Reactions of β-Carotene with Cigarette Smoke Oxidants. Identification of Carotenoid Oxidation Products and Evaluation of the Prooxidant/Antioxidant Effect

Daniel L. Baker,[†,‡] Ed S. Krol,[†] Neil Jacobsen,[§] and Daniel C. Liebler*[,†]

Department of Pharmacology and Toxicology, College of Pharmacy, and Department of Chemistry, University of Arizona, Tucson, Arizona 85721-0207

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Recent intervention trials reported that smokers given dietary β-carotene supplementation exhibited an increased risk of lung cancer and overall mortality. β-Carotene has been hypothesized to promote lung carcinogenesis by acting as a prooxidant in the smoke-exposed lung. We have examined the interactions of cigarette smoke with β-carotene in model systems. Both whole smoke and gas-phase smoke oxidized β-carotene in toluene to several products, including carbonyl-containing polyene chain cleavage products and β-carotene epoxides. A major product of the reaction was identified as 4-nitro-β-carotene, which was formed by nitrogen oxides in smoke. Both cis and all-trans isomers of 4-nitro-β-carotene were detected. The hypothesis that smoke-driven β-carotene autoxidation exerts prooxidant effects was tested in a liposome system. Lipid peroxidation in dilinoleoylphosphatidylcholine liposomes exposed to gas-phase smoke was modestly inhibited by the incorporation of 0.1 mol % β-carotene. Both the lipid soluble antioxidant α-tocopherol and the water soluble antioxidant ascorbate were oxidized more slowly by gas-phase smoke exposure in liposomes containing β-carotene. These data indicate that β-carotene exerts weak antioxidant effects against smoke-induced oxidative damage in vitro. It is unlikely that a prooxidant effect of β-carotene occurs under biologically relevent conditions or is responsible for an increased incidence of lung cancer observed in smokers who consume β-carotene supplements.

Introduction

Lung cancer is the leading cause of cancer death in the United States for both men and women (*1*). Observational epidemiology has shown that increased dietary intakes and elevated blood levels of β-carotene are associated with a decreased risk of lung cancer (*2*−*4*). However, recent intervention trials in heavy smokers indicated increased lung cancer incidences and deaths in those participants receiving β-carotene supplementation versus placebo (*5*, *6*). These results raise questions about the mechanism(s) by which supplemental β-carotene enhanced lung cancer in these cohorts.

Cigarette smoke is a complex mixture of literally thousands of compounds, many of which are known or suspected human carcinogens (*7*, *8*). Smoke may be divided into two main fractions on the basis of separation by a glass fiber filter (*8*). Tar is defined as that portion of whole smoke trapped by the filter and contains quinone/hydroquinone redox couples (*8*−*10*) that are capable of generating superoxide anion catalytically (*8*). Gas-phase smoke passes through the filter and contains a steady-state concentration of radicals resulting from nitrogen oxide-driven autoxidation of low-molecular weight hydrocarbons present in the smoke stream (*11*).

Despite considerable interest in β-carotene and lung cancer in the past decade, little is known about the chemistry and consequences of interactions between cigarette smoke and β-carotene. Handelman et al. have shown that β-carotene is depleted from human plasma exposed to gas-phase cigarette smoke, but the resulting products have not been examined (*12*). β-Carotene is known to react with NO_2 in hexane solution, although oxidation products have not been characterized (*13*). Everett and co-workers have shown by pulse radiolysis that NO_2 oxidizes β-carotene in solution via the β-carotene radical cation (eq 1) (*14*, *15*).

$$\mathrm{Car} + \mathrm{NO_2} \rightarrow \mathrm{Car^{+\bullet}} + \mathrm{NO_2^-} \qquad (1)$$

Peroxyl radicals, which are abundant in smoke (*8*, *11*), have been shown previously to oxidize β-carotene to epoxides and to aldehyde and ketone chain cleavage products (*16*−*19*) (see Figure 1 for structures discussed in the text).

β-Carotene is known to exert antioxidant effects in model systems, apparently by acting as a chain-breaking antioxidant. However, Burton and Ingold reported that β-carotene antioxidant activity is diminished at high oxygen tensions (*20*). This apparently reflects the ability of oxygen to add reversibly to β-carotene-derived radicals, thus generating peroxyl radicals that facilitate carotenoid

* To whom correspondence should be addressed: Department of Pharmacology and Toxicology, College of Pharmacy, The University of Arizona, P.O. Box 210207, Tucson, AZ 85721-0207. Telephone: (520) 626-4488. Fax: (520) 626-2466. E-mail: liebler@pharmacy.arizona.edu.
† Department of Pharmacology and Toxicology, College of Pharmacy.
‡ Present address: Department of Physiology and Biophysics, University of Tennessee at Memphis, 894 Union Ave., Memphis, TN 38163.
§ Department of Chemistry.

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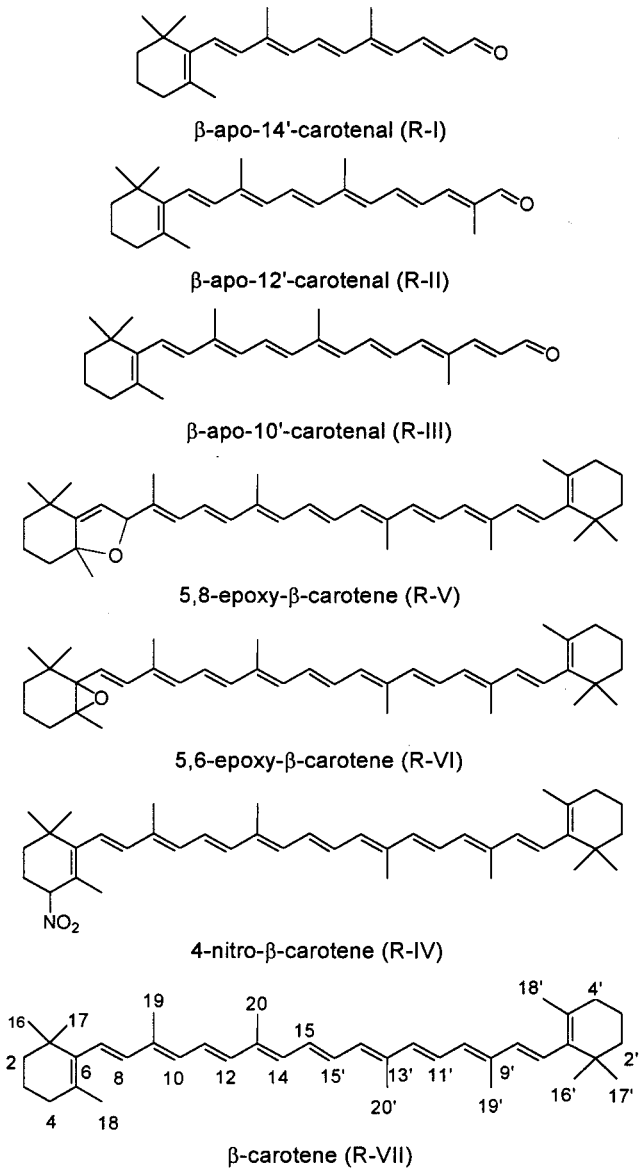


Figure 1. Structures and numbering schemes for all-trans *β*-carotene and the *β*-carotene oxidation products that are discussed herein.

autoxidation (eqs 2−4).

$$\text{Car} + \text{R}^{\bullet} \rightarrow \text{R-Car}^{\bullet} \quad (2)$$

$$\text{R-Car}^{\bullet} + \text{O}_2 \rightarrow \text{R-Car-OO}^{\bullet} \quad (3)$$

$$\text{R-Car-OO}^{\bullet} + \text{other molecules} \rightarrow \text{oxidative damage} \quad (4)$$

This oxygen-driven *β*-carotene autoxidation has been offered as the basis for possible *prooxidant* effects of *β*-carotene. Recently, Mayne et al. proposed that smoke-driven *β*-carotene autoxidation may enhance smoke-induced oxidative damage in the lungs of smokers taking supplemental *β*-carotene (*21*).

To test the hypothesis that *β*-carotene amplifies smoke-induced oxidative damage, we have investigated the oxidation of *β*-carotene by smoke and the effect of *β*-carotene on smoke-induced lipid peroxidation and antioxidant consumption in homogeneous solution and liposomal model systems. We have characterized the products of cigarette smoke-dependent oxidation of *β*-carotene in toluene and identified 4-nitro-*β*-carotene as a novel smoke-dependent oxidation product.

Experimental Procedures

Chemicals. All-trans *β*-carotene was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). Research grade cigarettes (1R3) were obtained from the University of Kentucky Tobacco and Health Research Institute (Lexington, KY). Cambridge filter pads were obtained from Performance Systematics (Caledonia, MI). Linoleic acid was purchased from Nu Chek Prep (Elysian, MN). 12-Hydroxylauric acid, soy lipoxidase (type I, EC 1.13.11.12), L-ascorbic acid, and ferrozine were obtained from Sigma Chemical Co. (St. Louis, MO). Rhodium (5% on alumina), PtO_2, pentafluorobenzoyl chloride, and diisopropylethylamine were purchased from Aldrich Chemical Co. (Milwaukee, WI). D-α-Tocopherol was a gift from Henkel Fine Chemicals (LaGrange, IL). BLPC[1] and DLPC were obtained from Avanti Polar Lipids (Alabaster, AL). All other chemicals were of the highest purity available and were used without further purification.

Instrumentation. Product analyses were performed with a Hewlett-Packard model 1040M diode-array detector coupled to a Hewlett-Packard 1050 four-channel gradient pump (Hewlett-Packard, Palo Alto, CA). Hewlett-Packard Chemstation software (version A.02.02) was utilized for system control and data analysis. LC/MS was performed on a Finnigan TSQ-7000 triple-quadrupole mass spectrometer with an atmospheric pressure chemical ionization source (Finnigan MAT, San Jose, CA).

All NMR spectra were acquired on a Bruker AM-500 spectrometer operating at a ^{1}H frequency of 500.13 MHz, using an inverse broad-band 5 mm probe (*22*). Samples were dissolved in 0.6 mL of benzene-d_6 at low light levels, and spectra were acquired at 30 °C with lights off and the spectrometer bore capped. All two-dimensional (2D) spectra were acquired without spinning in the TPPI (*23*) mode with 64 repetitions per t_1 value by collecting 2048 complex data points in t_2 and 750 real data points in t_1. For inverse experiments, no ^{13}C decoupling was used during acquisition. NMR data were analyzed using the Felix95 software package (BIOSYM/Molecular Simulations, San Diego, CA). A skewed, 45°-shifted sine-bell window was used for HMQC (*24*, *25*) spectra, and a sine-bell window function was used for DQF-COSY (*26*) spectra. In both cases, zero-filling was employed to give a final matrix of 2048 (F_2) × 1024 (F_1) real data points. The 2D spectra included a ^{1}H chemical shift range from 8 to 0 ppm and a ^{13}C chemical shift range from 150 to 0 ppm. In HMQC spectra of U-^{13}C samples, the cross-peaks were visibly broadened in the F_1 dimension by $^1J_{CC}$ coupling, but this did not significantly affect peak overlap or assignments. The residual C_6H_5D peak was used as a reference for ^{1}H (7.158 ppm) and ^{13}C (128.33 ppm).

Capillary gas chromatography was performed on a Hewlett-Packard 5890 series II gas chromatograph equipped with a Hewlett-Packard 7673 autosampler, a cool on-column injector, and an electron capture detector (Hewlett-Packard, Avondale, PA). Chemstation software 3365 series II was used for system control and data processing. Samples were analyzed on a 0.53 mm × 20 m DB-5 capillary column (J&W Scientific, Folsom, CA).

GC/MS was performed on a Fisons-VG MD-800 analyzer coupled to a Carlo Erba 8000 series gas chromatograph fitted with a Fisons A200S autosampler and on-column injector (Fisons Instruments, Beverly, MA). Analyses were carried out in the electron ionization mode at 70 eV. Fisons-VG Lab-Base system software was used for system control and data processing. Samples were analyzed on a 0.25 mm × 30 m DB-5ms capillary column (J&W Scientific).

[1] Abbreviations: APCI, atmospheric-pressure chemical ionization; BLPC, bovine liver phosphatidylcholine; DLPC, dilinoleoylphosphatidylcholine; DQF-COSY, double-quantum-filtered correlation spectroscopy; GC/MS, gas chromatography/mass spectrometry; HFAME, hydroxy fatty acid methyl ester; HMQC, heteronuclear multiple-quantum coherence; LC/MS, liquid chromatography/mass spectrometry.

Cigarette Smoke Oxidation of *β*-Carotene in Solution. *β*-Carotene (250 *μ*M in toluene) was exposed to whole or gas-phase 1R3 cigarette smoke, 200 mL every 15 min at 40 °C for 3 h using a smoking apparatus consisting of a 20 mL syringe, a three-way stopcock, and an in-line filter. Gas-phase smoke was that fraction of smoke that passed through a Cambridge glass fiber filter. Unoxidized *β*-carotene and products resulting from the oxidation of *β*-carotene in solution by whole or gas-phase 1R3 cigarette smoke were separated on a 4.6 mm × 250 mm Allsphere ODS-2 5 *μ*m HPLC column (Alltech Associates, Deerfield, IL) with gradient elution at a rate of 1.0 mL/min (*19*). Initially, the mobile phase consisted of 100% solvent A (85:15: 0.1 v/v/w acetonitrile/methanol/ammonium acetate). At 8 min, solvent B (2-propanol) was introduced in a linear gradient to 30% by 12 min. From 25 to 30 min, the solvent was returned to 100% solvent A using a linear gradient. Compounds were detected by monitoring the UV/vis absorbance.

Identification of *β*-Carotene Oxidation Products. *β*-Carotene (500 *μ*M in toluene) was oxidized with gas-phase smoke (200 mL every 5 min) at 40 °C for 1 h. Products were separated by reverse-phase HPLC on a 10 mm × 300 mm Allsphere ODS-2 5 *μ*m column using a mobile phase of acetonitrile/methanol/2-propanol (59.5:10.5:30 v/v/v) at a rate of 4 mL/min with detection at 450 nm. Fraction R−IV was collected for further analysis by cyano HPLC. Cyano HPLC was carried out on a 10 mm × 300 mm Allsphere cyano column, and compounds were eluted with hexane/ethyl acetate (99:1 v/v) at a rate of 4.0 mL/min with detection at 450 nm (*17*). Individual products were isolated and characterized by LC/MS and NMR.

Atmospheric-pressure chemical ionization LC/MS was performed on a 4.6 mm × 250 mm Allsphere cyano 5 *μ*m column, with a mobile phase of hexane/ethyl acetate (99:1 v/v) at a rate of 1.0 mL/min. Mass spectra were obtained in both the positive and negative modes. Double-quantum-filtered ^{1}H COSY spectra were obtained in benzene-d_6 at 30 °C.

Oxidation of [^{13}C]-*β*-Carotene. *β*-Carotene (98% ^{13}C) was obtained from Martek Corp. (Columbia, MD) as a crude algal hexane extract, which contained all-trans *β*-carotene as well as several mono cis *β*-carotene isomers. Mono cis isomers of *β*-carotene were converted to the all-trans form using the method of Foote et al. (*27*). All-trans *β*-carotene was purified by reverse-phase HPLC on a 10 mm × 300 mm Allsphere ODS-2 5 *μ*m column with a mobile phase of methanol/hexane (85:15 v/v) at a rate of 4.5 mL/min. The structure and isotopic purity of all-trans *per*-[^{13}C]-*β*-carotene were analyzed by UV/vis absorbance, LC/MS, and NMR. Purified all-trans *per*-[^{13}C]-*β*-carotene (150−200 *μ*M in toluene) was oxidized with 100 mL of gas-phase smoke every 5 min at 40 °C for 1 h. Products were anlayzed by reverse-phase and cyano HPLC as described above. Individual ^{13}C-labeled *β*-carotene oxidation products were collected for NMR characterization by HMQC (*28*) in benzene-d_6 at 30 °C.

Analysis of HFAME in DLPC Liposomes Oxidized with Cigarette Smoke. DLPC liposomes were prepared by ethanol injection (*29*) with or without incorporation of 0.1 mol % *β*-carotene. The liposomal suspensions were oxidized with 200 mL of whole or gas-phase cigarette smoke every 15 min for 3 h at 40 °C. Samples (250 *μ*L) were drawn and added to 750 *μ*L of water and 100 *μ*L of BHT (10 mM in ethanol). Lipids were extracted with 4.5 mL of chloroform/methanol (2:1 v/v). The pooled organic fractions were evaporated under N_2. Lipid hydroperoxides were reduced to corresponding alcohols with $NaBH_4$ (ca. 1 mg) in 0.5 mL of methanol. The reaction was terminated with the addition of 1.0 mL of water and the product extracted with 4.5 mL of chloroform/methanol (2:1 v/v). The organic layer was evaporated under N_2 in screw-cap Reactivials (Pierce, Rockford, IL). The extent of fatty acyl unsaturation was reduced with 5% rhodium on alumina (ca. 1 mg) and H_2 in 0.5 mL of methanol at room temperature for 30 min. Following centrifugation, the methanol supernatant was supplemented with 2.5 nmol of 12-hydroxymethyl laurate internal standard and removed and the catalyst was washed twice with 1.0 mL of 2:1 v/v chloroform/methanol. The supernatants were evaporated under N_2, and the fatty acids were transesterified with 0.5 M KOH in methanol (0.5 mL) at room temperature for 45 min. The reaction was terminated by the addition of 2.0 mL of 2.0 M phosphate buffer, and hydroxy fatty acid methyl esters were extracted with 4.5 mL of hexane. The extracts were evaporated under N_2 and derivatized with 1.5% pentafluorobenzoyl chloride and 0.75% diisopropylethylamine in toluene (325 *μ*L) at 65 °C for 60 min. Samples were analyzed by capillary gas chromatography with electron capture detection; 1.0 *μ*L injections were made on-column at 100 °C. After 1 min, the oven temperature was increased to 210 °C at a rate of 25 °C/min and finally to 270 °C at a rate of 4.5 °C/min for a total analysis time of 33 min. The injector temperature was maintained 3 °C above the oven temperature, and the detector was maintained at 275 °C throughout the run.

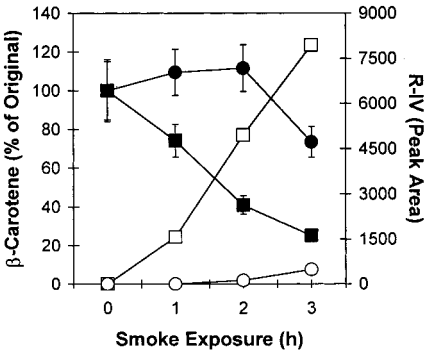


Figure 2. Smoke oxidation of *β*-carotene in toluene solution. Depletion of *β*-carotene (250 *μ*M) by gas-phase (■) and whole smoke (●) and generation of product R-IV by gas-phase (□) and whole smoke (○) exposure.

Analysis of Antioxidants in BLPC Liposomes Oxidized with Cigarette Smoke. Stock solutions of bovine liver phosphatidylcholine (30 *μ*mol) and appropriate amounts of *β*-carotene or α-tocopherol were evaporated together under N_2 and resuspended in 250 *μ*L of ethanol (*30*). The ethanol mixture was injected into phosphate-buffered saline that was being rapidly stirred to generate small unilamellar vesicles. In some experiments, 1 mmol of ascorbate was added to each 10 mL of phosphate-buffered saline solution immediately prior to ethanol injection.

β-Carotene was analyzed by reverse-phase HPLC using a Spherisorb ODS-2 5 *μ*m column (Alltech Associates) eluted with methanol/hexane (85:15 v/v) at a rate of 1.5 mL/min. Detection was by UV/vis absorbance at 450 nm. Samples (0.5 mL) were extracted with 1.5 mL of ethyl acetate, supplemented with α-tocopherol propionate as an internal standard, and dried under N_2. α-Tocopherol was assessed by GC/MS as described previously (*31*). Ascorbate was assessed spectrophotometrically through a modification of the method of Butts and Mulvihill (*32*). Samples consisted of 200 *μ*L of liposomal suspension, 450 *μ*L of 2.2 mM ferrozine, 50 *μ*L of 8.3 mM ferric ammonium sulfate, 400 *μ*L of water, and 900 *μ*L of ethanol. Samples were analyzed by UV/vis absorbance at 562 nm.

Statistical Analysis. Data were tested for statistical significance ($p < 0.05$) using ANOVA.

Results

Cigarette Smoke Oxidation of *β*-Carotene in Toluene. *β*-Carotene was consumed by cigarette smoke exposure in toluene solution. Unfiltered whole smoke depleted the *β*-carotene level by 27% over 3 h at 40 °C, whereas gas-phase smoke depleted the *β*-carotene level by 75% under the same conditions (Figure 2). Both gas-phase and whole cigarette smoke treatment of *β*-carotene in solution generated several oxidation products (Figures 3 and 4). The distribution of products appeared to be

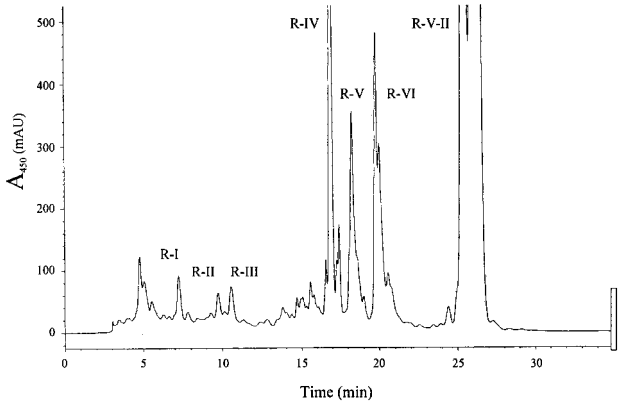


Figure 3. Reverse-phase HPLC product profile from gas-phase smoke oxidation of β-carotene (500 μM) in toluene solution.

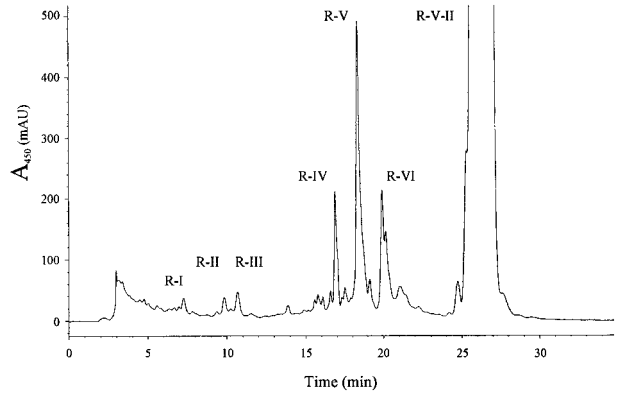


Figure 4. Reverse-phase HPLC product profile from whole smoke oxidation of β-carotene (500 μM) in toluene solution.

Table 1. Major Products Identified by Reverse-Phase HPLC Analysis of Gas-Phase Smoke Oxidations of β-Carotene in Toluene Solution

peak	identity	retention time (min)	UV/vis λ_{max} (nm)	positive APCI (m/z, $[M + H]^+$)
R-I	β-apo-14′-carotenal	7.2	398[a]	311
R-II	β-apo-12′-carotenal	9.8	422	351
R-III	β-apo-10′-carotenal	10.6	442	377
R-IV	4-nitro-β-carotene	16.9	454	582
R-V	5,8-epoxy-β-carotene	18.3	428, 452	553
R-VI	5,6-epoxy-β-carotene	19.8	446, 474	553
R-VII	β-carotene	25.2	452, 476	537

[a] The most intense absorbance bands are underlined.

similar with both treatments. However, gas-phase smoke formed greater quantities of these products than did whole smoke. Among the β-carotene oxidation products identified by HPLC retention, UV/vis absorbance, and LC/MS were β-carotene chain cleavage products and β-carotene epoxides (Table 1). Spectral data and HPLC retention times corresponded to previously published data for these compounds (*16*, *17*, *19*). In addition, a novel product, R-IV, eluted at 16.9 min in this system (Figures 3 and 4). R-IV eluted prior to the β-carotene epoxides, but after the polar chain cleavage products, suggesting a compound with intermediate polarity. The initial characterization of R-IV by positive ion LC/MS showed a signal at m/z 582, which corresponds to a $[M + H]^+$ ion for nitro-β-carotene, in which a β-carotene hydrogen is replaced by a nitro group. CID of this ion resulted in a prominent ion at m/z 536 corresponding to the loss of the nitro group, m/z 46 (data not shown). The time course of formation of this product fraction by whole and gas-phase smoke exposures is shown in Figure 2. In experiments

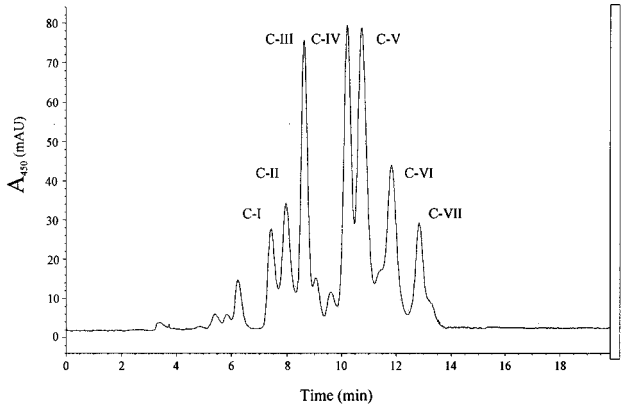


Figure 5. Cyano HPLC separation of reverse-phase HPLC-purified product R-IV generated from gas-phase smoke oxidation of β-carotene (500 μM) in toluene solution.

Table 2. UV/Vis and LC/MS Data for Compounds C-I−VII Purified by Cyano Chromatography from Product R-IV

peak	UV/vis λ_{max} (nm)	positive APCI (m/z, $[M + H]^+$)	negative APCI (m/z, $[M - H]^-$)
C-I	338, 445,[a] 471	ND[b]	580
C-II	339, 445, 471	ND	580
C-III	339, 445, 476	ND	580
C-IV	460	582	ND
C-V	342, 456	582	ND
C-VI	342, 454	582	ND
C-VII	346, 452	582	ND

[a] The most intense absorbance bands are underlined. [b] Not detected.

where β-carotene solutions were exposed to NO_2 in air (30 ppm), product profiles were virtually identical to those from smoke exposures (data not shown).

Peak R-IV was collected and subjected to further fractionation by cyano HPLC which resolved peak R-IV into two distinct groups of products (Figure 5 and Table 2). The first consisted of compounds C-I−III, which all produced strong signals at m/z 580 in negative ion APCI LC/MS analysis and weak signals at m/z 582 in the positive ion mode. The second group contained compounds C-IV−VI, and C-VII, which exhibited signals at m/z 582 in positive ion APCI LC/MS, but weak signals at m/z 580 in the negative ion mode. Thus, all of these products appeared to result from substitution of one β-carotene hydrogen with two oxygens and one nitrogen.

Products C-I−VII were treated with methylene blue and light according to the method of Foote et al. (*27*) to convert cis isomers to the thermodynamically favored all-trans form. C-I and C-II both converted to C-III, as assessed by retention time and UV/vis absorbance changes (Table 3). Likewise, methylene blue/light treatment converted compounds C-VI and C-VII to C-V (Table 3). Thus, C-I and C-II were identified as cis isomers of C-III, and C-VI and C-VII were identified as cis isomers of C-V.

Unfortunately, these compounds proved to be relatively unstable, and generating sufficient quantities (1 mg) of all seven for NMR analyses was not possible. To increase sensitivity in heteronuclear experiments, *per*-[^{13}C]-β-carotene was used to generate products labeled at all carbon positions with ^{13}C. Analysis of the oxidation products by HMQC permitted ^{13}C shifts of all nonquaternary carbons to be observed in samples containing ca. 100 μg of material. Because C-III was both the most abundant and apparently the most stable of the compounds, both HMQC (on *per*-^{13}C-labeled material) and

Table 3. Methylene Blue/Light Treatment of Compounds Isolated by Cyano Chromatography from Compound R-IV

	untreated		methylene blue/light-treated	
peak	retention time (min)	UV/vis λ_{max} (nm)	retention time (min)	UV/vis λ_{max} (nm)
C-I	5.8	336, 442,[a] 468	6.7	450, 476
C-II	6.2	338, 444, 470	6.7	450, 476
C-III	6.7	450, 476	6.7	450, 476
C-IV	7.0	462	7.0	460, 486
C-V	7.4	460, 486	7.3	460, 488
C-VI	8.0	452	7.2	460, 488
C-VII	9.3	454	7.3	458, 486

[a] The most intense absorbance bands are underlined.

Table 4. NMR Characterization of Product C-III and Comparison with All-Trans β-Carotene

	^{1}H (ppm)				^{13}C (ppm)		$^1J_{CH}$	
position[a]	C-III	βC[b]	mult[c]	J_{HH} (Hz)	C-III	βC[d]	(Hz)	COSY
2a	1.56	1.50	m		34.5	40.0	z	2b, 3b
2b	1.08		m				z	2a, 3a
3a	2.02	1.61	m		25.2	19.7	128	2b, 3b, 4
3b	1.67		m				123	2a, 3a, 4
4	4.58	1.98	br t	5	88.2	33.4	147	3a, 3b
7	6.03	6.33	d	16	124.3	126.9	153	8, 18
8	6.26	6.39	d	16	140.5	138.7	153	7
10	6.23	6.34	d	11	133.5	131.9	139	11, 19
11	6.72	6.78	dd	11, 15	125.0	125.5	147	10, 12
12	6.50	6.47	d	15	139.1	138.1	152	11
14	6.35	6.32	ol		133.9	133.2	145	15, 20
15	6.67	6.68	m		130.7	130.7	144	14
16	1.00	1.15	s		28.8	29.2	123	
17	0.86	1.15	s		27.4	29.2	123	
18	1.69	1.89	s		19.2	22.0	125	7
19	1.83	1.94	s		12.6	12.9	124	10
20	1.87	1.88	s		12.8	12.9	122	14
2′ab	1.50	1.50	m		40.0	40.0	121	3′ab
3′ab	1.61	1.61	m		19.7	19.7	121	2′ab, 4′ab
4′ab	1.98	1.98	br t	6	33.3	33.4	122	3′ab, 18′
7′	6.33	6.33	ol		127.0	126.9	148	4′ab, 18′
8′	6.39	6.39	d	16	138.6	138.7	152	7′
10′	6.34	6.34	ol		131.8	131.9	149	11′, 19′
11′	6.79	6.78	dd	11, 15	125.7	125.5	143	10′, 12′
12′	6.47	6.47	d	15	138.0	138.1	148	11′
14′	6.32	6.32	ol		133.1	133.2	144	15′, 20′
15′	6.68	6.68	m		130.7	130.7	144	14′
16′/17′	1.15	1.15	s		29.2	29.2	120	
18′	1.81	1.81	s		22.0	22.0	122	4′ab, 7′
19′	1.94	19.4	s		12.8	12.9	123	10′
20′	1.88	1.88	s		12.8	12.9	122	14′

[a] Refer to Figure 5. [b] *per*-[^{13}C]-β-Carotene shifts from HMQC included for comparison. [c] Multiplicity from one-dimensional ^{1}H spectrum of unlabled material: s, singlet; d, doublet; t, triplet; br, broad; ol, overlapped; and m, multiplet. [d] J_{CH}.

DQF-COSY (on natural abundance material) were used to assess this product. β-Carotene exhibits C-2 symmetry through the 15,15′ double bond, so both ^{1}H and ^{13}C NMR resonances are identical for corresponding positions on either side of the structure. However, once nitration occurs, this symmetry is lost and the spectra become very complex, particularly if substitution creates a chiral center. HMQC data for *per*-[^{13}C]C-III are shown in Table 4.

Nonquaternary positions on the nonadducted half of the molecule in product *per*-[^{13}C]C-III (positions 2′−20′) show ^{1}H and ^{13}C chemical shifts which are nearly identical to those in all-trans β-carotene. For consistency, the HMQC spectrum of all-trans *per*-[^{13}C]-β-carotene was used for comparison. The most notable change in the HMQC spectrum of *per*-[^{13}C]C-III is the cross-peak at 4.58 ppm (^{1}H) and 87.9 ppm (^{13}C) with a one-bond J_{CH} value of 147 Hz, typical of a CH group with a single electronegative substituent. In the DQF-COSY spectrum of natural abundance C-III, this resonance (4.58 ppm) is correlated to two peaks at 1.67 and 2.02 ppm. These two resonances correlate in the HMQC spectrum of *per*-[^{13}C]C-III to a single ^{13}C position (24.8 ppm), indicating an X-CH-CH_2 fragment. The resonance at 2.02 ppm is also correlated in the DQF-COSY spectrum to a resonance at 1.08 ppm which is part of another CH_2 fragment. The fragment X-CH-CH_2-CH_2 can only result from nitration of β-carotene at the 4-position, and this is the basis of all further assignments of C-III (see Table 4). Consistent with this conclusion is the fact that HMQC cross-peaks are not found near the 2′, 3′, and 4′ cross-peaks for C-III, while all other cross-peaks are located near and have structures similar to their counterparts in the unsubstituted half of the structure. The observation of five distinct ^{1}H chemical shifts for the five protons in the spin system (2a, 2b, 3a, 3b, and 4) is consistent with the creation of a chiral center at C-4. These assignments were confirmed by the DQF-COSY spectrum of natural abundance C-III, which exhibits long-range correlations from the methyl signals at positions 18, 19, and 20 to olefinic resonances 7, 10, and 14, respectively. Similar correlations were observed on the unsubsituted half of the molecule (18′, 19′, and 20′ to 7′, 10′, and 14′, respectively). Comparison of chemical shifts of C-III with those of *per*-[^{13}C]-β-carotene shows that the only positions with ^{13}C chemical shift differences of >2 ppm are 4, 2, 3, 18, and 7 (in order of decreasing differences), all within or directly attached to the six-membered ring. ^{13}C shift differences of >1 ppm are also observed for carbons 17, 8, and 10, the latter two indicating a change in the conjugation of the unsaturated system. Likewise, the only positions with ^{1}H chemical shift differences of >0.15 ppm are 4, 2b, 3a, 7, and 17 (in order of decreasing differences). The ^{1}H and ^{13}C chemical shifts on the unsubstituted half of the molecule are essentially identical to those for all-trans β-carotene.

DQF-COSY experiments performed on natural abundence C-I and C-II confirmed that these compounds were cis isomers of 4-nitro-β-carotene, as was suggested previously. Each peak appeared to be a mixture of mono cis isomers where the cis bond was on both the same and opposite halves of the structure with respect to the nitro adduct. C-III constituted up to 30% and C-I−III up to 60% of the cyano HPLC-purified material resulting from gas-phase cigarette smoke oxidation of β-carotene in toluene solution (200 mL every 5 min at 40 °C for 1 h).

Lipid Oxidation in DLPC Liposomes Exposed to Cigarette Smoke. To test the hypothesis that smoke-driven β-carotene autoxidation exerts prooxidant effects, we studied the effects of β-carotene on smoke-induced lipid peroxidation of phospholipids in a liposome system. Gas-phase smoke exposure resulted in a significant increase in the extent of peroxidation of DLPC liposomes as measured by the formation of HFAME (Figure 6). This result was consistent for both the 9- and 13-OH isomers of linoleic and total HFAME. Interestingly, the extent of formation of the 9-OH appeared to exceed that of the 13-OH in experiments with both gas-phase and whole smoke. Incorporation of 0.1 mol % β-carotene in DLPC liposomes slightly attenuated lipid peroxidation, but the reduction in the extent of HFAME formation was not statistically significant.

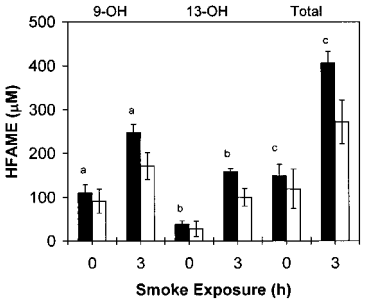


Figure 6. Gas-phase smoke-dependent lipid peroxidation, determined as hydroxy fatty acid methyl esters (HFAME), in DLPC liposomes containing 0.0 mol % β-carotene (■) or 0.1 mol % β-carotene (□). Groups with the same letters are significantly different ($p < 0.05$).

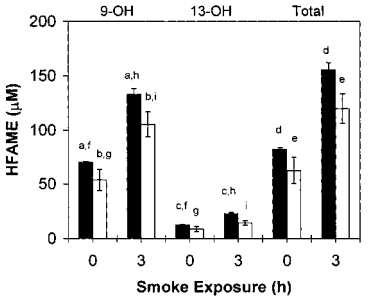


Figure 7. Whole smoke-dependent lipid peroxidation, determined as hydroxy fatty acid methyl esters (HFAME), in DLPC liposomes containing 0.0 mol % β-carotene (■) or 0.1 mol % β-carotene (□). Groups with the same letters are significantly different ($p < 0.05$).

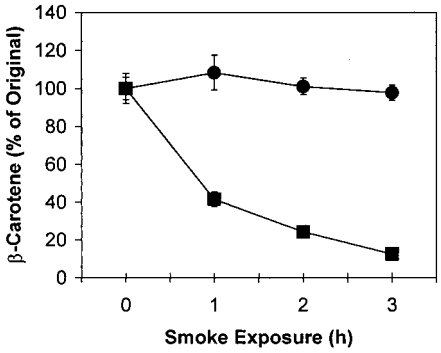


Figure 8. Depletion of β-carotene (0.1 mol %) by gas-phase (■) and whole smoke (●) in BLPC liposomes.

Whole smoke exposure also significantly increased the extent of lipid peroxidation of DLPC liposomes over that in control liposomes (Figure 7). Incorporation of 0.1 mol % β-carotene into the liposomal membrane resulted in a small but statistically significant decrease in the extent of formation of HFAME. These results indicate that β-carotene did not increase the extent of HFAME formation in DLPC liposomes oxidized with either gas-phase or whole smoke.

Antioxidant Depletion in BLPC Liposomes Exposed to Cigarette Smoke. We next examined the possibility that smoke-driven β-carotene autoxidation accelerates the depletion of other antioxidants by smoke. The depletion of β-carotene (0.1 mol %) in BLPC liposomes was measured over time in both whole and gas-phase smoke exposures. Three hours of exposure to whole smoke (600 mL total) did not significantly deplete the β-carotene level (Figure 8), whereas gas-phase smoke (600 mL total) under the same conditions consistently

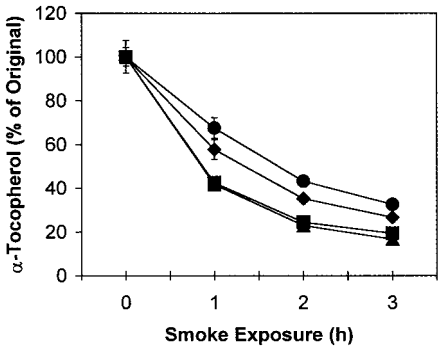


Figure 9. Gas-phase smoke depletion of α-tocopherol from BLPC liposomes containing 0.0 mol % β-carotene (■), 0.01 mol % β-carotene (●), 0.1 mol % β-carotene (▲), or 0.4 mol % β-carotene (◆).

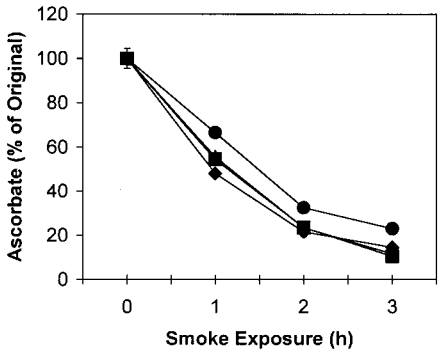


Figure 10. Gas-phase smoke depletion of ascorbate from BLPC liposomes containing 0.0 mol % β-carotene (■), 0.01 mol % β-carotene (●), 0.1 mol % β-carotene (▲), or 0.4 mol % β-carotene (◆).

decreased β-carotene levels by 88% within 3 h. In BLPC liposomes supplemented with α-tocopherol and ascorbate, little or no depletion of either antioxidant was observed upon whole smoke exposure (data not shown), whereas significant depletion occurred with gas-phase exposures (see below).

The oxidation of α-tocopherol and ascorbate was examined in BLPC liposomes exposed to gas-phase smoke in the presence of varying concentrations of β-carotene. The carotenoid produced a modest α-tocopherol sparing effect that was statistically significant at 0.01 and 0.4 mol % but not at 0.1 mol % β-carotene (Figure 9). Likewise, 100 mM ascorbate was modestly spared from oxidation when BLPC liposomes were supplemented with 0.01 mol % β-carotene, but not 0.1 or 0.4 mol % β-carotene (Figure 10). These data collectively indicate that β-carotene did not accelerate the smoke-induced oxidation of α-tocopherol and ascorbate, but produced instead a marginal sparing of the two antioxidants.

Discussion

β-Carotene autoxidation driven by smoke-borne oxidants has been hypothesized to amplify smoke-induced oxidative damage and contribute to increased lung cancer incidence in β-carotene-supplemented smokers. Two questions are at the center of this hypothesis. First, what is the chemistry of smoke-driven β-carotene oxidation? Second, do radical intermediates in β-carotene oxidation facilitate the oxidation of other biomolecules? Here we have characterized the principal products of smoke-driven β-carotene oxidation, including 4-nitro-β-carotene, and we have demonstrated that, despite facile smoke-

dependent oxidation, β-carotene does not enhance the peroxidation of membrane lipids or the depletion of the levels of other antioxidants in a liposomal model. The data strongly suggest that, although β-carotene is readily oxidized by smoke, prooxidant effects are unlikely to account for the apparent enhancement of lung cancer in smokers taking this supplement.

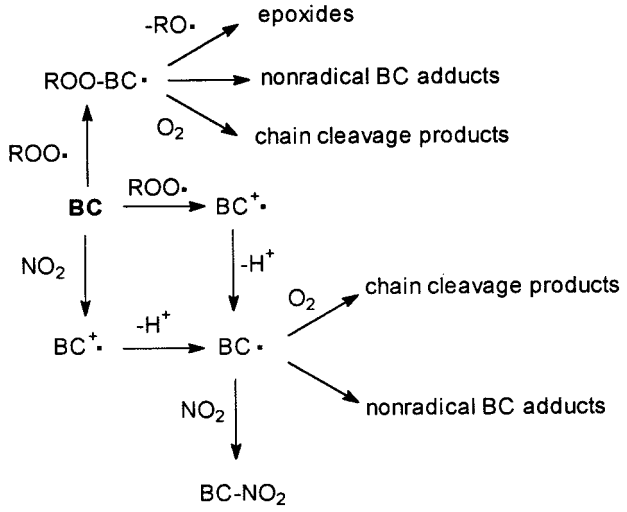
β-Carotene is readily oxidized in toluene solution by cigarette smoke. Several products result from the oxidation of β-carotene in solution, the most prominent of which is the novel product 4-nitro-β-carotene. The chain cleavage products β-apo-10′-, 12′-, and 14′-carotenals and the β-carotene epoxides 5,6-epoxy-β-carotene and 5,8-epoxy-β-carotene also were identified on the basis of retention times in reverse-phase HPLC, UV/vis spectra, and molecular ions detected by APCI LC/MS (Table 1). These compounds have been previously identified as β-carotene oxidation products in several systems (*17*−*19*, *33*). All have been reported previously to arise from peroxyl radical oxidation of β-carotene, and their appearance in this system is consistent with the presence of peroxyl radicals in smoke (*8*, *11*). The reaction probably generated chain cleavage products other than those reported in Table 1 (e.g., ketone products), although they evidently were not formed in high yield.

Gas-phase cigarette smoke is more effective at depleting the β-carotene level in solution (Figure 2) and generating β-carotene oxidation products than is unfiltered whole smoke (Figures 3 and 4). Similar effects have been noted previously in studies of the effect of smoke and smoke fractions. It has been shown that the tar fraction of smoke is a complex mixture of quinone/hydroquine redox couples (*8*−*10*). This fraction of smoke is reducing in nature and therefore can attenuate some of the oxidizing characteristics of gas-phase smoke. Smoke contains NO_2 (*8*, *11*) and peroxynitrite, both of which carry out diverse oxidations. β-Carotene oxidation by NO_2/air produced the same products seen with cigarette smoke exposures (data not shown). Taken together, these considerations suggest that some combination of volatile peroxyl radicals, nitrogen oxides, and peroxynitrite are the principal smoke-borne oxidants of β-carotene.

The reverse-phase HPLC product fraction R-IV contained several nitrogen oxide-modified β-carotene products (Figure 5). Compound C-III was identified as all-trans 4-nitro-β-carotene which accounted for up to 30% of these products under some oxidation conditions. Products C-I−III and C-IV−VII comprised two distinct compound classes. C-I−III exhibited UV/vis absorbance maxima near 450 nm with fine structure similar to that of β-carotene, which suggested that the β-carotene polyene was intact. Fractions C-I and -II also exhibited an absorbance maximum at 340 nm characteristic of cis carotenoids. In contrast, compounds C-IV−VI and C-VII displayed absorbance maxima above 450 nm and lacked fine structure. C-V−VII also exhibited cis peaks at 340 nm.

LC/MS analysis also suggested differences between these two distinct families of products. Negative ion APCI LC/MS detected C-I−III at *m*/*z* 580. However, signals at *m*/*z* 580 for the remaining four compounds were weak or absent. Likewise, positive APCI LC/MS yielded strong signals at *m*/*z* 582 for C-IV−VII, but signals at *m*/*z* 582 were weak or absent for C-I−III. Treatment of individual cyano HPLC-purified compounds with methylene blue and light (*27*) led to rapid conversion of cis isomers to the corresponding all-trans forms. Accordingly, products C-I and -II were converted to C-III, whereas C-VI and -VII were converted to C-V.

Scheme 1. Proposed Mechanism for the Consumption of β-Carotene and Generation of β-Carotene Oxidation Products by Cigarette Smoke Oxidants



Thus, the two product groups resolved by cyano HPLC include (1) all-trans 4-nitro-β-carotene (C-III) and its two cis isomers, C-I and -II, and (2) an apparently all-trans product C-V and its cis isomers, C-VI and -VII, all of which contain one nitrogen and two oxygens. Although the latter group exhibits apparent molecular masses identical to that of 4-nitro-β-carotene, they evidently are different, perhaps in the arrangement of the nitrogen oxide substituent. These products could be nitroso adducts of β-carotene epoxides, although this possibility seems unlikely because epoxidation would necessarily consume one polyene double bond and decrease the UV/vis absorbance maximum well below the observed 452−462 nm. These latter products may be nitrosooxy-substituted β-carotene oxidation products formed either by NO_2/N_2O_4 or by peroxynitrite (*34*, *35*). The nitrosooxy substituent would be more easily protonated in APCI LC/MS to generate the observed strong $[M + H]^+$ ion and correspondingly less able to stabilize an $[M - H]^-$ ion. Products C-IV−VII are formed in smaller amounts and appear less stable than C-I−III, and further work will be required to unambiguously establish their identities.

A mechanistic scheme for the smoke-mediated oxidation of β-carotene is proposed in Scheme 1. β-Carotene can react with either NO_2 or peroxyl radicals to generate a β-carotene radical (*15*, *36*, *37*), likely through the formation of the cation radical (eq 1) (*14*, *15*). Deprotonation and subsequent reaction with a second NO_2 can lead to formation of a nitro or nitrosooxy product, whereas other reactions of β-carotene radical intermediates with peroxyl radicals and oxygen form epoxides, chain cleavage products, and β-carotene nonradical adducts as described previously (*17*, *18*, *36*).

The reactivity of β-carotene at the 4 position has been noted previously. El-Tinay and Chichester showed that oxidation of β-carotene in solution by *N*-bromosuccinimide generated 4-oxo-β-carotene (*38*). Woodall et al. (*39*) reported 4-methoxy- and 4-ethoxy-β-carotene as products of peroxyl radical oxidations of β-carotene in solutions

containing methanol and ethanol, respectively. Likewise, Samokyszyn and Marnett showed that hydroperoxide-dependent cooxidation of 13-*cis*-retinoic acid by prostaglandin H synthase generated 4-hydroxy-13-*cis*-retinoic acid (*40*). Carbon 4 is one of four positions in β-carotene allylic to the polyene and is the most electron rich, as it resides at the terminus of the polyene structure (*38*).

This work is the first to describe 4-nitro-β-carotene as a characteristic marker product for the interactions of β-carotene with cigarette smoke. This product also is formed in NO_2-mediated oxidations of β-carotene and is, to our knowledge, the first reported oxidation product of β-carotene resulting specifically from exposure to a reactive nitrogen species. Other recent reports have shown that γ-tocopherol is an effective scavenger of reactive nitrogen species (*41*−*43*). These interactions generate 5-nitro-γ-tocopherol, a marker product for interactions of γ-tocopherol with peroxynitrite (*42*), NO_2 (*41*, *43*), and smoke (D. L. Baker and D. C. Liebler, unpublished observation). Although β-carotene may also function as a trap for reactive nitrogen species, as well as reactive oxygen species in vivo, β-carotene autoxidation may limit its antioxidant effectiveness. Indeed, the key question underlying this investigation is whether β-carotene autoxidation drives oxidation of other biomolecules.

Our experiments permitted a clear test of the hypothesis that β-carotene functions as a prooxidant when exposed to cigarette smoke under ambient air. Our data indicate that β-carotene acts as a weak antioxidant in smoke-exposed liposomes. Exposures to both gas-phase (Figure 6) and whole smoke (Figure 7) significantly increased HFAME levels in DLPC liposomes. β-Carotene incorporation into the membranes at 0.1 mol % modestly decreased HFAME levels over those of control liposomes without β-carotene. β-Carotene levels of 0.1 mol % are similar to those achieved by dietary supplementation in animals (*44*). In all cases, liposomes with β-carotene exhibited lower levels of HFAME than did liposomes without β-carotene. Thus, β-carotene did not increase the level of smoke-induced lipid peroxidation. The oxidation of other antioxidants in the presence and absence of β-carotene was also examined. Both α-tocopherol in BLPC liposomes and ascorbate in the liposome buffer were depleted by smoke exposures. The presence of β-carotene at 0.01, 0.1, and 0.4 mol % either modestly spared or had no effect on either α-tocopherol (Figure 9) or ascorbate (Figure 10) levels upon exposure to gas-phase smoke. These results indicate that β-carotene autoxidation, which occurs under these conditions (Figure 8), does not accelerate the depletion of other antioxidants by smoke.

While this paper was in review, Wang et al. reported that ferrets supplemented with β-carotene exhibited squamous metaplasia in the lung, which was enhanced by smoke exposure (*45*). Levels of β-carotene oxidation products were elevated in ferret lung postnuclear fractions from animals supplemented with β-carotene and exposed to smoke in corresponding in vitro experiments (*45*). Thus, although it is unlikely that a direct prooxidant effect of β-carotene occurs in the smoke-exposed lung, β-carotene oxidation products formed by smoke may themselves exert procarcinogenic effects. This intriguing hypothesis merits further investigation.

Conclusions

Cigarette smoke induces rapid β-carotene autoxidation with the concomitant formation of 4-nitro-β-carotene, a novel product derived from smoke-borne reactive nitrogen species. This product may serve as a useful marker for the interaction of β-carotene with smoke in biological systems. Although smoke induces rapid β-carotene autoxidation, a β-carotene "prooxidant effect" does not amplify smoke-induced oxidation of liposomal membrane lipids or the other antioxidants α-tocopherol or ascorbic acid. It is unlikely that prooxidant effects of β-carotene account for the increased incidence of lung cancer in smokers taking β-carotene supplements.

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