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Carotenoids exclusively synthesized in red pepper (capsanthin and capsorubin) protect human dermal fibroblasts against UVB induced DNA damage

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Abstract

Photoprotection with dietary carotenoids has been mechanistically linked to their antioxidant properties, especially quenching of singlet molecular oxygen and scavenging of peroxy radicals. DNA-protection and antioxidant effects of selected carotenoids exclusively synthesized in red pepper (capsanthin and capsorubin) were studied in comparison to those of the xanthophyll, lutein. Preincubation of human dermal fibroblasts (hdf) with capsanthin and capsorubin significantly counteracted UVB induced cytotoxicity at doses between 0 and 300 mJ/cm². Also, preincubation of hdf with all carotenoids (1µM) significantly decreased the formation of DNA strand breaks following irradiation with UVB light. Caspase-3 cleavage (which was employed as a marker for apoptosis after UVB exposure) was decreased after preincubation with all carotenoids studied. However, caspase dependent PARP-1 cleavage was not affected. It is likely that the remaining caspase activity is sufficient to promote UVB-induced apoptosis. UV-irradiation causes several kinds of damage and triggers different pathways of cellular response and carotenoids apparently interfere selectively. In this context, capsanthin and capsorubin exhibit similar properties as lutein and may be used as substitutes or complementary compounds for photoprotection with dietary constituents.

Keywords

Fibroblasts, carotenoids, capsanthin, capsorubin, UVB light, DNA damage.

44 Introduction

45

46 Human skin is a complex tissue responsible for the performance of important
47 biological tasks. It provides a specific barrier between the organism and the
48 environment, protects the body against mechanical damage, penetration of toxic
49 substances, invasion by microorganisms, and is the primary line of defense against
50 radiation.¹

51

52 An optimal nutritional status is essential for the maintenance of a healthy skin and
53 fosters all its relevant functions. Macronutrients, vitamins and minerals are required in
54 a balanced supply but also secondary plant constituents contribute to skin tasks. Due
55 to their physicochemical and biological properties some micronutrients are part of the
56 dermal defense system against UV-irradiation.²

57

58 Living organisms are exposed to the UVA (320-400 nm) and UVB (290-320 nm) light
59 which is linked to inflammatory responses (sunburn), photoaging, and initiation as
60 well as progression of different types of skin cancer.³⁻⁵ All these pathological states
61 are originally caused by direct or indirect UV-induced damages to DNA, proteins or
62 lipids. Photochemical cyclization of DNA bases to pyrimidine dimers is a typical
63 example for direct damage. Upon UV-irradiation reaction sequences are initiated
64 which result in the generation of reactive intermediates including oxygen centered
65 radicals (peroxyl radicals), hydroperoxides, or excited state molecules (singlet
66 oxygen). All these species are capable of chemical modifications of biologically
67 important molecules (photooxidative damage) or interfere with cellular signaling.⁶ As
68 a consequence of DNA damage following acute UV radiation, cell cycle is arrested

69 for repair of damaged DNA. If DNA damage is severe and can not be properly
70 repaired, apoptosis pathways are activated to eliminate damaged cells.⁷

71

72 Photoprotection is provided by skin specific defense strategies like increasing
73 epidermal thickness or, stimulation of melanogenesis, and general defense systems
74 comprising the entire antioxidant network of enzymes and low molecular weight
75 antioxidants.¹ Dietary antioxidants complement and support the endogenous
76 photoprotection system.⁸ In this context carotenoids, polyphenols, vitamin E and
77 vitamin C contribute to antioxidant defense and thus foster endogenous
78 photoprotection.^{9,10}

79

80 Carotenoids are natural isoprenoid compounds frequently found in all kind of plants.
81 They are decorative pigments but more important is their role in the light harvesting
82 system where they extend energy yield and protect against photooxidative damage.
83 Due to their unique structure with an extended linear system of conjugated double
84 bonds, carotenoids are capable to quench singlet oxygen ($^1\text{O}_2$) and excited triplet
85 state molecules. In chemical reactions they also scavenge lipid peroxy radicals, the
86 superoxide anion, hydroxyl radicals or hydrogen peroxide.¹¹

87

88 Lutein, zeaxanthin, cryptoxanthin, lycopene, α - and β -carotene are the major
89 carotenoids in the Western diet and frequently found in human blood and tissues.
90^{12,13} The pattern is determined by the composition of the diet and represents the
91 consumption of different fruit and vegetables. Apart from the main carotenoids, other
92 yet less studied carotenoids may contribute to photoprotection among them are
93 capsanthin and capsorubin.^{14,15} Red pepper (*Capsicum annuum* L.) and its dietary
94 products contain a variety of carotenoids. Its pigment pattern includes seven

carotenoids of which capsanthin, epoxycapsanthin and capsorubin (Fig. 1) are exclusively synthesized in this plant.¹⁴ Red pepper carotenoids exhibit a higher antioxidant capacity than other xanthophylls which was attributed to structure-related properties, particularly the presence of the keto groups providing protection against autoxidation.^{15, 16}

The aim of the present study was to test for the first time in a cell model, the photoprotective effect of red pepper-specific oxocarotenoids (capsanthin and capsorubin) against UVB radiation induced DNA damage. In this work, was employed lutein as reference carotenoid due to the photoprotective effects demonstrated in previous reports.¹⁷

Materials and Methods

Chemicals

Paprika oleoresin was kindly provided by EVESA (La Línea de la Concepción, Cádiz, Spain). Lutein was obtained from BASF (Ludwigshafen, Germany). Low-melting-point agarose was purchased from USB Corp. (Cleveland, OH, USA). Other chemicals were obtained from Sigma (Deisenhofen, Germany) unless stated otherwise.

Isolation of Carotenoids

Procedures for the isolation of carotenoid pigments from paprika oleoresin have been already described in detail in previous publications.^{18,19} Briefly, the method comprised: separation, isolation and purification of capsanthin and capsorubin from paprika oleoresin by thin layer chromatography on silicagel 60GF₂₅₄ first using, hexane/ethyl acetate/ethanol/acetone (95:3:2:2) as developer. Bands containing

capsanthin and capsorubin esters were scraped off, extracted with diethyl ether and subsequently saponified with KOH in methanol (10%, w/v). Saponification was for 1 h, in order to obtain the free oxocarotenoid and eliminate remaining oily matters. Subsequently, 200 mL of aqueous NaCl solution (10%, w/v) was added. The organic phase was separated, and washed with distilled water until neutral pH was reached. It was further washed twice with 200 mL of aqueous Na₂SO₄ (2%, w/v), filtered through a solid bed of Na₂SO₄ and the solvent was evaporated in a vacuum rotary evaporator. The residue was dissolved with a defined volume of hexane. Spectrophotometric quantification of capsanthin in the solution was performed at $\lambda_{\max}$ = 470 nm using an extinction coefficient of $E^{1\%}_{1\text{cm}} = 2072$; capsorubin was measured at $\lambda_{\max} = 479$ nm ($E^{1\%}_{1\text{cm}} = 2200$).²⁰ Purity of stock solutions was checked by HPLC as described below.

Carotenoid quantification by HPLC

HPLC was performed on a reversed-phase column (ODS2-C18, 250x4.6 mm, 5 μ m particle size, Mediterranea sea, Teknokroma, Barcelona, Spain). The eluent comprised a binary gradient at a constant flow of 1.5 mL/min, and the detection wavelength was set to 450 nm. The initial composition of the eluent (acetone-H₂O (75:25, v/v) was held for 5 min. A linear gradient was then applied for 5 min to yield a final composition of acetone-H₂O of 95:5 (v/v). This composition is held for 7 min. Finally the column was washed for 3 min with acetone. For quantification the internal standard method was applied, using β -apo-8'-carotenal. Chromatographic separation and carotenoid quantification are described in detail.¹⁸

Cell culture

Human dermal fibroblasts CCD-1064Sk were from the American Type Culture Collection (CRL-2076; LGC Standards GmbH, Wesel, Germany) and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine (Glutamax; Invitrogen, Karlsruhe, Germany) and penicillin-streptomycin (100 U per mL for penicillin and 100 µg per mL for streptomycin; PAA Laboratories GmbH, Cölbe, Germany).

Cells were kept at 37 °C in a humidified atmosphere with 5% CO₂ and used at a confluence of 80-90%. Before the experiments, cells were cultured for 24 h in FBS-free DMEM. Stock solutions were prepared in tetrahydrofuran (THF) and immediately before using were checked (see Fig suppl. 1) and further diluted (1:1000) with FBS-free DMEM. Control cells were treated with 0.1 % THF alone.

Cell viability and UVB irradiation

Cell viability was determined with the sulforhodamine B assay (SRB).²¹ Cells were plated in 48-well plates containing 0.5 mL growth medium (DMEM 10% FBS) per well. Cells were pre-treated with test compounds (capsanthin, capsorubin or lutein, 1 µM) 24 h before UVB irradiation. Prior to the irradiation, cells were washed twice with phosphate saline buffer (PBS) and once with Hanks' balanced salt solution (HBSS). Irradiation was performed in HBSS using the BioSun irradiation system (Vilber Lourmat, France). The lamp intensity was of the average of 1.5 mW/cm². Post-incubation was performed in FBS-free DMEM for 24 h. Cells were fixed with trichloroacetic acid (10% v/v) and stained for 10 min with sulforhodamine B (0.4% w/v), absorption was measured at 490 nm and 620 nm. For background correction the difference of the absorption values at both wavelengths was calculated.²¹ Each experiment was repeated independently five times.

Alkaline comet assay

The alkaline comet assay (single-cell gel electrophoresis) was used to measure DNA single- and double-strand breaks together with alkali-labile sites within the cell. Cells were plated in 6-cm dishes and preincubated with carotenoids. To induce strand breaks, human dermal fibroblasts were exposed to 100, 200 or 300 mJ/cm² UVB light. Cells were harvested immediately after the irradiation, centrifuged, and suspended in 200 µL low-melting-point agarose and kept at 37 °C. The suspension was transferred to prepared microscope slides containing a layer of 10% agarose and then cooled for 4 min at 4°C. Coverslips were gently dropped off and the microscope slides were placed overnight at 4°C in a lysis buffer (2 M NaCl, 30 mM EDTA, 10 mM Tris, 0.2 M NaOH, 1% Triton X-100, 10% DMSO, pH 10) to lyse cells and enable DNA unfolding. Slides were washed with water and placed on a horizontal gel electrophoresis chamber, which was filled with high-pH electrophoresis buffer (300 Mm NaOH, 1 Mm EDTA, pH > 13) until the slides were covered. Slides were kept in the buffer for 25 min to denature DNA before electrophoresis. Electrophoresis was conducted for 25 min at 25 V and 300 mA (Bio-Rad, PowerPac HCA). After electrophoresis the slides were washed three times with neutralizing buffer (0.4 M Tris-HCl, pH 7.5). For final fixation, the slides were kept in ethanol (80%) for 5 min and then dried overnight.^{22, 23} Directly before fluorescence microscopy, cells were stained with Midori green (Nippon Genetics Europe, Dueren, Germany). DNA damage was evaluated by determining the percentage of DNA in the comet tail compared to the total amount of DNA. At least 30 stained comets were selected (ex. 470 nm, em. 527 nm, 250-fold magnification) and analyzed with CometScore.

Carotenoid uptake

Cellular uptake of the carotenoid was determined by HPLC. After 24 h of incubation with the carotenoids, cells were harvested, suspended in 4 mL of HBSS, and snap-frozen at -80 °C until further processing. After sonication, an aliquot of the sample was centrifuged and the protein content was determined in the supernatant (Bradford protein assay). For HPLC analysis, 0.5 mL of THF and 0.2 nmol of β -apo-8'-carotenal (internal standard) were added to 2.5 mL of the supernatant and sonicated for 5 min. Carotenoids were extracted with 3 mL of n-hexane, the organic layer was collected, and the solvent was evaporated under nitrogen.¹⁷ The residue was dissolved in 200 μ L of acetone and 50 μ L of the solution was injected for HPLC analysis as described above.

UVB induced cellular apoptosis

Caspases, a family of cysteine acid proteases, are central regulators of apoptosis. Initiator caspases (including 8, 9, 10 and 12) are closely coupled to proapoptotic signals. Once activated, these caspases cleave and activate downstream effector caspases (including 3, 6, and 7), which in turn cleave cytoskeletal and nuclear proteins.²⁴⁻²⁶

To study the effect of capsanthin, capsorubin and lutein on UVB-induced apoptosis, hdf were preincubated with these compounds for 24 h and then irradiated with UVB light (100 mJ/cm²). Post-incubation was in FBS-free DMEM for 6 h. Cell lysis, determination of protein, SDS-PAGE and Western blotting were according to²⁷. In this assay a cleaved caspase antibody sampler kit (Cell Signaling Technology, Inc) was employed. Caspase-3 executes apoptosis and is responsible for the proteolytic cleavage of many key proteins including PARP. For activation caspase-3 must be

proteolytically processed from the inactive zymogen into activated p17 and p12 fragments. For Western blot analysis of cell extracts a cleaved Caspase-3 (Asp175) antibody and cleaved PARP (Asp214) were applied. We evaluate the activation status of caspases by detecting their cleaved forms. GAPDH antibody (Millipore Merck, Darmstadt, Germany) as a loading control was used. Densitometric analysis of the blots was performed with ImageJ analysis software (Wayne Rasband, National Institute of Health, Bethesda, MD, <http://rsbweb.nih.gov/ij/>).

Statistical analysis

Values are expressed as the mean $\pm$ SD of five independent experiments and were analyzed by using one-way analysis of variance (ANOVA). p values < 0.05 were considered statistically significant.

Results

Cell viability and carotenoid uptake

The SRB assay provides a measure for cell viability and was applied to investigate cytotoxic effects of capsanthin, capsorubin, and lutein with and without UVB irradiation. Fig. 2 shows the results of the pre-treatment of hdf with the single compounds (1 μ M) irradiated with different doses of UVB-light. Compared to the nonirradiated solvent control (THF), none of the carotenoids was toxic without irradiation (Fig. 2, black bars). UVB irradiation alone decreased cell viability in a dose dependent manner. After irradiation with (100, 200 or 300) mJ/cm^2 , cell viability lowered to 67%, 57% and 47% of control, respectively. These data are in agreement with observations from previous studies in the same cell type.^{17, 28}

As a major result it could be shown that the preincubation of hdf with capsanthin or capsorubin (1 μ M) significantly counteracted the toxic effects of UVB irradiation at all doses analyzed (100, 200 or 300 mJ/cm^2) (Fig. 2). However, for lutein only a statistically significant photoprotection was observed at the lowest UVB dose (100 mJ/cm^2).

To determine cellular uptake of carotenoids, hdf were pretreated with capsanthin, capsorubin or lutein for 24 h and carotenoid levels were analyzed by HPLC with or without UVB irradiation (Fig. 3). The results shown that all carotenoids analyzed were absorbed. The order was the following: capsorubin > lutein > capsanthin. Statistically significant between carotenoids was detected (see fig. 3). Upon irradiation, capsanthin and lutein cellular levels are decreased in all doses (100 and 300 mJ/cm^2). Surprisingly, capsorubin absorption only was decreased after high irradiation UVB (300 mJ/cm^2).

Comet assay

In this study, the Comet assay was used to evaluate DNA damage caused by UVB irradiation. With the alkaline comet assay DNA single- and double-strand are detected.

DNA strand breaks are detected after UVB radiation (100 and 300 mJ/cm^2) (Fig. 4) which is in accordance with previous reports.²⁹ To examine antioxidant and photoprotective properties of carotenoids on the formation of strand breaks, cells were preincubated with single carotenoids (capsanthin, capsorubin or lutein, 1 μ M) and after irradiated with UVB (100 and 300 mJ/cm^2). Under these conditions, a

significant decrease in DNA strand breaks was detected. Further, the results demonstrate that protection provided by capsorubin is significantly higher than lutein (Fig. 4).

UVB induced apoptosis

To study the implication of apoptotic processes after UVB irradiation, the modification of two key proteins, PARP1 and caspase-3 cleavage were examined.

Caspase-3 cleavage

Western blot analyses for caspase-3 cleavage were performed with non-irradiated and UVB irradiated (100 mJ/cm²) (Fig. 5). For each experiment, one representative Western blot out five independent experiments is shown. Caspase-3 cleavage is corrected for protein loading (GAPDH). Experiments were performed with capsanthin, capsorubin or lutein (1 µM).

UVB irradiation induced caspase-3 cleavage in hdf (Fig. 5, line 2). Preincubation of hdf with capsanthin or lutein significantly counteracted this effect (Fig. 5, lines 4 and 8). However, no effect was observed after treatment with capsorubin (Fig. 5, line 6).

PARP cleavage

Western blot analyses for PARP cleavage were performed with non-irradiated and UVB irradiated (100 mJ/cm²). For each experiment, one representative Western blot out of five independent experiments is shown. PARP cleavage is corrected for protein loading (GAPDH). Experiments were performed with capsanthin, capsorubin and lutein (1 µM).

UVB irradiation induced PARP cleavage in hdf (Fig. 5 b, lane 2). In this assay no protective effect was observed after preincubation with capsanthin, capsorubin and lutein (Fig. 5 b, lines 4, 6 and 8).

Discussion

Carotenoids are a family of compounds of over 600 fat-soluble plant pigments. Fruits and vegetables are major sources of carotenoids.³⁰ Their key role is protection against photooxidative damage mechanistically linked to the antioxidant properties of these compounds.¹¹ Capsanthin and capsorubin, carotenoids exclusively synthesized in red pepper, exhibit a higher antioxidant capacity than other xanthophylls which was attributed to structure-related properties, particularly the presence of the keto groups providing protection against autooxidation.¹⁵ In this paper we test for the first time, the protective effect of red pepper-specific oxocarotenoids (capsanthin and capsorubin) against UVB radiation induced DNA damage.

Results obtained in cell viability indicated that compared to the un-irradiated control cells treated with solvent alone, none of the carotenoids analyzed (capsanthin, capsorubin or lutein) were toxic without irradiation. Both capsanthin as capsorubin significantly protected hdf against the toxic effects of UVB irradiation at all doses employed. However, lutein was inability of protecting at high UVB dose (Fig. 2).

All carotenoids analyzed were absorbed by cells. However, oxo-carotenoids show different taken up by hdf after UVB irradiation. At lower UV dose, capsanthin absorption was decreased to 25%. Surprisingly, capsorubin absorption only was

diminished after highest irradiation. Polar carotenoids influence membrane properties and stabilize structure of the lipid bilayer,³¹ and this effect is particularly related with the stereochemistry of polar groups located at both ends of the polyene chain. Thus, the terminal keto groups of capsorubin in addition to the hydroxyl groups of both κ rings ensure a close interaction with the polar zones of the cellular membrane and would facilitate the distribution of the pigment into the cellular membrane. However the asymmetric distribution of polar groups and sort of rings in the case of capsanthin may contribute in a different way to the properties of the cellular membrane of hdf. It is like the stereochemistry of capsanthin impairs its anchoring to the polar zones of the membrane, limiting the uptake of the pigment. In a lesser extent the same effect is observed for lutein, which although presents hydroxyl groups at both sides of the polyene chain, the terminal ϵ ring produces a different distribution and interaction with the lipid membrane of the cells.

Preincubation of hdf with capsanthin, capsorubin or lutein significantly decreased the formation of DNA strand breaks induced by irradiation with UVB light. Photoprotection provided by capsorubin was significantly higher than other carotenoids analyzed. The protective effect is probably due to their high antioxidant capacity.²⁷ As was mentioned before, differences observed between capsorubin and lutein in the assay may be attributed to structure.

Finally, only preincubation of hdf with capsanthin or lutein counteracted caspase-3 cleavage in hdf after UVB irradiation. In caspase dependent PARP-1 cleavage, neither protective effect was observed. It is likely that the remaining caspase activity is enough to promote UVB-induced apoptosis.³² UV-irradiation causes several kinds

of damage and triggers different pathways of cellular response and carotenoids apparently interfere selectively.

The results obtained in this paper highlight for first time DNA-protecting and antioxidant properties of carotenoids exclusively synthesized in red pepper as capsanthin and capsorubin. These compounds exhibit similar properties as lutein and could be used as substitutes or complementary compounds for photoprotection with dietary constituents.

Abbreviations

DMEM, Dulbecco's modified Eagle's medium.

FBS, heat inactivated fetal bovine serum.

HBSS, Hanks' balanced salt solution.

Hdf, human dermal fibroblasts.

PBS, phosphate saline buffer.

SRB, sulforhodamine B.

THF, tetrahydrofuran.

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Figure 1.- Chemical structures of capsanthin, capsorubin and lutein.

Figure 2.- Cell viability of hdf after treatment with UVB light, measured with the SRB assay ($n=5$). Viability is given as a % of non-irradiated control cells treated with solvent alone. Capsanthin, capsorubin, and lutein individually at same concentration ($1\text{ }\mu\text{M}$). $p < 0.05$ related to control (solvent alone) in each UVB dose.

Figure 3.- Carotenoid uptake by the cells. Human dermal fibroblasts were treated with capsanthin, capsorubin and lutein for 24 hours and after irradiated at different UVB doses. Carotenoid levels were measured by HPLC ($n=5$). ^a indicates a significant difference between capsanthin and lutein in each UVB dose $p<0.05$. ^b indicates a significant difference between capsorubin and lutein in each UVB dose, $p<0.05$, ($n=5$).

Figure 4.- Alkaline comet assay. Incubation with capsanthin, capsorubin and lutein ($1\text{ }\mu\text{M}$): strand breaks were induced by UVB irradiation (100 and 300 mJ/cm^2). ^a indicates a significant difference related to control in each UVB dose, $p<0.05$, ($n=5$). ^b indicates a significant difference between capsorubin and lutein in each UVB dose, $p<0.05$, ($n=5$).

Figure 5.- Effect of capsanthin, capsorubin and lutein on apoptotic proteins induced by UVB exposure. Cells were pretreated with capsanthin, capsorubin and lutein ($1\text{ }\mu\text{M}$) for 24 h prior UVB exposure. After 24 h, the cells were collected for apoptosis analysis. Total protein was extracted for examination of expression levels of cleaved caspase-3 (a) and cleaved PARP-1 (b). * indicates a significant difference related to control, $p<0.05$, ($n=5$).

Figure 1 suppl.- UV-Vis absorption spectra of capsanthin (a) and capsorubin (b).

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CELL VIABILITY

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capsanthin and capsorubin ($1 \mu\text{M}$) significantly counteracted the toxic effects of UVB irradiation at all doses analyzed.

CASPASE-3 CLEAVAGE

Preincubation of hdf with capsanthin significantly counteracted UVB irradiation induced caspase-3 cleavage.

COMET ASSAY

A significant decrease in DNA strand breaks was detected after preincubation with capsanthin and capsorubin.

CAROTENOID UPTAKE

Capsanthin and capsorubin were absorbed by cells. Capsorubin is the carotenoid most stable after irradiation UVB.

Capsanthin and capsorubin, carotenoids exclusively synthesized in red pepper, protect human dermal fibroblasts against oxidation generated by UVB.