Intestinal absorption of epoxy- β -carotenes by humans

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An increased intake of fruits and vegetables has been shown to be associated with reduced risk of cancer. In epidemiological studies, supplements of β -carotene, which is abundant in fruits and vegetables, were not found to be beneficial in reducing the incidence of lung cancer in high-risk groups. Epoxycarotenoids are abundant in nature. 5,6-Epoxy- β -carotene was much more active than β -carotene in the induction of differentiation of NB4 cells [Duitsman, Becker, Barua and Olson (1996) FASEB J. 10, A732]. Epoxycarotenoids may, therefore, have protective effects against cancer. In order to do this, however, epoxycarotenoids

must be absorbed by the human body. There is no evidence that epoxycarotenoids, despite their abundance in dietary fruits and vegetables, are absorbed by humans. In this paper, it is demonstrated that orally administered dietary or synthetic epoxy- β -carotenes are absorbed by humans, as indicated by their appearance in the circulating blood.

Key words: 5,6-epoxy- β -carotene, epoxyretinyl palmitate, HPLC, mango, plasma.

INTRODUCTION

Epidemiological studies show that an increased intake of fruits and vegetables is associated with decreased risk of cancer [1]. Fruits and vegetables contain a myriad of chemical components, both nutrient and non-nutrient by classification. Peto et al. [2] hypothesized that increased intake of β -carotene, a major carotenoid in many fruits and vegetables, might reduce rates of human cancer. The hypothesis that β -carotene plays a role in cancer prevention is based on studies suggesting that carotenoids function as antioxidants, as enhancers of immune function, as precursors of retinoids and/or by stimulation of cell–cell communication. In order to determine whether β -carotene is the active phytochemical in cancer prevention, four major intervention trials in humans have been completed, all of which included β -carotene supplementation with or without other nutrients. Two studies provided evidence suggesting that β -carotene increased the risk of lung cancer in high-risk groups of smokers and/or workers who had been occupationally exposed to asbestos [3,4]. Another study showed neither positive nor negative effects of 12 years of β -carotene supplementation on the incidence of cancer [5]. One study, which involved combined supplementation of β -carotene with vitamin E and selenium to marginally malnourished people, showed lower mortality from gastric and total cancers in the supplemented group [6]. Thus these intervention trials indicated that β -carotene alone is probably not the active phytochemical in the prevention of cancer. The beneficial effects of fruits and vegetables in the prevention of cancer may, therefore, be due to phytochemicals or carotenoids other than β -carotene. Attention has now been diverted to other carotenoids [7], and protective effects of lycopene against prostate cancer have been reported [8,9].

Epoxycarotenoids are widely distributed in nature, and constitute major dietary carotenoids in a number of fruits and vegetables [10]. 5,6-Epoxy- β -carotene was shown to be highly active in a dose-dependent manner in the induction of differentiation of NB4 cells, as compared with β -carotene [11].

Epoxycarotenoids may, therefore, possess protective effects against cancer. In order to do this, however, epoxycarotenoids must be absorbed by the human body. There is no evidence that epoxycarotenoids, despite their abundance in dietary fruits and vegetables, are absorbed by humans. In this paper, evidence is presented for the absorption of orally administered 5,6-epoxy- β -carotene and 5,8-epoxy- β -carotene by humans.

MATERIALS AND METHODS

Chemicals and solvents

Crystalline synthetic all-*trans*- β -carotene was purchased from Sigma (St. Louis, MO, U.S.A.). Anhydrous diethyl ether, hexane, methanol and dichloromethane were supplied by Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). 3-Chloroperoxybenzoic acid and neutral Al_2O_3 (Brockman activity I) were purchased from Aldrich (Milwaukee, WI, U.S.A.). TLC was carried out on Polygram silica-gel G/UV 254 plates (4 cm $\times$ 8 cm and 20 cm $\times$ 20 cm) (Macherey–Nagel, Duren, Germany; supplied by Brinkmann Instruments, Westbury, NY, U.S.A.). A solvent mixture of hexane/acetone (95:5, v/v) was used during TLC.

5,6-Epoxyretinyl palmitate was prepared from retinyl palmitate by reaction with 3-chloroperoxybenzoic acid, as described in the preparation of 5,6-epoxymethyl retinoate [12], and purified by TLC.

Preparation of epoxy- β -carotenes

Epoxy- β -carotenes were prepared by slight modification of a published procedure [13], as follows. β -Carotene (2 mmol) was dissolved in diethyl ether (100 ml) to which 3-chloroperoxybenzoic acid (2 mmol) was added. The mixture was stirred at room temperature until a test solution during TLC showed that most of the β -carotene had reacted. Water was added, followed

Abbreviation used: DCI, direct chemical ionization.

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by dil. NaOH solution to neutralize excess 3-chloroperoxybenzoic acid. The ether layer was washed with water, dried over anhydrous Na_2SO_4 , and then evaporated to dryness in a rotary evaporator. The residue was dissolved in a minimum volume of diethyl ether (~ 1.5 ml), and subjected to either TLC (to obtain 5,6-epoxy- β -carotene) or column chromatography (to obtain 5,8-epoxy- β -carotene).

For TLC, the crude solution was applied as strips to two 0.5 μm TLC plates (20 cm $\times$ 20 cm). Three bands were separated: a fast-moving minor band of β -carotene (R_f 0.92), a major band of 5,6-epoxy- β -carotene (R_f 0.73), and a band of 5,6,5',6'-diepoxy- β -carotene (R_f 0.56). The 5,6-epoxy- β -carotene band was extruded, and extracted with diethyl ether until all the colour had been extracted. The extract was evaporated to dryness in a rotary evaporator. The residue was dissolved in a few drops of diethyl ether. Hexane (0.5 ml) was added to the solution, which was then kept at -70°C . The mother liquor was removed from the needle-shaped crystals of 5,6-epoxy- β -carotene, and the crystals were dried under argon. 5,6-Epoxy- β -carotene exhibited λ_{max} values of 473, 447 and ~ 423 nm (in HPLC solvent and in hexane). Direct chemical ionization (DCI)-MS (reactant gas methane) of 5,6-epoxy- β -carotene (M_r 552) showed a m/z value of 552 (M^+ ; 100%). Additional fragments were seen at m/z 568 ($M + \text{CH}_4$; 1%), 553 ($M + 1$; 50%), 537 ($M - \text{CH}_3$; 4%) and 472 ($M - 80$; 2%).

Further characterization of the epoxy function in 5,6-epoxy- β -carotene was carried out as described by Karrer and Jucker [14] by rapid isomerization of the 5,6-epoxy ring to a 5,8-furanoid ring, as follows. To a solution of 5,6-epoxy- β -carotene in methanol was added a trace of 0.01 M aq. HCl. The solution was shaken, and the absorption spectrum was recorded. A hypsochromic shift of the absorption maximum of 5,6-epoxy- β -carotene by 19 nm to 428 nm showed that a monoepoxy function was present [13–15]. The absorption spectrum of the 5,8-furanoid compound (λ_{max} 454, 428 and ~ 400 nm) and the retention time on HPLC were identical with those of 5,8-epoxy- β -carotene, as described below. On shaking a solution of 5,6-epoxy- β -carotene in diethyl ether with conc. HCl, the acid layer developed a pale blue colour which was not very stable [13,14].

Although the use of neutral alumina has been reported for the purification of 5,6-epoxy- β -carotene [13,15], it was found in the present study that most of 5,6-epoxy- β -carotene was converted into 5,8-epoxy- β -carotene during passage through the neutral alumina column, due possibly to the presence of traces of HCl. 5,6,5',6'-Diepoxy- β -carotene seen during TLC of the crude product was similarly isomerized to 5,8,5',8'-diepoxy- β -carotene. Therefore, in order to obtain 5,8-epoxy- β -carotene, the crude oxidation product of β -carotene was subjected to column chromatography on neutral Al_2O_3 [de-activated by addition of 2% (w/w) water]. Development with hexane resulted in the elution of β -carotene. Development with 1–2% (v/v) diethyl ether in hexane resulted in some 5,6-epoxy- β -carotene, followed immediately by a major band of 5,8-epoxy- β -carotene. Fractions of 5–10 ml were collected, and the absorption spectrum of each fraction was recorded. Fractions containing 5,8-epoxy- β -carotene were examined by TLC for purity. The pure fractions of 5,8-epoxy- β -carotenes were pooled, evaporated to dryness and dissolved in diethyl ether/hexane as described above. Crystalline 5,8-epoxy- β -carotene showed λ_{max} values of 454, 428 and ~ 400 nm (in hexane and in HPLC solvent), and a single peak on HPLC. DCI-MS (reactant gas methane) of 5,8-epoxy- β -carotene (M_r 552) showed an m/z value of 552 (M^+ ; 100%). Additional fragments were seen at m/z 568 ($M + \text{CH}_4$; 5%), 553 ($M + 1$; 73%), 537 ($M - \text{CH}_3$; 6%) and 472 ($M - 80$; 20%). On shaking a solution of 5,8-epoxy- β -carotene in diethyl ether with conc.

HCl, the acid layer developed a pale blue colour which was not very stable [13,14].

HPLC procedure

Details of the HPLC procedure have been published previously [16]. In brief, gradient reverse-phase HPLC was carried out on a Rainin (Woburn, MA, U.S.A.) Microsorb 3 μm C_{18} column (3.6 cm $\times$ 10 cm) by using a 20-min linear gradient of methanol/water (3:1, v/v) containing 10 mM ammonium acetate to methanol/dichloromethane (4:1, v/v) at a flow rate of 0.8 ml/min. This was followed by isocratic elution with the latter solvent for another 20 min. A photodiode array detector (model 996; Waters Associates, Milford, MA, U.S.A.) was used and set for wavelengths 280–520 nm to record data during HPLC. The chromatogram was monitored at 445 nm for 5,6-epoxy- β -carotene, β -carotene and other plasma carotenoids. Chromatograms were also recorded later at 425 nm and 400 nm (for other carotenoids) and at 340 nm, 325 nm and 315 nm for retinoids. The spectrum index plots of chromatograms at each of the above-mentioned wavelengths of detection were also recorded.

Preparation of oral dose

Crystalline 5,6-epoxy- β -carotene or 5,8-epoxy- β -carotene (each 5 mg; 9.1 μmol) was transferred on to a small mortar, and ground with 1 ml of corn oil until the crystals dissolved. The oily solution was soaked into a small piece of bread, and eaten by a volunteer. Another 1 ml of oil was added to the mortar, ground, and the oil was again transferred to a piece of bread and eaten by the volunteer.

Another volunteer consumed one ripe mango with his breakfast, which consisted of bread-and-butter and tea. Analysis of ripe mango fruit (grown in Mexico; purchased from a local grocery store) showed that it was rich in epoxycarotenoids [16], and that 5,6-epoxy- β -carotene ($\sim 9 \mu\text{g/g}$ of pulp) was one of the major carotenoids present.

Collection of blood

Capillary blood (200 μl) was collected from volunteers before and 3 h after consuming a mango at breakfast or after receiving the oral dose of 5,8-epoxy- β -carotene. The blood was centrifuged immediately in a Readacrit centrifuge (Clay Adams, Parsippany, NJ, U.S.A.) at 500 g for 4 min. The plasma was transferred into a polypropylene microcentrifuge tube (1.5 ml) (USA/Scientific Plastics, Ocala, FL, U.S.A.), and analysed immediately or stored at -20°C .

Venous blood was collected from the volunteer before (0 h) and 2, 4, 6, 8 and 24 h after an oral dose of 5,6-epoxy- β -carotene. The blood was centrifuged at 500 g (1500 rev./min) for 20 min at 4°C , and the plasma was stored at -20°C until analysis by HPLC.

Extraction of carotenoids and retinoids from plasma

A slight modification of the previously published procedure [17] was used for the simultaneous extraction of carotenoids and retinoids from plasma. Plasma (0.1–0.5 ml; 1 vol.) was treated with ethanol (2 vol.) and ethyl acetate (1 vol.) in a 1.5 ml microcentrifuge tube (0.1 ml of plasma) or a 13 cm test tube (0.5 ml of plasma). Retinyl acetate (internal standard) in ethanol containing butylated hydroxytoluene was added, followed by dilute acetic acid (10%, v/v; 0.1 vol.). The mixture was vortexed (30 s), then centrifuged (1 min at 500 g). The supernatant solution

was separated, and kept cold. The pellet was vortexed and centrifuged as before with a mixture of hexane (2 vol.) and ethyl acetate (2 vol.). The two supernatant solutions were pooled, and water (1 vol.) was added. The solution was vortexed and centrifuged as before. The upper organic layer was removed using a pipette, and evaporated to dryness under a gentle stream of argon. The residue was dissolved in a mixture of propan-2-ol/dichloromethane (2:1, v/v; 70 μ l). A 50 μ l sample of the extract was injected on to the HPLC system.

RESULTS AND DISCUSSION

5,6-Monoepoxy- β -carotene and 5,8-monoepoxy- β -carotene were prepared and characterized using published procedures. Karrer and Jucker [14] reported the use of monoperphthalic acid for the epoxidation of β -carotene, and the procedure was successfully used by us in the past for the preparation of epoxides of β -carotene [15]. In recent years, monoperphthalic acid has been replaced by 3-chloroperoxybenzoic acid for the epoxidation of the β -ionone ring of retinoids [12] and β -carotene [13], because 3-chloroperoxybenzoic acid is more stable than monoperphthalic acid and is commercially available. The identities of 5,6-monoepoxy- β -carotene and 5,8-monoepoxy- β -carotene were confirmed by studying their UV-visible absorption spectra, mass spectra and behaviour in the presence of traces of dilute and concentrated HCl. The UV-visible spectral data and the be-

haviour of the epoxides of β -carotene in the presence of traces of dilute and concentrated acid agreed very well with the published data. The MS data for 5,6-monoepoxy- β -carotene and 5,8-monoepoxy- β -carotene obtained during the present study by DCI using methane as a carrier gas also agreed very well with the electron-impact-MS data reported by Liebler and Kennedy [18] for the epoxides of β -carotene. Although the UV-visible spectra of the two epoxides were very different, the mass spectra of the 5,6- and 5,8-epoxides were almost identical, suggesting that rearrangement might occur under the conditions employed during MS analysis [18].

Analysis of extracts of capillary plasma showed that a small but significant peak, which was absent from plasma before consumption of mango, was present in the plasma after mango consumption. The retention time during HPLC of this compound was the same as that of 5,6-epoxy- β -carotene (29.9 min), and the spectrum index plot showed that the absorption spectrum of the compound in the peak was similar to that of 5,6-epoxy- β -carotene. Although other epoxycarotenoids were also present in the mango, no other epoxycarotenoid peak was detected in the plasma.

Analysis of capillary plasma extracts before and 3 h after an oral dose of 5,8-epoxy- β -carotene (9.1 μ mol) showed a major peak whose retention time and absorption spectrum were similar to those of synthetic 5,8-epoxy- β -carotene. The concentration of 5,8-epoxy- β -carotene in plasma 3 h after the dose was found to be approx. 1.2 μ mol/litre of plasma.

Analysis of plasma extracts after an oral dose of 5,6-epoxy- β -carotene (9.1 μ mol) showed that 5,6-epoxy- β -carotene was a major carotenoid in the plasma. The HPLC profiles of carotenoids present in the plasma before and 6 h after the oral dose of 5,6-epoxy- β -carotene are shown in Figures 1(a) and 1(b) respectively. The concentrations of 5,6-epoxy- β -carotene in the plasma at various times after the dose are shown in Figure 2. The concentration of 5,6-epoxy- β -carotene was highest (2.29 μ mol/litre of plasma) 6 h after the dose. The volunteer receiving the dose of 5,6-epoxy- β -carotene weighed 60 kg. Based on the assumption that 4% of the body weight is plasma, the volunteer would have had 2.4 litres of total plasma, and thus a total of 2.29 μ mol/litre $\times$ 2.4 litres = 5.5 μ mol (61% of the administered dose) of 5,6-epoxy- β -carotene in the circulation 6 h after the dose. This showed that the administered dose of 5,6-epoxy- β -carotene was efficiently absorbed. The identity of 5,6-epoxy- β -carotene was confirmed by comparison of its retention time during HPLC under several different conditions, and by study of the absorption spectrum recorded by the photodiode array detector with that of synthetic 5,6-epoxy- β -carotene. Furthermore, when the plasma extract was treated with a trace of dilute HCl and then analysed by HPLC, the absorption spectrum of 5,6-epoxy- β -carotene changed to that of 5,8-epoxy- β -carotene.

HPLC analysis of retinoids in the plasma after the dose of 5,6-epoxy- β -carotene showed the presence of retinyl palmitate in the plasma, but no other major metabolite of 5,6-epoxy- β -carotene. However, scrutiny of the spectrum index plot of the chromatogram monitored at 313 nm for 5,6-epoxyretinol showed that a minor peak with the characteristic UV spectrum of 5,6-epoxyretinyl palmitate (λ_{max} 325, 313 and $\sim$ 295 nm in HPLC solvent) was also present. The retention time and UV spectrum of the metabolite of 5,6-epoxy- β -carotene were identical with those of synthetic 5,6-epoxyretinyl palmitate. Quantification of 5,6-epoxyretinyl palmitate formed from orally administered 5,6-epoxy- β -carotene was not possible due to very small amount of the metabolite that was present in the plasma. Although retinol was the predominant retinoid present in the plasma before and after the dose, no 5,6-epoxyretinol could be detected in the plasma at

Figure 1 Reverse-phase gradient HPLC profiles of carotenoids in human plasma

A human volunteer was given an oral dose of 5,6-epoxy- β -carotene (9.1 μ mol). Plasma was analysed for carotenoids before (a) and 6 h after (b) the oral dose. Peak identification: 1, bilirubin; 2, lutein; 3, zeaxanthin; 4, β -cryptoxanthin; 5, 5,6-epoxy- β -carotene; 6, lycopene; 7, β -carotene. The detection wavelength was 445 nm. AU, absorbance units.

Figure 2 Time course of 5,6-epoxy- β -carotene concentration in human plasma after a single oral dose of 5,6-epoxy- β -carotene

A human volunteer was given an oral dose of 5,6-epoxy- β -carotene (9.1 μ mol). Plasma was analysed for 5,6-epoxy- β -carotene at various times after the dose.

any time after the dose. 5,6-Epoxyretinoic acid was also not detected.

It is not known whether epoxy- β -carotenes or epoxyretinoids derived from them can serve as modulators of physiological processes *in vivo*. The fate of epoxyxanthophylls, such as violaxanthin and neoxanthin, which are major carotenoids in green vegetables, is not known. In view of the present finding that

epoxy- β -carotenes are absorbed efficiently by humans, further studies are warranted to determine whether other epoxycarotenoids are absorbed by humans and metabolized into hitherto unknown carotenoids and retinoids that might also play role in cancer prevention.

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