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Cytotoxic and genotoxic effects of β-carotene breakdown products on primary rat hepatocytes

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According to Siems and colleagues, free radical attack on β-carotene results in the formation of high amounts of cleavage products with prooxidant activities towards subcellular organelles such as mitochondria. This finding may be an explanation for the contradictory results obtained with β-carotene in clinical efficacy and cancer prevention trials. Since primary hepatocytes proved to be very sensitive indicators of the genotoxic action of suspect mutagens/carcinogens we therefore investigated a β-carotene cleavage products mixture (CP), apo8′-carotenal (apo8′) and β-carotene utilizing primary cultures of rat hepatocytes. The end-points tested were: the mitotic index, the percentage of necrotic and apoptotic cells, micronucleated cells, chromosomal aberrations and sister chromatid exchanges (SCE). Our results indicate a genotoxic potential of both CP and apo8′ already at the concentrations 100 nM and 1 μM, i.e. at pathophysiologically relevant levels of β-carotene and β-carotene breakdown products. A 3 h treatment with CP induced statistically significant levels of micronuclei at concentrations of 0.1, 1 and 10 μM and chromosomal aberrations at concentrations of 1, 5 and 10 μM. Apo8′ induced statistically significant levels of micronuclei at concentrations of 0.1, 1 and 5 μM and chromosomal aberrations at concentrations of 0.1, 1 and 10 μM. Statistically significant increases in SCE induction were only observed at a concentration of 10 μM CP and apo8′. In contrast, no significant cytotoxic effects of these substances were observed. Since β-carotene induced neither significant cytotoxic nor genotoxic effects at concentrations ranging from 0.01 up to 10 μM, these observations indicate that most likely β-carotene breakdown products are responsible for the occurrence of carcinogenic effects found in the Alpha-Tocopherol Beta-Carotene Cancer Prevention (ATBC) Study and the Beta-CArotene and RETinol Efficacy Trial (CARET).

Abbreviations: BrdU, bromodeoxyuridine; apo8′, apo8′-carotenal; CP, β-carotene cleavage products; EGF, epidermal growth factor; MEM, Minimum essential medium.

Introduction

Many diseases, such as certain forms of cancer and cardiovascular diseases, are associated with oxidative stress (1). Therefore, as a strategy to prevent their development, antioxidant and radical scavenging properties of carotenoids, especially of β-carotene, are widely used (2–6). The use of β-carotene and carotenoids is further supported by their demonstrated anti-genotoxic effects (7–12).

However, prooxidant and co-carcinogenic effects have also been reported (13–15). Furthermore, the Alpha-Tocopherol Beta-Carotene Cancer Prevention (ATBC) Study and the Beta-CArotene and RETinol Efficacy Trial (CARET) unexpectedly showed an increased risk of lung cancer in smokers (16,17). Wang and Russell (18) showed that β-carotene decreases the level of retinoic acid in the lungs and this reduces the inhibitory effect of retinoids on activator protein-1. As a consequence, lung cell proliferation and, potentially, tumor formation is enhanced (18). The same authors suggested that β-carotene metabolites are responsible for the carcinogenic response in the lungs of cigarette smokers. These data are supported by Leo and Lieber (19), who showed that β-carotene supplementation in smokers who also consume alcohol promotes pulmonary cancer and possibly also cardiovascular diseases.

In their investigations, Van Poppel *et al*. (20) demonstrated the lack of a protective effect of β-carotene on smoking-induced DNA damage in lymphocytes of heavy smokers.

Effects of β-carotene have been reported to be modified under certain conditions and at certain concentrations. Zhang and Omaye (21) demonstrated that antioxidant and prooxidant effects of β-carotene are dependent on oxygen tension and the concentration of β-carotene.

Furthermore, free radical attack on carotenoids results in the formation of numerous breakdown products, β-carotene cleavage products (CP), which could contribute to the carcinogenic effects (22). In addition, it was demonstrated, that apo8′-carotenal (apo8′), a metabolite of β-carotene, acts as a strong inducer of liver cytochromes P450 1A1 and 1A2 (23).

Since a genotoxic potential of CP cannot be excluded from these observations, the effects of CP, apo8′ and β-carotene were investigated in primary cultures of rat hepatocytes. These cells proved to be a highly sensitive and reliable test system for the evaluation of the genotoxic potential of mutagens/promutagens (24–26). The end-points tested were: the mitotic index, the percentage of necrotic and apoptotic cells, micronucleated cells, chromosomal aberrations and sister chromatid exchanges (SCE).

Materials and methods

Materials

Minimum essential medium (MEM) with Earle's salts and non-essential amino acids and antibiotics were obtained from Life Technologies (Vienna, Austria).

Plastic culture dishes were from Sarstedt (Austria). Epidermal growth factor (EGF), collagenase and other cell culture chemicals, unless otherwise specified, were purchased from Sigma Chemical Company via Biotrade (Vienna). Apo8′ was a gift from BASF AG (Ludwigshafen, Germany).

Since crystalline β-carotene is not water-soluble the carotenoid had to be emulsified in a soybean oil carrier to enable physiological activity. The water-dispersable β-carotene in soybean suspension (2% w/v) and the soybean suspension used as blank matrix in the experiments were a gift of Cognis Australia Pty Ltd (Australia). The carotenoid emulsion was made from a starting material of 30% β-carotene (derived from an algal extract) in soybean oil which was emulsified into a 30% water, 70% glycerol aqueous phase using a glyceryl mono-oleate emulsifier. The fine emulsion provides the carotenoid in a lipid globule size of ~1 μm or less so that interaction can occur at the cellular level.

The generation of CP was performed as described by Siems *et al*. (22) and Sommerburg *et al*. (27) by C.-D.Langhans, who is gratefully acknowledged. For degradation, β-carotene was dissolved in methanol containing 2% (v/v) trichloromethane to achieve sufficient solubility of the carotenoid. Chemical destruction of β-carotene was done by bleaching with hypochlorite by adding NaOCl in a 100-fold concentration relative to the carotenoid. The samples reacted at room temperature and in daylight for 10 min. After hexane extraction, different CP were identified (HPLC and GC–MS) and partially quantified (HPLC) in the aliquots obtained. The CP mixture obtained from a 0.5 mM β-carotene stock solution contained β-carotene (0.16 mM), apo15′-carotenal (0.08 mM), apo12′-carotenal (0.12 mM) and apo8′-carotenal (0.006 mM) and a number of products which could not be identified by HPLC. Further products could be identified by GC–MS, but not quantified because of the extraordinary technical difficulty. Related to all peaks detected during GC–MS analysis was a peak area of ~4.8% accounting for β-cyclocitral, 0.1% for ionene, 9.9% for β-ionone, 1.9% for β-ionone-5,6-epoxide and 4.5% for dihydroactinidiolide. Furthermore, 4-oxo-β-ionone was detected in trace amounts.

Animals

Female Fischer 344 rats weighing ~100 g were obtained from Harlan (Winkelman, Germany). They were kept in a temperature and humidity controlled room with a 12 h light–dark cycle. Water was provided *ad libitum*. The animals were allowed to acclimatize for at least 2 weeks prior to hepatocyte isolation.

Hepatocyte isolation and culture

Hepatocytes were isolated from female Fischer 344 rats by the *in situ* two-step collagenase perfusion technique as described by Michalopoulos *et al*. (28). The isolated hepatocytes were plated at a density of 20 000 viable cells/cm^2 on collagen-coated 60 mm diameter plastic culture dishes. According to Eckl *et al*. (29), the hepatocytes were plated in 5 ml of serum-free MEM containing 1.8 mM calcium supplemented with non-essential amino acids, pyruvate (1 mM), aspartate (0.2 mM), serine (0.2 mM) and penicillin (100 U)/streptomocin (100 μg/ml). The cultures were incubated at 37°C, 5% CO_2 and 95% relative humidity. After an incubation period of 3 h, the medium was exchanged for fresh MEM and the cultures were returned to the incubator.

Treatment

Approximately 20 h after the first exchange of the medium, the test substances were added to the cultures at concentrations of 0.01, 0.1, 1, 5 and 10 μM CP, apo8′ and β-carotene and incubated for 3 h. Then the medium was aspirated and the plates were washed twice with fresh medium to completely remove the applied substances. Finally, fresh MEM containing 0.4 mM Ca^{2+}, supplemented as described above with the further addition of insulin (0.1 μM) and EGF (40 ng/ml), was added. To determine SCE induction, bromodeoxyuridine (BrdU) (10 μM) was added to three dishes of each concentration. Thereafter, cells were incubated for an additional 48 h.

Fixation, staining and cytogenetic analysis

Cytogenetic studies were performed as described by Eckl *et al*. (24). After 48 h colcemid (0.4 μg/ml) was added to three dishes (where BrdU was added) per concentration and the cultures were incubated for a further 3 h. No colcemid was added to the cultures for the micronucleus assay.

For the micronucleus assay, cells were fixed in the dishes with methanol: glacial acetic acid (3:1) for 15 min, briefly rinsed with distilled water and air dried.

For chromosome preparations cells were harvested by replacing the medium with 2 ml of collagenase solution (0.5 mg collagenase/ml) and incubation for 10 min. The detached cells were collected by centrifugation (44 *g*), treated with hypotonic KCl solution (0.02 M) for 10 min at 37°C and fixed in freshly prepared methanol:glacial acetic acid (3:1) overnight. Preparations were made by dropping the cell suspension on precleaned frosted slides.

For micronucleus determination the fixed cells were stained with the fluorescent dye DAPI (4,6′,6-diamidino-2-phenylindole) in McIlvaine buffer (0.2 M Na_2HPO_4 buffer adjusted with 0.1 M citric acid to pH 7.0) for 30 min in the dark at room temperature. After washing with McIlvaine buffer, the dishes were rinsed with distilled water followed by air drying. For microscopic observation, fixed and stained cells were mounted in glycerol. To determine the mitotic index, the frequencies of apoptotic and necrotic cells and the number of cells with micronuclei per 1000 cells per dish were analysed under a fluorescence microscope (Leitz Aristoplan).

The slides for studying chromosomal aberrations and SCE induction were stained with Hoechst 33258 (4.5 μg/ml) in Sörensen phosphate buffer pH 6.8 for 20 min, rinsed with Sörensen phosphate buffer and exposed to black light (General Electric F 40 BLB Black light) for 15 min on a warming plate kept at 50°C. After removal of the coverslips the slides were briefly washed with distilled water and stained in 5% Giemsa solution. Twenty well-spread first division metaphases were analysed for chromosomal aberrations under a phase contrast microscope (Leitz Laborlux 11). Twenty well-spread second division metaphases were analysed for SCE. The number of aberrations is given per diploid cell, i.e. 42 chromosomes. The SCEs are reported per chromosome.

Statistical analysis

Student's double-sided *t*-test for independent samples was used to calculate the levels of significance.

Results

Cytotoxicity

As shown in Table I, no significant changes in the mitotic index and the frequencies of necrotic and apoptotic cells were induced by the two substances tested (CP and apo8′).

Genotoxicity

Micronucleus induction. As demonstrated in Figure 1, CP and apo8′ had significant effects on the frequency of micronucleated cells. For CP, micronucleus induction was significant at concentrations of 100 nM ($P < 0.005$) and 1 and 10 μM ($P < 0.05$). At higher concentrations, the efficiency of CP in inducing micronucleated cells declined. Similar data were obtained for apo8′, which induced significantly increased levels of micronucleated cells at concentrations of 100 nM, and 1 and 5 μM ($P < 0.005$). In contrast to CP, apo8′ also gave rise to significant formation ($P < 0.05$) of micronucleated cells at a concentration of 5 μM.

Although both CP and apo8′ proved to be potent inducers of chromosomal aberrations, a different dose–response effect was obtained, as demonstrated in Figure 2. Chromosomal aberrations induced by CP showed a maximum value at a concentration of 100 nM, however, due to wide variations between the single experiments this value was not significant.

Table I. Effects of CP and apo8′-carotenal on cell proliferation (mitotic index) and cell death (necrosis and apoptosis)

Compound	Conc.	Mitotic cells (%)	Necrotic cells (%)	Apoptotic cells (%)
Control	1% DMSO[a]	1.73 ± 0.30	0.02 ± 0.01	0.06 ± 0.03
CP	0.01 μM	1.33 ± 0.25	0.07 ± 0.03	0.08 ± 0.03
	0.1 μM	1.58 ± 0.42	0.02 ± 0.01	0.05 ± 0.00
	1 μM	1.56 ± 0.23	0.02 ± 0.01	0.05 ± 0.00
	5 μM	1.63 ± 0.29	0.02 ± 0.01	0.05 ± 0.05
	10 μM	1.33 ± 0.25	0.08 ± 0.03	0.08 ± 0.03
apo8′-carotenal	0.01 μM	1.27 ± 0.20	0.05 ± 0.05	0.07 ± 0.03
	0.1 μM	1.78 ± 0.26	0.02 ± 0.01	0.06 ± 0.03
	1 μM	1.68 ± 0.19	0.02 ± 0.01	0.07 ± 0.03
	5 μM	1.52 ± 0.08	0.02 ± 0.04	0.05 ± 0.00
	10 μM	1.82 ± 0.25	0.01 ± 0.02	0.05 ± 0.00

Data represent the means ± SD of three independent experiments.
[a]The concentration of DMSO at the highest concentrations applied.

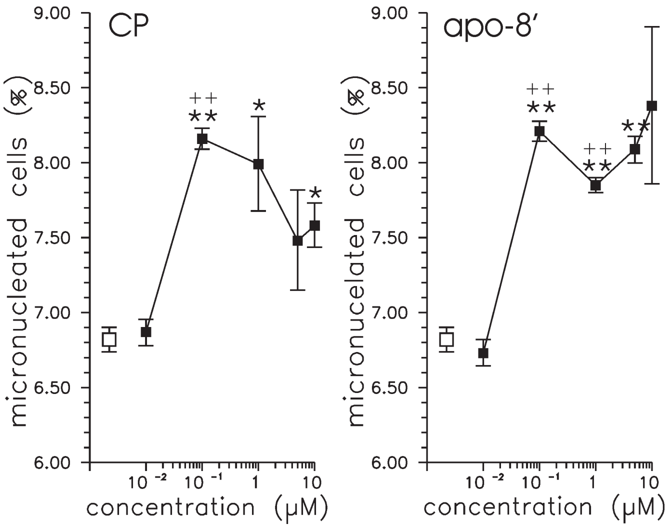

Fig. 1. Frequencies of micronucleated cells in control cultures (open squares) and cultures treated with CP and apo8′-carotenal (closed squares). Data represent the means ± SEM of three independent experiments. $^{*}P \leq 0.05$; $^{**}P \leq 0.005$ compared with the control. $^{++}P \leq 0.005$ compared with the preceding concentration.

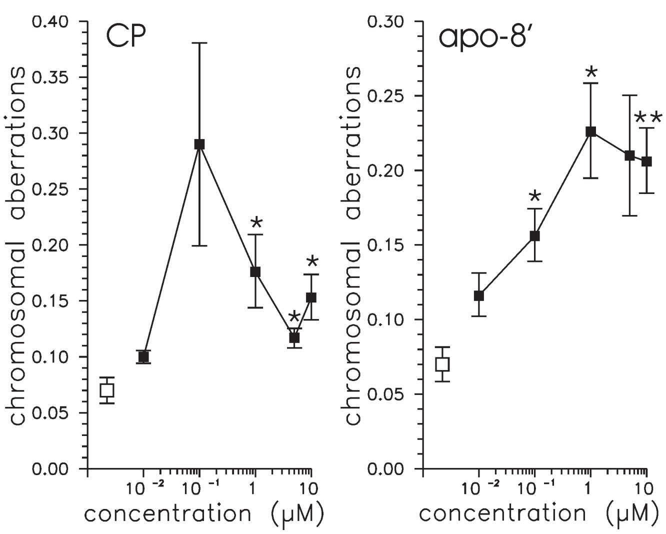

Fig. 2. Frequencies of chromosomal aberrations in control cultures (open squares) and cultures treated with CP and apo8′-carotenal. Data represent the means ± SEM of three independent experiments. $^{*}P \leq 0.05$; $^{**}P \leq 0.005$.

A statistically significant induction of chromosomal aberrations by CP was observed at concentrations of 1, 5 and 10 μM ($P < 0.05$). In contrast, apo8′ induced significantly increased levels of chromosomal aberrations at 100 nM, 1 μM ($P < 0.05$) and 10 μM ($P < 0.005$).

Induction of SCE. As demonstrated in Figure 3, CP and apo8′ gave rise to a concentration-dependent increase in the rate of SCEs which became significant ($P < 0.05$) at a concentration of 10 μM. β-Carotene tested for comparison induced no significant cytotoxic or genotoxic effects (Table II).

Discussion

Most toxicological investigations of β-carotene and carotenoids have focused on the antimutagenic properties.

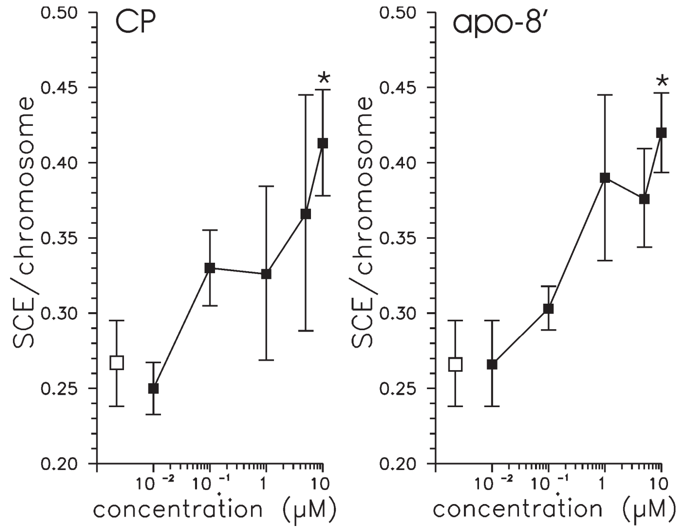

Fig. 3. Frequencies of sister chromatid exchanges in control cultures (open squares) and cultures treated with CP and apo8′-carotenal. Data represent the means ± SEM of three independent experiments. $^{*}P \leq 0.05$; $^{**}P \leq 0.005$.

Antimutagenic effects of β-carotene and carotenoids have been described mainly for bone marrow cells of mice (7,8,10,11) or have been demonstrated in the *Salmonella typhimurium* test (11,12). The same applies to the breakdown product apo8′. Antimutagenic activity of apo8′ against benzo[*a*]pyrene and aflatoxin B1 were found in the Ames test and by investigating micronucleated polychromatic erythrocytes in bone marrow cells of mice (11). Additionally, Durnev *et al*. (10) revealed antimutagenic properties of apo8′ when applied at a dose of 50 mg/kg. Lower concentrations did not induce a significant antimutagenic effect against cyclophosphamide- and dioxidine-induced mutagenicity in bone marrow cells taken from mice.

Based on the observation of an increased risk of lung cancer in smokers (16,17) in the ATBC Study and CARET Siems *et al*. (22) hypothesized that degradation of β-carotene leads to the formation of high amounts of cleavage products with prooxidant properties (CP) under heavy oxidative stress, while under conditions of moderate oxidative stress, antioxidant effects of β-carotene are dominant. The results of our study do not directly support this assumption, but demonstrate a genotoxic effect which could either be due to the prooxidant properties of CP or to a direct or indirect (involving metabolism in hepatocytes or other metabolically competent cells such as those in the lung) action on DNA. Such an action has not been described before and appears to have relevance for the *in vivo* situation, since the effects are already seen at nanomolar concentrations which can be produced *in vivo* under defined conditions.

Apo8′ has been reported to be a strong inducer of liver cytochromes P450 1A1 and 1A2 (23). Our data obtained with apo8′ revealed a significant mutagenic potential with respect to micronucleus induction, induction of chromosomal aberrations and SCE. Whether these observations are interrelated or not cannot be answered. However, the dose–response effects obtained in this study could indicate a link between these observations:

Induction of micronuclei and chromosomal aberrations by apo8′ follow saturation-type dose–response characteristics,

Table II. Cytotoxic and genotoxic effects of β-carotene

		Mitotic index (%)	Necrotic cells (%)	Apoptotic cells (%)	Micronuclei (%)	CA/metaphase	SCE/chromosome
Control	Matrix in 1% DMSO[a]	1.50 ± 0.26	0.13 ± 0.12	0.20 ± 0.00	7.90 ± 0.90	0.12 ± 0.03	0.49 ± 0.05
β-carotene	0.01 μM	1.87 ± 0.51	0.13 ± 0.12	0.10 ± 0.10	7.40 ± 1.37	0.13 ± 0.04	0.50 ± 0.09
	0.1 μM	1.60 ± 0.20	0.17 ± 0.06	0.27 ± 0.15	7.63 ± 0.74	0.15 ± 0.05	0.53 ± 0.05
	1 μM	1.46 ± 0.12	0.13 ± 0.12	0.05 ± 0.00	9.50 ± 0.56	0.16 ± 0.08	0.56 ± 0.07
	10 μM	1.63 ± 0.35	0.13 ± 0.12	0.13 ± 0.12	8.93 ± 0.99	0.23 ± 0.12	0.64 ± 0.10

Data represent the means ± SD of three independent experiments.
[a]The concentration of the matrix plus DMSO at the highest concentrations applied.

whereas the dose–response effect obtained with CP appears to be bell-shaped. For both CP and apo8′ the efficiency of micronucleus induction decreases at concentrations >0.1 μM, with the induction of chromosomal aberrations decreasing at concentrations >0.1 and >1 μM, respectively. Treatment with either CP or apo8′ did not influence the mitotic index and the frequencies of necrotic and apoptotic cells remained unchanged at all concentrations tested, thus both substances showed no cytotoxic effects. Therefore, the shapes of the dose–response curves cannot be explained by an increased toxicity of CP. Since it has been demonstrated that apo8′ acts as a strong inducer of liver cytochromes P450 1A1 and 1A2 (23), the plateau in the dose–response curve may reflect these changes. In other words, the induced drug-metabolizing or other enzymes could reduce the genotoxic action of apo8′. The dose–response curve for CP treatment may also include such a component, due to the presence of apo8′. In addition, one can also expect effects from other breakdown products, the combined effect of which is a decrease at higher CP concentrations. This behaviour is not uncommon and has been observed in the hepatocyte test after treatment with complex environmental mixtures (30,31).

Since micronuclei are the result of either chromosome breaks or disturbances of the mitotic spindle (32) and chromosome aberrations result from clastogenic events with and without chromosomal rearrangements (33), these parameters are usually considered to be clear evidence for mutagenicity. On the other hand, SCE may not represent actual damage to chromosomes, but could instead be considered a result of damage repair. Thus, the different dose–response effects obtained for the different end-points tested indicate that both CP and apo8′ primarily induce clastogenic events while the SCE-inducing activity becomes significant at high concentrations. This observation could eventually be linked to the shapes of the dose–response curves for chromosomal aberrations and micronuclei, and could indicate the generation of differently acting metabolites.

During the perfusion, washing steps and medium changes hepatocytes are exposed to increased oxygen tension and it has been shown that elevated oxygen concentrations may lead to an increase in the formation of free radicals, in other words may exert oxidative stress which particularly influences the frequencies of micronucleated cells and the occurrence of SCEs (32). This effect is taken into consideration with the appropriate controls, however, it cannot be excluded that the genotoxic effect of β-carotene breakdown products (CP and apo8′) is influenced to some extent. Further investigations under hyperoxic conditions and the use of antioxidants will help to clarify a potential combinational effect.

Summarizing, the results obtained in this study indicate that β-carotene breakdown products are capable of inducing genotoxic effects at concentrations which may occur under conditions of intense oxidative stress, e.g. induced by heavy smoking. Therefore, our findings could be helpful in explaining the adverse side effects reported in the ATBC study and CARET (16,17).

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