

# Enhancement of the UVA induction of haem oxygenase-1 expression by $\beta$ -carotene in human skin fibroblasts

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**Abstract**  $\beta$ -Carotene has often been discussed as a means to reduce the risk of skin photodamage. We studied the antioxidative potential of  $\beta$ -carotene in human skin fibroblasts exposed to ultraviolet A light. Surprisingly, we found a pro-oxidative effect of  $\beta$ -carotene. Using the induction of haem oxygenase-1 as a marker for oxidative stress, we found a strong enhancement of gene expression by  $\beta$ -carotene in ultraviolet A-irradiated cells. This effect was clearly suppressed by concomitant addition of vitamin E but only moderately by vitamin C. The results show that  $\beta$ -carotene has pro-oxidative properties in human skin fibroblasts exposed to ultraviolet-A light.

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**Key words:**  $\beta$ -Carotene; Vitamin E; Vitamin C; Hemoxygenase; Ultraviolet A; Oxidative stress

## 1. Introduction

Carotenoids have been extensively studied as chemopreventive agents that may reduce the risk of skin photodamage [1].  $\beta$ -Carotene supplementation was found to be a successful therapy against certain photosensitivity diseases, e.g. erythropoietic protoporphyria [2]. Regarding normal skin, several reports exist showing short-term photoprotective effects of  $\beta$ -carotene supplementation, e.g. decreased formation of erythema [3,4], whereas others show no beneficial effects [5–7]. The influence of  $\beta$ -carotene on photocarcinogenesis was reported to be preventive [8], but in contrast to these findings, some authors described ineffectiveness [9] or even exacerbation of tumour development by  $\beta$ -carotene [10]. As exposure to natural or artificial sunlight is steadily increasing as a result of changing leisure habits and environmental conditions, the intake of dietary  $\beta$ -carotene supplements to support the effect of sunscreens has been recommended [3].

Few data exist about how  $\beta$ -carotene acts in vitro in skin exposed to ultraviolet (UV) light. It has been supposed that  $\beta$ -carotene might act as an antioxidant [3] defeating UVA-generated cutaneous oxidative stress [11]. Although knowledge about the modulation of UV effects in skin cells by antioxidants is limited, cellular antioxidant defence mechanisms seem to be crucial to the prevention of oxidative UV skin damage [12]. An accepted marker for the onset of oxidative damage to skin is the expression of haem oxygenase-1 (HO-1) [13], which appears to be redox-regulated [14]. Haem oxygen-

ase is a microsomal enzyme which catalyses the rate-limiting step in haem catabolism leading to the formation of carbon monoxide, ferrous iron and biliverdin, which is subsequently converted to bilirubin. The HO-1 isoenzyme is ubiquitously expressed and is inducible by the substrate haem itself, as well as a variety of agents leading to the formation of reactive oxygen species, e.g. UVA light [14]. Induction of HO-1 by irradiation with UVA light seems to be involved in an adaptive cellular defense mechanism against oxidative damage through increasing cellular levels of antioxidant biliverdin and bilirubin, as well as inducing ferritin synthesis [15]. The effector species for UVA-mediated HO-1 up-regulation seems to be photochemically generated singlet oxygen [16], which causes damaging effects on biomolecules [17].  $\beta$ -Carotene is accepted to be an efficient singlet oxygen quencher [18]. However, the extent to which this property plays a role in protecting against skin photodamage is unknown.

In the present study we investigated the antioxidant potential of  $\beta$ -carotene in human skin fibroblasts exposed to UVA light in vitro. Surprisingly, we found, in contrast to the expected antioxidative effect, that  $\beta$ -carotene had a pro-oxidative effect. Using the induction of HO-1 as a marker for oxidative stress, we found a strong enhancement of HO-1 mRNA and protein induction by  $\beta$ -carotene in UVA-irradiated cells. This effect was clearly suppressed by concomitant addition of vitamin E, but only moderately by vitamin C.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated. Tissue culture media, serum and supplements were obtained from Biochrom (Berlin, Germany) unless otherwise indicated. Methyl- $\beta$ -cyclodextrin (M $\beta$ CD) was purchased from RBI (Natick, MA, USA), RRR- $\alpha$ -tocopherol from Hoffmann-La Roche (Basel, Switzerland) and L-ascorbic acid from Merck (Darmstadt, Germany).

### 2.2. Cell culture

HFP-1 normal human skin fibroblasts were derived from biopsy material of the lower abdomen and cultured as described previously [19]. For all experiments confluent, contact-inhibited cell populations were used at passages 9–18, corresponding to cumulative population doubling levels (CPDLs) 12–23.

### 2.3. Preparation of supplement solutions

Lipophilic supplements were prepared as water-soluble aqueous complexes of M $\beta$ CD in order to avoid organic solvents. All-*trans*  $\beta$ -carotene was prepared as a 1 mM M $\beta$ CD stock solution similar to the method described previously [19]. RRR- $\alpha$ -tocopherol was prepared as a 57 mM M $\beta$ CD stock solution by dissolving 1 g M $\beta$ CD in 2 ml bidistilled water, adding 250 mg RRR- $\alpha$ -tocopherol and mixing for 24 h under N<sub>2</sub> atmosphere. L-Ascorbic acid was prepared as a 0.1 M stock in phosphate-buffered saline (PBS) [20]. All supplement solutions were prepared with a minimum of light exposure, filtered sterile and stored at –80°C, except for the L-ascorbic acid stock solution,

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**Abbreviations:** HO-1, haem oxygenase-1; UVA, ultraviolet A; UVB, ultraviolet B; M $\beta$ CD, methyl- $\beta$ -cyclodextrin; p.i., post irradiation

which was prepared fresh. All-*trans*  $\beta$ -carotene and RRR- $\alpha$ -tocopherol stock solutions were tested for concentrations by spectrophotometric means. The purity of the RRR- $\alpha$ -tocopherol stock solution was determined by high-performance liquid chromatography (HPLC) according to a modification of the previously described method [21]. A 250 $\times$ 4 mm Grom-Sil 100 Cyano-2 PR column (Grom, Herrenberg, Germany) was used with a flow rate of 1.5 ml/min. The eluent was *n*-hexane:2-propanol (98.5:1.5). The purity of the all-*trans*  $\beta$ -carotene stock solution was determined by HPLC using a 250 $\times$ 4.6 mm YMC30 column (YMC, Kyoto, Japan). Eluent A was methanol:*tert*-butylmethylether:water (81:15:4), eluent B was methanol:*tert*-butylmethylether:water (6:90:4). A gradient was performed from 100% A to 0% A in 90 min with a flow rate of 1 ml/min.

#### 2.4. Treatment with supplements

Cells were seeded at a density of  $3 \times 10^5$  or  $1 \times 10^6$  cells per 21 cm<sup>2</sup> or 58 cm<sup>2</sup> tissue culture dish, respectively. Twenty-four hours after inoculation the medium was renewed and the cells were grown to 100% confluence for 1 week. On day 7 (i.e. 7 days before UV irradiation) media were replaced by fresh medium without phenol red as described previously [19], and cells were preincubated for 7 days with  $\beta$ -carotene (0.5 or 5.0  $\mu$ M), or vehicle alone.

If required, cells were concomitantly preincubated with  $\alpha$ -tocopherol (10 or 25  $\mu$ M) 4 days before UV irradiation, and/or with ascorbic acid (50 or 100  $\mu$ M) 90 min before UV irradiation. All cells received the same amounts of vehicle (final concentration below 0.2% M $\beta$ CD). On day 14 after inoculation cells were UV-irradiated or sham-irradiated.

#### 2.5. UV irradiation

Cells were irradiated with 20 J/cm<sup>2</sup> UVA light as described previously [19]. In brief, cells were washed (twice) and 21 cm<sup>2</sup> dishes were covered with 2 ml and 58 cm<sup>2</sup> dishes with 5 ml Hanks' balanced salt solution (HBSS). Irradiation was carried out on a heating platform at 37°C in a UV irradiation chamber (Dr Gröbel UV Elektronik, Ettlingen, Germany). The UVA light source (TLD15W/05 lamps, Philips, Hamburg, Germany) emitted radiation in the range of 300–460 nm with a maximum at 365 nm and with a fluence of 2.1 W UVA and <0.01 W UVB light, according to the manufacturer. The UV dose was measured with a UV-MAT dosimeter and cosine-corrected sensors RM-11UVA and RM-11UVB. After irradiation, cells were fed with the saved conditioned medium. Sham-irradiated cells were treated in the same manner except that they were not irradiated.

#### 2.6. Western blot analysis

Immediately on irradiation or 48 h p.i. cells were rinsed twice and harvested with ice-cold PBS. Cell pellets were lysed in 60 mM Tris, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, benzamide (1:5000) and protease inhibitors Pefabloc SC (2 mM; Bio-mol, Hamburg, Germany), leupeptin (1  $\mu$ M) and pepstatin A (1  $\mu$ M; Calbiochem, CA, USA). The protein content was determined in an aliquot after dilution (1:100) with the Bio-Rad protein assay (Bio-Rad, Munich, Germany) and bovine serum albumin (BSA) for calibration. 20  $\mu$ g of total cell lysate proteins were electrophoresed on a 12% SDS-polyacrylamide gel and then blotted onto PVDF membranes (Boehringer, Mannheim, Germany). The blots were blocked in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TTBS) and 3% BSA (w/v) at room temperature for 30 min. Blots were incubated with rabbit polyclonal anti-HO-1 antibody (dilution 1:20 000) for 1 h at room temperature in TTBS containing 1% BSA (w/v). After two washes in TTBS, blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (dilution 1:3000) for 30 min at room temperature. After three washes in TTBS and two washes in TBS the colour was developed using NBT/BCIP as the substrate.

#### 2.7. HO-1 cDNA probe

A 365 bp cDNA probe for HO-1 was generated by polymerase chain reaction (PCR). Oligonucleotide primers for HO-1 (forward, 5'-TTC TTC ACC TTC CCC AAC-3'; reverse, 5'-GCA TAA AGC CCT ACA GCA AC-3') were designed based on the complete coding sequence of the human HO-1 mRNA (accession number X06985).

Total RNA was extracted from human microvascular endothelial cells (HMEC-1) [22] and used for poly(A)<sup>+</sup> selection to isolate mRNA according to the manufacturer's instruction for the Oligotex mRNA-kit (Qiagen, Hilden, Germany). The mRNA was used to synthesise

cDNA by means of the SuperScript kit (Gibco-BRL, Eggenstein, Germany). For the PCR amplification a 50  $\mu$ l reaction contained 2  $\mu$ l ss cDNA solution from the reverse transcription step, 3 units of Vent DNA polymerase (Biolabs, Schwalbach, Germany), 1 mM of each of the four deoxynucleoside triphosphates, and 50 pmol/l of each HO-1 primer. After denaturing at 94.5°C for 5 min the thermocycling was carried out at 94.5°C for 1 min, 60°C for 2 min and 72°C for 3 min. Forty-five cycles were employed, followed by a final extension step at 72°C for 10 min. The specific amplified DNA fragment was cloned into the pZeroTM-1 vector (Invitrogen, Groningen, The Netherlands) and sequenced by 4base lab GmbH (Reutlingen, Germany). The nucleotide sequence of the cloned PCR product was found to be 100% identical to human HO-1 mRNA.

#### 2.8. RNA extraction and Northern blot analysis

Total RNA was isolated according to the manufacturer's instructions for the RNeasy Mini Kit (Qiagen, Hilden, Germany) and analysed by Northern blot hybridisation with specific cDNA probes for HO-1 and  $\beta$ -actin as described elsewhere [23,24]. 10  $\mu$ g of total RNA was denatured with 10% formaldehyde and 65% formamide, stained with 1.5 ng/lane ethidium bromide and separated through 1% agarose formaldehyde gels. Transfer of RNA to positively charged nylon membranes (Boehringer, Mannheim, Germany) was carried out by capillary blotting with 20 $\times$ SSC for 15–17 h. DNA probes were labeled with [<sup>32</sup>P]dCTP (Freiburg, Germany) by primer extension using random hexanucleotides (High Prime Labeling System, Boehringer, Mannheim, Germany). Filters were preincubated for 2 h at 68°C in a solution containing 0.25 M Na<sub>2</sub>HPO<sub>4</sub>, 1% BSA, 1 mM EDTA, and 7% SDS. The hybridisation was carried out for 16–20 h at 68°C in the same solution containing 2.5–4 ng/ml of labeled cDNA. After hybridisation filters were washed at the final stringency of 20 mM phosphate buffer, 1 mM EDTA, and 1% SDS at 68°C. Filters were exposed at –80°C for up to 24 h depending on the level of expression. mRNA transcripts were quantified by densitometric scanning of the blots with the Herolab E.A.S.Y enhanced analysis system (Herolab, Wiesloch, Germany). To correct for differences in gel loading, integrated optical densities were normalised to human  $\beta$ -actin, obtained on the same blot.

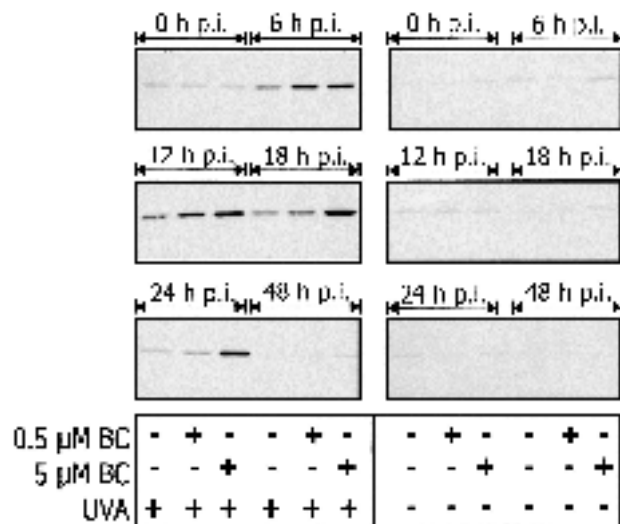


Fig. 1. Enhanced UVA induction of HO-1 protein by  $\beta$ -carotene. HFP-1 fibroblasts were preincubated with (+) or without (–)  $\beta$ -carotene (BC) as indicated and then irradiated with 20 J/cm<sup>2</sup> UVA light (+) or sham-irradiated (–). Cells were harvested at different time intervals p.i. and analysed for HO-1 protein levels by Western blotting. Blots shown are representative of three independent experiments.

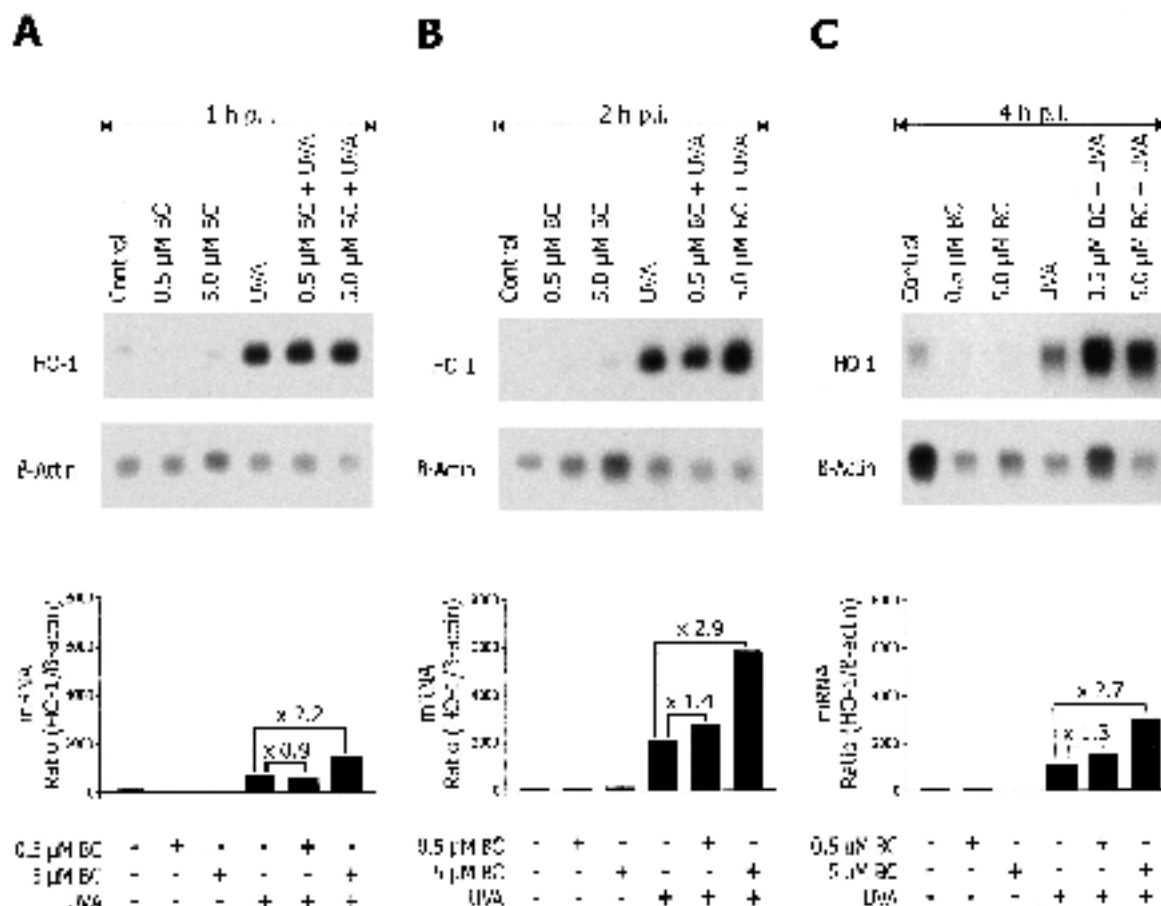


Fig. 2. Enhanced UVA induction of HO-1 mRNA by  $\beta$ -carotene. Cells were treated as described in Fig. 1. Total RNA was isolated 1 h p.i. (A), 2 h p.i. (B) or 4 h p.i. (C) and analysed for HO-1 mRNA levels by Northern blotting. Densitometric values obtained for the HO-1 mRNA transcripts were normalised to values for  $\beta$ -actin which were obtained from the same blot. Controls were set 100%. Data presented are the result of one experiment.

### 3. Results

#### 3.1. Enhanced UVA induction of HO-1 by $\beta$ -carotene

HFP-1 normal human skin fibroblasts were preincubated with 0.5 or 5.0  $\mu$ M  $\beta$ -carotene and irradiated with 20 J/cm<sup>2</sup> UVA light or sham-irradiated. The kinetics of HO-1 induction was analysed at the protein (Fig. 1) and mRNA (Fig. 2) levels.  $\beta$ -Carotene preincubation showed no influence on basal levels of HO-1 protein and mRNA in sham-irradiated cells. UVA irradiation clearly induced HO-1 expression at both levels. This is in keeping with previous reports [15,25]. Preincubation of HFP-1 with  $\beta$ -carotene strongly enhanced the UVA induction of HO-1 with effects dependent on both dose and time.

At the protein level (Fig. 1), UVA-irradiated cells showed a maximum HO-1 induction at 12 h p.i. and declined to basal levels at 24 h p.i. In contrast, cells preincubated with  $\beta$ -carotene before irradiation showed a maximum HO-1 protein induction 18 h p.i. and the up-regulation was prolonged until 24 h p.i. The  $\beta$ -carotene enhanced UVA induction of HO-1 was confirmed at the mRNA level (Fig. 2A–C). Preincubation with 0.5  $\mu$ M or 5.0  $\mu$ M  $\beta$ -carotene before UVA irradiation resulted in a 1.4- or 2.9-fold HO-1 mRNA up-regulation, respectively, within 2 h p.i., compared to cells not treated with  $\beta$ -carotene (Fig. 2B). These levels remained approximately constant until at least 6 h p.i. (data not shown).

#### 3.2. Modulation of $\beta$ -carotene-enhanced HO-1 induction by vitamins E and C

Ascorbic acid and  $\alpha$ -tocopherol were concomitantly added to  $\beta$ -carotene- or vehicle-preincubated fibroblasts. The cells were then irradiated with 20 J/cm<sup>2</sup> UVA light or sham-irradiated.

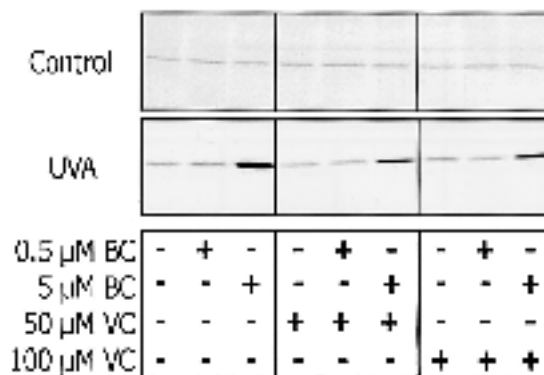


Fig. 3. Effect of ascorbic acid on  $\beta$ -carotene enhanced HO-1 protein induction. HFP-1 fibroblasts were preincubated with (+) or without (–)  $\beta$ -carotene (BC) and ascorbic acid (VC) as indicated. Then cells were irradiated with 20 J/cm<sup>2</sup> UVA light or sham-irradiated (control). Cells were harvested 12 h p.i. and analysed for HO-1 protein levels by Western blotting. Blots shown are representative of three independent experiments.

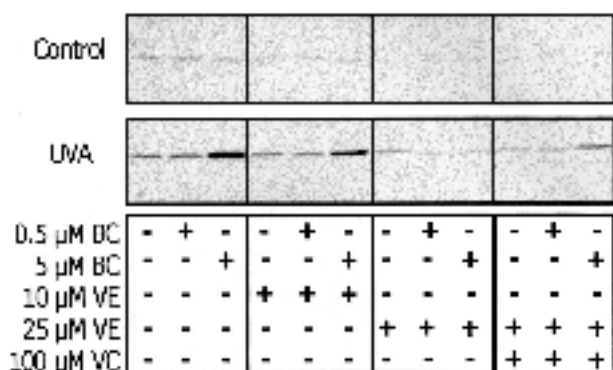


Fig. 4. Effect of  $\alpha$ -tocopherol on  $\beta$ -carotene-enhanced HO-1 protein induction. HFP-1 fibroblasts were preincubated with (+) or without (-)  $\beta$ -carotene (BC),  $\alpha$ -tocopherol (VE) and ascorbic acid (VC) as indicated. Then cells were irradiated with 20 J/cm<sup>2</sup> UVA light or sham-irradiated (control). Cells were harvested 12 h p.i. and analysed for HO-1 protein levels by Western blotting. Blots shown are representative of three independent experiments.

diated. HO-1 induction was analysed 12 h p.i. at the protein level. We found in sham-irradiated cells (Fig. 3) that 50  $\mu$ M or 100  $\mu$ M ascorbic acid had no influence on HO-1 protein basal levels with or without  $\beta$ -carotene preincubation. In UVA-irradiated cells ascorbic acid showed a moderate suppressing effect on HO-1 protein expression.

Incubation with 10  $\mu$ M or 25  $\mu$ M  $\alpha$ -tocopherol (Fig. 4) resulted in a moderate decrease of HO-1 protein basal levels in sham-irradiated cells, regardless of  $\beta$ -carotene preincubation. In contrast to the ascorbic acid treatment, in UVA-irradiated cells  $\alpha$ -tocopherol clearly suppressed HO-1 protein expression in a dose-dependent manner.

Cells treated with a combination of 25  $\mu$ M  $\alpha$ -tocopherol and 100  $\mu$ M ascorbic acid (Fig. 4) showed a moderate decrease of HO-1 protein basal levels with or without  $\beta$ -carotene preincubation. After UVA irradiation the HO-1 induction was clearly suppressed in cells treated with a combination of  $\alpha$ -tocopherol and ascorbic acid when preincubated with 0 or 0.5  $\mu$ M  $\beta$ -carotene whereas preincubation with 5.0  $\mu$ M  $\beta$ -carotene resulted in a diminished suppression of HO-1.

#### 4. Discussion

In the present study we investigated the effect of  $\beta$ -carotene on induction of the stress protein HO-1 in human skin fibroblasts exposed to UVA light. From the well-known antioxidative action of  $\beta$ -carotene [26,27] we expected a dose-dependent suppression of the UV-induced stress response but, surprisingly, we found a strong enhancement of HO-1 expression by  $\beta$ -carotene on mRNA and protein levels. This indicates a pro-oxidative potential of  $\beta$ -carotene in human skin fibroblasts exposed to UVA light. In order to further address the question whether the generation of reactive oxygen species by  $\beta$ -carotene that might result in lipid peroxidation was indeed responsible for the increase of HO-1 stress response, we concomitantly added antioxidant vitamins C and E. In our experiments vitamin C had no clear modulating effect on the  $\beta$ -carotene enhancement of HO-1 induction. In contrast, lipophilic vitamin E suppressed HO-1 induction dose-dependently to basal levels in  $\beta$ -carotene- and vehicle-preincubated cells.

These observations provide evidence that  $\beta$ -carotene does act as a pro-oxidant. To our knowledge this is the first report of a pro-oxidative action of  $\beta$ -carotene in skin cells exposed to UV light. Previous indications of pro-oxidative properties of  $\beta$ -carotene were described in vitro and in vivo under rather non-physiological conditions regarding oxygen tension,  $\beta$ -carotene concentration and interactions with other antioxidants [28]. In our system we simulated cutaneous UVA exposure using physiologically relevant doses of micronutrients and UVA radiation. For preincubation of cells with  $\beta$ -carotene 0.5  $\mu$ M or 5.0  $\mu$ M solutions were used, corresponding to human plasma levels without or with moderate oral  $\beta$ -carotene supplementation, respectively [29]. Irradiation was achieved with a full spectrum of suberythemal 20 J/cm<sup>2</sup> UVA light [30].

It might be questioned whether the observed pro-oxidative effect of  $\beta$ -carotene could be attributed to any oxidation or degradation products which could be inherent in a commercial  $\beta$ -carotene product. This is rather unlikely because via analysis of the used  $\beta$ -carotene and M $\beta$ CD-stock solution by HPLC no oxidation or degradation products of  $\beta$ -carotene were detected. The M $\beta$ CD-stock solution of  $\beta$ -carotene consisted of 96% all-*trans*  $\beta$ -carotene and the remaining 4% were identified as 15-*cis*, 13-*cis* and 9-*cis*  $\beta$ -carotene. Additionally, we carried out a liquid chromatography mass spectrometric (LC/MS) analysis (data not shown) which confirmed the results of the HPLC analysis. From the analytical methods applied, we conclude that the  $\beta$ -carotene used is of sufficient purity and the described pro-oxidative effect can be attributed to  $\beta$ -carotene, and mainly to all-*trans*  $\beta$ -carotene.

We assume that the pro-oxidative effect of  $\beta$ -carotene occurs mainly in the membrane region of the cell which might be concluded from the fact that carotenoids are detected predominantly in cell membranes [31]. This is supported by our findings that vitamin E, which is mainly located in the membrane, influences the action of  $\beta$ -carotene.

Concerning the discussed beneficial effect of  $\beta$ -carotene in skin exposed to sunlight, it has been reported that  $\beta$ -carotene acts only in part as an optical filter [32].  $\beta$ -Carotene is supposed to act mainly as quencher of activated oxygen species in the skin and this property was attributed to its photoprotective effects in vitro [20] and in vivo [3]. In contrast, instead of a suppression of the oxidative stress-derived HO-1 cascade, we identified an increase in cutaneous UVA stress response.

Why  $\beta$ -carotene has a pro-oxidative effect under UVA light exposure may only be hypothesised to date. UVA irradiation results in lipid peroxidation in human skin fibroblasts [19] and subsequent activation of the HO-1 cascade [33]. This is confirmed by our finding that vitamin E, a chain-breaking antioxidant [34], reduces HO-1 response. It can be assumed that after UVA exposure either  $\beta$ -carotene itself or any degradation products (apo-carotenals) enhance lipid peroxidation which again is abolished via the antioxidative action of vitamin E. This might explain why in our experiments vitamin E had a strong suppressing effect on HO-1 induction in contrast to vitamin C.  $\beta$ -Carotene itself seems unlikely to induce lipid peroxidation in the skin [35].

Interestingly, we found the same pro-oxidative effect for the non-provitamin A carotenoid lycopene (unpublished data). Consequently, the oxidising properties seem to be inherent in the carotenoid molecule itself or in any photodegradation products, regardless of provitamin A activity. Further inves-

tigations are necessary to find out precisely what happens to the  $\beta$ -carotene molecule under UVA exposure and to identify the effector species for the HO-1 induction in this respect.

A further possible sun-protective mechanism of action of  $\beta$ -carotene might result from partial metabolism into the vitamin A metabolite retinoic acid. It has been reported that retinoic acid prevents and repairs skin photodamage [36] and thus elicits anti-photoaging and anti-photocarcinogenic effects. As a result, vitamin A has recently been called 'anti-sunshine vitamin A' [37]. However, to our knowledge no data on retinoic acid formation from  $\beta$ -carotene in skin fibroblasts are yet available.

As regards our findings,  $\beta$ -carotene might combine beneficial and detrimental effects under light exposure to skin that could depend on the wavelength and on the indicator of photodamage investigated [38]. Such a paradox of a molecule causing both toxic and protective cellular effects has also been observed in other effectors, e.g. nitric oxide [39].

An accumulation of  $\beta$ -carotene in the human skin following oral supplementation or fortification of foods might lead to an imbalance in the cutaneous antioxidative system and therefore cause oxidative damage in UVA light-exposed skin. Our results emphasise that skin photoprotection seems to be complex and cannot be managed by the administration of  $\beta$ -carotene alone. We strongly recommend that dietary supplements of  $\beta$ -carotene should be taken only in combination with vitamin E, if at all.

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