivalent to 1.5 packages of cigarettes/d in humans based upon the concentrations of urinary cotinine equivalents (13).

Preparation of postnuclear fractions from lung tissue. We used the postnuclear fraction of lung tissue in the in vitro incubations because this fraction contains excentric cleavage activity (29). The postnuclear fractions of lung tissue were prepared according to the procedures described previously with minor modification (29,30). Briefly, ferret lung tissue was homogenized on ice in a Brinkmann Polytron homogenizer for 15 s at speed 10 with cold Hepes buffer (w/v, 1:5), pH 7.4, containing 20 mmol/L Hepes, 1.15% KCl, 1 mmol/L EDTA, and 0.1 mmol/L DTT. The homogenate was centrifuged in a Sorval RT6000 refrigerated centrifuge (Du Pont) at 2100 × g at 4°C for 30 min. The supernatant fractions were collected and stored at −80°C. The protein concentration was determined using the bicinchoninic acid protein assay (Pierce).

Incubation and extraction procedure. β-Carotene was purified using a 5% water-weakened alumina column. The standard incubation mixture contained 1 mg of postnuclear protein, 10 μmol/L β-carotene (dissolved in DMSO), 50 μmol/L α-tocopherol (dissolved in DMSO), 10 or 50 μmol/L ascorbic acid (dissolved in deionized and distilled water), 20 mmol/L Hepes buffer (pH 7.4), 2 mmol/L NAD⁺, 150 mmol/L KCl, 2 mmol/L DTT, and 1 mmol/L EDTA in a final volume of 1 mL. The concentration of 50 μmol/L of ascorbic acid used here was similar to the mean plasma levels in U.S. adults ≥19 y old (44 μmol/L) in the third National Health and Nutrition Examination Survey (31). The amounts of α-tocopherol and β-carotene used here were higher than the normal plasma levels in the U.S. adults ≥19 y old (26 μmol/L for α-tocopherol, 0.4 μmol/L for β-carotene) (31), but they were similar to the serum levels in the intervention groups [40 μmol/L (50th percentile) to 49 μmol/L (80th percentile) for α-tocopherol; 6 μmol/L (50th percentile) to 9 μmol/L (80th percentile) for β-carotene] in the Alpha-Tocopherol, Beta Carotene Cancer Prevention Study (ATBC Study), a large clinical trial among smokers (32). The incubation mixtures were incubated under red light in glass vials at 37°C in a shaking water bath for 60 min. Two control vials that lacked either substrate or postnuclear protein were assayed. The vials were recovered and the incubation mixtures were exposed to room air as the gas phase. After incubation, β-apo-carotenals (β-apo-14′, β-apo-12′, β-apo-10′, and β-apo-8′), RA, and retinal were extracted and analyzed by the method described previously with minor modification (29,30). Briefly, 100 μL of an ethanol solution of 0.5 mol/L KOH was added to the 1-mL incubation mixture to stop the reaction, followed by the addition of retinyl acetate and echinenone (internal standard) in 50 μL of ethanol. The mixture was extracted by adding 3 mL of hexane, mixing on a vortex, and then centrifuging for 10 min at 800 × g at 4°C. The hexane layer was removed. The residue was then acidified by adding 50 μL of 6 mol/L HCl. A second extraction was performed with 3 mL hexane. The two extractions were pooled, dried under N₂, and resuspended in 50 μL of ethanol for injection into the HPLC system as described below. HPLC analysis for β-apo-carotenals, RA, and retinal. A gradient reversed-phase HPLC was used for the analysis of β-apo-carotenals (β-apo-14′, β-apo-12′, β-apo-10′, and β-apo-8′), RA, and retinal as described previously with minor modification (13). Briefly, the gradient reversed-phase HPLC system consisted of a Waters 615 Pump (Waters Chromatography Division/Millipore), a Waters 717 Plus autosampler, a Waters 996 photodiode array detector, a pecosphere-3 C18 4.6 × 3.3 cm reverse phase column (Perkin-Elmer) and a Waters 840-Digital 350 data station. The HPLC mobile phase was acetonitrile/tetrahydrofuran:H₂O (50:20:30 by vol, 1% ammonium acetate in H₂O for solvent A and 50:44:6 by vol, 1% ammonium acetate in H₂O for solvent B). The gradient procedure (at a flow rate of 1 mL/min) was as follows: 100% solvent A was used for 3 min followed by a 6-min linear gradient to 40% solvent A and 60% solvent B, then a 12-min hold at 40% solvent A and 60% solvent B, and then a 7-min gradient back to 100% solvent A. In this HPLC system, RA, retinal, β-apo-14′-carotenal, β-apo-12′-carotenal, β-apo-10′-carotenal, β-apo-8′-carotenal, and β-carotene eluted at 6.8, 8.6, 11.6, 16.3, 17.2, 19.9, and 22.5 min, respectively.

<table>
<thead>
<tr>
<th>β-Apo-carotenals</th>
<th>β-Apo-14′</th>
<th>β-Apo-12′</th>
<th>β-Apo-10′</th>
<th>β-Apo-8′</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmol/(mg protein · h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not smoke-exposed</td>
<td>19 ± 5a</td>
<td>45 ± 4a</td>
<td>32 ± 6a</td>
<td>11 ± 2a</td>
</tr>
<tr>
<td>Smoke exposed</td>
<td>106 ± 36b</td>
<td>88 ± 13b</td>
<td>90 ± 16c</td>
<td>60 ± 14c</td>
</tr>
<tr>
<td>Smoke exposed + 10 μmol/L vitamin C</td>
<td>124 ± 32b</td>
<td>97 ± 27b</td>
<td>86 ± 28b</td>
<td>65 ± 16c</td>
</tr>
<tr>
<td>Smoke exposed + 50 μmol/L vitamin C</td>
<td>104 ± 39b</td>
<td>92 ± 23b</td>
<td>81 ± 23c</td>
<td>55 ± 11c</td>
</tr>
<tr>
<td>Smoke exposed + 50 μmol/L vitamin E</td>
<td>90 ± 24b</td>
<td>70 ± 12b</td>
<td>56 ± 13b</td>
<td>27 ± 5b</td>
</tr>
<tr>
<td>Smoke exposed + 10 μmol/L vitamin C + 50 μmol/L vitamin E</td>
<td>83 ± 29b</td>
<td>80 ± 17b</td>
<td>48 ± 17b</td>
<td>18 ± 5a</td>
</tr>
<tr>
<td>Smoke exposed + 50 μmol/L vitamin C + 50 μmol/L vitamin E</td>
<td>34 ± 9a</td>
<td>56 ± 11a</td>
<td>36 ± 8a</td>
<td>14 ± 4a</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 6.
2 Means in a column with different letters differ, P < 0.05.
29.5 min, respectively. Retinyl acetate (internal standard for retinoids) and echinenone (internal standard for β-apo-carotenals) eluted at 10.1 and 25.2 min, respectively. Individual retinoids and carotenoids were identified by co-elution with standards and absorption spectrum analysis. Retinoids and carotenoids were quantified relative to the internal standard, by determining peak areas calibrated against known amounts of standards.

Statistical analysis. Results were expressed as means ± SD and data were analyzed by one-way ANOVA followed by Tukey’s honest significant difference test. Differences were considered significant at P < 0.05.

RESULTS

After the incubation of β-carotene alone with lung postnuclear fractions, the formation of β-apo-carotenals in smoke-exposed ferrets was approximately fivefold higher for β-apo-14’ and β-apo-8’, onefold higher for β-apo-12’, and twofold higher for β-apo-10’ compared with control ferrets (Table 1). The enhanced formation of β-apo-carotenals after smoke exposure was 68% lower for β-apo-14’, 36% lower for β-apo-12’, 60% lower for β-apo-10’, and 77% lower for β-apo-8’ when 50 μmol/L of α-tocopherol and 50 μmol/L of ascorbic acid were added to the incubation mixture together (Table 1). In the presence of α-tocopherol, ascorbic acid inhibited the formation of β-apo-carotenals in a dose-dependent manner, although ascorbic acid alone had no effect (Fig. 1). In addition, 50 μmol/L of α-tocopherol alone significantly lowered the production of β-apo-10’ (by 38%) and β-apo-8’ (by 55%), but not β-apo-14’ and β-apo-12’-carotenal. Higher concentrations of α-tocopherol in the incubation mixture also did not affect their production (data not shown). Similarly, the combination of 50 μmol/L of α-tocopherol and 10 μmol/L of ascorbic acid significantly lowered the production of β-apo-10’ (by 47%), and β-apo-8’-carotenal (by 70%).

Smoke exposure significantly lowered the production of RA from β-carotene, but not retinal compared with controls not exposed to smoke (Table 2). However, the production of RA in the lung postnuclear fractions of smoke-exposed ferrets was significantly higher (by approximately twofold) when 50 μmol/L of α-tocopherol combined with either 10 or 50 μmol/L of ascorbic acid was added to the incubation mixtures (Table 2). When 50 μmol/L of α-tocopherol alone or ascorbic acid alone at either 10 or 50 μmol/L concentrations was added to the incubation mixtures, the production of RA in the lung postnuclear fractions of smoke-exposed ferrets was also significantly higher (~80%) compared with that in the lung postnuclear fractions of smoke-exposed ferrets without additions of either α-tocopherol or ascorbic acid. However, the increased production of RA by α-tocopherol or ascorbic acid alone reached levels that were similar to that of control ferrets. In addition, α-tocopherol alone or the combination of α-tocopherol and ascorbic acid at either 10 or 50 μmol/L also significantly increased the production of retinal compared with control ferrets (by 60%, 2.2-fold, and 2.3-fold, respectively) (Table 2).

DISCUSSION

In the present incubation study, we demonstrated that the smoke-enhanced production of β-apo-carotenals, excentric cleavage oxidative metabolites of β-carotene, was decreased by the combination of α-tocopherol and ascorbic acid. In contrast, the production of RA among smoke-exposed ferrets was substantially elevated by the combination of α-tocopherol and ascorbic acid. These findings may have potential implications in cancer prevention because excentric cleavage oxidative metabolites of β-carotene could be related to carcinogenesis. β-Carotene metabolites were shown to facilitate the binding of benzo[a]pyrene metabolites to DNA (14). Furthermore, Perocco et al. (15) found that the induction of BALB/c 3T3 cell transformation by benzo[a]pyrene was markedly enhanced by the addition of β-carotene, although from their study it is uncertain whether the enhancement of cell-transforming activity of β-carotene was due to β-carotene itself or its metabolites. Excentric cleavage oxidative metabolites of β-carotene may also be associated with carcinogenesis through their induction of cytochrome P450 enzymes, which can convert protocarcinogens into activated carcinogens, facilitate lipid peroxidation, or enhance RA catabolism, thereby diminishing retinoid signaling (18). For example, β-apo-8’-carotenal was shown to be a strong inducer of cytochrome P450 enzymes (17). Several studies have shown that β-apo-carotenals inhibit mitochondria respiration and elevate the accumulation of mal-
In an in vivo experiment of ferrets perfused with and without smoke exposure was evaluated in two previous studies. By the fact that the excentric cleavage metabolites of 
\[ \text{H9252} \]
alone. Our observed synergistic protective effects of 
\[ \text{H9251} \]
exposed to environmental tobacco smoke (38) supplemented with high doses of 
\[ \text{H9251} \]
carotenal in intestinal mucosa was gradually decreased with increasing concentrations of 
\[ \alpha \text{-tocopherol}, \text{ascorbic acid, and } \beta \text{-carotene in combination against oxidative damage in an in vitro cell system (23,26,36,37) and in humans exposed to environmental tobacco smoke (38–41).}

The effect of 
\[ \alpha \text{-tocopherol alone on } \beta \text{-carotene metabolism without smoke exposure was evaluated in two previous studies. In an in vivo experiment of ferrets perfused with } \beta \text{-carotene and various concentrations of } \alpha \text{-tocopherol, the production of } \beta \text{-apo-12'} \text{-carotenal in intestinal mucosa was gradually decreased with increasing concentrations of } \alpha \text{-tocopherol, albeit this change was not significant. However, the production of retinal, a central cleavage product of } \beta \text{-carotene, was significantly increased (42). In the present study, we observed that } \alpha \text{-tocopherol alone decreased the production of other the smoke-induced } \beta \text{-apo-carotenals, including } \beta \text{-apo-14', } \beta \text{-apo-12', } \beta \text{-apo-10' and } \beta \text{-apo-8'. The reduction was significant for the latter two metabolites. In an in vitro incubation experiment using intestinal mucosa from apparently healthy rats, with the addition of } \alpha \text{-tocopherol, } \beta \text{-carotene's excentric cleavage products were almost completely blocked and } \beta \text{-carotene was converted exclusively into the central cleavage product of retinal (43). In the present study, } \alpha \text{-tocopherol alone did not completely block the formation of excentric cleavage metabolites of } \beta \text{-carotene, which may be explained by the fact that the excentric cleavage metabolites of } \beta \text{-carotene were substantially induced by smoke exposure in the present study (Table 1). Several other factors such as different species and organs of animals may also contribute to the discrepancy in the magnitudes of the inhibitory effects of } \alpha \text{-tocopherol on the production of } \beta \text{-apo-carotenals from } \beta \text{-carotene in the various studies.}

Ascorbic acid, which can facilitate both } \alpha \text{-tocopherol recycling and } \beta \text{-carotene stability (23), is an important part of antioxidant defense under conditions of smoke exposure. Ascorbic acid itself was shown to protect lipids against peroxidative damage in human plasma by scavenging oxygen-derived free radicals (20). Epidemiologic studies have demonstrated consistently that diets high in fruit and vegetables, and hence high in ascorbic acid, are associated with a lower risk for cancers of the lung, oral cavity, esophagus, and stomach (44). Smoking, however, substantially lowers circulating ascorbic acid levels (4–7). In the ATBC Study, a randomized clinical trial among 29,133 male smokers, 50 mg/d } \alpha \text{-tocopherol supplementation combined with 20 mg/d } \beta \text{-carotene for 5–8 y did not protect against smoke-related lung cancer (32). Although plasma ascorbic acid levels were not reported in the ATBC study, lower levels of plasma ascorbic acid would be expected among the smoking participants in that study than in a non-smoking population. This may have resulted in a loss of protection usually conferred by ascorbic acid on } \alpha \text{-tocopherol and } \beta \text{-carotene. Although, in the present study, ascorbic acid alone had no effect on the inhibition of the formation of } \beta \text{-apo-carotenals, it appeared to interact with } \alpha \text{-tocopherol to protect against the formation of excentric cleavage products from } \beta \text{-carotene because the inhibitory effects of the combination of } \alpha \text{-tocopherol and ascorbic acid on excentric cleavage production of } \beta \text{-carotene were significantly greater than with } \alpha \text{-tocopherol alone.}

Data from the present study suggest that adding } \alpha \text{-tocopherol and ascorbic acid when using high doses of } \beta \text{-carotene may prevent excessive formation of excentric cleavage products of } \beta \text{-carotene and the subsequent cascade of events that may result from them (i.e., bioactivating carcinogens, destroying RA via induction of cytochrome P450 enzymes, and facilitating the binding of benzo[a]pyrene metabolites to DNA). Using a combination of antioxidant nutrients that can provide mutual protection and regeneration might prove to be an effective chemopreventive strategy against lung cancer in smokers.}

### Table 2

<table>
<thead>
<tr>
<th>Incubation of ( \beta \text{-carotene (10 } \mu \text{mol/L) }</th>
<th>RA</th>
<th>Retinal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not smoke-exposed</td>
<td>RA: 3.20 ± 1.02(^a)</td>
<td>Retinal: 25 ± 8(^a)</td>
</tr>
<tr>
<td>Smoke exposed</td>
<td>RA: 1.94 ± 0.61(^a)</td>
<td>Retinal: 29 ± 11(^a)</td>
</tr>
<tr>
<td>Smoke exposed + 10 } \mu \text{mol/L vitamin C}</td>
<td>RA: 3.55 ± 1.91(^b)</td>
<td>Retinal: 28 ± 9(^a)</td>
</tr>
<tr>
<td>Smoke exposed + 50 } \mu \text{mol/L vitamin C}</td>
<td>RA: 3.31 ± 1.32(^b)</td>
<td>Retinal: 21 ± 9(^a)</td>
</tr>
<tr>
<td>Smoke exposed + 50 } \mu \text{mol/L vitamin E}</td>
<td>RA: 3.60 ± 0.49(^b)</td>
<td>Retinal: 41 ± 12(^b)</td>
</tr>
<tr>
<td>Smoke exposed + 10 } \mu \text{mol/L vitamin C} + 50 } \mu \text{mol/L vitamin E}</td>
<td>RA: 5.53 ± 1.29(^c)</td>
<td>Retinal: 79 ± 7(^c)</td>
</tr>
<tr>
<td>Smoke exposed + 50 } \mu \text{mol/L vitamin C} + 50 } \mu \text{mol/L vitamin E}</td>
<td>RA: 5.84 ± 1.08(^c)</td>
<td>Retinal: 82 ± 9(^c)</td>
</tr>
</tbody>
</table>

1. \(^a\) Values are means ± SD, \( n = 6 \).  
2. \(^a\) Means in a column with different letters differ, \( P < 0.05 \).


