Carotenoids
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Chapter 16

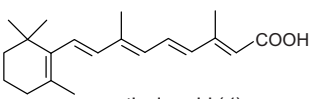
Skin Photoprotection by Carotenoids

Regina Goralczyk and Karin Wertz

A. Introduction

In Western populations, a lifestyle favouring tanned skin leads to increased exposure to natural and artificial sources of UV-radiation (UVR). To keep the adverse effects of this exposure, such as sunburn, immunosuppression, photoaging and photocarcinogenesis, to a minimum, nutritional manipulation of the basic endogenous protective properties of skin is an attractive target. In this respect, considerable interest has been directed for many years towards the dietary carotenoids, because of their radical scavenging and singlet oxygen quenching properties and thus their putative role in photochemistry, photobiology and photomedicine.

Hypothetically, carotenoids could be involved in several ways to protect skin from sunlight damage, namely by increasing optical density, quenching singlet oxygen ($^1O_2$) or, for provitamin A carotenoids, *via* formation of retinoic acid (*1*), a known topical therapeutic agent against photodermatoses. The role of $^1O_2$ in UVA-induced oxidative stress is well established and has been reviewed extensively [1,2]. Carotenoids can also scavenge other reactive oxygen species [3,4], such as superoxide anions, hydroxyl radicals or hydrogen peroxide. Under certain conditions, however, *i.e.* higher oxygen partial pressure, carotenoids may act as pro-oxidants [5,6] (*Chapter 12*).



retinoic acid (*1*)

Figure 1 shows the various structural layers of the skin, and the depth of penetration of radiation of different wavelengths; the shorter the wavelength, the greater is the energy of the radiation. Chronic exposure to UV radiation leads to epidermal and dermal damage, such as hyperkeratosis, keratinocyte dysplasia and dermal elastosis, in affected skin areas, clinically presenting as photoaged skin with actinic or solar keratosis. These precancerous lesions show an increased risk for the development of squamous cell carcinoma (SCC).

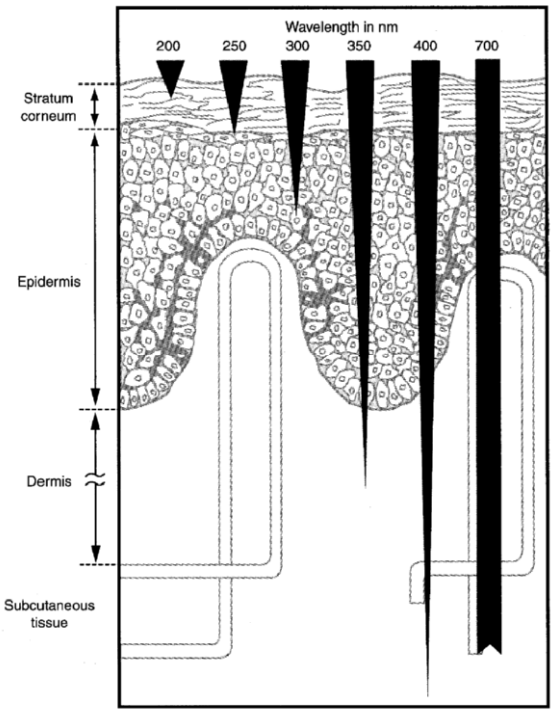


Fig. 1. Scheme of the structural layers of the skin, *i.e.* the stratum corneum, epidermis, dermis and subcutis. The black arrows show the penetration depth of increasing wavelengths [7]. In contrast to UVB (290-320 nm), which only penetrates through the epidermis, UVA (320-400nm) can penetrate deep into the dermis and subcutis.

The molecular mechanisms of skin photodamage and photoaging have been subjects of extensive research [8] (Fig. 2). UV radiation activates a wide range of cell-surface growth factors and cytokine receptors [9]. This ligand-independent receptor activation induces multiple downstream signalling pathways that converge to stimulate the transcription factor AP-1. Among the genes that are up-regulated by AP-1 are several members of the matrix metalloprotease (MMP) family. Increased MMP expression and activity causes enhanced collagen proteolysis and, together with reduced collagen expression, results in skin elastosis

and wrinkling [10]. Under chronic UV exposure, the clinical condition is accompanied by dilated and twisted microvasculature, *i.e.* teleangiectasia and hyper-pigmentation (clinical features of photoaging [11]).

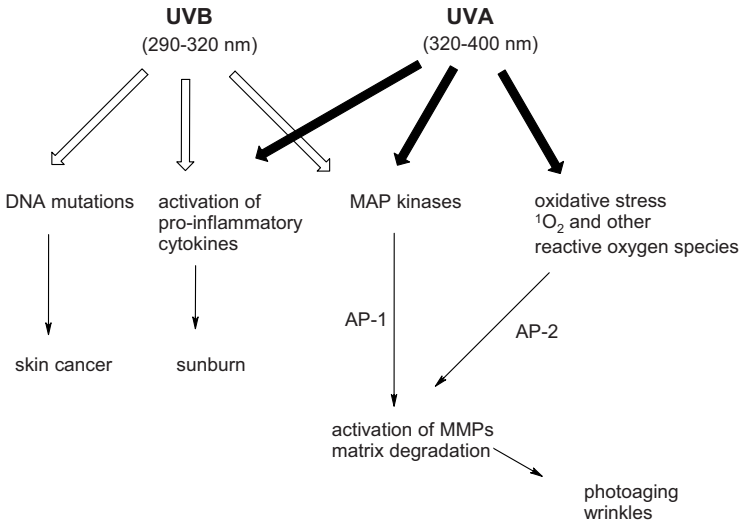
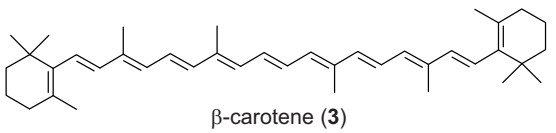


Fig. 2. Major mechanisms for the involvement of UVB and UVA in photocarcinogenesis and photoaging

UVB (290-320 nm) is mainly absorbed by keratinocytes in the epidermis. By direct interaction with the DNA, it causes mutations and skin cancer. UVB also leads to sunburn, which is an erythema resulting from an inflammatory response to the photodamage to the skin.

UVA (320-400 nm) plays a major role in photoaging. UVA can penetrate into the deeper dermis and induces the generation of reactive oxygen species (ROS), which can induce mutations in the mitochondrial DNA, thus leading to losses of enzymes involved in oxidative phosphorylation (see Section **D**.3.) and deficiencies in energy metabolism. The defects in the respiratory chain lead to further inductions of ROS. Singlet oxygen can also induce up-regulation of MMPs directly, independent of the AP-1 pathway (see Section **D**.4.).

In this *Chapter*, photoprotective effects of dietary carotenoids, especially β-carotene (**3**), towards skin damage induced by UVA and UVB are reviewed, underlying molecular mechanisms are discussed, and the availability of carotenoids at the target skin tissue is summarized.



β-carotene (**3**)

B. Uptake and Metabolism of Carotenoids in Skin Cells

1. Humans and mouse models

The effectiveness of photoprotection will largely depend on the local concentration of the carotenoids in the specific skin compartment at the site of UV-induced radical formation.

β-Carotene and other carotenoids are transported to the skin and accumulate mainly in the epidermal layers. High β-carotene concentration in skin leads to increased reflection and scattering of light. Thus, penetration of photons into deeper skin layers is lessened. Reflection of light has also been utilized to measure β-carotene concentration in skin by non-invasive reflection spectroscopy. β-Carotene does not, however, act as an optical UV filter [12], since its main absorption maximum, like that of most carotenoids, is around 460 nm and not in the UVB/UVA range of wavelengths.

The amount of carotenoids deposited in skin correlates with dietary intake and bioavailability from the food source (see *Chapter 7*). After absorption, carotenoids are transported in the bloodstream *via* lipoproteins to the various target tissues [13-16]. Recently, cholesterol transporters such as SR-B1 and CD 36 were shown to mediate a facilitated absorption of carotenoids in the gut [17-19]. It is likely that carotenoids are taken up by these transporters also in the epidermis, which is an active site of cholesterol accumulation for maintenance of permeability barrier function. SR-B1 is expressed in human epidermis [20], predominantly in the basal layers.

Unfortunately, reports on carotenoid concentrations in skin of humans or laboratory animals are rare, many of them old and most referring to β-carotene only. Comparisons across publications are complicated by the fact that different methods were applied, such as simple absorption spectroscopy of skin extracts, non-invasive reflectance spectroscopy or HPLC of skin biopsies. The latter method can be regarded as the most appropriate technique, but it requires analysis of skin biopsies, which are often collected in different ways, *i.e.* blister, scrape or punch, and result in different fractions of dermis/epidermis or even contamination with subcutaneous fat. Furthermore, absorption spectroscopy, non-invasive reflection spectroscopy and earlier HPLC of skin extracts were only able to detect total carotenoids, and did not differentiate between the various carotenes, xanthophylls and their isomers. In addition, efficiency of extraction of skin samples varies, thus leading to large differences in carotenoid recoveries. In general, however, the correlation of skin to plasma carotenoid concentration is very good [21-23]. A compilation of reported β-carotene/carotenoid concentrations in skin is shown in Table 1.

Table 1. β-Carotene or carotenoid concentration in human skin, normal and after dietary supplementation.

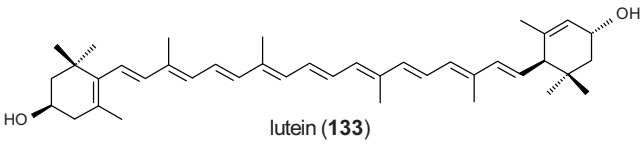
Treatment	Analysis Method	Tissue	Value nmol/g	Ref.
Normal skin	Extraction/ absorption spectrum	Scrapings Epidermis Dermis	 0.39 0.01	[24]
β-Carotene (beadlets) 180 mg/day, 10 weeks	Extraction/ absorption spectrum	Epidermis blister	1.7	[24]
Normal skin	Extraction/ absorption spectrum shave biopsy	Epidermis Dermis Subcutis Surface lipid Comedones	4.1 1.3 3.5 10.0 14.5	[25]
Normal skin	HPLC	Whole skin	0.09	[21]
a) Baseline b) 120 mg β-carotene, single dose	HPLC	Whole skin	a) 1.41 b) 1.74	[26]
a) Baseline b) 30 mg/day β-carotene (beadlets), 10 weeks	HPLC	Punch biopsy (Dermis/ Epidermis)	a) 8.3 b) 24.2	[27]
β-Carotene (24 mg/day) from algal extract, 12 weeks	Reflection spectroscopy, total carotenoids	Forehead	1.4	[28]
Tomato paste, 16 mg lycopene, 20 weeks	Reflection spectroscopy, total carotenoids	Hand palm	Control: 0.33- 0.19 Treated: 0.26-0.3	[29]
Combination of vitamin E, β-carotene, lycopene, selenium, proantho-cyanidins (Seresis), 16 weeks	HPLC	Punch biopsy (Dermis/ Epidermis)	β-Carotene: nmol/mg protein baseline: 0.007 56 days: 0.022 112 days: 0.012	[30]
a) β-Carotene, 24 mg/day b) mixed carotenoids from algae, 24 mg/day, 12 weeks	Reflection spectroscopy, total carotenoids	Hand palm	a) ~ 1.1 b) ~ 1.5 controls ~0.5	[31]

Apparently, carotenes are present at higher concentration in the epidermis and in surface lipids than in the dermis, consistent with the distribution of lipid transporters. Physiological levels between 0.09 and 4 nmol/g wet weight are reported. Upon supplementation with β-carotene, reported values vary widely, *i.e*. 1.7 nmol/g (determined by absorption spectrophotometry) [24] in the epidermis after administration of supplements of 180 mg/day over 10 weeks, or 8 nmol/g in punch biopsies at baseline compared to 24 nmol/g after supplementation with 30 mg β-carotene/day over 10 weeks [27]. In contrast, a lower concentration of 1.4 nmol/g was determined by reflectometry after a 12-week supplementation with β-carotene from an algal source [28]. The variability of skin carotenoid concentrations across human studies may be due to differences in the bioavailability of the supplemented product and/or to the use of different analytical methods.

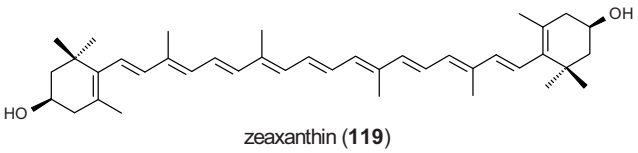
The level of β-carotene in plasma and in epithelial cells (oral mucosa cells, OMC) is dependent on skin-type [32]; individuals with Type I, *i.e*. fair skin and hair, and high UV-sensitivity, have significantly lower β-carotene levels than Type IV individuals, who have strong pigmentation, dark hair and low UV-sensitivity.

Similar large variations in skin β-carotene concentrations have been reported in studies with rodent models. Depending on the protocols used for intervention and the bioavailability of the β-carotene supplement, values in mice vary as extremely as from 0.27 to 8 nmol/g [33-35]. This demonstrates the difficulty of establishing the absolute β-carotene concentration in the target tissue for correlation with its photoprotective effects. Nevertheless, although much higher doses are required, skin levels of β-carotene in mice are in the same order of magnitude as in humans, making the mice relevant models for studying the interactions of carotenoids with UV-induced processes in skin.

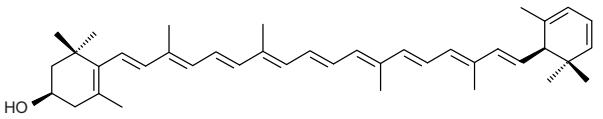
There are fewer HPLC data on skin levels of other dietary carotenoids. In normal skin, xanthophylls such as lutein (**133**), zeaxanthin (**119**), 2′,3′-anhydrolutein (**59.1**), and α-cryptoxanthin (**62**) and β-cryptoxanthin (**55**) were detected, as well as low amounts of their monoacyl and diacyl esters [36].



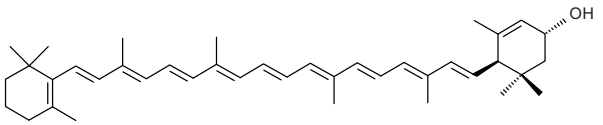
lutein (**133**)



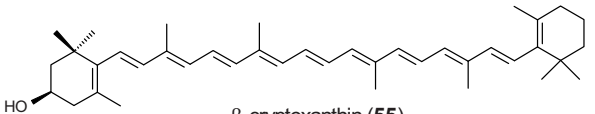
zeaxanthin (**119**)



2',3'-anhydrolutein (**59.1**)

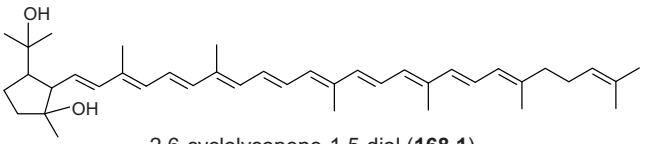


α-cryptoxanthin (**62**)



β-cryptoxanthin (**55**)

Supplementation with lycopene-rich products, *i.e.* carrot juice (from the variety 'Nutrired', containing 2.5 mg lycopene and 1.3 mg β-carotene/100 ml), a lycopene supplement from tomato extract, a lycopene-containing drink or a supplement of synthetic lycopene, for 12 weeks led to about 20-40% increases in total skin carotenoid levels as measured by reflection spectroscopy [37]. Daily supplementation with 40 g tomato paste (providing 16 mg lycopene) for 10 weeks, however, did not lead to significant increases in skin total carotenoid levels as determined by reflection spectroscopy [29]. Lycopenodermia, a rare reversible cutaneous condition similar to carotenodermia, can be observed after excessive dietary ingestion of lycopene-containing products [38]. Two oxidative metabolites of lycopene, namely the stereo-isomeric 2,6-cyclolycopene-1,5-diols A and B (**168.1**), which are only present in tomatoes in extremely low concentrations, have been isolated and identified in human skin [39].



2,6-cyclolycopene-1,5-diol (**168.1**)

Recently, a novel approach for non-invasive, laser optical detection of carotenoid levels in skin by Raman spectroscopy has been established [40]. The Raman scattering method monitors the presence of carotenoids in human skin and is highly reproducible. Evaluation of five anatomical regions demonstrated significant differences in carotenoid concentration by body region, with the highest carotenoid concentration noted in the palm of the hand.

Comparison of carotenoid concentrations in basal cell carcinomas, actinic keratosis, and their peri-lesional skin demonstrate a significantly lower carotenoid concentration than in region-matched skin of healthy subjects. Furthermore, the method reveals that carotenoids are a good indicator of antioxidant status. People with high oxidative stress, *e.g.* smokers, and subjects with high exposure to sunlight, in general, have reduced skin carotenoid levels, independent of their dietary carotenoid consumption. Portable versions of the Raman spectroscopy instruments are now available and could have a broad application in dermatology and cosmetics.

The levels of β-carotene in serum decreased in unsupplemented but not in supplemented individuals on chronic UV exposure [32,41]. Depletion of skin carotenes and retinol after UV irradiation, and restoration by carotene supplementation were also observed in hairless mouse models [35]. When skin is subjected to UV light stress, more lycopene is destroyed than β-carotene, suggesting a role of lycopene as first defence line towards oxidative damage in tissues [26].

In conclusion, carotenoids from dietary intake accumulate in skin, thus allowing them to exert their photoprotective function at the target site. The levels correlate with bioavailability of the supplement, UV-exposure, and genetic factors such as skin type.

2. Carotenoids in skin cell models

a) Culture conditions

A precondition for carotenoid efficacy in photoprotection is that the carotenoids are taken up by the cells. Since the amount of carotenoid accumulated depends on many factors, such as cell line [42], carotenoid concentration in the cell culture media, vehicle used to bring the carotenoid into solution, treatment period *etc*., it is essential to analyse the uptake and metabolism in cultures, before drawing conclusions on the efficacy of the carotenoid.

The major difficulty concerns the choice of the vehicle to be used to solubilize the highly lipophilic carotenoids without adverse effects on the cells. The vehicle should also prevent oxidative degradation of the carotenoids without affecting the UV response of the cells. Among the vehicles that have been used are organic solvents such as tetrahydrofuran (THF) [43], cyclodextrins [44], liposomes [45] or adsorption on nanoparticles [46]. When carotenoids were supplied in the latter three vehicles, deleterious pro-oxidative rather than protective effects were observed, in particular in the absence of stabilizing antioxidants such as vitamin E. Caution has to be exercised in interpreting such negative results because, for example, cyclodextrins were shown to deplete cholesterol from cells and alter the UV-response [47,48]. In liposomes, carotenoids are soluble only to a limited degree, leading to lower loading of the cells. In addition, enhanced pro-oxidative reactions can occur due to peroxidation of the liposomal lipids.

Use of THF as a vehicle leads to reliable results. It requires, however, removal of peroxides from the solvent by column chromatography on alumina. Carotenoid stock solution

should always be prepared fresh before each experiment, to avoid oxidative degradation. Even then, carotenoids degrade rapidly in medium under cell culture conditions, *i.e*. within 24 hours. Thus, the medium must be changed daily to avoid accumulation of degradation products in the cells [43]. The concentration of the carotenoid in media and cells should always be monitored carefully by HPLC.

b) Uptake and metabolism of carotenoids in skin cells

It has been demonstrated that HaCaT keratinocytes take up β-carotene in a time-dependent and dose-dependent manner (Table 2). The HaCaT cells had to be supplemented for at least two days to achieve significant β-carotene accumulation. The cells continued to take up β-carotene thereafter, and maximum β-carotene levels were found after three days of supplementation. If no fresh β-carotene was added, the β-carotene content decreased, demonstrating that a daily supply of fresh β-carotene is crucially required to maintain the cellular β-carotene content.

Table 2. β-Carotene uptake and metabolism in HaCaT skin keratinocytes.
HaCaT cells were treated with 0.5, 1.5 or 3 μM β-carotene for 2 days. Cellular contents of β-carotene and β-carotene metabolites were determined by HPLC. <LOD: below limit of detection. Retinol and retinyl palmitate concentrations were below the limit of detection in all cases.

β-Carotene supplementation (μM)	**(all-*E*)-β-Carotene (pmol/10^6 cells)**	**(*Z*)-β-Carotene (pmol/10^6 cells)**	**Apocarotenals (pmol/10^6 cells)**
Placebo	<LOD	<LOD	<LOD
0.5	9.70 ± 0.09	0.20 ± 0.07	1.18 ± 0.04
1.5	34.30 ± 0.05	0.41 ± 0.02	3.21 ± 0.19
3.0	63.90 ± 0.22	0.82 ± 0.16	5.04 ± 0.11

As a provitamin A, β-carotene may act *via* retinoid pathways through local metabolism to retinol or apocarotenals and further to retinoic acid. Human skin fibroblasts *in vitro* increased their intracellular retinol after β-carotene supplementation [49]. HaCaT keratinocytes expressed β-carotene 15,15′-monooxygenase at low levels, but the retinol content in HaCaT cells was below the HPLC detection limit. Also, only marginal amounts of retinoic acid (RA) were formed from β-carotene, as detected indirectly by the induction of the RA target gene RARβ (Fig. 3, right). In contrast, expression of the β-carotene 9,10-oxygenase was much higher, and apocarotenals were detected in cells [43].

In keratinocytes (Fig. 3, left) [43] and similarly in skin fibroblasts [48], UVA irradiation destroyed β-carotene so that only 13% remained of the content before irradiation. Consistent with this finding, the retinoic acid response element (RARE)-dependent gene activation by β-carotene was reduced if the cells were irradiated with UVA (Fig. 3, right) [43].

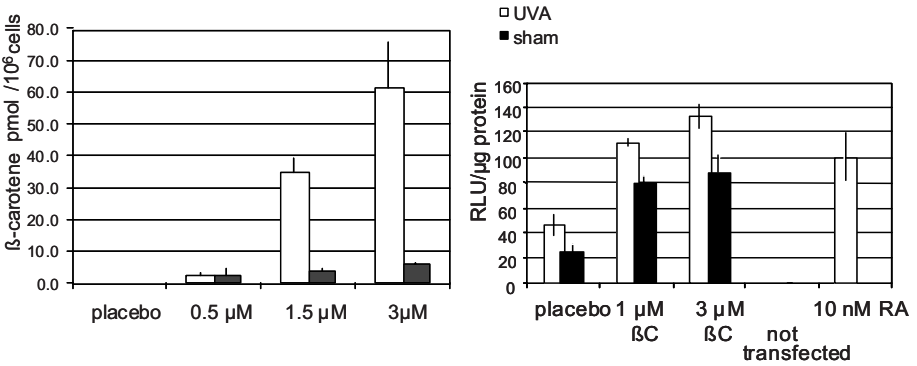


Fig. 3. Left: Dose-dependent uptake of β-carotene in HaCaT skin keratinocytes and depletion of cellular β-carotene stores by UVA irradiation. HaCaT cells were supplemented with 0.5, 1.5 or 3 µM β-carotene for 2 days prior to UVA irradiation (270 kJ/m^2). Cellular β-carotene content was analysed by HPLC. Right: Effect of β-carotene on transactivation of a retinoic acid-dependent reporter construct: HaCaT cells were transiently transfected with a reporter gene construct containing 5 direct repeats of the wild type. Luciferase activity was determined after 40 h treatment with β-carotene. RLU: random luminescence units, RA: retinoic acid.

UVA caused down-regulation of all retinoid receptors about 2-fold, except for RARα, which was not influenced by UVA. Apparently, regulation of RARα and RARγ expression, as well as regulation of RXRα and RXRγ, has a $^1O_2$-dependent component, as UVA irradiation in the presence of D_2O, which is known to extend the lifetime of $^1O_2$, had a significant effect on these transcripts. β-Carotene had no significant effect on the basal or UVA-regulated expression levels of the RARs and RXRs. Of note, β-carotene non-significantly induced RARβ in a dose-dependent manner, an effect observed predominantly in unirradiated cells. It shows that weak retinoid activity is generated from β-carotene in HaCaT cells; this may be attributed to the products of excentric cleavage of β-carotene, apocarotenals, which are present at detectable concentrations in HaCaT cells.

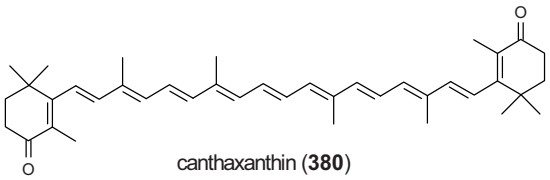
These findings are in agreement with observations *in vivo*, which also show that UVA exposure depleted epidermal vitamin A stores [35,50]. It has been reported [51] that retinoid content and RXRα expression were reduced in UV-irradiated SKH-1 hairless mice, and β-carotene 15,15′-monooxygenase activity was induced in response to this UV-induced depletion.

In conclusion, the observation of a depletion of vitamin A and provitamin A stores by UV light calls for an awareness of an increased requirement for vitamin A and carotenoid in situations of extensive sun exposure, in view of the role of vitamin A in maintaining skin integrity.

C. Photoprotection *in vivo*

1. Photosensitivity disorders

Elucidation of the function of carotenoids in singlet oxygen quenching in photosynthetic plants, algae and bacteria has led to the assumption that similar protection might be relevant in human skin, where UV light in the presence of endogenous photosensitizers can also induce formation of excited triplet species. The accumulation of large amounts of protoporphyrin, an endogenous photosensitizer, in the blood and skin of patients with inherited erythropoietic protoporphyria (EPP) gives rise to symptoms of itching and burning of the skin when patients are exposed to sun light.



canthaxanthin (**380**)

In particular, β-carotene and canthaxanthin (**380**) have been shown to be beneficial in alleviating the symptoms of erythropoietic protoporphyria and other conditions such as polymorphous light eruptions [52-56]. These findings are mainly based on uncontrolled human studies performed in the 1960s and 1970s, usually with low case numbers. About 84% of the patients responded to successively increasing doses of oral β-carotene (formulated as beadlets) of up to 180-300 mg/day by showing increased tolerance to sunlight exposure. The US Food and Drug Administration approved the use of β-carotene for the treatment of EPP in 1975. This high dose β-carotene treatment did not lead to any adverse side effects other than a discolouration of the skin.

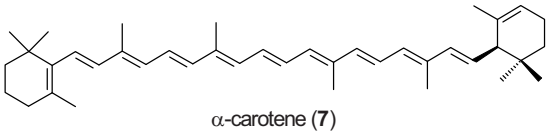
In conclusion, some patients react with improvement of skin symptoms in erythropoietic protoporphyria after oral supplementation with β-carotene, but extremely high doses over several months or years, leading to plasma levels of about 8 μmol/L, are required to achieve an effect.

2. Photocarcinogenesis

The encouraging results with β-carotene on erythropoietic protoporphyria led to further speculation that β-carotene might also have a protective role against photocarcinogenesis. Although several studies in rodent models initially showed promising results with high doses of β-carotene, these effects could not be reproduced later, and even an exacerbation of UVB-induced skin carcinogenesis was observed [57-59]. These contradictory findings remain unexplained; an influence of the specific diet was discussed, however. In humans, subsequent

large randomized skin cancer prevention trials did not find a risk reduction in non-melanoma skin cancer by β-carotene (50 mg β-carotene/day for 5 years [60]; 20 mg β-carotene/day for 4.5 years [61]; 50 mg β-carotene every other day for 12 years [62]). A possible explanation of these results could be that supplementation would be necessary to increase carotene content during earlier phases of life, before the initial pathogenic events. Yet, from a mechanistic point of view, it seems rather unlikely that β-carotene is able to interact with the direct mutagenic and carcinogenic actions of UVB. This process involves absorption of short wavelength radiation by DNA, formation of the major types of DNA damage photoproducts, *i.e.* cyclobutane pyrimidine dimers and pyrimidine-6-4-pyrimidone photoproducts which are formed between adjacent pyrimidine nucleotides on the same strand of DNA. The resulting DNA mutations consequently lead to activation of oncogenes or inactivation of tumour suppressor genes.

Observational studies do not support a role of dietary carotenoids in non-melanoma skin cancer risk reduction [63-67]. Results from a prospective nested case control study embedded in the Nambour Skin Cancer Trial in Australia [67] suggested a positive association of basal cell carcinoma development with intake of lutein, but not of other carotenoids, selenium or vitamin E. In another recent observational study within the Isotretinoin-Basal Cell Carcinoma Prevention Trial [68], serum lutein, zeaxanthin and β-cryptoxanthin were positively related to risk of squamous cell carcinomas; risk ratios for subjects in the highest *versus* lowest tertiles were for lutein 1.63 [95% confidence interval (95% CI) 0.88-3.01; P for trend = 0.01], for zeaxanthin 2.40 (95% CI 1.30-4.42; P for trend = 0.01), and for β-cryptoxanthin 2.15 (95% CI 1.21-3.83; P for trend = 0.09), respectively. These observations would imply a detrimental effect of higher carotenoid intake rather than a protective effect.



α-carotene (**7**)

A case control study for assessment of melanoma risk found that individuals in high *versus* low quintiles of energy-adjusted vitamin D, α-carotene (**7**), β-carotene, β-cryptoxanthin, lutein, and lycopene had significantly reduced risk for melanoma [Odds Ratios (a measure of the degree of association, *e.g.* the odds of exposure among the cases compared with the odds of exposure among the controls) ≤ 0.67], which remained significant after adjustment for the presence of dysplastic nevi, education, and skin response to repeated sun exposure. Larger prospective population studies would be required to substantiate such a protective effect.

Together, these studies provide only little or no evidence for a role of β-carotene and other dietary carotenoids in prevention of melanoma and non-melanoma skin cancer in humans.

3. Sunburn

Sunburn is the inflammatory reaction of the skin in response to excessive exposure to natural or artificial solar light of UVB wavelength. It is characterized by reddening of the skin, and, depending on the severity, by blister formation and ablation of the epidermis. On a histological level, sunburn cells, *i.e*. keratinocytes undergoing programmed cell death, form within hours after exposure. The minimal dose of UVB required to produce an erythema (MED) is dependent on the skin type. The MED is assessed by chromametry and used routinely to determine the sun protection factor (SPF) of sun screens.

Human dietary intervention studies of the effect of carotenoids on sun erythema formation have recently been reviewed comprehensively [23,69]. The effect of the carotenoid on the endpoint minimal erythema dose was investigated at various doses ranging from 24 to 180 mg per day β-carotene, or mixed carotenoid/micronutrient combinations, or carotenoids supplied as vegetable juices. Supplements were administered for between 3 days and 24 weeks. In eight of the ten studies reviewed, the MED was increased or sun erythema was less pronounced, indicating a protective effect. Two studies, where supplementation was very short, *i.e*. 3 days [70] to 4 weeks [71] showed no protective effect. Recently, another study with 15 mg β-carotene over 8 weeks also showed no effect on MED, but there was also no increase in skin β-carotene levels after the supplementation [72].

The evidence for a protective effect of β-carotene against sunburn was confirmed in a recent meta-analysis of the literature up to June 2007 on human supplementation studies and dietary protection against sunburn by β-carotene [73] (Fig. 4).

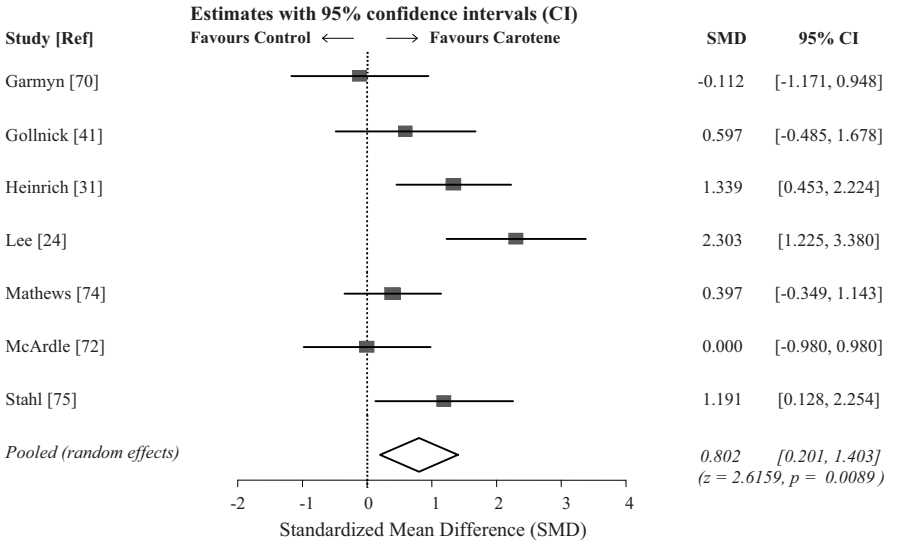


Fig. 4. Results of seven studies of effects of β-carotene *versus* placebo on protection against sunburn evaluated in a meta-analysis. (From [73] with permission).

Seven studies which evaluated the effectiveness of β-carotene in protection against sunburn were identified in Pubmed, ISI Web of Science and EBM Cochrane library [73]. Data were abstracted from these studies by means of a standardized data collection protocol. Although two of the studies considered showed no protective effect of β-carotene, the other five all showed varying levels of protection, with Standardized Mean Difference (SMD) ranging from 0.397 (95% CI -0.349, 1.143) to 2.303 (95% CI 1.225, 3.380). When the results were pooled, this gave an overall SMD of 0.802 (95% CI 0.201, 1.403, p = 0.0089). The meta-analysis showed that (i) β-carotene supplementation protects against sunburn and that (ii) the study duration had a significant influence on the size of the effect. Regression plot analysis revealed that protection required a minimum of 10 weeks of supplementation with a mean increase in the protective effect of 0.5 standard deviations with every additional month of supplementation. Thus, dietary supplementation of humans with β-carotene provides protection against sunburn in a time-dependent manner. These studies taken together show that erythema reduction is the photoprotection parameter which is most consistently affected by carotenoids. The effect seems not to be specific for a particular carotenoid, since a mixture of 6 mg each of lutein, lycopene and β-carotene was as effective as 24 mg β-carotene alone. Similarly, a mixture of antioxidants consisting of lycopene, β-carotene (6 mg/d each), vitamin E (10 mg/d) and selenium (75 μg/d) for 7 weeks increased erythema threshold significantly [76].

It should be noted that erythema reduction by carotenoid is mild and correlates with a Sun Protection Factor (SPF) of 2, putting into question the clinical relevance. In no case should oral supplementation with carotenoids replace the use of UV filters. On the other hand, orally supplemented β-carotene was shown to enhance the effectiveness of topical sun lotions [41]. Overall, dietary carotenoids may find their use and are important as part of a basic skin protection, in particular upon occasional sun exposure, when a UV filter is not applied.

4. Photoaging

No large human intervention studies have yet been conducted to address the effects of carotenoids on clinical parameters of premature photoaging, such as wrinkling, pigmentation, teleangiectasia (a widening of the fine capillaries in skin), dryness and inelasticity. The Nambour Skin Cancer Trial in Australia [61] addressed photoaging only to a limited extent. The subjects, 556 adults aged 25-50 years, were randomized in a 2 x 2 factorial trial to a daily sunscreen with Sun Protection Factor (SPF)-15 *vs*. usual (occasional) sunscreen use, and β-carotene (30 mg daily) *vs*. placebo treatment over a period of 4.5 years. Participants were exposed to the natural sunlight during the course of the trial. Silicone impressions of skin texture of the back of the hand were evaluated before and after treatment. There was a significant interaction effect of sunscreen and β-carotene on photoaging. Relative to the placebo group, the adjusted odds ratio (the odds of the occurrence of an event or disease is compared between the unexposed and exposed groups) for photoaging was about two-thirds

for those on sunscreen, about one-third for those on β-carotene but slightly increased for those on both treatments. This was taken to suggest independent roles for sunscreen and β-carotene in the prevention of photoaging of the skin in sun-exposed white populations [77]. The negative interaction observed for the combination of sunscreen with β-carotene remained unexplained. Although the study had some limitations with parameter assessments and statistical analyses, it could be considered to provide the first evidence of a preventive effect of β-carotene on clinical photoaging caused by sunlight, including UVA.

In the Seresis study on molecular markers for photoaging, the effect of an antioxidant mixture containing β-carotene and lycopene [vitamin E (10 mg), β-carotene (2.4 mg), standardized tomato extract (25 mg lycopene), selenium yeast (25 mg), and proantho-cyanidins from grape seed extract (25 mg)] was addressed [30]. In a 2 x 2 factorial design, 48 volunteers who had received either the antioxidant medication or placebo for 10 weeks were exposed to low dose UVB for 2 weeks and MED measurements taken. Before and after irradiation, the proteins MMP-1 and MMP-9, two major metalloproteases which degrade various collagens and other interstitial matrix proteins and also cleave the cytokine IL1β from its propeptide, were analysed in skin biopsies. After 2 weeks of UVB exposure, MMP-1 was slightly increased in the placebo group ($p<0.03$) and decreased in the Seresis group ($p<0.044$). MMP-9 did not change significantly. The MED was increased in the Seresis-treated group, *i.e.* sunburn induction was reduced by the antioxidant mixture.

Recently, it was demonstrated [78] that long-term supplementation with antioxidant micronutrients was able to improve parameters related to skin structure. Thirteen volunteers per group received a daily supplement consisting of either (i) lycopene (3 mg), lutein (3 mg), β-carotene (4.8 mg), α-tocopherol (10 mg), and selenium (75 μg), or (ii) lycopene (6 mg), β-carotene (4.8 mg), α-tocopherol (10 mg), and selenium (75 μg), or (iii) placebo, for 12 weeks. Skin density and thickness were assessed by ultrasound measurement, and roughness, scaling, smoothness and wrinkling assessed by Surface Evaluation of Living Skin. Both supplement mixtures containing carotenoids, vitamin E and selenium increased skin density and thickness over the treatment period, and skin surface, including roughness and scaling, was significantly improved.

A recently published human study [79] demonstrated that oral supplementation with 10 mg/day lutein and 0.6 mg/day zeaxanthin in combination with a topical treatment (50 ppm lutein, 3 ppm zeaxanthin) for 12 weeks provided better photoprotection and skin hydration, and an increase in superficial skin lipids than did the individual treatments. The reduction in lipid peroxidation following oral supplementation alone was equal to that given by the combined treatment. Skin elasticity was improved significantly by the topical treatment, and to a lesser extent by the combined and oral treatments. These results also show that, in addition to a photoprotective action, carotenoids, in this case the xanthophylls lutein and zeaxanthin, are able to improve physiological cosmetic skin parameters.

Modern optical non-invasive methods were used to investigate the structures of furrows and wrinkles *in vivo* and to correlate them with the concentration of lycopene, analysed by

resonance Raman spectroscopy, in the forehead skin of 20 volunteers aged between 40 and 50 years [80]. In a first step, no significant correlation was found between the age of the volunteers and their skin roughness. In a second step, a significant correlation was obtained between the skin roughness and the lycopene concentration ($R = 0.843$, $p<0.01$). The indication from these findings is that higher levels of lycopene in the skin effectively lead to lower levels of skin roughness.

The results of these studies provide the first evidence to support the hypothesis that antioxidant mixtures containing carotenoids can reduce UV-induced molecular markers of premature photoaging in humans and also improve skin structure parameters. Complementary mixtures of low dose micronutrients constituting a synergistic antioxidant network are as effective as moderate to high-dose supplements of a single carotenoid.

5. Photoimmune modulation

UV-radiation has been shown consistently to induce a number of immunological changes to the immune system. Continuous alleviation of photoimmune suppression by protective dietary micronutrients is warranted in vulnerable populations, *i.e.* children and the elderly [81]. Whilst there is evidence from preclinical and clinical studies about general immune modulatory effects of carotenoids [82-84] (*Chapter 17*), few studies in humans and animals have addressed the protection against photoimmune suppression. β-Carotene supplements (30 mg/day) given to healthy young volunteers for four weeks protected against suppression of delayed type hypersensitivity (DHT) induced by UVA [85]. In the Eilath study [41], the same dose of 30 mg/day β-carotene over 10 weeks prevented the UV-exposure-induced loss of Langerhans cell density in the epidermis.

A diet enriched with 0.4% lutein and 0.04% zeaxanthin for 2 weeks decreased significantly the UVB-induced inflammatory oedematous cutaneous response and the hyper-proliferative rebound in female hairless Skh-1 mice [86].

Mice fed dietary lutein demonstrated significant inhibition of ear swelling induced by UVB radiation compared to controls on a standard laboratory diet. Suppression of contact hypersensitivity response by a lower, repeated dose of UVB radiation was also significantly inhibited by feeding lutein. When UVB radiation was given at a single dose of 10,000 J/m^2 to inhibit the induction of contact hypersensitivity at a distant, non-irradiated site, no effect of lutein was seen. Lutein accumulated in the skin of the mice following diet supplementation and was also shown to decrease UVR-induced ROS generation [87].

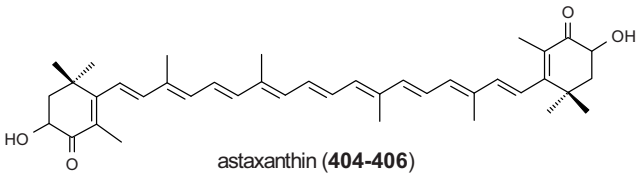
D. Mechanistic Aspects of Photoprotection by Carotenoids

UV radiation induces reactive oxygen species (ROS) including singlet oxygen, $^1O_2$ [1], which can damage lipids, proteins and DNA. The UVA wavelengths between 320 and 400 nm are considered the part of the light spectrum most likely to cause this oxidation.

Singlet oxygen, induced mainly by UVA, can regulate the expression level of a variety of genes involved in the cell cycle or apoptosis (see *Chapter 11*). Furthermore, genes involved in photoaging (such as MMPs, [88,89], haem oxygenase (HO)-1 [90], and intracellular adhesion molecule 1 [91]) have been reported to be regulated by UVA and/or $^1O_2$. Inhibition or moderation of these molecular events could confer photoprotection on target cells.

1. Inhibition of lipid peroxidation

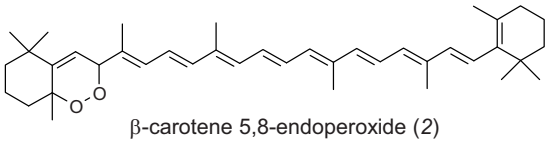
Several studies have used cultured human or other skin fibroblasts to examine the protective effects of carotenoids on UV-induced lipid peroxidation. β-Carotene prevented UVA-induced membrane damage of human skin fibroblasts [92]. Lycopene, β-carotene and lutein, applied in liposomes as the vehicle, decreased UVB-induced formation of thiobarbituric acid-reactive substances (TBARS, see *Chapter 12*) at 1 hour to levels 40-50% of those of controls free of carotenoids [45]. The amounts of carotenoid needed for optimal protection were 0.05, 0.40 and 0.30 nmol/mg protein for lycopene, β-carotene and lutein, respectively. Further increases of carotenoid content in cells beyond the optimum levels led to pro-oxidant effects. In another study, the depletion of catalase and superoxide dismutase (SOD) by UVA was restored, and TBARS reduced by culturing rat kidney fibroblasts with β-carotene or lutein (1 μM each), or with astaxanthin (**404-406**), which was reported to give superior protective activity at concentrations as low as 10 nM [93]. Cultivation of human skin fibroblasts and melanocytes with pure astaxanthin or an astaxanthin-containing algal extract prevented UVA-induced oxidative DNA damage, and restored also UVA-induced alterations in SOD activity and glutathione content [94].



astaxanthin (**404-406**)

In humans, a mixture of antioxidants consisting of lycopene (6 mg), β-carotene (6 mg), vitamin E (10 mg) and selenium (75 μg) per day for 7 weeks reduced lipid peroxide levels, and also improved parameters of the epidermal defence system against UV-induced damage such as sunburn cell formation and pigmentation [76].

Studies in mouse models confirm the prevention by carotenoids of oxidative stress induction by UV irradiation in skin [35]. Baseline TBARS were lower than in controls in hairless mice receiving β-carotene or palm fruit carotenoids (α-carotene 30%, β-carotene 60%, other carotenoids including lycopene 10%) at 0.005% dispersed as emulsions in drinking water. The palm fruit carotenoids accumulated in skin to a higher degree than β-carotene alone. UV irradiation-induced TBARS were decreased by palm fruit carotenes, but not by β-carotene, which may be explained by the differences in the bioavailability of the supplemented products. β-Carotene reduced the degree of lipid peroxidation in UVA-irradiated skin homogenates *ex vivo* from Balb/c mice, which had been supplemented for three weeks with 50 mg β-carotene/100 g diet [95]. β-Carotene 5,8-endoperoxide (*2*), a marker for the $^1O_2$ reaction, increased in the homogenates.



β-carotene 5,8-endoperoxide (*2*)

In healthy volunteers who had been supplemented with 15 mg β-carotene daily for 8 weeks, skin malondialdehyde concentrations after UVR (270-400 nm) were not reduced, whereas the effect of 400 mg vitamin E supplementation was significant [72]. No effects were observed on other indicators of oxidation. The lack of efficacy in this study may be explained by the low skin levels of β-carotene.

Overall, these studies *in vitro* and *in vivo* show that carotenoids can exert their protective antioxidant function when present at sufficiently high concentration in the skin cells.

2. Inhibition of UVA-induced expression of haem oxygenase 1

The human haem oxygenase 1 (HO-1) enzyme catalyses the first and rate-limiting step in haem degradation. The HO-1 gene is strongly activated within the first hours that follow UVA irradiation of normal human dermal fibroblasts and this response is being used as a marker of oxidative stress in cells. It has been shown that the induction of this gene occurs *via* $^1O_2$ produced on interaction of UVA radiation with an as yet undefined cellular chromophore. Carotenoids could be expected to suppress the UVA induced HO-1 gene activation in human cells. Unexpectedly, two studies with skin fibroblasts *in vitro* found an opposite effect. The first study applied β-carotene in cyclodextrins at levels of 0.5 and 5 μM [44]. A significant pro-oxidative effect and enhancement of UVA-induced HO-1 expression were observed. Combined application of β-carotene with vitamin E prevented the pro-oxidative effect, but did not exhibit a protective effect. In the second study, β-carotene or lycopene (0.5-1.0 μM) were prepared in nanoparticle formulations together with vitamin C and/or vitamin E. As in the

study above, either β-carotene or lycopene led to a further 1.5-fold rise in the UVA-induced HO-1 mRNA levels [46].

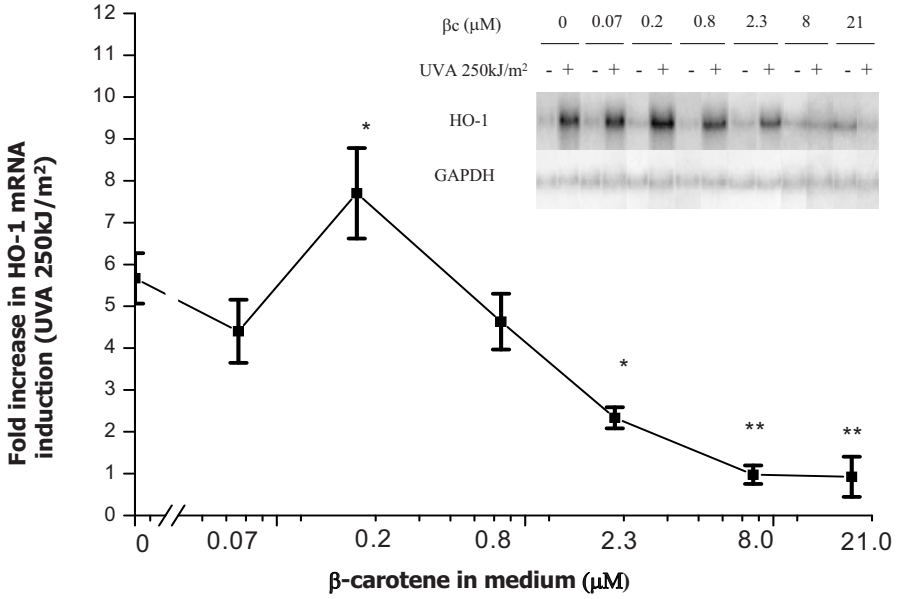


Fig. 5. Main graph: Modulation by β-carotene of UVA-induced haemoxygenase-1 (HO-1) mRNA accumulation [48]. Insert: The modulation, by 0.07, 0.2, 0.8, 2.3, 8.0 and 21 μM β-carotene (in THF), of UVA-induced HO-1 mRNA levels (UVA 250kJ/m^2) normalized over glyceraldehyde-3-phosphate dehydrogenase **(**GAPDH) mRNA in FEK4 skin fibroblasts, as measured by Northern Blot Analysis.

In another study (Fig. 5), the suppression of UVA-induced levels of HO-1 mRNA was measured after addition of a series of six β-carotene concentrations to the culture medium of FEK4 skin fibroblasts for three days, under the conditions described in Section **B**.2.a.

A concentration-dependent inhibition of UVA-induced transcriptional activation of HO-1 in exponentially growing FEK4 cells by β-carotene was observed, despite a UVA-induced increase of apocarotenals, indicators for oxidative degradation. Inhibition occurred at concentrations observed in human plasma after dietary supplementation with β-carotene.

These results also demonstrate, as mentioned earlier, the importance of culture conditions to avoid secondary influences *in vitro* that may cause altered responsiveness to UV and oxidative stress in cells.

3. Prevention of mitochondrial DNA deletions

Mutations of mitochondrial (mt) DNA have been reported to play a causative role in processes such as carcinogenesis, normal aging and premature photoaging of the skin [96-98].

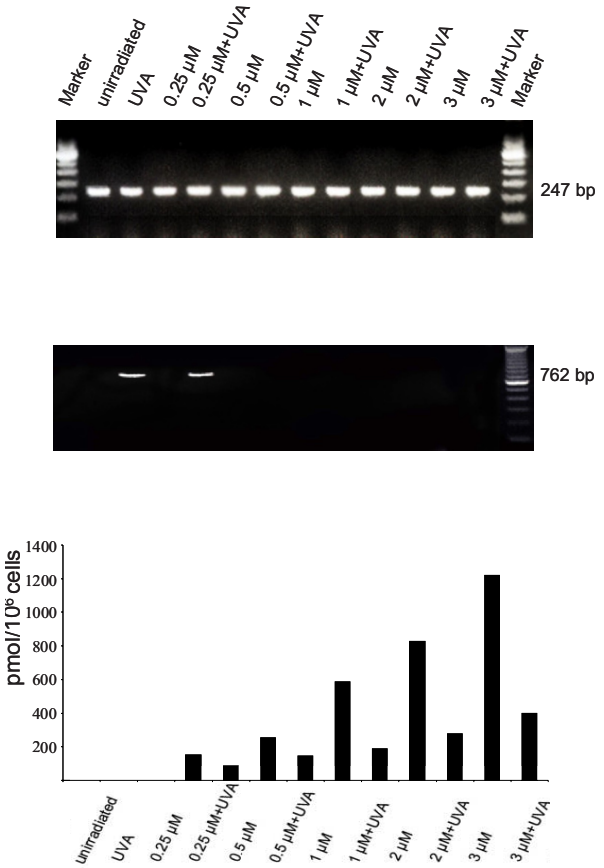


Fig. 6. Protective effect of β-carotene against photoaging-associated mtDNA deletion. Human dermal fibroblasts were repetitively exposed to UVA in the presence or absence of β-carotene at concentrations ranging from 0.25 to 3.0 µM, with HPLC assessment of β-carotene levels and PCR amplification of the common deletion and the reference fragment after each week of irradiation [96].
Top) Representative agarose gel of PCR amplifications of the reference fragment.
Middle) Representative agarose gel of PCR amplifications of the common deletion.
Bottom) Levels of β-carotene (pmol/10^6 cells).

Skin showing clinical signs of photoaging is characterized by an increase of mitochondrial mutations. The most frequent mutation of mtDNA is a 4977bp deletion, also called 'common deletion', which is considered to be a marker for alterations of the mt genome. Repetitive exposure of normal human fibroblasts to sublethal doses of UVA radiation leads to the induction of the common deletion and this is mediated in a singlet oxygen dependent fashion. The ability of β-carotene to protect normal human fibroblasts from the induction of photoaging-associated mtDNA deletions was investigated [99]. (all-*E*)-β-Carotene was tested at doses from 0.25 to 3.0 μM for uptake into cells as well as for its protective capacity. Assessment of cellular uptake of (all-*E*)-β-carotene, measured by HPLC, revealed a dose-dependent increase of intracellular concentration, as well as an increase in oxidative metabolites, *i.e*. apocarotenals and epoxides. UVA exposure led to a decrease of (all-*E*)-β-carotene, its *Z* isomers and oxidative metabolites. Assessment of mtDNA deletions by polymerase chain reaction (PCR) revealed reduced levels of mtDNA mutagenesis in cells incubated with β-carotene at concentrations of 0.5 μM and higher (Fig. 6). Taken together, these results indicate that β-carotene is taken up into the skin fibroblasts in a dose-dependent manner, interacts with UVA radiation in the cell and shows protective properties against the induction of a photoaging-associated mtDNA mutation.

4. Metalloprotease inhibition

Matrix metalloproteases (MMPs) are among the most important photoaging-associated genes induced by $^1O_2$. Investigation of the effect of carotenoids on suppression of UVA-induced MMPs is therefore of major relevance for establishing the protective effects of the carotenoids against photoaging.

In a detailed investigation, HaCaT keratinocytes were precultured with β-carotene at physiological concentrations (0.5, 1.5 and 3.0 μM) prior to UVA exposure from a Hönle solar simulator (270 kJ/m^2) [43]. The lifespan of $^1O_2$ was enhanced by irradiation in the presence of deuterium dioxide (D_2O). Expression levels of target genes such as MMP-1 were determined by TaqMan® Quantitative Real Time RT-PCR (Fig. 7).

β-Carotene suppressed the UVA-induction of MMP-1, MMP-3, and MMP-10, three major MMPs involved in photoaging (Fig. 7). Not only MMP-1, but also MMP-10 regulation was demonstrated to involve $^1O_2$-dependent mechanisms. β-Carotene quenched $^1O_2$-mediated induction of MMP-1 and MMP-10 dose-dependently with an approximately 50% reduction compared to cells treated with vehicle alone without β-carotene.

In contrast to this, in another study [46] an enhancement effect of β-carotene and lycopene on MMP-1 induction by UVA in fibroblasts was observed. As discussed above for HO-1, it is likely that the mode of β-carotene application is responsible for the differences in effects.

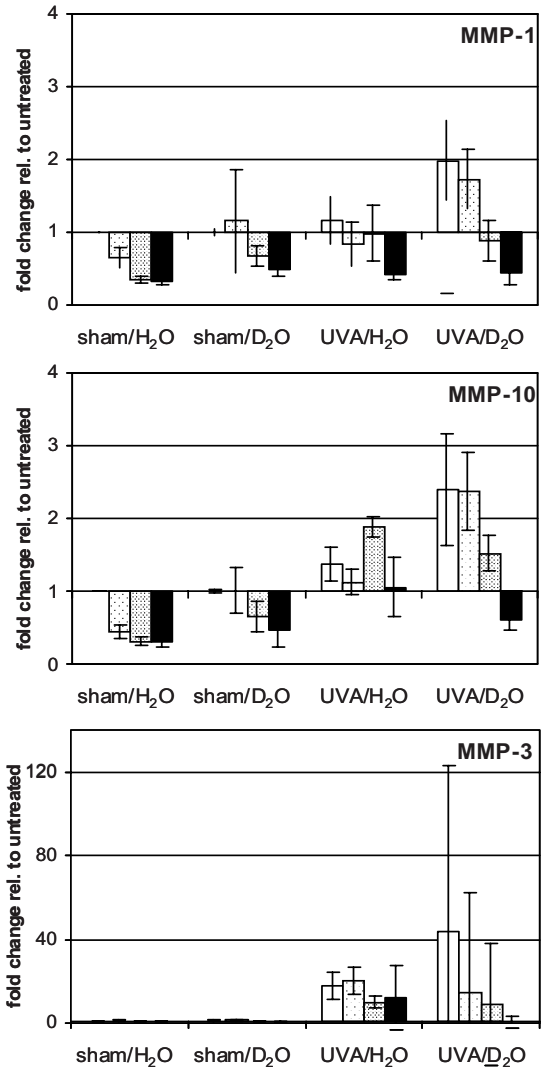


Fig. 7. Effect of β-carotene on ^2H_2O-enhanced UVA induction of (top) MMP-1, (middle) MMP-10, and (bottom) MMP-3. HaCaT cells were pretreated for 2 days with 0.5, 1.5 or 3.0 μM β-carotene. The cells were irradiated with UVA (270 kJ/m^2) in phosphate-buffered saline (PBS) made with ^2H_2O or H_2O, to analyse $^1O_2$ inducibility of genes. Gene expression 5 hours after UVA irradiation was analysd by TaqMan® Quantitative Real Time PCR. Values are geometric means ± standard error from three independent experiments [42].

5. Use of microarray analysis to profile gene expression

The introduction of modern molecular techniques and tools such as gene expression microarrays, proteomics and metabolomics (in nutrition research, termed 'nutrigenomics') created unique opportunities to identify the modes of action of nutritional compounds and study their influences on disease prevention beyond the commonly established functions. Microarrays allow genome-wide monitoring of gene expression in one step in small samples and their clustering to biological pathways. Some technical aspects of the methodology are summarized below.

RNA is extracted from treated cells or from control cells. Biotin-labelled probes are generated from these RNAs and incubated with microarrays. Microarrays carry oligonucleotides which recognize and bind probe molecules corresponding to a specific RNA ('riboprobes'). Riboprobes binding to their respective oligonucleotides are made visible by a fluorophore coupled to streptavidin. Nowadays, microarrays are available that can detect genes of selected pathways or which cover the entire (*ca*. 30 000 genes) of the genome. Gene activity is defined by the number of transcripts derived from a gene. In microarrays, this signal is converted to signal intensity of the fluorophore. Gene regulation by, for example, a carotenoid, is detected by comparing the signal intensity for a given RNA in treated *vs* control cells. Bioinformatics programs are used to analyse the vast amount of data, and translate the regulation of thousands of genes into biological meanings.

To analyse overall gene expression and identify specific processes influenced by β-carotene, Affymetrix® Gene Chip technology was applied in studies similar to those for MMPs (Section **D**.4) [100]. HaCaT cells were pre-cultured with β-carotene at physiological dose levels (0.5, 1.5. and 3.0 μmol/L) before exposure to UVA from a solar light lamp.

The results from Gene Chip hybridizations show that β-carotene altered UVA-induced changes in gene expression, in some cases reducing, in others enhancing the specific UVA effect. Downregulation of growth factor signalling, moderate induction of pro-inflammatory genes, upregulation of immediate early genes including apoptotic regulators, and suppression of cell cycle genes were hallmarks of the UVA effect. Of the 568 genes that were regulated by UVA, β-carotene reduced the UVA effect for 143, enhanced it for 180, and did not alter the UVA effect for 245 genes.

In unirradiated keratinocytes, gene regulations suggested that β-carotene reduced stress signals and extracellular matrix (ECM) degradation, and promoted keratinocyte differentiation. In UVA-irradiated cells, β-carotene inhibited those gene regulations by UVA that promote ECM degradation, suggesting a photoprotective effect of β-carotene. β-Carotene enhanced UVA-induced expression of tanning-associated protease-activated receptor 2, suggesting that β-carotene enhances tanning after UVA exposure. The combination of β-carotene-induced differentiation with the cellular 'UV response' led to a synergistic induction of cell cycle arrest and apoptosis by UVA and β-carotene. The different interaction modes imply that β-carotene/UVA interactions involve multiple mechanisms.

The 'transcriptomics' results, *i.e.* the expression profiles of retinoic acid target genes, confirmed the finding (Section **B**.2.b) that the retinoid-mediated effect of β-carotene in this cell system was minor, indicating that the β-carotene effects reported here were predominantly mediated through vitamin A-independent pathways.

A model of the interactions of β-carotene and UVA is shown in Fig. 8. It is proposed that β-carotene reduced the UVA-induction of genes involved in ECM degradation and inflammation by acting as a $^1O_2$ quencher. The mild photoprotective effect of β-carotene is suggested to be based on inhibition of these $^1O_2$-induced gene regulations, rather than on a physical filter effect, since the absorption maximum of β-carotene, *e.g.* 460 nm, lies outside the UVB/UVA range. β-Carotene, if scavenging ROS other than $^1O_2$, is irreversibly damaged and converted into radicals, if not rescued by other antioxidants. Thus, β-carotene did not inhibit UVA-induced stress signals, and enhanced some. UVA exposure suppressed several retinoic acid target genes. Since HaCaT cells produce marginal amounts of retinoid activity from β-carotene, the provitamin A activity of β-carotene did not translate into restored expression of RA target genes in this system.

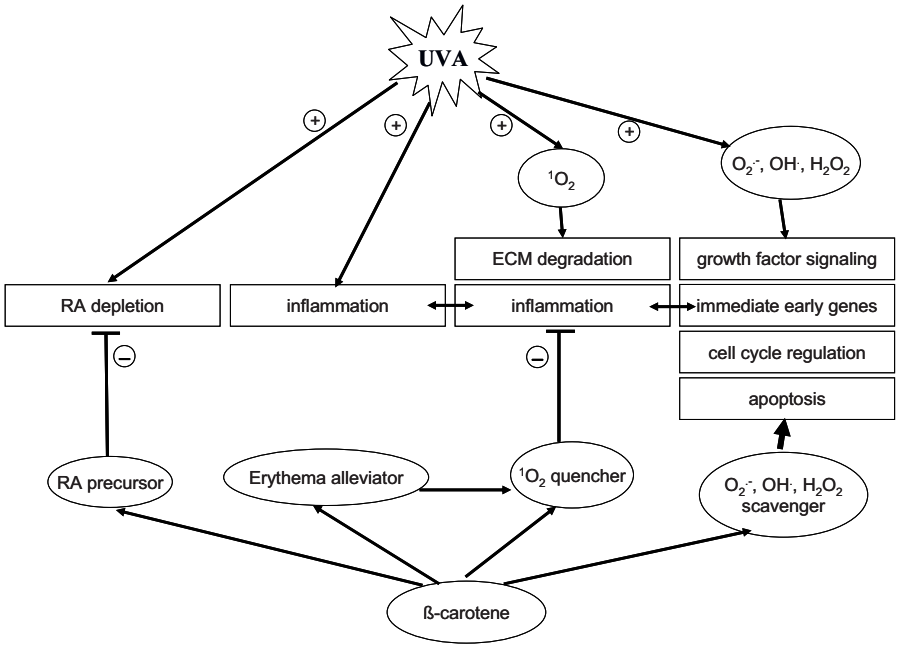


Fig. 8. Proposed relationship of the modes of action of β-carotene to its influence on UVA-induced biological processes. **+** indicates upregulation, **–** downregulation of processes.

E. Summary and Conclusion

Besides alleviation of symptoms in photosensitivity disorders by β-carotene, data obtained from human trials with carotenoids consistently show a moderate reduction in the development of sun-induced erythema. Some human studies also point to a possible beneficial effect of single carotenoids or of antioxidant compositions containing carotenoids in reducing the effects of premature skin aging. Chronic supplementation for more than 10 weeks is required to achieve these effects. The required doses, mainly established for β-carotene, lycopene and lutein, are between 10 and 20 mg/day, but can be lowered below 10 mg/day when the carotenoid is applied as part of an antioxidant composition containing mixed carotenoids and/or vitamins E, C and selenium. That the function of carotenoids in skin is strongly linked to their $^1O_2$ quenching properties is supported by studies *in vivo* and *in vitro*. Dietary intake of carotenoids can prevent the UV-induced losses in antioxidant defence systems and stores of skin retinol. Recent research elucidating the molecular modes of action shows that β-carotene can reduce up-regulation of UVA-induced pathways that are strongly involved in photoaging processes.

In conclusion, a considerable body of evidence, mostly from experiments with β-carotene, has emerged over the past 30-40 years on the benefits of carotenoids in photoprotection of human skin. Therefore, nutritional manipulation of carotenoid levels in skin, in conjunction with other antioxidants, has its importance as part of a concept of basic lifetime photoprotection to complement topical sun protection.

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