Free Radical Research, March 2009; 43(3): 280–286

informa
healthcare

The degradation of (all-E)-β-carotene by cigarette smoke

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(Received 12 November 2008; revised 12 December 2008)

Abstract
The effects of cigarette smoke in promoting the degradation of (all-E)-β-carotene have been studied, but some conflicting results promoted a further study. β-Carotene was solubilized in hexane and challenged with filtered cigarette smoke both at room temperature and at −20°C. The products arising from smoke-induced oxidation were assessed using a combination of HLPC-DAD, LC-MS and GC-MS. At room temperature the degradation of β-carotene was very rapid, with only a few products being detected using HPLC-DAD. A range of volatile products including β-ionone, β-cyclocitral and 5,6-epoxy-β-ionone were detected using GC-MS. In contrast, when the reaction was slowed (by reducing the reaction temperature), a much wider range of products could be detected by HPLC-DAD, including 4-nitro-β-carotene and several of its geometric isomers. These degradation products suggest that the C4 position on the β-carotene end-group plays a key role in initiating free radical attack.

Keywords: *β-carotene, HPLC-DAD, 4-nitro-β-carotene, cigarette smoke*

Introduction

β-Carotene is arguably one of the most important carotenoids in human nutrition as it has distinct antioxidant properties and is a major precursor of vitamin A [1,2]. Several *in vitro* studies have indicated that β-carotene is able to inhibit the growth of transformed human cell lines and also upregulate the expression of connexins [3]. Such observations have led to suggestions that this carotenoid may have anti-carcinogenic effects. However, the results of two efficacy trials, the ATBC and the CARET studies [4,5], indicated that β-carotene instead led to the development of lung neoplasms in ~26% of individuals who smoked more than a pack of 20 cigarettes per day. A number of hypotheses have been put forward in order to explain this apparent contradiction, such that the dose of β-carotene used in the trials was too high. *In vitro* studies have shown that whilst a protective effect of β-carotene is evident at lower concentrations, this response is lost at high concentrations and that the carotenoid failed to protect either HT29 or HepG2 cells from damage by oxidants generated by a xanthine/xanthine oxidase [6] or H_2O_2 [7], respectively. This observation has also been substantiated by others, using different cell types and oxidants [8]. An alternative hypothesis suggests that when high levels of β-carotene in the human lung are challenged with oxidant-rich cigarette smoke in an antioxidant poor environment, this could lead to the production of a range of oxidation products such as epoxides and apo-carotenals that may, themselves, have biological function. Although a number of β-carotene cleavage products have been identified, there is still limited knowledge about the

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ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760802691497

fate of carotenoids when challenged with complex oxidants such as cigarette smoke.

Indeed, very few studies have directly studied the interaction of β-carotene and cigarette smoke. Baker et al. [9] indicated that the gaseous phase of cigarette smoke can interact with β-carotene to generate 4-nitro-β-carotene. This was an interesting observation, as it was the first to characterize a 4-nitro-carotenoid which strengthens the argument first put forward by Woodall et al. [10] that the allylic C-4 position of β-carotene is quite reactive and is susceptible to hydrogen abstraction by free radicals, such as peroxyl radicals. This observation was consistent with electron density maps that indicate that the C4 position is a very reactive position on the β-carotene molecule [10]. The resulting carotene radical can then interact with reactive nitrogen species (RNS) (found in cigarette smoke) to form 4-nitro-β-carotene.

In a separate study, Rahman et al. [11] indicated that the gaseous phase of cigarette smoke can oxidize β-carotene very effectively, generating many products that displayed strong absorbance in the UV region of the spectrum. However, the study did not find any evidence to support the formation of 4-nitro-β-carotene.

The importance of the C4 and C4′ positions on the β-ionone rings of β-carotene have largely been overlooked in many of the degradative pathways that have been reported. In this study we observed a range of products that were formed when β-carotene is degraded by cigarette smoke, particularly those that involve an addition reaction at the C4 or C4′ position and we will investigate some novel products that are generated during the process.

Materials and methods

Oxidation of β-carotene following challenge with cigarette smoke

A 30 mL solution of 7 μM β-carotene in hexane was placed in a 100 mL volumetric flask and purged with 50 mL of filtered (2.5 μm Acrodisk filter) smoke from a single cigarette. The flask was then sealed and gently mixed at room temperature or at −20°C. This process was repeated every 20 min for up to 7 h. Samples (2 mL) were taken regularly throughout the experiment and butyl-hydroxytoluene (BHT) was added at 20 μg/mL to preserve the sample. Samples were rapidly dried under a steady stream of oxygen-free nitrogen and stored at −80°C prior to analysis using a combination of UV/VIS spectroscopy, HPLC, LC-MS or GC-MS. Samples were analysed as rapidly as possible after collection.

UV/VIS spectroscopy

Dried samples were resuspended to 2 mL in benzene and placed at an appropriate dilution in a silica cuvette and scanned at 300–600 nm using a MR 3000 Milton Roy Diode array spectrophotometer.

HPLC

Separation of β-carotene and its oxidation products was achieved on a C30 reversed-phase carotenoid column (5 m spherical size, YMC Inc., Wilmington, NC). The mobile phase (A: acetonitrile/water (9:1 v/v); B: ethyl acetate) was delivered at 1.0 mL/min using a binary pump (Hewlett Packard G1328A), in combination with a vacuum degasser (Agilent 1100 series G1379A). At time 0, 100% A:0% B this followed a linear gradient such that at 10 min the solvent mixture was 52% A and 48% B. This was held for 7.5 min, then a linear gradient was followed such that at 35 min there was 0% A and 100% B. Compounds were detected over a wavelength range of 280–600 nm using a diode array detector (Hewlett Packard HPLC detection system 1040 M series 2). Preliminary identification of compounds was performed by analysis of t_R and spectral properties (λ_{max} and fine structure).

GC-MS

GC separation of putative oxidation products was carried out on a Varian Model 3400 equipped with a programmable temperature vapourizer injector coupled to an ion-trap mass spectrometer (Magnum ITD; Finnigan Mat, UK). A DB/5MG column (26 m × 0.18 mm × 0.18 m, J&W Scientific Inc, UK) was used with helium as the carrier gas. The initial injector temperature was 40°C, which was then raised to 280°C at a rate of 200°C/min. The transfer line was kept at 280°C and the initial column temperature was 40°C (held for 4 min). Column temperature was raised to 280°C at 10°C/min and held for 2 min. Thereafter, the conditions were held for 30 min. The ion trap mass spectrometer was scanned from 50–650 amu with a scan rate of 0.6 s per scan. Oxidation products were identified by their t_R and by their MS spectra (including comparison against the spectral library of the National Institute of Standards and Technology). For identification of a substance a fit of the MS spectra of the sample and the library or literature data of more than 85% was required. Further, substances having a match greater than 75% were considered as candidates of additional metabolites as their potential structures were consistent with oxidative metabolites of β-carotene.

LC-MS

HPLC-mass spectrometry (LC-MS) was carried out using a method adapted from that described in Caris-Veyrat et al. [12]. HPLC analysis was carried out with a HP 1050 (Hewlett-Packard GmbH, Waldbronn, Germany) equipped with a quaternary pump solvent

delivery system, an autosampler and a diode array detector. This was coupled to a LCZ 4000 mass spectrometer (Micromass Platform, Manchester, UK). For separation, a 250 × 4.6 mm i.d., 5 μm, YMC Pack C30 (YMC Inc., Wilmington, NC) equipped with a 20 × 4.6 mm, 5 μm, C-30 precolumn was used and kept at 27°C. The mobile phase consisted of: solvent A—water containing 2% (v/v) ammonium acetate; solvent B—methanol containing 2% (v/v) ammonium acetate; solvent C—methyl-*tert*-butyl ether. The following gradient system was used: 0–2 min, 40/60/0 (% solvent A/% solvent B/% solvent C); 5 min, 20/80/0; 10 min, 4/81/15; 60 min, 4/11/85; 70 min, 4/11/85; 70.01 min, 0/100/0. The flow rate was 1 mL/min and the injection volume was 10 μL. Absorption spectra were recorded between 190 and 900 nm. Positive electrospray mode was used as mass ionization technique. Mass parameters: capillary voltage 3.2 V, cone voltage 40 V, source block temperature 120°C, desolvation temperature 150°C. Nitrogen was used as drying gas at 300 L/h. Mass spectra were acquired with a scan range of *m/z* 300–800. Data were acquired and processed with MassLynx 3.4 software.

Partial synthesis of 4-nitro-β-carotene

4-Nitro-β-carotene was prepared using a modified nitrosylation reaction [13]. Nitrosylation was performed by dissolving 1.0 mg of (all-E)-β-carotene in 1.0 mL of chloroform. This was then mixed with 1.0 mL of phosphate buffer (pH 3.0). The reaction was initiated by the addition of 40 μl of 2% (w/v) sodium nitrite. The reaction vial was vented and mixed continuously for 2 h before extraction of the products with hexane. The organic layer was removed and dried under a stream of oxygen-free nitrogen. The residue was re-suspended in ethanol and analysed using HPLC and LC-MS.

Results

At room temperature, the deleterious effects of cigarette smoke on β-carotene were marked. Rapid bleaching of β-carotene was observed, resulting in approximately an 80% decrease in the concentration of carotenoid after 7 h (Figure 1). This was accompanied by a 10 nm hypsochromic shift in the λ_{max} from 465 nm to 455 nm. Spectroscopic analysis of the reaction mixture also showed the presence of a number of products that absorbed strongly in the UV region. The degradation of β-carotene followed first order reaction kinetics and this was very similar to that obtained when β-carotene was oxidized using 2,2′-azobis(cyclohexane) cyanonitrile (unpublished data).

Whilst HPLC analysis revealed the production of a range of geometic isomers of β-carotene (notably the

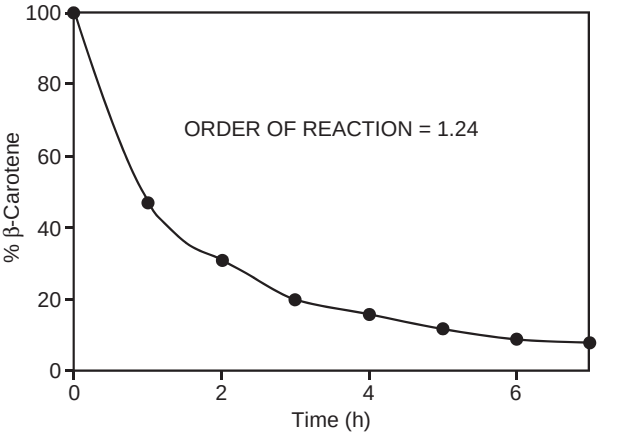

Figure 1. A 30 mL hexane solution of 7 μM β-carotene was placed in a 100 mL volumetric flask and was challenged with 50 mL of filtered cigarette smoke. This process was repeated every 20 min for a total of 7 h. At certain time points, 1 mL of the hexane solution was removed and the sample was scanned using a UV/VIS diode spectrophotometer between 200–650 nm. The decrease in the absorbance λ_{max} of β-carotene was recorded. The rates of depletion were used then to determine the rate of reaction. This process was repeated six times and indicated an order of reaction of 1.24. Data from one such experiment is shown in the figure.

(9Z) form—as identified by comparison to R_t λ_{max} and fine structure (III/II%) of an authentic standard) few apo-carotenals or epoxides were observed (Figure 2). There was no indication of the formation of any nitrated products. When the oxidative challenge was conducted at room temperature the rate of degradation of (all-E)-β-carotene was so rapid that any short-lived reaction products were not detected. In order to slow the reaction down the challenge was repeated at −20°C. Under these conditions a much broader spectrum of products was observed compared to that seen when β-carotene was challenged with cigarette smoke at the higher temperature. After 1 h following initial exposure with cigarette smoke, HPLC revealed the presence of more than 20 separate compounds which showed strong absorbance at 450 nm (Figure 3). The spectral characteristics of the main com-

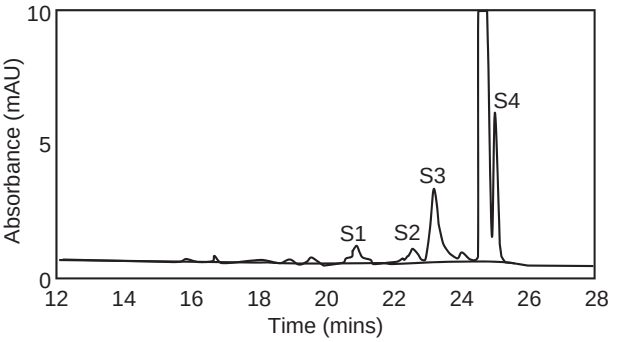

Figure 2. HPLC profile at 460 nm when β-carotene was challenged with cigarette smoke at room temperature over a 5-h period. Only a few products were detected despite a rapid depletion of the carotenoid. S1 and S2 are epoxide derivatives, whilst S3 and S4 are isomers of β-carotene.

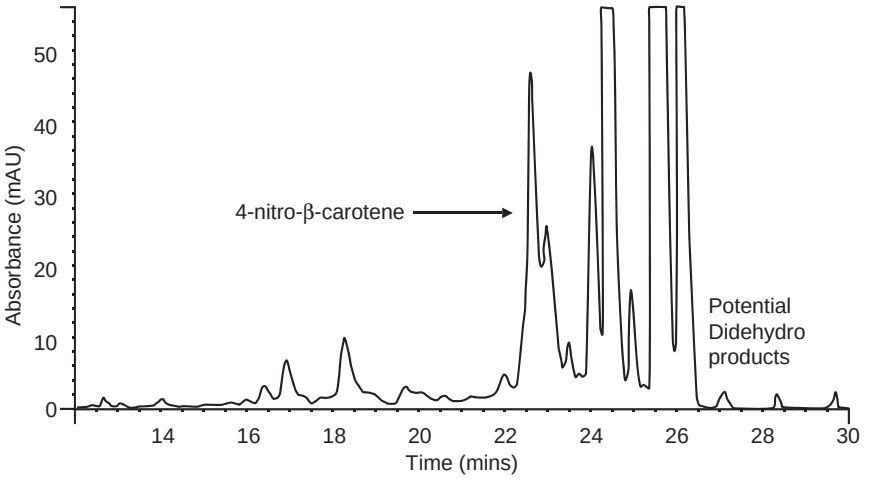

Figure 3. β-Carotene was exposed to cigarette smoke then rapidly cooled to −20°C. The degradation products were detected by HPLC using a C30 column. Over 20 compounds were observed.

pounds detected by HPLC are presented in Table I. The presence of 4-nitro-β-carotene as one of the major reaction products was confirmed by comparison to the synthesized product and the structure confirmed by LC-MS, which indicated a mass ion of [M^+ 583]. The λ_{max} in the HPLC solvent was 452 nm, which is in agreement with that published by Baker et al. [9]. A range of other compounds with similar t_R to 4-nitro-β-carotene were also observed. These had the same mass ion as 4-nitro-β-carotene, but they differed in their spectral characteristics by displaying a distinctive *Z*-peak (at ~350 nm) combined with a hyposchromic shift of 2–6 nm in the λ_{max}. Together, these data strongly suggest that these products are geometric (Z)-isomers of 4-nitro-β-carotene. The spectral characteristics of these products are compared to established data for both (all-E)-β-carotene and a range of (Z)-forms of this carotenoid (Table I; [14,15]).

In addition to the presence of 4-nitro-β-carotene, the interaction of (all-E)-β-carotene with cigarette smoke resulted in the formation of two other compounds in relatively high levels. On the basis of their chromatographic behaviour and spectral characteristics with the published literature [10,16,17] these are provisionally identified as 3,4-didehydro-β,β-carotene (t_R 27 min, λ_{max} 462 nm) and 3,4;3′,4′-tetradehydro-β,β-carotene (t_R 28 min, λ_{max} 472 nm). Whilst HPLC and LC-MS analysis permitted the detection of a wide range of products formed as a result of challenging (all-E)-β-carotene with cigarette smoke, a number of short chain degradation products were identified using GC-MS and these oxidation products are shown in Table II. Most of these have been previously reported as oxidation products of β-carotene (including β-ionone, β-cyclocitral and β-ionone-5,6-epoxide [18]), but additionally the presence of two novel products were detected. The fragmentation patterns were consistent with nitro-β-ionone and nitro-β-cyclocitral (Figure 4). Since C4 on the cyclohexene ring is the most reactive it is tempting to speculate that the nitro group on each of these compounds will be located at that position.

The detection of nitro-β-ionone and nitro-β-cyclocitral indicates that nitrated β-carotene is degraded in a similar pattern to that of β-carotene. Alternatively it may suggest that nitration at the C4 position can occur at any stage during the oxidation of the β-carotene molecule.

Discussion

Filtered cigarette smoke contains many radicals and reactive species including superoxide, hydrogen

Table I. Major products detected by HPLC following oxidation of (all-E)-β-carotene by cigarette smoke at −20°C.

t_R (min)	Isomer	(Z)-peak (nm)	Max (II) (nm)	Max (III) (nm)	Q-ratio	Q-ratio reported[a]
21.5	(13Z)-4-nitro-β-carotene	345	447	470	3.5	NR
22.4	4-nitro-β-carotene		451	477	16.8	NR
23.4	(9Z)-4-nitro-β-carotene	351	445	471	11.8	NR
24.4	(13Z)-β-carotene	340	445	471	2.4	2.8
25.6	(all-E)-β-carotene		453	477	16.7	17.4
26.2	(9Z)-β-carotene	345	447	473	11	11.5

[a] [14,15].

Table II. Molecular masses and mass fragment ions for volatile products derived from cigarette smoke oxidation of (all-E)-β-carotene determined by GC-MS.

Name	Mass $[M]^+$	Scan number	Fragment ions (m/z)
β-Cyclocitral	152	1282–1286	67,81, 91, 109, 123, 137, 152
5,6-Epoxy-β-ionone	208	1315–1319	67, 91, 121, 136
β-Ionone	192	1655–1659	91, 107, 123, 135, 149, 177
Dihydroactinidiolide	180	1735–1739	67, 81, 95, 111, 124, 137, 152, 180
4,6,6-Trimethyl-2-(3-methylbuta-1,3-dienyl)-3-oxatricyclo[5.1.0.0(2,4)] octane	218	2027–2031	79, 91, 105, 107, 119, 129, 133, 147, 161, 175, 218
2,6,6-Trimethyl-1-cyclo-hexene-1-acetaldehyde	164	1870–1874	67, 79, 91, 108, 123
3,7,7-Trimethyl-1-penta-1,3-dienyl-2-oxabicyclo[3.2.0]hept-3-ene	205	2595–2599	76, 91, 104, 123, 133, 149
Nitro-β-ionone	238	1943–1947	77, 91, 107, 121, 135, 149, 195, 223, 238
Nitro-β-cyclocitral	198	1961–1965	55, 69, 83, 98, 111, 125, 137, 165, 180

peroxide and peroxynitrite [19]. It has recently been demonstrated [20] that oxidants in the gaseous phase of cigarette smoke can pass through the lung alveolar wall and into the blood of rats. This led to an increase in nitration of low density lipoproteins and may lead to endothelial cell dysfunction. The complex mixture of oxidants would mean that any interaction with antioxidants such as the carotenoids may lead to the rapid degradation and the formation of potentially deleterious biological agents.

The reactivity of cyclic carotenoids such as β-carotene is heavily influenced by the extension of the conjugated double bond system into the end-group at C5–C6 and the resulting influence of steric hindrance on the capacity for C4 to donate an unpaired electron into the conjugated polyene chain. This, in turn, permits hydrogen abstraction at C4 by peroxyl radicals. In the case of (all-E)-β-carotene this would be expected to yield a β-carotene radical that could either undergo a similar reaction at C3 to yield 3,4-didehydro-β-carotene or may react with oxygen to produce 4-oxo-β-carotene. Alternatively it could react with RNS (Reactive Nitrogen Species) to instead produce 4-nitro-β-carotene. Evidence for the formation of 3,4-didehydro-β-carotene and 4-nitro-β-carotene is presented within this study. However, we did not detect 4-oxo-β-carotene as might have been predicted. 4-Nitro-β-carotene is formed as a product of the cigarette smoke-induced degradation of (all-E)-β-carotene. However, this particular product was difficult to detect and was only observed once the reaction itself had been slowed. It is therefore likely that the failure of some earlier studies [11] to detect 4-nitro-β-carotene may be due to its

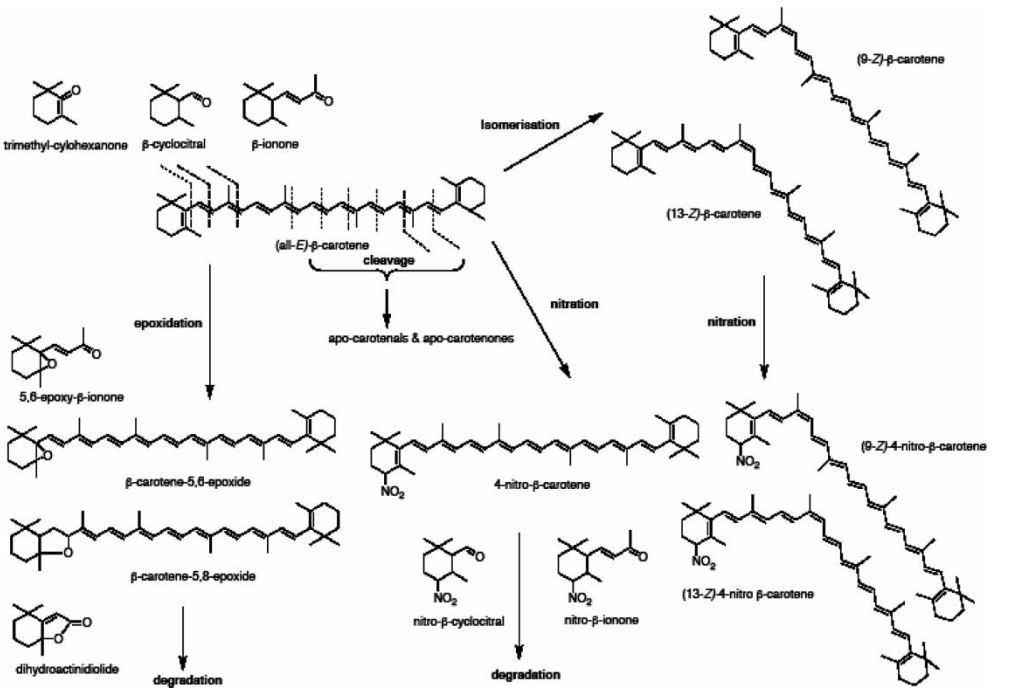

Figure 4. Suggested degradative pathway for β-carotene exposed to cigarette smoke.

transient nature. Baker et al. [9], by using high concentrations of β-carotene, did detect 4-nitro-β-carotene.

The degradation rate and order of reaction in this study (1.24) is also in keeping with that estimated by Rahman et al. [11]. A number of geometric isomers of β-carotene (e.g. (9Z) and (13Z) see Table I) were also detected (both at room temperature and at -20°C) as a result of exposure to filtered cigarette smoke. Such (Z)-isomers are often regarded as transient, but were detected throughout the degradation period. The accumulation of such geometric isomers would suggest either that isomerization may be a fundamental step during the degradative pathway or that isomerization slows down the degradative pathway. Baker et al. [9] indicated that isomers of 4-nitro-β-carotene may be present as a result of the degradation of β-carotene with cigarette smoke. Evidence in this study suggests that (9Z)-4-nitro-β-carotene and (13Z)-4-nitro-β-carotene are formed. These correspond to the dominant (Z)-isomers of β-carotene formed as both the (9Z) and (13Z)-isomers are thermodynamically favoured, but it raises the question as to whether nitration occurs before or after isomerization. Certainly following isomerization, the delocalization of electrons within the polyene chain would be shifted and this may be expected to reduce the reactivity at C4.

At room temperature, the degradation of β-carotene by cigarette smoke was very rapid and lack of products detected by HPLC-DAD would indicate an increased rate of eccentric cleavage of the carotenoid. This would yield structures such as β-ionone or nitro-β-ionone and the corresponding apo-β-carotenal and even this degradative species was probably subject to immediate eccentric cleavage to yield more short chain volatile products. In an *in vivo* situation it would be expected that any carotenoids in the interstitial cell spaces of the lung would probably yield insignificant concentrations of any nitrated products. Within cellular membranes, however, the reaction rates may differ, as there is a natural barrier to the cigarette smoke, perhaps promoting the accumulation of nitrated products. Arora et al. [21] have indeed demonstrated that 4-nitro-β-carotene was detected in the plasma membranes of a human lung cell line following challenge with cigarette smoke. The biological importance of oxidation products from β-carotene and lycopene have recently been reported [22,23]. One such compound, Apo-14′-carotenoic acid, an oxidation product of β-carotene, was found to inhibit the growth of a human bronchial epithelial cell line [24]. This effect was explained by the conversion of the oxidation production and the parent carotenoid to retinoic acid and inducing an up-regulation in the retinoic acid receptor (RARβ). Cigarette smoke can generate a range of oxidation products derived from β-carotene. If these compounds are stable within the lung, some may then offer protection by inhibiting proliferation of transformed cells, whereas others may act to encourage proliferation and cell growth. Certainly cigarette smoke can induce the addition of radicals to β-carotene and at room temperature it will cleave the carotenoid both centrally and eccentrically to yield short chain compounds with potential metabolic activity.

Acknowledgements

We would like to Mrs Nicola Bateman for her technical expertise regarding GC-MS measurements.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on iFirst on 27 January 2009.