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β-Carotene Degradation Products – Formation, Toxicity and Prevention of Toxicity

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Abstract

Carotenoids are widely used as important micronutrients in food. Furthermore, carotenoid supplementation has been used in the treatment of diseases associated with oxidative stress such as various types of cancer, inflammatory diseases or cystic fibrosis. However, in some clinical studies harmful effects have been observed, e.g. a higher incidence of lung cancer in individuals exposed to extraordinary oxidative stress. The causal mechanisms of harmful effects are still unclear. Carotenoid breakdown products (CBPs) including highly reactive aldehydes and epoxides are formed during oxidative attacks in the course of antioxidative action. We investigated the formation of CBPs by stimulated neutrophils (and at further conditions), tested the hypothesis that CBPs may exert mitochondriotoxicity and tried to prevent toxicity in the presence of members of the antioxidative network. Stimulated neutrophils are able to degrade β-carotene and to generate a number of CBPs. Concerning mitochondriotoxicity, we found that CBPs strongly inhibit state 3 respiration of rat liver mitochondria at concentrations between 0.5 and 20 μM. This was true for retinal, β-ionone, and for mixtures of cleavage/breakdown products. The inhibition of mitochondrial respiration was accompanied by a reduction in protein sulfhydryl content, decreasing GSH levels and redox state, and elevated accumulation of malondialdehyde. Changes in mitochondrial membrane potential favor functional deterioration in the adenine nucleotide translocator as a sensitive target. The presence of additional antioxidants such as α-tocopherol, ascorbic acid, N-acetyl-cysteine or others could mitigate mitochondriotoxicity. The findings reflect a basic mechanism of increasing the risk of cancer induced by carotenoid degradation products.

Carotenoids are known as biologically important micronutrients with a large number of functions. Of all known carotenoids around fifty display provitamin A activity [1]. Carotenoids are also precursors of retinoids. It has been suggested that the antioxidant potency of β-carotene is transformed by scavenging oxygen radicals, thus protecting against cancer [2, 3]. Therefore, the intake of β-carotene was recommended,

especially in the form of supplements. Carotenoid supplementation has been further used for prevention and treatment of diseases with oxidative stress [4], such as cancer, UV-mediated skin diseases, neurodegenerative diseases, and cystic fibrosis. The majority of epidemiological studies consistently showed that increased consumption of food rich in β-carotene is associated with a reduced risk of lung and some other types of cancer [5]. A similar relationship has been found between levels of β-carotene in blood plasma and risk of cancer [5, 6].

In contrast, the ATBC and CARET studies indicated that supplementation of β-carotene and/or vitamin A in individuals at high risk of lung cancer increased the incidence of lung cancer [7, 8]. Pro-oxidant activity of β-carotene and procarcinogenic action in the case of preexisting premalignant lesions were discussed as possible reasons for these unexpected effects [7–14].

Murata and Kawanishi [15] reported that low concentrations of retinal (vitamin A aldehyde) and retinol (vitamin A) cause cellular DNA cleavage and induction of 8-oxo-7,8-dihydro-2-deoxyguanosine formation in HL-60 and HP100 cells. Superoxide radical anions generated by autoxidation of carotenoid derivatives were dismutated to H_2O_2, which was responsible for DNA damage. Retinal has a pro-oxidant capability, which could lead to carcinogenesis.

β-Carotene supplementation seems to be absolutely necessary for several diseases such as cystic fibrosis [16]. Patients with cystic fibrosis were recommended to take a daily dose of about 1 mg β-carotene per kilogram body weight, which is much higher than that given to the patients in the CARET trial. Therefore, the causal mechanism of increased cancer risk mediated by β-carotene intake has to be elucidated to establish safe conditions for supplementation.

In a previous work, we provided evidence that carotenoid cleavage products (CCPs) inhibit Na^+-K^+-ATPase activity [17]. Interestingly, CBPs were stronger inhibitors of Na^+-K^+-ATPase activity than the endogenous major lipid peroxidation product 4-hydroxynonenal (HNE) [17].

Here, we investigate the questions (a) whether CBPs are generated not only in the presence of hypochlorite and under other – more or less – artificial conditions, but also by stimulated neutrophils, (b) which mitochondriotoxic effects CBPs have, and (c) whether the presence of other members of the antioxidative network such as α-tocopherol, ascorbic acid etc. can mitigate or even prevent toxic effects of CBPs.

We argue that pro-oxidative actions of CBPs – especially in mitochondria and nucleus – are responsible for the harmful effects of high-dosage supplementation of β-carotene in patients suffering from extraordinary oxidative stress (due to cigarette smoking or working with asbestos). In our opinion, those conditions may also exist if other inflammatory conditions exist in the lungs (or other tissues). Impairment of mitochondrial function including changes in calcium homeostasis [18] can cause an increase in the formation of superoxide, thus promoting oxidative stress resulting in the oxidation of lipids, proteins and DNA molecules. DNA damage seems to be possible also by direct reactions of CBPs with DNA molecules. Oxidative DNA damage is

a hallmark of cancerogenesis. We have found that CBPs inhibit state 3 respiration in mitochondria. Furthermore, we provide evidence that CBPs increase oxidative stress in mitochondria, which is characterized by increased malondialdehyde (MDA) formation and decreases in mitochondrial GSH and protein SH [13].

Methods

Preparation and Formation of CBPs

The generation and analysis of the CBPs was performed as previously described [19]. Mixtures of breakdown products from β-carotene, retinal, and β-ionone were produced by mixing samples of a methanolic stock solution of these compounds at a concentration of 0.04 mM, each with 1 mM hypochlorous acid at room temperature. CBP collection was carried out after bleaching reaction was finished, indicated by a stable color of the stock solution. The extraction was carried out twice with hexane. The hexane phases were combined and evaporated with nitrogen until dry. The residue was redissolved in aliquots of hexane, adjusted to 1 or 0.5 mM stock solutions of CCPs, which were stored at –80°C.

Incubation of Carotenoids with Stimulated Neutrophils

Leukocytes were purified from heparinized human blood, freshly drawn from healthy donors according to a method of Ferrante and Thong [20, 21]. Leukocyte preparation containing 90–98% of polymorphonuclear leucocytes (PMLs) and apparently free of contaminating erythrocytes was obtained by a one-step procedure involving centrifugation of blood samples layered on Ficoll-Hypaque medium. Cells were suspended in 0.5 mM Ca^{2+}-containing phosphate-buffered saline at 37°C, pH 7.4, and stimulated in the presence of 1 μg/ml phorbal myristate acid. After 5 min of preincubation, β-carotene was added to the medium, leading to a final concentration of 1 μM for the degradation experiments and of 100 μM and 10 mM for the identification experiments, respectively. For the degradation experiments at time points 0 and 30 min, fractions of the culture media were extracted for measurements of absorption spectra. Similar experiments without phorbal myristate acid stimulation of the cells were carried out as controls. For calculation of the degradation rate, further experiments were carried out in which degradation of β-carotene (initial concentration 1 μM) by PMLs (10×10^6 cells/ml) was measured after 10, 30, and 60 min. For the identification experiments, fractions of the culture media were extracted after 0 and 30 min, and the resulting fractions were analyzed by HPLC and gas chromatography mass spectrometry (GCMS). HPLC and GCMS procedures have been described in detail in the study by Sommerburg et al. [21].

Preparation of Mitochondria

For the experiments, freshly isolated liver mitochondria from 180- to 220-gram male Wistar rats were used. The mitochondria were prepared in ice-cold medium containing 250 mM sucrose, 1 mM EGTA and 1% (w/v) bovine serum albumin at pH 7.4 (isolation medium) using a standard procedure. After the initial isolation, Percoll was used for purifying mitochondria from a fraction containing some endoplasmic reticulum, Golgi apparatus, and plasma membranes. The mitochondria were well coupled, as indicated by a respiratory control index greater than 5 with glutamate plus malate as substrates.

Incubation of Mitochondria and Measurement of Mitochondrial Respiration

For measurements of mitochondrial respiration, aliquots of CBP solutions (retinal, β-ionone, retinal breakdown products, β-ionone breakdown products, and β-carotene breakdown products) were transferred into reaction vials and evaporated with argon until dry. Afterwards, 2 ml of the incubation medium (containing 10 mM sucrose, 120 mM KCl, 15 mM NaCl, 20 mM Tris, 2 mM $MgCl_2$, 5 mM NaH_2PO_4, pH 7.4) was added to dissolve CBPs. The solution was transferred into a thermostat-controlled chamber equipped with a Clark-type electrode. Then the mitochondrial suspension (final concentration 1 mg protein/ml) was added. After 3 min of preincubation, 5 mM glutamate and 5 mM malate were added, and the state 4 respiration was measured. State 3 respiration was adjusted by adding 200 μM ADP. Uncoupled respiration was accomplished by addition of 0.1 μM carbonyl cyanide p-(tri-fluoromethoxy) phenylhydrazone to the mitochondrial suspension in the presence of hydrogen-supplying substrates. For control, the incubation medium without any carotenoid breakdown products was transferred into the incubation chamber followed by preincubation of mitochondria (1 mg/ml) for 3 min. The addition of substrates and the time schedule were identical to incubations in the presence of CBPs.

Furthermore, incubations of up to 20 min in the presence and absence of CBPs were carried out, and samples were withdrawn for GSH, GSSG, MDA and protein SH measurements.

For experiments on possible prevention of toxicity exerted by CBPs, all incubations were repeated in the absence and in the presence of various antioxidants (α-tocopherol 1, 5, 10, 20, 100 μM; ascorbic acid 10, 50, 100, 250 μM; urate 20, 100 μM, NAC 100 μM; DHLA 10 μM; SOD, catalase, ebselen 100 μM, and various combinations).

Assays for GSH, GSSG, Protein SH and MDA

GSH and GSSG were analyzed by a microtiter plate assay according to Baker et al. [22]. The content of protein SH was determined according to Ellman [23].

TBA-MDA conjugates were measured using an HPLC-based method [24].

Mitochondrial Membrane Potential

The dissipation of the mitochondrial membrane potential was followed at 30°C in a thermostat-controlled chamber equipped with a tetra phenyl phosponium cation-sensitive electrode [25].

Statistical Analysis

Significant differences were determined using Student's t test. A probability of $p < 0.05$ was accepted as significant. Data are presented as mean ± SE.

Results

Degradation of β-Carotene by Activated Neutrophils (PMLs)

It could be demonstrated that β-carotene was degraded in culture medium of activated PMLs, but not in medium of nonactivated PMLs. The degradation rate, calculated

Table 1. CBPs identified after oxidation of β-carotene in the presence of hypochlorite and after incubation with primary cultures with activated human PMLs

Apocarotenals, long-chain products	Short-chain products
Apo-4′-carotenal	β-Ionone
Apo-8′-carotenal	Ionene
Apo-12′-carotenal	β-Cyclocitral
Retinal (Apo-15′-carotenal)	β-Ionone-5,6-epoxide
Hexanedioic acid, mono(2-ethylhexyl)ester	Dihydroactinidiolide 4-oxo-β-ionone 1,5,5-trimethyl-6-acetomethyl-cyclohexene 3,7,7-trimethyl-1-penta-1,3-dienyl-2-oxabicyclo[3.2.0]hept-3-ene 2,6,6-trimethyl-1-cyclo-hexene-1-acetaldehyde 4,6,6-trimethyl-2-(3-methylbuta-1,3-dienyl)-3-oxatricyclo[5.1.0.0(2,4)]octane

Apocarotenals were identified by HPLC, short-chain products by GCMS.

from separate experiments in which the time dependence was investigated, was 730 pmol/ml × (10×10^6 cells) × h after addition of 1 nmol/ml of β-carotene.

Identification of CBPs

After treatment of β-carotene with hypochlorite and after its incubation with activated primary cultures of human PMLs, various CBPs could be identified by HPLC (apocarotenals) and GCMS (so-called short-chain products of carotenoids). Those CBPs are listed in table 1 [see also 21].

Inhibition of Mitochondrial State 3 Respiration

All types of CBPs investigated strongly inhibited the state 3 respiration in a dose-dependent manner (fig. 1). State 4 respiration was hardly affected [13]. The presence of 20 μM CBPs led to a 30–50% decrease. Also low concentrations of CBPs, such as 1 μM and even 0.5 μM, exerted clear inhibitions. The inhibition by retinal was 12.4 ± 0.5% at 1 μM and 6.3 ± 2.9% at a concentration of 0.5 μM. The ranges of inhibition for the different CCPs were 5–12% at 1 μM and 3–6% at 0.5 μM.

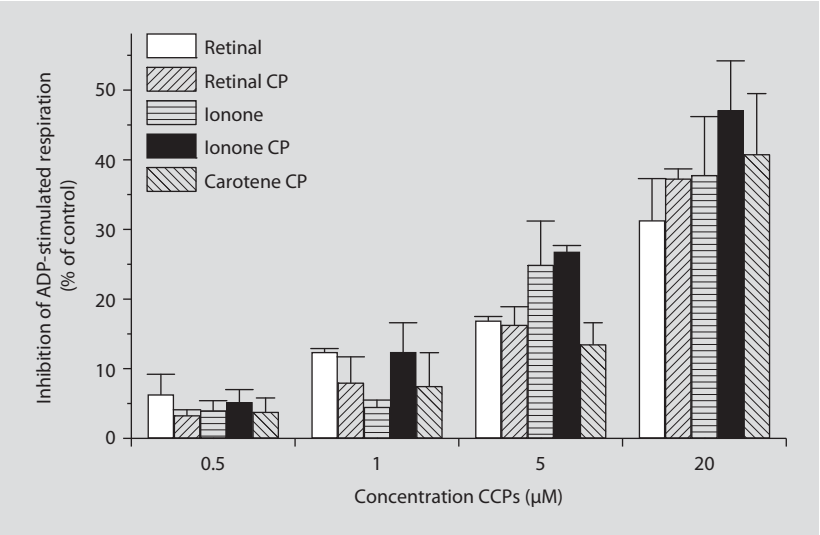

Fig. 1. Influence of carotenoid cleavage/breakdown products on respiration. Rat liver mitochondria were incubated at 30°C in a medium containing 10 mM sucrose, 120 mM KCl, 15 mM NaCl, 20 mM Tris, 2 mM $MgCl_2$, 5 mM NaH_2PO_4, pH 7.4 (incubation medium). Five different types of cleavage products were used: retinal, β-ionone, mixtures of retinal (retinal CP) or β-ionone (ionone CP) or β-carotene cleavage products (carotene CP). Inhibition of respiration is presented as a decrease in the difference of respiration after and before ADP addition (state 3 minus state 4 respiration) in percent of complete inhibition. 100% inhibition corresponds to a decrease in respiration of 53.4 ± 3.5 nmol O_2/mg/min, which is the ADP-induced increase in respiration of controls.

Adenine Nucleotide Translocator as a Sensitive Target of Oxidation by CBPs

The dissipation of the mitochondrial membrane potential was measured in order to distinguish between inhibition of the adenine nucleotide translocator and inhibition of the F_0F_1-ATPase by the decomposition products of carotenoids. After blocking the electron transport within the respiratory chain by cyanide, the F_0F_1-ATPase splits ATP to ADP and inorganic phosphate, paralleled by pumping protons into the extramitochondrial space. Therefore, inhibition of the F_0F_1-ATPase results in the acceleration of depolarization. For CBPs which inhibited significantly the ADP-induced increase in respiration (state 3 minus state 4), it was shown that they had no effect on the kinetics of membrane depolarization [13]. This observation supports the suggestion that the inhibition of respiration by degradation products of carotenoids is mainly caused by impairing adenine nucleotide translocation.

Changes in GSH, GSSG, Protein SH, and MDA Concentrations

The mitochondrial GSH content rapidly decreased in the presence of retinal and other β-carotene derivatives. Strongest decreases were observed in the presence of retinal

and retinal breakdown products. 20 μM CBPs led to a GSH decrease from 6.2 ± 0.5 nmol/mg protein (control) to 1.7 ± 0.3 (retinal) and 2.51 ± 0.44 (retinal breakdown products). Parallel to this, GSSG content increased. The GSSG increase in combination with GSH decrease resulted in increasing ratios of GSSG/total GSH. 20-min incubation of mitochondria in the presence of 20 μM retinal led to a threefold increased ratio compared with controls. Moreover, most of CBPs caused a decrease in total GSH pool (GSH + GSSG) due to GSSG efflux through mitochondrial membranes.

Loss of protein SH was found during the 20-min incubation with either of the CBP mixtures at a concentration of 20 μM. The mitochondrial protein SH content decreased from 86 ± 4.6 (control) to 68 ± 1.9, and 78 ± 7.7 nmol SH/mg protein in the presence of 20 μM of retinal breakdown products and retinal, respectively. Even when only a small share of protein SH of mitochondria is lost (usually less than 20% of total), the absolute protein SH loss is markedly higher than the absolute loss of GSH. In the presence of 20 μM of retinal breakdown products, the total SH loss was 22 ± 2.8 nmol/mg protein. Taking into account the final protein concentration of about 1 mg mitochondrial protein/ml of suspension, the bulk of retinal breakdown products may be bound to SH groups.

The levels of MDA are enhanced more than tenfold after 20 min of incubation in the presence of 20 μM CBPs in comparison with control incubations.

Discussion

Formation of β-Carotene Degradation Products (CBPs)

There are various conditions for CBP formation (table 2). Some of these conditions were studied by our research group [13, 14, 17, 21, 26]. It was clearly shown that β-carotene is degraded by stimulated PMLs in vitro. This gave the pathophysiological meaning to our experiments on formation and identification of CBPs by hypochlorous acid (table 1). While formation of apocarotenals under these conditions has been studied before [19, 27–29], this was not the case for short-chain products. When performing gas chromatography mass spectrometry, the CBPs 5,6-epoxi-β-ionone, ionene, β-cyclocitral, β-ionone, dihydroactinidiolide, and 4-oxo-β-ionone were found to be formed during degradation of β-carotene by hypochlorous acid. This may be of biological and medicinal relevance because many CBPs are carbonyls and epoxides and highly reactive and, therefore, potentially toxic.

Toxicity of CBPs: CBPs Exert Pro-Oxidant Effects

Many of the CBPs were identified as aldehydes. Retinal, the different apocarotenals and also a certain number of newly identified short-chain derivatives are of aldehydic

Table 2. Conditions leading to β-carotene degradation and formation of CBPs

Endogenous enzymatic degradation steps
Radical reactions (e.g. AIBN, AMVN)
UV light (artificial light, sunlight)
Heat
Cigarette smoke
Phagocytosing cells
Hypochlorite
Autoxidation in dependence on pO_2
All physiological antioxidative reactions

nature. It is well known that aldehydes react rapidly with sulfhydryl groups and lysyl and histidine residues even at low cellular levels.

Recently, we demonstrated the inhibition of the Na^+-K^+-ATPase by a mixture of β-carotene cleavage products derived from hypochlorite treatment [17]. We found that CBPs exerted a much higher in vitro toxicity than HNE, nonenal, and nonanal, which are also aldehydes and react with nucleophilic groups. This means that CBPs are very reactive and that they may be of particularly high relevance under pathophysiological conditions. Thus, the depletion of mitochondrial protein SH and GSH after exposure of isolated rat liver mitochondria may be caused by direct reactions of aldehydes with mitochondrial sulfhydryl groups. The data presented further demonstrate that the decrease in SH groups under the influence of CBPs was paralleled by the inhibition of state 3 respiration (predominantly the ADP-induced increase in respiration) due to impairment of adenine nucleotide translocation. This could be expected since the adenine nucleotide translocator has been shown to be sensitive to fatty acid CoA derivatives and to lipid peroxidation products such as HNE [30]. Inhibition of electron transfer by the respiratory chain due to the inhibition of adenine nucleotide transport leads to a rise in superoxide radical anion production by the respiratory chain and subsequently to formation of H_2O_2 and hydroxyl radicals [31]. Accordingly, an increase in oxidative stress is induced in mitochondria. This suggestion is in line with our observation that CBPs caused additional MDA accumulation.

Biological Impact of CCPs

Several conditions result in the rapid nonenzymatic oxidative cleavage of β-carotene, i.e. heavy oxidative stress (smoking, asbestos) and photoirradiation in the skin and eyes [32]. The same should be true for hypochlorite-mediated carotenoid cleavage in the neighborhood of activated PMLs. Hypochlorite released by phagocytic cells

is, at least temporarily, present at high concentrations ranging from 5 to 50 μM in the tissue [33]. These levels are high enough to initiate the nonenzymatic cleavage of β-carotene. Carotenoid levels are markedly higher than 1 nmol/g in human tissues. Therefore, these concentrations are high enough for CBP formation leading to toxic effects. Additionally, due to the lipophilic properties of carotenoids, even significantly higher concentrations can be measured in mitochondrial and plasma membranes in comparison with the values in total tissue [34]. Furthermore, higher concentrations of CBPs may be expected in mitochondria because of their capability to produce oxygen free radicals themselves [35]. Supplementation of β-carotene in combination with heavy oxidative stress (smoking, inflammation) may further increase the in vivo concentrations of CBPs. Therefore, CBP concentrations in mitochondria which correspond to the concentrations used in our in vitro experiments (0.5–20 μM) are very likely in such situations. Our data provide evidence that CBPs deplete mitochondrial sulfhydryl groups and impair oxidative phosphorylation in mitochondria at the level of adenine nucleotide translocation. Oxidative stress resulting from the impairment of the mitochondrial energy metabolism in the presence of CBPs and indicated by enhanced MDA formation may induce oxidative damage to DNA molecules in the mitochondria and nucleus. Mitochondrial DNA has a pronounced susceptibility to oxidative stress because of the absence of histones and a lower capacity of DNA repair in comparison with the nucleus. Marques et al. [36] have shown the DNA-damaging potential of CBPs. Treatment of calf thymus DNA with CBPs significantly increased levels of 1,N2-etheno-2′-deoxyguanosine and 8-oxo-7,8-dihydro-2′-deoxyguanosine, known mutagenic DNA adducts. Furthermore, formation of apo-14-carotenal augments inflammatory response mediated by cytokines such as TNF-α. If apo-14-carotenal binds to peroxisome proliferator-activated receptor-α (PPARα), the PPARα cannot inhibit NFκB-mediated inflammation anymore. Additionally, retinaldehyde and retionoid acid signal functions in carotenoid breakdown under oxidative stress are under discussion [37, 38]. Oxidative DNA damage, in general, increases the risk of cancer. Thus, our data may indicate a basic mechanism of the harmful effects of carotenoids in situations of increased oxidative stress (fig. 2). Under conditions of mild oxidative stress, antioxidative effects of β-carotene supplementation will dominate. But under conditions of extraordinary oxidative stress, there exists a potential risk of cancerogenesis due to the rapid accumulation of pro-oxidative-acting CBPs (fig. 2).

Prevention of CBP-Induced Mitochondriotoxicity by Antioxidants

All antioxidants used were able to reduce β-carotene degradation by human neutrophils and, therefore, the rapid formation of β-carotene breakdown products. In the presence of antioxidants, the impairment of mitochondrial respiration and mitochondrial SH system by CBPs was drastically reduced, and in some cases almost completely

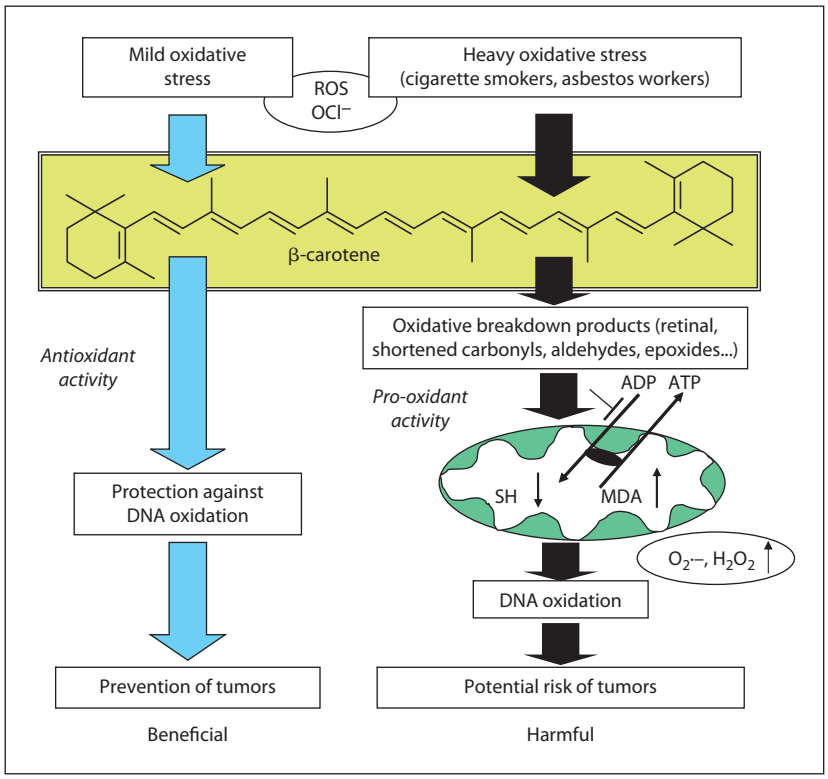

Fig. 2. β-Carotene supplementation. Antioxidant and pro-oxidant activities under different conditions. Predominant antioxidant and tumor-preventing activity of β-carotene supplementation under physiological conditions and conditions of mild oxidative stress, but dominating pro-oxidative effects of such supplementation under conditions of extraordinary oxidative stress.

prevented. α-Tocopherol was the most effective member of the antioxidative network under those conditions.

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