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# β-Carotene breakdown products may impair mitochondrial functions—potential side effects of high-dose β-carotene supplementation☆

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**Abstract**

β-Carotene (BC) and other carotenoids are mainly considered as belonging to the group of micronutrients. As they are contained in fruit and vegetables and thus part of human diet, a regular low-dose intake from natural sources is normally assured. In the last decade high-dose supplementation with synthetic carotenoids has been used successfully in the treatment of diseases believed to be associated with oxidative stress. However, in a few clinical studies harmful effects have been observed as well, e.g., a higher incidence of lung cancer after BC was given in high doses to smokers. Our studies aim at shedding light on the causal mechanisms of the known side effects that we have investigated. Possibilities of preventing them are discussed. Obviously, on certain conditions of high-dose carotenoid supplementation, both the antioxidant and prooxidant reactions may arise. Carotenoid breakdown products (CBP) including very reactive aldehydes and epoxides are formed during oxidative attack in the course of antioxidative action. Carotenoid breakdown products inhibit state 3 respiration of isolated rat liver mitochondria at concentrations between 0.5 and 20 μM. In vivo stimulated neutrophils might represent an important source for the generation of CBP, and the lung might be a critical organ in CBP formation. The inhibition of mitochondrial state 3 respiration by CBP is accompanied by a reduced content of protein sulfhydryl groups, decreasing glutathione levels and redox state, and also elevated accumulation of malondialdehyde. Changes in mitochondrial membrane potential favour functional deterioration of the adenine nucleotide translocator (ANT). The findings reflect a basic mechanism of the side effects of BC supplementation in circumstances of severe oxidative stress induced by CBP representing a class of lipid oxidation products. We are striving for safe conditions of carotenoid supplementation in order to protect patients in need of this kind of medical treatment from possible side effects, such as unwanted prooxidative reactions.


*Keywords:* β-Carotene; Carotenoid; Carotenoid breakdown products; Oxidative stress; Respiration; Mitochondria; Antioxidant; Aldehydes; Malondialdehyde (MDA); Free radicals; Neutrophils; Lung

## 1. Introduction

Of all known carotenoids—about 600 different types are found in nature—about 40 are regularly consumed by humans. Carotenoids are known to be biologically important micronutrients with a large number of functions. Around 50 carotenoids exhibit provitamin A activity and may serve as precursors of retinoids as reviewed in Refs. [1–3]. It has been suggested by Slaga [4] and by Burton and Ingold [5] that β-carotene (BC) may provide protection against cancer development in human. Therefore, intake of BC is recommended, especially in the form of high-dose supplements. Carotenoid supplementation has been further recommended for the prevention and treatment of degenerative diseases

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related to oxidative stress [6,7], such as UV-mediated skin or eye diseases, neurodegenerative diseases and cystic fibrosis. The majority of epidemiological studies consistently showed that increased consumption of food rich in BC is associated with a reduced risk of lung and some types of cancer [8]. A similar relationship has been found between plasma levels of BC and risk for some types of cancer [8,9].

However, a few intervention trials with a large number of patients showed a different result. In contrast to the epidemiological observations, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) study and the Beta-Carotene and Retinol Efficacy Trial (CARET) indicated that supplementation of BC and/or vitamin A in subjects having a high risk of lung cancer increased the incidence of lung cancer overall mortality [10,11]. Prooxidant activity of BC and procarcinogenic action in case of preexisting premalignant lesions were discussed as possible reasons for these unexpected effects [10,12–17].

Edge and Truscott [18] discussed the switch from an antioxidant to a prooxidant behaviour of carotenoids using results based on pulsed radiation techniques. They suggested (a) an antioxidant protection by carotenoids only as a synergistic effect together with antioxidants, such as vitamins E and C, and (b) the switch from antioxidant to prooxidant behaviour as a function of oxygen concentration. Furthermore, they speculated that the key prooxidant step involves the oxygen being "carried" from a peroxyl radical to the lipid via the carotenoid.

Recently, Murata and Kawanishi [19] reported that low concentrations of retinal (vitamin A aldehyde) and retinol (vitamin A) cause cellular DNA degradation and the induction of 8-oxo-7,8-dihydro-2-deoxyguanosine formation in HL-60 and HP100 cells. They concluded that superoxide radical anions generated by autoxidation of carotenoid derivatives were dismutated to $H_2O_2$, which was responsible for DNA damage. They also concluded that retinal has prooxidant capability, which may lead to carcinogenesis as well.

Attempts to use carotenoids for cancer chemoprevention and treatment continue [20,21]. On the other hand, supplementation with BC (and other carotenoids) seems to be absolutely necessary for several diseases, such as cystic fibrosis [22]. Patients with cystic fibrosis were recommended to take a daily dose of about 0.5 to 1.0 mg BC per kg body weight, which, for some patients, is indeed much higher than the dose given in the ATBC or CARET trial. Carotenoid supplementation is furthermore necessary for babies being fed formula preparation [23]. Therefore, the causal mechanism of increased risk of cancer mediated by BC intake has to be elucidated to establish safe conditions for carotenoid supplementation.

In a previous work we provided evidence that carotenoid breakdown products (CBP) inhibit $Na^+$–$K^+$–ATPase activity [24]. Interestingly, CBP inhibit $Na^+$–$K^+$–ATPase activity stronger than the endogenous major lipid peroxidation product 4-hydroxynonenal (HNE) [24].

Previously, we investigated the question whether CBP, namely, retinal, β-ionone and mixtures of breakdown products, which were generated in the presence of hypochlorite, increase oxidative stress by impairing mitochondrial function [25]. In pathophysiological situations, mitochondria are the main producers of superoxide radical anions and $H_2O_2$ within the cell [26]. Changes in calcium homeostasis and impairment of mitochondrial function [27] can cause an increase in the formation of "superoxide" [28], thus promoting oxidative stress resulting in the oxidation of lipids, proteins and DNA molecules. Oxidative DNA damage is a hallmark of cancer genesis. We have found that CBP inhibit state 3 respiration in isolated rat liver mitochondria, which is accompanied by increased oxidative stress in the mitochondria, as reflected by decreases in mitochondrial GSH and protein SH and increased formation of malondialdehyde.

## 2. Ways of BC degradation and variety of oxidative breakdown products

The underlying chemical and physical character enabling the carotenoid to act as an antioxidant is determined by its molecular structure. β-Carotene is characterized by a chain of 11 conjugated double bonds with methyl branches spaced along the chain. Cyclohexenyl terminal groups with 1,1,5-trimethyl substitution are typical for BC and for a number of other carotenoids. The most important structural feature is the extensive delocalisation of the polyene π-electrons, which enables carotenoids to absorb visible light. Thus carotenoids show intense colours from yellow to red. However, the electron-rich system makes carotenoid molecules also highly susceptible to attacks by electrophilic reagents. This results in increased instability of these substances towards oxidation. Oxidation of BC and the consecutive degradation of the molecule have been intensively studied under different conditions mimicking more or less pathophysiologically relevant situations in vivo and in vitro (see Fig. 1).

One important cause of the destruction of carotenoids is UV-light exposure, which has been associated with carotenoid oxidation in eye tissues, in the skin or simply in food. As corresponding in vitro models, rose bengal, methylene blue or 12-(pyrene)dodecanoic acid was used to study the photosensitized oxidation of BC [24,29,30]. In other studies, initiators of radical reactions such as 2,2′-azo-bis-isobutyronitril (AIBN) and 2,2′-azo-bis(2,4-dimethylvaleronitril) (AMVN) were used for carotenoid degradation in the presence of oxygen. These substances were used to model the radical trapping properties of the carotenoid [24,31–35]. In a more recent study, the direct effect of ozone and also of molecular oxygen on carotenoids in an aqueous model system was investigated [36].

Another pathophysiologically relevant oxidant able to degrade BC is hypochlorous acid [24,35]. This compound is

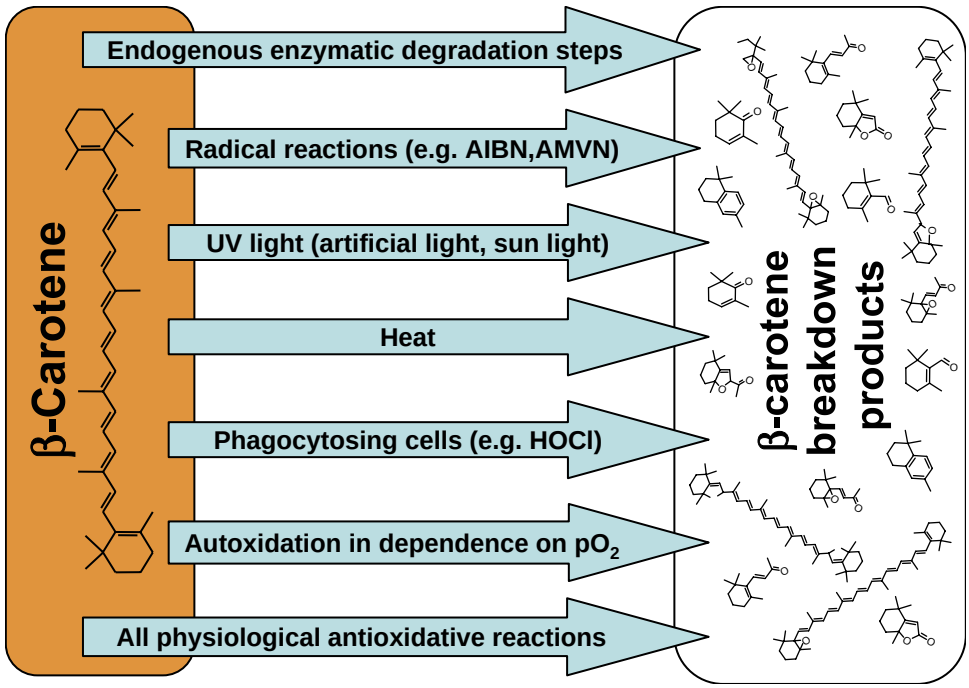


Fig. 1. Natural and experimental sources of carotenoid decomposition. Formation of different carotenoid breakdown products from one precursor compound such as BC.

used by phagocytic cells for antibacterial defense, and it can be assumed that hypochlorous acid plays a relevant role in BC degradation mediated by neutrophils in vivo [37]. These and other sources of oxidative carotenoid degradation are shown in Fig. 1.

Since carotenoids are mainly degraded by enzymes and also by antioxidative reactions of carotenoids leading to formation of various CBP, we have to expect a multitude of CBP under in vivo conditions.

In a number of studies mentioned above, successful attempts were made to identify those CBP. It became obvious that during mild oxidative stress, independent of its source, mostly high molecular weight products, such as apo-carotenals, are formed. However, further oxidation of these high molecular weight products to so-called short-chain breakdown products depends to a large extent on the concentration and the character of the oxidants used. However, many of the oxidation products are formed uniformly under the different conditions studied. Those products include the high molecular weight products β-apo-8′-carotenal, β-apo-10′-carotenal, β-apo-12′-carotenal, β-apo-14′-carotenal and β-apo-15′-carotenal [29,32,35,37–41]. Also, short-chain products, such as β-cyclocitral, β-ionone, ionene, 5,6-epoxi-β-ionone, dihydroactinidiolide and 4-oxo-ionone, are found constantly after oxidation of BC with different oxidants [29,32,37–39,42–46]. In contrast, a few products are formed only under certain conditions. This is true, for example, for β-ionylidenacetaldehyde, 4-oxo-β-ionylidenacetaldehyde and 5,8-epoxi-β-ionone, which were found only after reaction of BC with molecular oxygen [38,39].

## 3. Stimulated neutrophils: a contributor to oxidative BC breakdown

Stimulated neutrophils produce a number of electrophilic agents, which serve as host defence against invading microorganisms. These agents are also able to degrade carotenoids. As will be shown shortly, BC is degraded to colourless compounds in culture medium of human neutrophils [37] or rat peritoneal macrophages [47], which have been activated in the presence of phorbol myristate acetate (PMA), but not in medium of nonactivated cells. The degradation rate is about 0.7 nmol/(hour$\times 10^5$ cells) after addition of 1 μM BC to human neutrophil suspension in phosphate-buffered saline.

Experiments with ex vivo models show that BC prevents lymphocyte damage induced by PMA-activated monocytes [48], while neutrophils kill colourless mutant strain of *Sarcina lutea* more readily than a carotenoid-containing strain [49]. Another model system shows that viability of *Escherichia coli* transformants producing carotenoids is higher than that of wild-type bacterium in a medium containing myeloperoxidase, hydrogen peroxide and $Br^-$ at pH 4.5, which simulates the intracellular condition of polymorphonuclear leukocytes [50]. On the other hand, carotenoids (BC, lutein, bixin, canthaxanthin) suppress the luminol-dependent chemiluminescence of PMA-activated

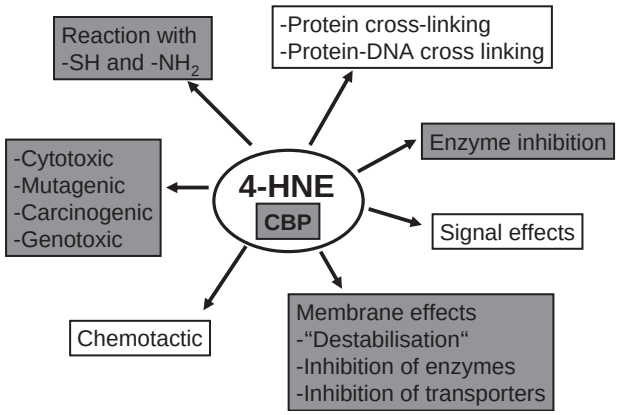


Fig. 2. Overview of the biological effects of the major aldehydic lipid peroxidation product HNE and of CBP. The fields representing those effects, which were already shown (in previously published papers) to be initiated not only by HNE, but also by CBP, are shaded.

macrophages [47], which reflects the complex cooxidative processes that follow the respiratory burst of the phagocytic cells [51].

Carotenoids may scavenge a variety of toxic oxygen metabolites released by macrophages during respiratory burst. The quenching activities of carotenoids against singlet oxygen from a hydrogen peroxide/hypochlorite system have been widely studied [52–55]. The ability of carotenoids to quench singlet oxygen decreases in the following order: lycopene, gamma-carotene, astaxanthin, canthaxanthin, α-carotene, BC, zeaxanthin, lutein. However, it has been observed [56] that BC readily reacts with hypochlorite with a rate constant of $2.3\times10^4$ mol/(L s), and this suggests that carotenoids may protect living cells from hypochlorite, rather than from singlet oxygen. Recent work reports that carotenoids react with hydrogen peroxide, nitric oxide and peroxynitrite in cell-free systems [47,57].

## 4. β-Carotene breakdown products affect biological processes

In clinical studies, the harmful effects of carotenoids have been described [10], e.g., a higher incidence of lung cancer in individuals exposed to extraordinary oxidative stress, such as heavy smokers or asbestos workers. These harmful effects may be caused by oxidatively formed carotene breakdown products including highly reactive aldehydes and epoxides. It should be mentioned that carotenoids are enzymatically cleaved by dioxygenases, epoxidases, hydroxylases, dehydrogenases and aldehyde oxidases [58]. Retinal is the primary cleavage product of BC, which is formed by 15,15′-dioxygenase [59,60]. Furthermore, CBP are formed non-enzymatically by the attack of different free radical species under conditions of enhanced oxidative stress [38,61–63]. Handelman et al. [35] performed a CBP generation in the presence of hypochlorite in order to mimic the in vivo formation in inflammatory regions following activation of neutrophils. Many of these products are aldehydes, carbonyls and epoxides [25,64–66]. Therefore, one has to expect similarities with other aldehydes, carbonyls or epoxides, such as the aldehydic lipid peroxidation products malondialdehyde (MDA) and 4-hydroxynonenal (HNE). As Fig. 2 shows for HNE, aldehydic compounds are able to react with amino acid residues of proteins and peptides, as well as with nucleic acids and lipids. Since many of the CBP are aldehydes, one should assume that a major part of their chemical reactions and biological effects will be similar to the reactions and effects of MDA and HNE due to the aldehydic function. Carotenoid breakdown products that are aldehydes, carbonyls and epoxides are indeed able to exert the typical chemical reactions of these functional groups with biomolecules. Those reactions are the chemical basis of the potential toxicity of CBP.

## 5. β-Carotene breakdown products impair mitochondrial functions

It was demonstrated that exogenously added CBP disturbed important mitochondrial functions [25]. Carotenoid breakdown products as a mixture obtained by a reaction of BC with hypochlorite, but also as a single substance, inhibited ADP-stimulated respiration in the concentration range between 0.5 and 20 μM in isolated rat liver, brain and lung mitochondria. Fig. 3 shows the influence of CBP on the ADP-stimulated respiration in rat liver mitochondria.

In previous experiments, we found that under almost identical incubation conditions lung mitochondria were

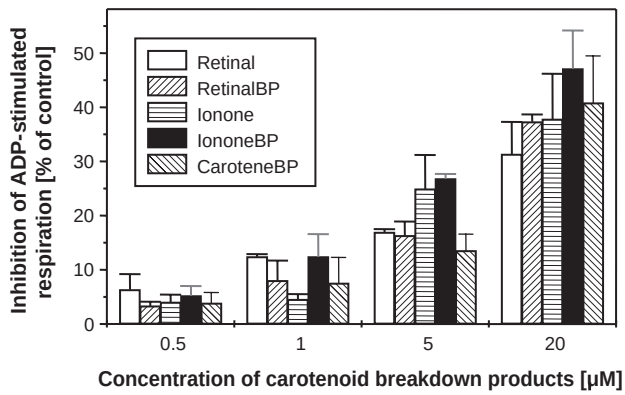


Fig. 3. The influence of CBP on the ADP-stimulated respiration in rat liver mitochondria. Rat liver mitochondria were incubated at 30 °C in 10 mM sucrose, 120 mM KCl, 15 mM NaCl, 20 mM Tris, 2 mM $MgCl_2$, 5 mM $NaH_2PO_4$ at pH 7.4. As CBP retinal, β-ionone, mixtures of retinal breakdown products (retinalBP), β-ionone breakdown products (iononeBP) or BC breakdown products (caroteneBP) were used. The concentrations were 0.5, 1, 5 or 20 μM, respectively. The inhibition of the respiration is presented as a decrease in the difference of respiration after and before ADP addition, i.e., state 3 respiration minus state 4 respiration, in % of complete inhibition. One hundred percent inhibition corresponds to a decrease in respiration of 53.4±3.5 nmol oxygen/(mg min), which is equal to the ADP-induced increase in respiration of the controls. Values are given as mean±S.E. from three independent experiments (data from Ref. [25]).

more susceptible than brain mitochondria, and brain mitochondria were more susceptible than liver mitochondria (unpublished results). During incubation with CBP, mitochondrial protein SH and glutathione levels were diminished, whereas oxidized glutathione and thiobarbituric acid reactive substances were accumulated [25]. The inhibition of the adenine nucleotide transport (ANT) seems to be the reason for the perturbation of mitochondrial functions by CBP (see also Fig. 4). This hypothesis is supported by three arguments: firstly, the ANT is very sensitive to oxidative stress because of its high content of SH groups, also in the active centre. Therefore, oxidation of SH impairs easily the ANT activity [67]. Secondly, the ATP synthetase could be mostly excluded as reason for the perturbation of mitochondrial functions by measurements of the dissipation of membrane potential [25]. Thirdly, for retinal a concentration-dependent inhibition of the activity of the adenine nucleotide translocase has been shown (I. Wiswedel, P. Schönfeld, personal communication, 2004).

Chen et al. [67] showed that the transporting activity of ANT was also inhibited by two unsaturated aldehydes, 4-hydroxynonenal (HNE) and 4-hydroxyhexenal (HHE). Concerning the underlying mechanism of this inhibition, they concluded from reconstitution experiments that the loss of sulfhydryl groups and/or the alteration of the physicochemical status of the lipid environment, in which the ANT is embedded, may be responsible for the impairment of ANT function by aldehydic products. It was already published that carotenoids as retinoic acids inhibit ANT activity in heart and liver mitochondria and induce mitochondrial permeability transition [68].

Aldehydes and structurally related compounds such as hydroxynonenal, trans-retinal, trans-retinoic acid and other 2-alkenals specifically induce uncoupling of mitochondria through the uncoupling proteins UCP1-3 and the adenine nucleotide translocase. The uncoupling was inhibited by potent inhibitors of ANT (CAT and bongkrekate) and UCP (GDP) [69]. The results suggest that these aldehydic products are not merely toxic, but may be a biological signal for decreasing mitochondrial ROS production. Uncoupling of oxidative phosphorylation was also reported by other authors [70] for retinal and retinoic acid (at levels above 0.5 and 0.25 nmol/mg protein, respectively). As potential cause, the membrane bilayer disrupting properties of these compounds were discussed.

From feeding experiments, it is known that BC, when fed (or supplemented), accumulates preferably in the mitochondrial fraction of cells. For example, BC-fed chicks had higher concentrations in all subcellular fractions relative to controls, but the mitochondrial fraction contained the highest levels of BC, followed by lysosomes, microsomes and nuclei, respectively [71]. Dietary BC increased retinal and α-tocopherol levels in the liver and in the mitochondrial fraction, while the plasma vitamin E level was decreased.

The preferred accumulation of BC in the mitochondria suggests mitochondrial generation of CBP, which in turn impairs mitochondrial function.

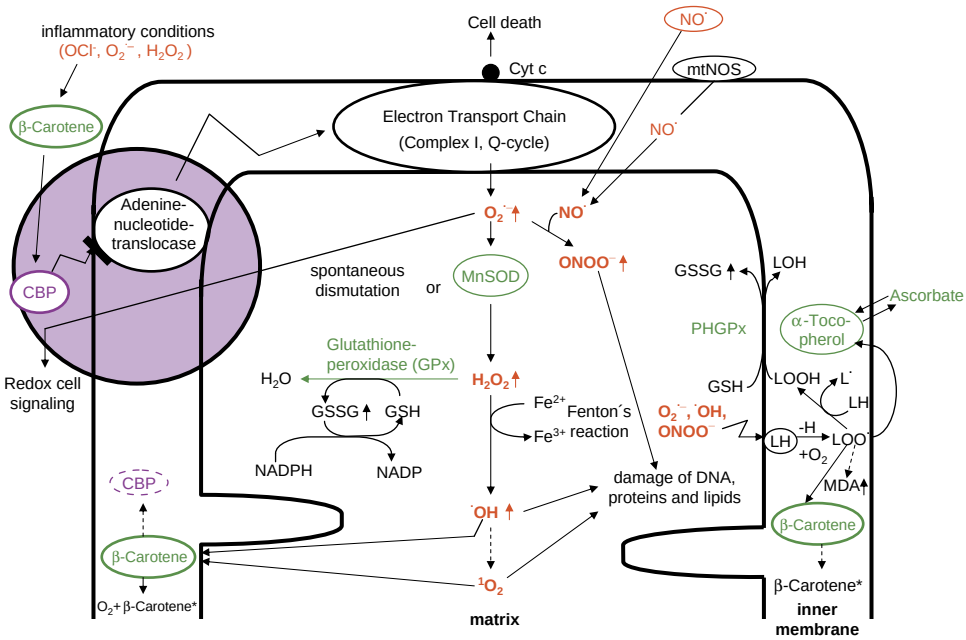


Fig. 4. Schematic representation concerning the role of BC and of CBP in the balance between prooxidative reactions and antioxidant protection against ROS and their secondary products in mitochondria. The schematic representation shows that the adenine nucleotide translocase is the main target of prooxidative CBP effects. The scheme includes the mitochondrial antioxidative enzymes and low molecular nonenzymatic antioxidants at one side, and ROS—mainly formed via the Q cycle within the respiratory chain and RNS with their potential targets at the other side. cyt c, cytochrome *c*; mtNOS, mitochondrial NO synthetase; PHGPx, phospholipid hydroperoxide glutathione peroxidase; CCP, BC cleavage products; β-carotene*, activated BC or BC peroxyl radical.

## 6. Influence on the balance between antioxidative and prooxidative mechanisms in mitochondria

### 6.1. Antioxidative role

Most of the evidence supports the notion that BC acts in vivo as an antioxidant. The polyene chain is responsible for the chemical reactivity toward oxidizing agents and free radicals and for their role as antioxidant. β-Carotene is a powerful scavenger of singlet oxygen and acts at low oxygen concentrations as chain-breaking antioxidant towards lipid peroxyl radicals. However, at higher oxygen concentrations carotenoid peroxyl radicals are generated, which could act as a prooxidant in an autoxidation process, as shown by Britton [72]. Thus, BC can act as a prooxidant as well as an antioxidant in the mitochondria. The scheme in Fig. 4 shows the role of BC in the antioxidant protection against reactive oxygen species (ROS) in the mitochondria. The representation shows on the side of antioxidants the mitochondrial antioxidative enzymes [manganese superoxide dismutase (Mn-SOD) which detoxifies superoxide anions, glutathione peroxidase removing hydrogen peroxide, and phospholipid hydroperoxide glutathione peroxidase (PHGPx) as lipohydroperoxide reducing system] and low molecular lipid-soluble nonenzymatic antioxidants (especially α-tocopherol and BC). On the other side, ROS and reactive nitrogen species (RNS) with their potential targets are illustrated in the figure.

Enhanced amounts of superoxide anions are formed, especially by complexes I and III of the respiratory chain under pathophysiological conditions, as shown for the inhibition of the adenine nucleotide translocase [25], which we also assume to be the primary target of CBP. Reactive oxygen species such as superoxide and hydrogen peroxide at low amounts are suggested to play a major role in redox signaling and apoptosis. At high levels of these reactive oxygen intermediates, the mitochondria are subjected to oxidative stress, leading to damage of DNA, proteins and lipids. β-Carotene as a singlet-oxygen-quenching molecule may also be able to scavenge lipid peroxyl radicals within the mitochondrial membrane, but the interactions with α-tocopherol are not completely understood. In liposomal membranes, the antioxidant activity of BC was found to be much smaller than that of α-tocopherol. When BC and α-tocopherol were present together in homogeneous solution, α-tocopherol was consumed predominantly and BC was spared. On the contrary, BC was consumed faster than α-tocopherol when the radicals were generated within the lipophilic compartment of the membranes [73]. In general, BC was found to be less potent as an antioxidant than α-tocopherol. The stable BC peroxyl radical reacts with oxygen to give BC-peroxyl radical that is not stable, but able to attack lipids to continue chain oxidation [74].

Depending on their structure, retinoids can either ameliorate or exacerbate stress-related damage [75]. Thus, retinol was protective, because retinol deprivation enhanced oxidative damage, as indicated by the rapid loss of mitochondrial membrane potential. Supplementation with a physiological concentration of retinol reversed this effect. Anhydroretinol, a known antagonist, working by displacing retinal from common binding sites on serine/threonine kinases, also caused mitochondrial membrane depolarisation. This effect was $Ca^{2+}$-dependent and cyclosporine-sensitive, suggesting an upstream signaling mechanism rather than a direct membrane effect. Retinoids are known to act as survival factors of cardiac cells, because retinal reduced the incidence of heart disease and protected the heart from inflammation, oxidative damage and degeneration. Conversely, chronic retinol deficiency in rats decreased the respiratory activity of their heart mitochondria [76]. Retinol is able to protect, at least in part, mitochondria from oxidative damage. Retinol and appropriate derivatives can be expected to find clinical application for stabilization of the cellular redox state after ischemia/reperfusion and other conditions of oxidative stress.

Carotenoids efficiently inhibit lipid peroxidation at low oxygen tension, and an interaction of BC with lipid peroxyl radical leading to a radical adduct was postulated [5]. Other free radicals such as nitrogen dioxide, thiyl and sulfonyl radicals are rapidly scavenged by BC.

It is known that retinoids modulate—independently of nuclear receptors—cell proliferation, differentiation and apoptosis [77], which is also dependent on ROS and RNS.

### 6.2. Prooxidative properties

Under heavy oxidative stress, supplemented BC and other carotenoids are oxidatively degraded leading to the formation of high amounts of breakdown products with prooxidant properties. These are—as already mentioned above—shortened carbonyls, aldehydes, epoxides and others, which are very reactive and may exert harmful effects.

A possible mechanism of antioxidative and prooxidative activities of BC and derived products is presented schematically in Fig. 5 (modified according to Ref. [25]). The hypothesis assumes that under circumstances of moderate oxidative stress (see left side of the figure), the antioxidant effects of BC predominate. Under circumstances of heavy oxidative stress (see the right side of the figure), however, the bulk of supplemented BC is rapidly oxidatively degraded, leading to the formation of high amounts of breakdown products with prooxidant activities. Under these circumstances, the prooxidative actions of CBP overcome the antioxidant activity of BC, resulting in harmful effects. These are, for example, the impairment of adenine nucleotide translocase, accompanied by an increase in oxidative stress, indicated by reduced protein sulfhydryl content, lowered glutathione levels and redox state, and elevated accumulation of malondialdehyde. The increased levels of ROS after inhibition of energy metabolism, particularly superoxide anion radicals and hydrogen peroxide, disturb the surrounding macromolecules, including proteins and nucleic acids thereby increasing the risk of cancer. That might be an explanation for the increased incidence of lung cancer in smokers with high-dose

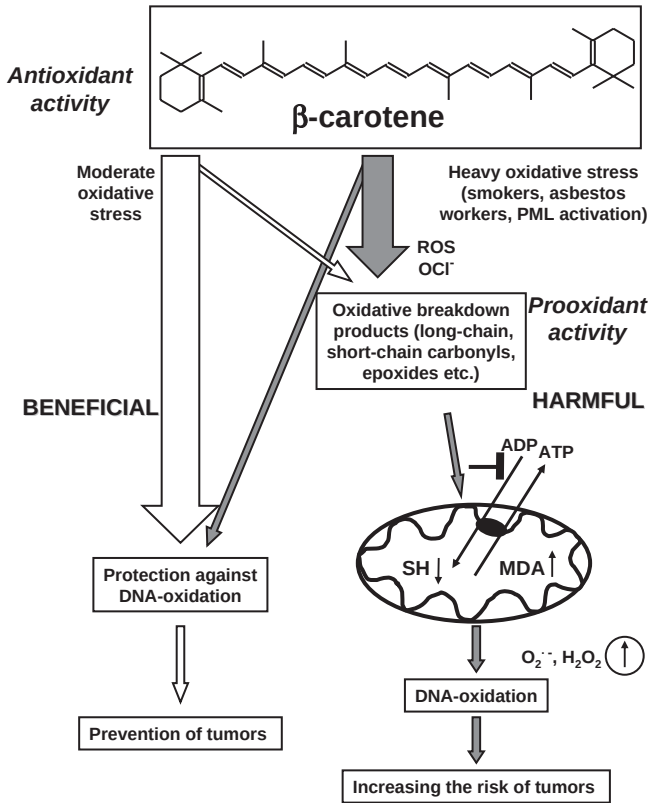


Fig. 5. Hypothetic scheme concerning the duality between prooxidant and antioxidant effects of BC. In this figure, both possibilities of BC effects are discussed: one with an overwhelming of antioxidative effects, and the other with an overwhelming of prooxidative effects. In our opinion the rate of BC breakdown and, therefore, the rate of CBP formation is the most important point. We suggested [25] that under conditions of moderate oxidative stress—shown at the left side—the formation of CBP is slow and the antioxidant effects of BC predominate. Under conditions of heavy oxidative stress—shown at the right side—the supplemented BC is rapidly decomposed leading to the rapid formation of CBP with their prooxidant effects. The remaining BC level will be low. Under these conditions the prooxidative actions of CBP overcome the antioxidant activity of BC resulting in harmful effects. These may include the impairment of ANT, reduction of protein SH content, decrease of GSH level and also elevated accumulation of MDA. The increased levels of ROS after inhibition of energy metabolism, particularly superoxide anion radicals and hydrogen peroxide, disturb the surrounding macromolecules, including proteins, peptides and nucleic acids, thereby increasing the risk of cancer. That might be one explanation for the increased incidence of lung cancer in smokers with high-dosage carotenoid supplementation as observed in the ATBC and CARET studies.

carotenoid supplementation as observed in the ATBC and CARET studies. This hypothesis underlines the opinion that at all circumstances without heavy oxidative stress the antioxidative reactions of carotenoids will not lead to harmful effects.

## 7. β-Carotene breakdown products modify respiratory burst and induce apoptosis in neutrophils

While neutrophils survive in the circulation only for a short time (8–20 h), their half life can increase several fold once they enter infected or inflamed tissues. In the absence of cytokines or other proinflammatory agents, aged neutrophils undergo programmed cell death (spontaneous apoptosis) mediated by caspases [78]. In acute inflammation, the constitutive apoptotic pathway is delayed. Under these circumstances, the potential for inflammatory neutrophils to cause tissue damage via the release of toxic ROS and granule enzymes such as proteases is high [79].

The pathways involved in signal transduction of neutrophils are overlapping and complex [80,81]. A common early downstream event after receptor binding is the activation of membrane phospholipid metabolism to generate two important second messengers, diacylglycerol and inositol 1,4,5-triphosphate, which promote calcium release from intracellular stores and activate protein kinas C [82–84]. Guanine-binding proteins also play a key role in this process [85]. Neutrophil activation causes a burst in cell respiration and a dramatic increase in ROS production. The stimuli, which trigger neutrophil response in vitro, include substances that bind to specific receptors (e.g., the chemotactic peptide f-Met-Leu-Phe) or substitute for diacylglycerol and activate protein kinase C directly (e.g., phorbol myristate acetate, PMA) [86].

In vitro experiments show that CBP exert opposite effects on neutrophil function in a restricted concentration range. Stimulation of superoxide production in PMA-activated cells is induced by low concentrations (1 μM) of CBP with aliphatic chains of different length (retinal, β-ionone, CBP), but not by the precursor carotenoids lacking carbonyl moiety (BC or lycopene) [87]. The stimulatory effect is accompanied by a reduction of the lag phase of cell response, which follows PMA stimulation. Indeed, it has been shown [88,89] that retinal stimulates phospholipase C activity and promotes redistribution of protein kinase C from soluble to a particulate neutrophil fraction. Thus, suboptimal doses of retinal induce minimal production of oxygen radicals, but prime the cells to release enhanced amounts of superoxide in response to appropriate stimuli [89].

In contrast, at concentrations slightly higher than those required to enhance the cell response, CBP inhibit superoxide production by PMA-activated neutrophils [87]. On the other hand, if the chemotactic peptide f-Met-Leu-Phe is used instead of PMA to trigger neutrophil response, inhibition of superoxide production is observed even at micromolar concentrations of CBP. The paradoxical inhibitory effect exerted by CBP correlates with the observation that relatively high amount of retinal inhibits protein kinase C from various sources [90,91] and blocks phosphorylation of the 47-kDa component of neutrophil NADPH oxidase [88,92]. Neutrophil apoptosis may be another reason for the diminished superoxide production by neutrophils in the presence of high CBP concentrations. Indeed, 20 μM CBP stimulate intracellular caspase 3 activity and chromatin fragmentation both in neutrophils [87] and in T-lymphoblast cell line [93].

These findings indicate that CBP may play a critical role in the modulation of neutrophil function. While micromolar concentrations of CBP may contribute to optimise neutrophil response in inflammatory processes, inhibition of superoxide production and premature activation of neutrophil apoptosis at high CBP levels may perturb neutrophil function and greatly increase the potential risk for host tissue injury by noxious agents.

## 8. Necessity to supplement BC

Krinsky [62] already in 1993 published that carotenoids play a preventive role in age-related diseases. The positive effects were explained by the biological actions carotenoids were believed to exert in human [7,63]. β-Carotene received special attention as a micronutrient with a wide spectrum of biological functions. Since BC is metabolized further to retinoic acid, it is understandable why the substance was seen and proven in vitro as a positive agent regarding immunoenhancement [94,95]. Apart from this, a number of observational trials showed that high intake of BC is related to a lower incidence of degenerative diseases and certain types of cancer [96,97]. However, the relevance and necessity of supplementation of pharmacological doses of BC are critically discussed since intervention trials failed [14]. Today, it seems clear that BC is not the only important carotenoid and that the substance acts only as part of the antioxidant network. That term, which was introduced in the literature by Packer [98–100], means that all antioxidants can work only in dependence from each other forming a "network". The question arises whether or not BC should be supplemented as a single substance or only together with other carotenoids and antioxidants as a cocktail. In the following, we focus on situations of BC deficiency under physiological and pathophysiological conditions in which BC supplementation seems to be necessary and useful.

Lipid-soluble vitamins were found markedly lower in the cord blood of neonates in comparison with the serum levels of their mothers [101–105]. Carotenoid concentrations in cord blood plasma were reported to be approximately 9–25% of the plasma levels of the mothers [103–105]. For newborns, breast milk or formula preparations are the only way to obtain carotenoids. As shown, mothers have a broad variety of carotenoids in their milk [106]. Like other nutrients, carotenoids are more concentrated in colostrum (by a factor 3.5–4.5) than in mature mother's milk [23]. Furthermore, concentration of BC in the milk of lactating women may be manipulated by the daily diet [107,108]. In 1986, Ostrea et al. [103] reported for BC an increase of plasma levels within 5 days in breastfed infants but not in infants fed formula preparation. However, when the study was conducted the formula preparations used did not contain supplemented BC. In a newer study, a total of eight formula preparations available on the European market were investigated for their content of different carotenoids [23]. The results of this study showed that still four out of eight formula preparations did not contain BC. Moreover, none of these formula preparations met the profile of the main carotenoids found in mother's milk. When measuring the plasma carotenoids of the infants a direct correlation could be seen compared to the carotenoid profile of the milk fed to the infants [23]. Three weeks after birth, BC was higher in breastfed infants ($P<.05$), but not in infants fed mother's milk as well as formula preparations. Moreover, in the group of infants fed formula only, BC was found even significantly lower than measured after birth ($P<.05$) [23]. Since a fast replenishment of plasma carotenoids is desired, supplementation of formula preparations with BC and other carotenoids seems to be necessary.

Vitamin A deficiency is another problem which may be prevented by a guaranteed intake of 600–1500 μg retinal or BC daily. The clinical consequences, which occur usually at the age of 2–3 years, are known and will not be discussed here. States of deficiency are rare in healthy children with a balanced diet. However, vitamin A deficiency is still a problem in underdeveloped countries especially in those in which rice is the staple food. A new approach to prevent the problem in those countries was the distribution of so-called golden rice. The rice is genetically manipulated so that BC is produced in the grains [109–111]. However, since absorption of BC depends also on the amount of fat in the diet, this factor should also be considered in the children's diet. Whether or not delivery of BC via rice might establish sufficient plasma levels of the carotenoid and so prevent the problem of vitamin A deficiency in these countries will be determined in the future.

Apart from this, diseases such as chronic intestinal disorders, celiac disease, hepatic and pancreatic disease, iron-deficiency anemia or chronic infectious disease may also lead to disturbances of absorbance and consumption of BC and, consequently, to a state of deficiency of the carotenoid as well as of vitamin A. In these cases, absorbance of lipids has to be improved but also the substrate BC in the diet has to be increased to reach sufficient plasma levels. Cystic fibrosis (CF) is an example of a disease with real carotenoid deficiency. Exocrine pancreas insufficiency leads to a malabsorption of lipids and lipid-soluble vitamins. Supplementation of vitamins A, D, E and K is already widely accepted today; however, this is not the case for carotenoids. Therefore, most CF patients suffer a distinctive carotenoid deficiency [112,113], and, in some cases, carotenoids are not detectable at all in the plasma of CF patients. To correct the BC levels in these patients, Rust et al. [114] and Winklhofer-Roob et al. [115] suggested supplementation of BC in pharmacological doses of approximately 0.5–1.0 mg/kg body weight [114,115]. These amounts of BC showed indeed a sufficient increase of the carotenoid in the plasma and also a decrease of lipid peroxidation parameters in the plasma [114,116,117]. Interestingly, patients under BC supplementation also had significant clinical benefits, e.g., a decrease in pulmonary exacerbations and an improvement in lung function [117,118]. This shows that BC deficiency in CF is

clinically relevant and the correction of carotenoid levels in those patients should be required as regular treatment of this disease.

## 9. The lung as a critical organ

As known from previous studies, BC accumulates in lung tissue in comparable amounts compared to other organs [119]. In the intervention studies ATBC and CARET, high doses of BC were given as supplements. As shown afterwards by Mayne [14], this resulted in carotenoid levels in the blood of 3.0 and 2.1 μg/ml, respectively, as compared to the average blood levels for the U.S. population (0.05–0.5 μg/ml). It might be assumed that a similar effect of an increase in BC concentration is also true for the lung tissue of persons taking the supplements.

In the respiratory system, oxygen enters the lung with a pressure of about 150 mm Hg as present in normal air. In the alveoli, an extremely thin barrier between air and capillaries allows oxygen to move from the alveoli into the blood and carbon dioxide to move from the blood in the capillaries into the alveoli. In that situation, BC present in lung tissue is exposed to a relatively high partial pressure of oxygen. Burton and Ingold [5] demonstrated already in 1984 that the antioxidant behaviour of BC was, in part, dependent upon the partial pressure of oxygen. As they showed, BC exhibits good radical-trapping antioxidant activity only at partial pressures of oxygen significantly less than 150 mm Hg. At higher oxygen pressure, the carotenoid loses its antioxidant activity and shows an autocatalytic, prooxidant effect, particularly at relatively high concentrations. Although these data were obtained only in vitro, the consequences of these effects for in vivo situations are still under discussion [17]. In case of high doses, BC supplementation tissue concentration might be reached, which consequently leads to conditions close to the in vitro conditions under which the prooxidative behaviour of BC was observed.

Another specificity of the lung is the powerful defence system. An average person who is moderately active breathes about 20,000 L of air every 24 h. The immune system protects the lung from foreign and possibly pathogenic microorganisms, such as bacteria, viruses, fungi and yeasts that infiltrate the lung with every breath. The organisms that reach the alveolar compartment via the airways first contact the pulmonary epithelium and the resident phagocytes (most notably alveolar macrophages). Phagocytes as well as lung epithelial cells are able to release cytokines and chemokines to recruit white blood cells in the circulation, such as neutrophils. In this way alveolar macrophages initiate and coordinate the host response to infection, including adaptive immunity.

During cellular defense, phagocytosing cells are able to liberate a number of oxidants to kill invading microorganisms. One of these oxidants is hypochlorite that is able to damage a high number of different biologically important macromolecules [120–122]. As revealed in vitro, the reaction rate of hypochlorite with BC is slow as compared to other reducing agents, e.g., reduced glutathione [123]. However, myeloperoxidase, the source of hypochlorite, is a highly cationic protein that becomes attached shortly to membranes after its release from stimulated neutrophils [124]. Thus, an interaction of hypochlorite with more lipophilic constituents such as carotenoids seems to be realistic under conditions where neutrophils accumulate extensively. For example, hypochlorite is able to destroy carotenoids in low-density lipoproteins [125]. Calculations revealed that $2–4\times10^6$ leukocytes/ml produce 100–140 μM hypochlorite during 1 h [126], leading to realistic BC/hypochlorite ratios between 1:100 and 1:10 [127]. If so, inflammatory cells present in the lung should be able to degrade BC. To prove the hypothesis, in vitro experiments with primary cultures of PML were done (with at least $4\times10^6$ cells/ml). Water-dispersible carotenoids were introduced to the culture medium, and the PML was activated by phorbol myristic acid (PMA). These experiments showed clearly that activation of the cells leads to degradation of substantial amounts of carotenoids by stimulated PML after 30 min.

Under normal conditions those effects might not be of relevance. However, several factors may alter the physiological balance regarding recruitment and activity of inflammatory cells in lung tissue. One of these factors is the smoking behaviour of a person. In a very recent study, Amin et al. [128] investigated bronchial biopsies of asymptomatic smokers (smokers without respiratory problems) and never-smokers. In their study, smokers compared to never-smokers showed a highly increased number of inflammatory cells. The number of neutrophils found in the biopsy specimen of smokers was about 12 cells/mm$^2$. In contrast to them, never-smokers only had about 2 cells/mm$^2$ [128]. These results are in agreement with the work of Eidelman et al. [129] who described a fivefold increased number of neutrophils in lung tissue of smokers compared to nonsmokers and others reporting significantly increased numbers of inflammatory cells in general in lung tissue of smokers [130,131].

However, smokers are not the only group showing increased numbers of inflammatory cells in the lung. In atopic and nonatopic asthmatics, eosinophils and mast cells—which are able to liberate oxidants as well—were shown to be increased compared to controls [132]. However, the number of neutrophils was found increased only in nonatopic asthmatics in the same study [132]. A more detailed overview about oxidative events, especially also about the role of inflammatory cells, in the pathogenesis of asthma is given in reviews recently published [133,134]. They describe how increased amounts of eosinophils and neutrophils have the potential to harm host tissue and contribute to inflammatory injury. During activation, NADPH oxidase is stimulated, and ROS such as superoxide radical and its dismutation product $H_2O_2$ are formed in high amounts. Furthermore, the leucocytes have

enzymes, e.g., peroxidases that amplify the oxidative property of $H_2O_2$. Another disease in which increased numbers of neutrophils was observed in lung tissue is the primary Sjogren's syndrome, which is connected to rheumatic disorders [135].

Patients with the mentioned disorders dot not necessarily have the need for a BC supplementation. However, as stated above, most patients with CF show pancreatic insufficiency leading to a malabsorption of carotenoids. Since these patients may benefit from an additional BC intake, it is interesting to have a look at them. Hubeau et al. [136] published in 2001 a comprehensive quantitative analysis of inflammatory cells of the airway mucosa of CF patients using immunohistochemistry. They found that in CF patients, neutrophils were particularly numerous at the level of the segmental bronchi [136]. They measured the number of neutrophils in lung tissue of CF patients, which was seven times higher than that of persons without CF. This shows that risk of oxidative stress mediated by neutrophils is at the same level as or even higher than that of smokers.

If partial oxygen pressure and a high number of inflammatory cells in lung tissue are considered as risk factors, BC supplementation in those patients has to be done in a line with a close monitoring of the blood levels of the carotenoid to avoid such high tissue concentrations as seen in the intervention trials (ATBC and CARET).

## 10. Conclusions: proposals for safe BC supplementation

Increased consumption of fruits and vegetables was always described to reduce markers of oxidative cellular damage that can be assessed in blood or urine as described by Thompson et al. [137]. Obviously, the problems seen in the mentioned intervention trials were caused by the high-dose supplementation under conditions of severe oxidative stress.

We propose the following strategies to avoid negative side effects by high-dose carotenoid supplementation: BC supplementation, if necessary, should be accompanied with the monitoring of BC plasma levels to avoid critically high levels. We propose to define upper plasma levels which should be a part of BC supplementation guidelines. Furthermore, at high-dose BC supplementation the so-called antioxidative network [98–100] as complex system of interacting antioxidants should be balanced. The strengthening of this complex system of antioxidants may contribute both to reduced rate of CBP formation by stimulated neutrophils and to detoxification of those potentially mitochondriotoxic and genotoxic compounds.

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