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The effect of β-carotene on the expression of interleukin-6 and heme oxygenase-1 in UV-irradiated human skin fibroblasts in vitro

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Abstract β-Carotene is discussed as an anti-oxidant micronutrient and singlet oxygen quencher in human skin, protecting against UV light-induced damage. However, we recently demonstrated that β-carotene has a pro-oxidant potential in cultured human skin fibroblasts because it enhances the UVA induction of heme oxygenase-1 (HO-1). Herein, we further show that β-carotene also strongly promotes the UVA induction of pro-inflammatory interleukin-6 (IL-6) in skin fibroblasts in vitro. Singlet oxygen quencher sodium azide abrogated up-regulation of IL-6, and likewise also of HO-1. In UVB-irradiated cells, β-carotene did not modulate levels of IL-6 and HO-1. The observed effects might be relevant for UV-induced inflammatory processes.

Key words: β-Carotene; Interleukin-6; Heme oxygenase-1; UVA; UVB; Singlet oxygen

1. Introduction

Ultraviolet radiation, and mainly long-wavelength UVA light (320–400 nm), is a strong oxidant, and overexposure to sunlight provokes an inflammatory stress response in skin which acutely manifests as erythema ('sunburn') [1–3]. Anti-oxidant micronutrients such as β-carotene (BC) have been intensively studied for their potential in preventing skin photodamage [4–6]. BC was found to protect from erythema formation in humans [7–9], however, the mechanism how BC modulates molecular events in UV-induced inflammatory processes in skin is unclear.

In UV-irradiated skin, photochemical reactions lead to the formation of reactive oxygen species, and singlet molecular oxygen ($^1O_2$) is considered the biologically most relevant one [1]. BC is a scavenger of free radicals such as peroxyl radicals, superoxide anions and hydroxyl radicals and, in particular, it is well-known as a potent quencher of $^1O_2$ [10–12]. $^1O_2$ is a highly reactive non-radical form of molecular oxygen generated in type II photodynamic reactions with photosensitizers (e.g. porphyrins, flavins, quinones) [13], and it oxidizes a large number of biological molecules, causing damage to lipids, proteins, and DNA [14,15]. Besides, $^1O_2$ mediates the UVA-activation of a variety of genes which are involved in cellular stress response and signal transduction pathways, and it has been shown that $^1O_2$ promotes UVA-induced apoptosis, inflammatory processes as well as early events in photoaging [15,16].

Among the genes which are activated in dermal fibroblasts by UVA radiation, heme oxygenase-1 (HO-1; EC 1.14.99.3) is the most strongly induced one that has been observed [17]. HO-1 is the inducible form of three isozymes of heme oxygenase, a microsomal enzyme which catalyzes the rate-limiting step in heme catabolism [18]. Moreover, HO-1 is a sensitive marker for oxidative stress induced by the substrate heme itself, as well as a wide variety of cellular stressors including reactive oxygen species such as hydrogen peroxide, hydroxyl radical, nitric oxide, and $^1O_2$ [17,19]. In dermal fibroblasts, photochemically generated $^1O_2$ seems to be the main effector species for UVA-mediated HO-1 up-regulation [20].

Previously, we observed that BC strongly enhances the stress response in UVA-irradiated skin fibroblasts as determined by induction of HO-1 [21]. Consequently, we hypothesized that BC does not significantly act as a $^1O_2$ quencher in UVA-irradiated skin fibroblasts and that the observed pro-oxidant potential of BC might also enhance inflammatory responses to UVA-induced oxidative stress such as production of pro-inflammatory cytokines. Dermal fibroblasts are a major target of UVA radiation in skin [22] and, in response to UV exposure, they release a network of cytokines participating in the onset of cutaneous inflammation, among them interleukin-6 (IL-6) [23–25]. It has been shown that, via interrelated autocrine loops, photochemically generated $^1O_2$ induces in cultured skin fibroblasts production and release of IL-1 and IL-6, which results in an activation of matrix-metalloproteinases and thus tissue degradation [26]. Besides, IL-6 is a pleiotropic cytokine with numerous biological activities in skin and an important mediator of inflammatory and immunologic reactions [27]. The physiological role of IL-6 in skin and in particular in skin fibroblasts is only partly understood. However, production and release of IL-6 seems to play a crucial role in the pathogenesis of local and systemic sunburn reactions as well as photoaging of skin, tumor development and autoimmune diseases such as lupus erythematosus [27–29].

In this study we investigated the effect of BC on the expression of IL-6 in human skin fibroblasts following irradiation

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Abbreviations: IL-6, interleukin-6; HO-1, heme oxygenase-1; $^1O_2$, singlet molecular oxygen; p.i., post irradiation; BC, β-carotene; NaN_3, sodium azide; DIG, digoxigenin

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with suberythemal doses of UVA light in vitro. Furthermore, we studied the role of BC as a $^1O_2$ quencher in UVA-irradiated cells modulating IL-6 and HO-1 inductions with sodium azide (NaN_3), and we determined whether the observed effects of BC occurred only following irradiation with UVA or also UVB light. BC was used at physiologically relevant doses of 0.5 μM or 5 μM corresponding to human plasma levels without or with moderate oral BC supplementation, respectively [30].

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma (Deisenhofen, Germany) unless otherwise indicated. Tissue culture media, serum and supplements were obtained either from Biochrom (Berlin, Germany) or from Life Technologies (Paisley, UK). Commercial kits were used according to the manufacturers' protocols.

2.2. Cell culture

Normal human skin fibroblasts (HFP-1), which had been derived from biopsy material of the lower abdomen of a 3.5-year-old male donor, were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 648 μg/ml L-alanyl-L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin [31]. For experiments, cell populations were used between cumulative population doubling levels 12–25.

2.3. Cell treatment

Fibroblasts were treated with BC as previously described [21]. In brief, BC (type II; Sigma, Deisenhofen, Germany) was prepared as a water-soluble methyl-β-cyclodextrin formulation. Confluent fibroblast monolayers were given phenol red-free medium which was supplemented with 0.5 or 5 μM BC, or vehicle alone. All cells obtained the same amounts of vehicle with a non-toxic final concentration of methyl-β-cyclodextrin below 0.2% [32]. Treatment with BC was carried out for 7 days until UV irradiation.

NaN_3 (Fluka, Buchs, Switzerland) was prepared as a 1 M stock solution in Hanks' balanced salt solution (HBSS) (Gibco BRL, Paisley, Scotland). Prior to UV irradiation, cells were washed twice and then covered with 0–100 mM NaN_3 [20,33] in HBSS. Treatment with NaN_3 was carried out for 15 min and continued during the UV irradiation period [34].

2.4. UV irradiation

Cells were irradiated with UV light as described [21]. In brief, irradiation was carried out in a UV irradiation chamber (Dr. Groebel UV Elektronik, Ettlingen, Germany) at 37°C. For irradiation with UVA light (20 J/cm^2), a UVA light source (TLD15W/05 lamps, Philips, Hamburg, Germany) was used which emitted radiation in the range of 300–460 nm with a maximum at 365 nm and an irradiance of 3.3 mW/cm^2. For irradiation with UVB light (50 mJ/cm^2), the light source (F15T8UVB lamps, Philips) emitted radiation at wavelengths between 280 and 315 nm with an irradiance of 0.4 mW/cm^2 [31]. The UV chamber was equipped with a UV-MAT dosimeter and cosine-corrected sensors RM-11UVA and RM-11UVB. The time periods of UVA and UVB irradiation were 101 min and 2 min, respectively.

2.5. Western blot analysis

Cells were lysed in 10 mM Tris, pH 7.5, 0.9% (w/v) NP-40, 0.1% (w/v) SDS, 1 mM Pefabloc (Biomol, Hamburg, Germany), 1 μM Leupeptin, 1 μM Pepstatin A (Calbiochem, CA, USA), and 50 U/ml Benzonase (Merck, Darmstadt, Germany). Equal amounts of total protein (20 μg) were electrophoresed on a 12% SDS–polyacrylamide gel (Laemmli system) under reducing conditions and electroblotted. Immunodetections of IL-6 or HO-1 were carried out using a biotinylated goat polyclonal anti-hIL-6 IgG antibody (R&D Systems, Wiesbaden-Nordenstadt, Germany) or a rabbit polyclonal anti-HO-1 IgG antibody (Stressgen, Victoria, BC, Canada). As secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-biotin IgG (New England Biolabs, Schwalbach/Taunus, Germany) or HRP-conjugated goat anti-rabbit IgG (New England Biolabs) were used. Immunoreactions were visualized by means of a chemiluminescent detection system with LumiGlo/Peroxide as the substrates (New England Biolabs).

2.6. Preparation of cDNA probe

A 559 bp cDNA probe for IL-6 was prepared with polyA$^+$ mRNA from HFP-1 dermal fibroblasts. cDNA was synthesized by means of a SuperScript kit (Gibco BRL). Based on the complete coding sequences of the human mRNA for IL-6 (accession no. M54894), oligonucleotide primers for IL-6 (forward, 5′-CCA GTA CCC CCA GGA GAA GA-3′; reverse, 5′-CAT GCT ACA TTT GCC GAA GAG-3′) were designed and purchased from Interactiva (Ulm, Germany). PCR amplification was carried out in a PTC-200 DNA engine (MJ Research Inc., Watertown, MA, USA) with a 50 μl reaction mixture containing 2 μl cDNA, 3 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania), 1 mM of each relevant deoxynucleoside triphosphate (Amersham, Freiburg, Germany) and 50 pmol of each primer. Amplification was performed after incubation for 15 min at 72°C running 45 cycles (1 min at 94.5°C, 2 min at 60°C, 3 min at 72°C), followed by 10 min at 72°C. The amplified DNA fragment of IL-6 was cloned into the pCRII-TOPO vector (Invitrogen, Groningen, The Netherlands). Sequencing of plasmids was performed by 4base lab GmbH (Reutlingen, Germany) and the nucleotide sequence of the cloned PCR product was found to be 100% identical to human IL-6 mRNA. The specific DNA fragment of IL-6 was isolated from 40 μg vector using *Eco*RI (Fermentas).

For detection of β-actin, a 680 bp cDNA probe which had been cloned into the pZeroTM-1 vector (Invitrogen) was obtained as a kind gift from PD Dr. Jürgen Frank, Department of Biological Chemistry and Nutrition, University of Hohenheim (Stuttgart, Germany). The two probes for IL-6 and β-actin were labeled with digoxigenin (DIG) using a DIG-High Prime kit (Boehringer Mannheim, Mannheim, Germany).

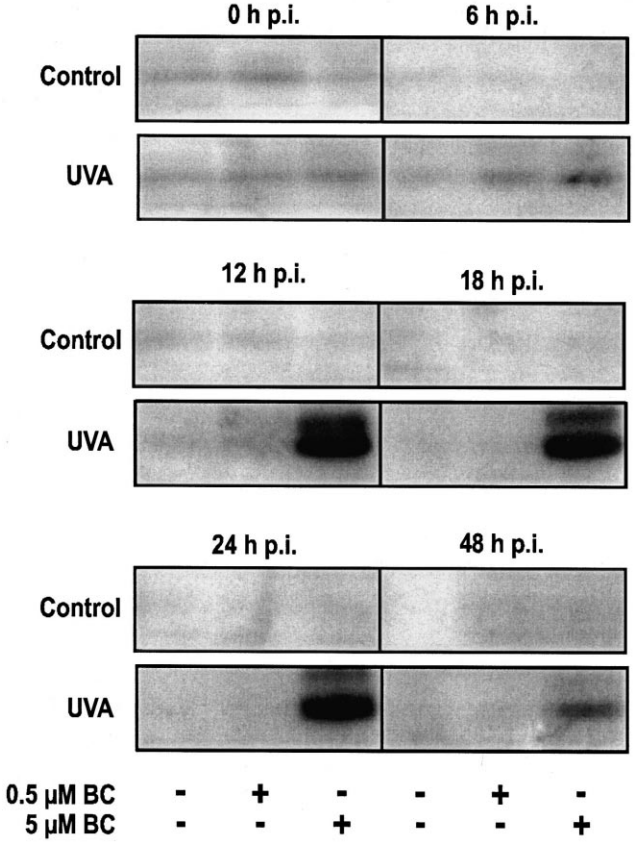

Fig. 1. BC stimulates UVA induction of IL-6 protein expression in HFP-1. Cells were treated with BC or vehicle alone as indicated and then irradiated with 20 J/cm^2 UVA light, or they were sham-irradiated (control). Cells were harvested at different time intervals p.i. and analyzed for protein levels of IL-6. Western blots shown are representatives of three independent experiments.

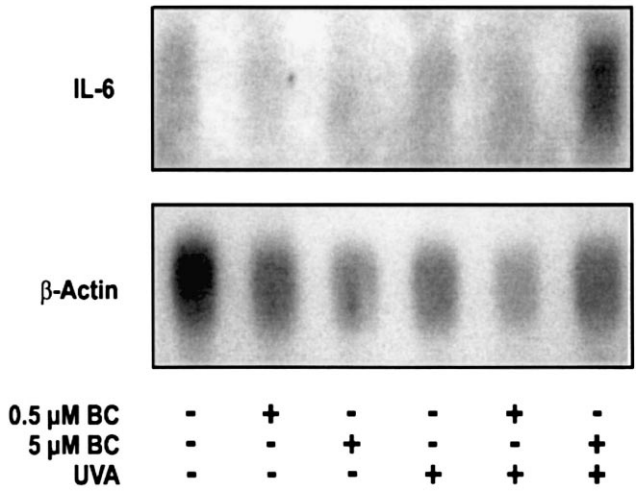

Fig. 2. BC stimulates UVA induction of IL-6 mRNA expression. HFP-1 skin fibroblasts were treated as described in Fig. 1. Total RNA was isolated at 2 h p.i. and mRNA levels were analyzed by Northern blotting using DIG-labeled cDNA probes for IL-6 and β-actin. Blots are representatives of two independent experiments.

2.7. Northern blot analysis

Total RNA was isolated by means of RNAWIZ™ reagent (Ambion, Austin, TX, USA). Northern blot hybridization was carried out as previously described [21] using 12 μg of total RNA. Hybridization of nylon membranes was performed with DIG-labeled cDNA. For chemiluminescent detection, a DIG-Luminescent Detection kit for Nucleic Acids (Boehringer Mannheim) was used with CSPD as the substrate.

3. Results and discussion

3.1. BC promotes UVA induction of IL-6

In irradiated cells, pre-incubation with 5 μM BC resulted in a strong and time-dependent up-regulation of IL-6 protein expression (Fig. 1). A significant increase in IL-6 expression was first measured 6 h post irradiation (p.i.) and peaked at 12 h p.i. In vehicle-treated control cells as well as in cells treated with a low dose of 0.5 μM BC, IL-6 protein expression was not significantly induced following UVA irradiation. In non-irradiated cells, a low constitutive protein expression of IL-6 could be detected which was not altered by BC treatment.

Similar effects of BC on UVA induction of IL-6 were found on the mRNA level (Fig. 2). Basal mRNA expression of IL-6 was not influenced by BC treatment in non-irradiated cells. In UVA-irradiated cells, IL-6 mRNA expression was not induced either in vehicle-treated control cells or in cells treated with 0.5 μM BC. However, treatment with 5 μM BC resulted in an induction of IL-6 mRNA expression at 2 h p.i.

These results demonstrate that BC modulates the expression of pro-inflammatory IL-6 in UVA-irradiated cells. Based on literature data, an increase of IL-6 expression in vehicle-treated control cells following UVA exposure on both protein and mRNA levels was expected in this study [23,24,26,35]. Differences to previous reports can be explained by other conditions of UVA exposure and time points investigated. In our system, UVA alone was not sufficient to obtain a measurable response. We observed induction of IL-6 by 20 J/cm^2 UVA light when cells were irradiated in the presence of deuterium oxide, which enhances the lifetime of the UVA effector species $^1O_2$ (data not shown). In conclusion, these data suggest that treatment with BC leads to an increased formation of reactive oxygen species in UVA-irradiated cells and it might be hypothesized that increased amounts of $^1O_2$ are generated.

3.2. NaN_3 attenuates the effect of BC on UVA induction of stress genes

In order to investigate the role of $^1O_2$ in the increase of IL-6 induction by BC, we used a for skin fibroblasts well-established system of NaN_3 treatment [26,36–38]. NaN_3 was used at non-toxic doses of 0–100 mM [34]. Induction of IL-6 was determined 12 h p.i. on the protein level (Fig. 3) and compared to UVA induction of HO-1. In sham-irradiated cells, NaN_3 had no influence on basal protein levels of both IL-6 and HO-1, neither in vehicle-treated control cells nor in BC-treated cells. In UVA-irradiated cells, NaN_3 substantially

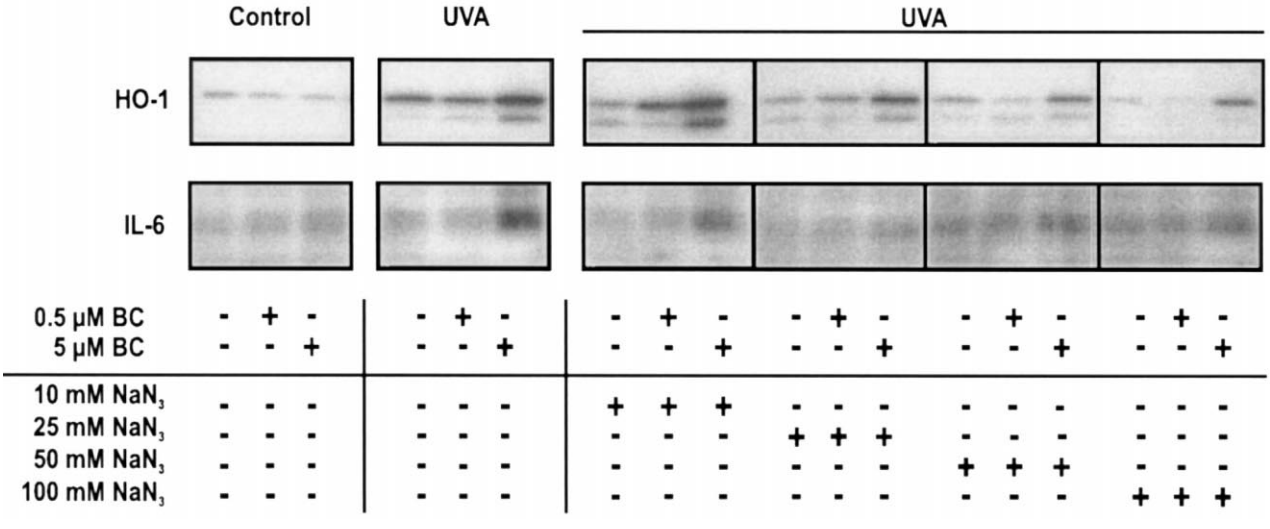

Fig. 3. NaN_3 reduces enhanced UVA induction of IL-6 and HO-1 protein expression. HFP-1 skin fibroblasts were treated with BC and/or NaN_3, or vehicles alone as indicated. Then cells were irradiated with 20 J/cm^2 UVA light, or were sham-irradiated (control). Cells were harvested at 12 h p.i. and analyzed for protein levels of IL-6 and HO-1. Western blots shown are representatives of three independent experiments.

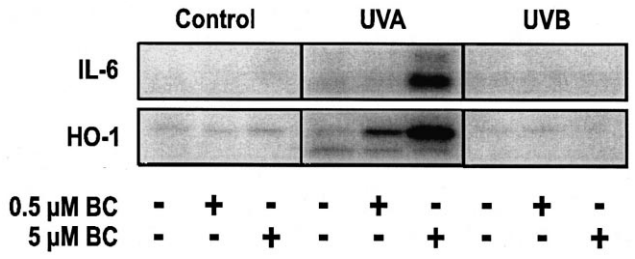

Fig. 4. BC does not enhance IL-6 and HO-1 expression in UVB-irradiated skin fibroblasts. Cells were treated with BC or vehicle alone as indicated and then irradiated with 20 J/cm^2 UVA or 50 mJ/cm^2 UVB light, or they were sham-irradiated (control). Cells were harvested at 12 h p.i. and analyzed for protein levels of IL-6 and HO-1. Western blots shown are representatives of three independent experiments.

decreased levels of IL-6 in vehicle-treated and BC-treated cells. Furthermore, our data show that UVA induction of stress genes which is stimulated by BC is not solely due to the action of $^1O_2$ but, at least in part, to other oxidants as NaN_3 was not able to entirely suppress IL-6 and HO-1 up-regulation in irradiated cells. And what is more, BC seems not to act as a $^1O_2$ quencher.

It is known that NaN_3 with equal efficiency scavenges not only $^1O_2$ but also hydroxyl radicals. However, Basu-Modak and Tyrrell [20] presented evidence that $^1O_2$ is the main effector of HO-1 induction, and that the hydroxyl radical, which is also generated by UVA light [39], has no significant effect on UVA-induced HO-1 induction [20]. Consequently, it seemed reasonable to use NaN_3 as specific $^1O_2$ quencher for the present study. A role of hydroxyl radicals in the UVA induction of IL-6 is not known and cannot be excluded. Preliminary studies using deuterium oxide to increase the lifetime of $^1O_2$ resulted in increased protein levels of IL-6 and HO-1 following UVA irradiation in vehicle-treated control cells and cells treated with 0.5 μM BC. In cells treated with 5 μM BC plus UVA light, the use of deuterium oxide caused cell death (data not shown). Further studies are necessary to clearly define the species involved in the UVA-induced stress response in BC-treated cells.

3.3. BC has no effect on IL-6 and HO-1 protein expressions in UVB-irradiated cells

Irradiation with UVB light did not result in a significant increase in protein levels of IL-6 and HO-1 (Fig. 4) neither with nor without pre-treatment with BC. Up-regulation of IL-6 and HO-1 in human skin fibroblasts by UVB light has been previously described by other authors. In literature, UVB light has been reported to be an important inducer of IL-6, but it has only weak effects on HO-1 [19,25].

UVA light is the main source of photo-oxidative stress in skin, however, UVB light also has an oxidative component [40]. Our data indicate that in UVB-irradiated cells, the presence of BC does not enhance generation of reactive oxygen species which could have resulted in an up-regulation of IL-6 or HO-1. Obviously, the described effects of BC on the induction of HO-1 and inflammatory IL-6 occur specifically when cells are irradiated with UVA light.

It should be noted that irradiation with UV light was carried out using light sources with irradiances comparable to the sunlight in a southern hemisphere summer [41]. Furthermore, absolute doses of UVA and UVB light were chosen which may not cause significant erythema in humans of the caucasian skin type [42], and which are considered non-toxic for cultured human skin fibroblasts [43]. The UVB dose was 400-fold smaller than the UVA dose, however, the same or similar doses are usually used to study UV-induced expression of IL-6 and HO-1 in skin cells [19,25,26,35,43]. This is due to the fact that humans are exposed to much less UVB radiation when compared to UVA as summer daylight comprises of approximately 5% UVB and 95% UVA light. Nonetheless, many biological effects of UV exposure are much greater at shorter wavelengths, and UVA light is estimated to contribute only around 20% to the detrimental effects of sun exposure [44].

At present, the role of BC in UV-light exposed skin cannot be defined clearly. As recently reviewed, human and experimental studies investigating various biological endpoints and using different model systems have resulted in a number of contradictory data [4]. However, it may be expected that BC exerts anti-oxidative and anti-inflammatory properties in human skin via quenching photochemically generated $^1O_2$, and thus provides photoprotection.

From the presented study we conclude that BC does not seem to act as an anti-oxidant and $^1O_2$ quencher under UVA exposure in vitro. In contrast, BC stimulates under cell culture conditions a stress response in UVA-irradiated skin fibroblasts, which involves HO-1 and the inflammatory cytokine IL-6. Further studies are necessary to clarify whether such an effect of BC also exists in animals and humans where BC acts as part of a complex anti-oxidant network [45]. If verified in vivo, a pro-inflammatory and pro-oxidative potential of BC might promote UV damage in skin.

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