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# Cytotoxicity of β-carotene cleavage products and its prevention by antioxidants

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**When we investigated the genotoxicity of β-carotene cleavage products (CPs) in primary rat hepatocytes stimulated to proliferate, we observed dose-dependent increases of chromosomal aberrations, sister chromatid exchanges and micronuclei. In contrast to other genotoxic substances, however, this increased genotoxicity was not accompanied by increased cytotoxicity. As a consequence we observed metaphases showing massive chromosomal damage, indicating inhibition of apoptosis by CPs enabling these cells to proceed in the cell cycle. Since proliferative stimulation by growth factors may support this effect, the *in vitro* toxicological effects of CPs were studied on proliferatively quiescent primary rat hepatocytes. A significant increase of both apoptosis and necrosis was found. Supplementation with antioxidants did not significantly lower the level of apoptosis, while the level of necrosis was significantly reduced by Trolox and *N*-acetylcysteine at all concentrations tested as well as ascorbic acid (50 μM) and a combination of Trolox (50 μM) and ascorbic acid (50 μM). These observations indicate that a) the cytotoxic potential in combination with the genotoxic potential of CPs may promote the initiation of cells due to compensatory cell division in exposed tissues and may aggravate inflammatory processes under chronic exposure, and b) the applied antioxidants may protect from cytotoxicity primarily *via* the detoxification of aldehydic β-carotene cleavage products.**

**Keywords:** β-carotene cleavage products, hepatocytes, apoptosis, necrosis, antioxidants

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## INTRODUCTION

β-Carotene is widely used in the prevention of diseases associated with oxidative stress (Sies *et al.*, 1992; Olson & Krinsky, 1995; Halliwell, 2000; Agarwal & Rao, 2000; Lu *et al.*, 2001) because of its demonstrated antioxidant and antimutagenic properties (Aidoo *et al.*, 1995; Rauscher *et al.,* 1998; Arriaga-Alba *et al.*, 2000). Unexpectedly, however, the Alpha-Tocopherol Beta-carotene-Cancer prevention (ATBC) study and the beta-CArotene and RETinol Efficacy (CARET) Trial showed an increased risk of lung cancer in smokers (Blumberg & Block, 1994; Omenn *et al.*, 1996). It was therefore suggested by Wang and Russell (1999) that β-carotene metabolites are responsible for the carcinogenic response, and Siems *et al.* (2002) were the first to demonstrate that β-carotene cleavage products induce oxidative stress *in vitro*. Based on this observation both a cleavage product (CP) mixture generated by hypochlorite bleaching of β-carotene and one of the major carotenals — apo8'-carotenal — were tested for their genotoxic potential in the primary hepatocyte assay, both in the presence and absence of oxidative stress (Alija *et al.*, 2004; 2006). Those investigations clearly demonstrated a dose-dependent genotoxic potential of the CPs which was further enhanced in the presence of oxidative stress by hypoxia/reoxygenation or DMNQ (2,3-dimethoxy-1,4-naphthoquinone) application. This genotoxic potential, however, was not accompanied by cytotoxicity: no significant increase of apoptosis or necrosis was observed, nor was there any significant influence on the cell proliferation rate, indicating that the CPs may not be toxic. Since the combined treatment with CPs and hypoxia/reoxygenation led to the appearance of highly abnormal metaphases, the lack of apoptosis may also indicate that cell cycle control is compromised allowing damaged cells to proceed through the cycle instead of becoming arrested for repair, or going into apoptosis.

Although the latter assumption appears to be reasonable in view of the present knowledge of cell cycle control mechanisms, there is also evidence from studies with hepatocytes that growth factors such as epidermal growth factor (EGF) used for proliferative stimulation of resting hepatocytes can inhibit apoptosis (Musallam *et al.*, 2001; Ethier *et al.*, 2003). The clarification whether the intrinsic cytotoxicity of CPs is masked after proliferative stimulation with EGF is of major importance because cell death *in vivo* in a given tissue will be compensated by cell division (Jaeschke & Bajt, 2006), which in turn increases the probability for the fixation of mutations as indicated by the genotoxic potential of CPs (Alija *et al.*, 2004). To evaluate the potential cytotoxicity, primary cultures of hepatocytes were therefore treated with CPs at a concentration of 1 μM for 3 h followed by incubation in the absence of EGF, and harvested 48 h later to determine endpoints of cytotoxicity.

Since Liu *et al.* (2004) had demonstrated that cigarette smoke enhanced oxidative excentric cleavage of β-carotene was significantly decreased when α-tocopherol (50 μM) and ascorbic acid (50 μM) were incubated together with β-carotene (10 μM) and lung postnuclear fractions of cigarette smoke-exposed ferrets, we further

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**Abbreviations:** CPs, β-carotene cleavage products; EGF, epidermal growth factor.

tested whether supplementation of the cultures with antioxidants could prevent the cytotoxicity of CPs.

## MATERIALS AND METHODS

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). EGF, collagenase and other cell culture chemicals — unless otherwise specified — were purchased from Sigma Chemical Company *via* Biotrade (Vienna, Austria).

The generation of β-carotene breakdown products (CP) was performed as described by Siems *et al.* (2000) and Sommerburg *et al.* (2003) by C.-D. Langhans. 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 (adding NaOCl in a 100 fold excess relative to the carotenoid). The samples were allowed to react at room temp. and daylight for 10 min. After hexane extraction different CPs were identified (HPLC and GCMS) 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), apo8'-carotenal (0.006 mM), and a number of products which could not be identified by HPLC. Further products could be identified by GCMS but not quantified because of the extraordinary technical difficulty. Relative to all peaks detected during GCMS analysis the peak area of roughly 4.8% accounted 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 traces. The concentration of the CPs applied refers to the original β-carotene concentration.

**Animals.** Female Fischer 344 rats weighing approx. 100 g were obtained from HARLAN, Winkelmann (Germany) and 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 acclimate for at least two weeks prior to hepatocyte isolation.

**Hepatocyte isolation and culture.** Hepatocytes were isolated from rats by the *in situ* two-step collagenase perfusion technique as described by Michalopoulos *et al.* (1982), plated at a density of 20000 viable cells/cm² on collagen-coated 60-mm diameter plastic culture dishes. Hepatocytes were plated in 5 ml of serum-free Minimum Essential Medium (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)/streptomycin (100 μg/ml). The cultures were incubated at 37 °C, 5% $CO_2$ and 95% relative humidity. The medium was exchanged for fresh MEM after an incubation period of 3 h and the cultures were returned to the incubator.

**Treatment.** Approximately 18 h after the first exchange of the medium the cultures were treated with the β-carotene cleavage product mixture at a concentration of 1 μM alone or in combination with antioxidants or antioxidant combinations (final concentrations: Trolox: 10, 50 or 100 μM; ascorbic acid: 50 μM; *N*-acetylcysteine: 10, 50 or 100 μM; Trolox: 10 μM plus ascorbic acid: 50 μM; Trolox: 50 μM plus ascorbic acid: 50 μM), and incubated for three hours. A concentration of 1 μM CPs was chosen since it had been demonstrated to be the lowest concentration inducing significant levels of micronucleated cells (Alija *et al.*, 2004). Furthermore, this concentration is closest to the level of β-carotene in normal human blood plasma (Hallfrisch *et al.,* 1994).

CPs were dissolved in DMSO (dimethylsulfoxide) yielding a final concentration of 1% in the medium. Therefore, control cultures were treated with 1% DMSO. For comparison cells were also treated with the antioxidant concentrations and combinations applied. Thereafter the medium was aspirated and the plates were washed twice with fresh medium to completely remove any traces of the test substances. Finally, fresh MEM containing 0.4 mM $Ca^{2+}$, supplemented as described above and insulin (0.1 μM) was added to the dishes.

**Fixation, staining and cytological analysis.** For the determination of apoptosis and necrosis, cells were fixed after an additional 48 h in the dishes with methanol/glacial acetic acid (3:1) for 15 min, briefly rinsed with distilled water and air dried. The fixed cells were stained with the fluorescent dye DAPI (4',6-diamidino-2-phenylindole) (0.2 μg/ml) in McIlvaine buffer (0.2 M $Na_2HPO_4$ adjusted with 0.1 M citric acid to pH 7.0) for 30 min in the dark at room temp. 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 frequencies of apoptotic, necrotic and mitotic cells as well as changes of cell densities due to loss of dead cells 35 optical fields (magnification: 400×) across the 60 mm diameter Petri dishes (two dishes per treatment) were scored under the fluorescence microscope (Leitz Aristoplan).

Since exact quantification of apoptotic events is not possible by use of electrophoretic methods (DNA-laddering), and the TUNEL assay fails to discriminate between apoptotic and necrotic cells in hepatocytes (Grasl-Kraupp *et al.*, 1995), the morphology of DAPI-stained nuclei was used to unequivocally discriminate between apoptosis (crescent-like condensed or fragmented chromatin) and necrosis (small, pyknotic nuclei revealing highly condensed chromatin) as suggested previously (Oberhammer *et al.*, 1992). This method has the further advantage that the frequency of apoptotic and necrotic events can be determined in parallel in a single culture. The identification of apoptotic and necrotic cells was confirmed by use of the annexinV/propidium iodide assay (Annexin-V-FLUOS Staining Kit from Boehringer Mannheim, Germany) but is not further mentioned in the text. From our experience with double staining with DAPI and Trypan Blue allowing a precise discrimination between viable, necrotic (primary and secondary) and different stages of apoptotic cells (Bresgen *et al.,* 2008), and those of others, i.e. Huerta *et al.* (2007), it is evident that morphological criteria most appropriately and unequivocally describe apoptosis and necrosis, while it is very risky to rely on results obtained with methodologies leading to errors due to harvesting of the cells from primary culture and inappropriate staining as pointed out above.

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

## RESULTS

When primary hepatocytes are treated for 3 hours with β-carotene cleavage products at a concentration of 1 μM without subsequent proliferative stimulation with

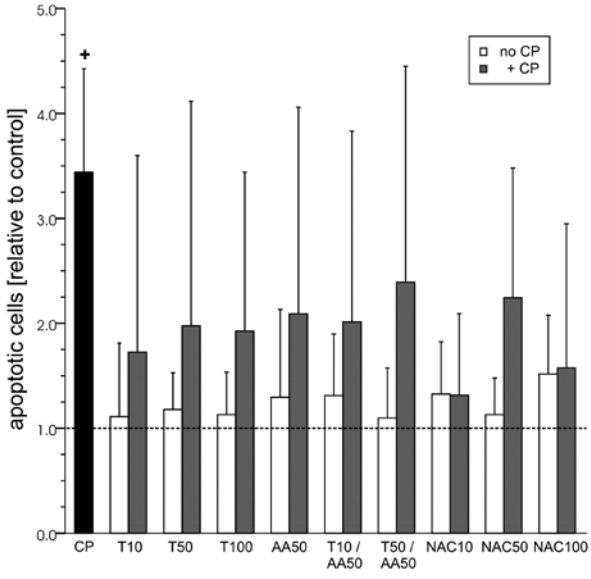


**Figure 1. Effects of treatment with CPs and combinations of CPs and antioxidants on apoptotic cell death in primary rat hepatocytes.**
Data are presented as changes relative to the DMSO control (dotted line) and represent the mean ±S.D. of three independent experiments. Non-transformed control values expressed as percentage of cell population: control: 0.22±0.08; CPs: 0.7±0.15; $^{+}P<0.05$ compared to untreated control. Student's double sided *t*-test for independent variables.

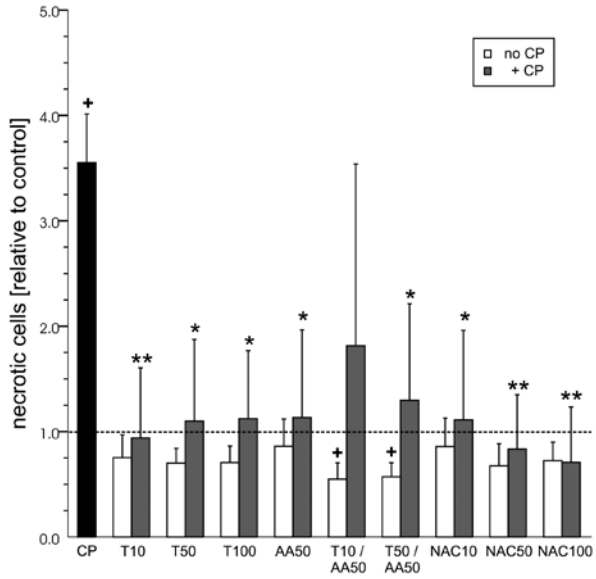


**Figure 2. Effects of treatment with CPs and combinations of CPs and several antioxidants on necrotic cell death in primary rat hepatocytes.**
Data are presented as changes relative to the DMSO control (dotted line) and represent the mean ±S.D. of three independent experiments. Non-transformed control values expressed as percentage of cell population: control: 1.2±0.58; CPs: 4.2±1.7 $^{+}P<0.05$ compared to the untreated control; $^{*}P \le 0.05$, $^{**}P \le 0.005$ compared to 1 μM CP. Student's double sided *t*-test for independent variables.

EGF, a significant ($P<0.05$) increase of both apoptosis (control: 0.22±0.08; CP: 0.7±0.15) and necrosis (control: 1.2±0.58; CP: 4.2±1.7) is observed after 48 h (Fig. 1 and 2). Simultaneous supplementation of antioxidants reduces the frequency of apoptosis and especially necrosis. Trolox at every concentration tested reduced necrosis to approximately control level (10 μM: $P<0.005$; 50 and 100 μM: $P<0.05$). Ascorbic acid had no significant effect on the rate of apoptosis but significantly reduced necrosis to the control level ($P<0.05$). Combinations of Trolox and ascorbic acid had no significant effect on apoptosis induction by CPs. However, a combination of 50 μM Trolox and 50 μM ascorbic acid significantly reduced necrosis. *N*-Acetylcysteine had no significant effect on the rate of apoptosis, but dose-dependently and significantly reduced necrosis (10 μM: $P<0.05$; 50 and 100 μM: $P<0.005$).

CP treatment significantly ($P<0.05$) increased the rate of background mitotic activity (Table 1). Although there was a tendency for Trolox (50 and 100 μM) and ascorbic acid to reduce the mitotic activity in combination with CPs, the antioxidant treatment had no significant effect on cell proliferation.

When the antioxidants were applied in the absence of CPs, there was a tendency for lower levels of necrosis compared to the untreated controls, however, this effect was significant only for the combinations of Trolox and ascorbic acid ($P<0.05$, Fig. 2).

**Table 1. Effect of treatment with CP and a combination of CP and different antioxidants on cell proliferation (percentage of mitotic cells) in primary rat hepatocytes**

| Treatment | no CP | + 1 μM CP |
|---|---|---|
| Control (1% DMSO) | 0.044 ± 0.034 | 0.116 ± 0.039* |
| + 10 μM Trolox | 0.017 ± 0.029 | 0.145 ± 0.109 |
| + 50 μM Trolox | 0.067 ± 0.076 | 0.039 ± 0.009 |
| + 100 μM Trolox | 0.067 ± 0.029 | 0.063 ± 0.031 |
| + 50 μM Ascorbic Acid | 0.1 ± 0.05 | 0.099 ± 0.038 |
| + 10 μM Trolox + 50 μM Ascorbic Acid | 0.067 ± 0.058 | 0.119 ± 0.013 |
| + 50 μM Trolox + 50 μM Ascorbic Acid | 0.1 ± 0.087 | 0.11 ± 0.039 |
| + 10 μM *N*-acetylcysteine | 0.117 ± 0.126 | 0.1 ± 0.028 |
| + 50 μM *N*-acetylcysteine | 0.033 ± 0.029 | 0.129 ± 0.055 |
| + 100 μM *N*-acetylcysteine | 0.033 ± 0.05 | 0.101 ± 0.042 |

Data represent the mean ± S.D. of three independent experiments. $^{*}P < 0.05$ compared to the control (no treatment). Student's double sided *t*-test for independent samples.

## DISCUSSION

### Cytotoxicity of CPs

In contrast to the observed lack of cytotoxicity of β-carotene cleavage products in primary cultures of rat hepatocytes proliferatively stimulated with EGF (Alija *et al.*, 2004; 2006), significant cytotoxic effects (both necrosis and apoptosis) were observed in non-stimulated cultures. The explanation for this observation has to take into account the most likely mode of CP action at the cellular level. According to Murata and Kawanishi (2000) autoxidation of retinoids causes the formation of superoxide which dismutates to hydrogen peroxide, which in turn in the presence

of transition metals causes DNA damage. Such a mode of action of retinoids will most likely also involve lipid peroxidation and the generation of aldehydic breakdown products such as 4-hydroxynonenal, since its formation can be considered to be proportional to the amount of reactive oxygen species formed within the cell. In fact, the formation of malondialdehyde and 4-hydroxynonenal by CPs of β-carotene has been shown in mitochondrial suspensions (Siems *et al.*, 2002). 4-Hydroxynonenal in turn is involved in apoptosis signalling in various ways often involving the p53 tumor suppressor protein (Sommerburg *et al.*, 2003) and Fas (Laurora *et al.*, 2005; Sharma *et al.*, 2008).

As demonstrated previously, CPs are also able to induce the generation of DNA adducts: Marques *et al.* (2004) investigated the reactions of retinal and β-apo-8'-carotenal, two β-carotene oxidation products, with 2'-deoxyguanosine to evaluate their DNA damaging potential. In those experiments significantly increased levels of 1,$N^2$-etheno-2'-deoxyguanosine and 8-oxo-7,8-dihydro-2'-deoxyguanosine, believed to be important in the development of human cancers, were induced by both β-carotene and CPs. Depending on the accumulated DNA damage one can therefore assume the initiation of the signalling cascade towards apoptosis involving p53 (Müller *et al.*, 1998) or necrotic cell death, when apoptosis is impaired (Zong *et al.*, 2004; Edinger & Thompson, 2004).

EGF has been demonstrated to inhibit apoptosis (Musallam *et al.*, 2001; Ethier *et al.*, 2003) by downregulation of the proapoptotic Bid protein involved in the transduction of the Fas apoptotic signal (Ethier *et al.*, 2003), and by upregulation of the antiapoptotic Bcl-$x_L$ protein (Musallam *et al.*, 2001). Therefore, the lack of apoptosis in proliferatively stimulated primary hepatocyte cultures appears to be due to signals mediated by EGF. The lack of necrosis thus could be understood as an indirect effect of EGF as described above: in the case of apoptosis impairment, cells have been shown to enter necrosis (Zong *et al.*, 2004). An anti-apoptotic signal of EGF will therefore most likely also have an influence on necrosis.

When considering the *in vivo* consequences of the cytotoxic potential of β-carotene cleavage products, one has to keep in mind that cell death in a given tissue such as the liver will trigger compensatory cell division (Jaeschke & Bajt, 2006). In context with our previous findings demonstrating a significant genotoxic potential of CPs when hepatocytes are proliferatively stimulated (Alija *et al.*, 2004; 2006), any intrinsic cytotoxicity will thus promote the initiation step of carcinogenesis. Furthermore, chronic cytotoxicity is known to cause inflammation, which is associated with the promotion of tumor growth (Vakkila & Lotze, 2004). Summarizing, these *in vitro* toxicological features of CPs further support the hypothesis that oxidative cleavage products of β-carotene are responsible for the carcinogenic response in the beta-Carotene and Retinol Efficacy Trial (CARET) and Alpha-Tocopherol Beta-carotene Cancer prevention (ATBC) chemoprevention trials.

### Antioxidant effects on CP-induced cytotoxicity

The mechanisms by which the applied antioxidants reduce cytotoxicity are manyfold and concern both reactive oxygen species formed *via* autoxidation of retinoids (Murata & Kawanishi, 2000), xenobiotic metabolism and detoxification of aldehydic products:

Trolox — representing a water-soluble vitamin E derivative, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carbonic acid — inhibits peroxynitrite-mediated oxidative stress (Salgo & Pryor, 1996), activates pregnane-X-receptor which regulates genes involved in xenobiotic detoxification (Traber, 2004), can protect from aldehyde toxicity (Watanabe *et al.*, 1992), and has been demonstrated to inhibit apoptosis and to reduce cytotoxicity in various cell types, such as thymocytes and lymphocytes (Wu *et al.*, 1990; McClain *et al.*, 1995). Similarly, ascorbic acid has been demonstrated to inhibit peroxynitrite-induced apoptosis (Sandoval *et al.*, 1997), to promote detoxification and elimination of 4-hydroxy-2(*E*)-nonenal (Miranda *et al.*, 2009) and, together with *N*-acetylcystein, to protect against aldehyde toxicity (Sprince *et al.*, 1975).

*N*-Acetylcysteine on the other hand protects SH groups. It is rapidly metabolized to intracellular glutathione and therefore increases the glutathione pool of cells. Glutathione itself contributes to the detoxification of many chemicals into less harmful compounds. *N*-Acetylcysteine additionally chelates heavy metals involved in ROS formation (Dekhuijzen & Beurden, 2006; Atkuri *et al.*, 2007; Fishbane, 2008). *N*-Acetylcysteine has been demonstrated to inhibit oxidative stress-induced apoptosis and necrosis (Zafarullah *et al.*, 2003; Li *et al.*, 2005).

When comparing the efficiency of necrosis inhibition by the antioxidants applied differences become evident which may depend on the potential protective mechanism involved. As described above, toxicity could involve reactive oxygen species due to autooxidation of retinoid compounds contained in the cleavage product mixture as well as aldehydic compounds such as carotenals. *N*-acetylcysteine as well as Trolox and ascorbic acid, also known to be capable of detoxifying aldehydes, significantly reduce necrotic cell death, the most efficient being *N*-acetylcysteine. Combinations of Trolox and ascorbic acid, interestingly, did not cause a significant reduction of necrosis at a Trolox concentration of 10 μM, although — independently applied — the same concentration led to a highly significant protection from necrotic cell death. Whether this effect is due to interactions between the two antioxidants or with compounds contained in the β-carotene cleavage product mixture cannot be explained at this stage and requires further in depth investigation.

Summarizing, the protective mechanism of the antioxidants applied appears to be their potential to detoxify aldehydes generated by the oxidative cleavage of β-carotene, i.e., apo-carotenals. In addition, they may protect from reactive oxygen species generated during autoxidation of retinoid compounds contained in the cleavage product mixture.

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