RESEARCH ARTICLE

3,3′-Dihydroxyisorenieratene prevents UV-induced formation of reactive oxygen species and the release of protein-bound zinc ions in human skin fibroblasts

Kaya Lutter[1], Silke De Spirt[1], Sebastian Kock[2], Klaus-Dietrich Kröncke[1], Hans-Dieter Martin[2], Tanja Wagener[1] and Wilhelm Stahl[1]

[1] Institute of Biochemistry and Molecular Biology I, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany
[2] Institute of Organic Chemistry and Macromolecular Chemistry, Natural Compounds and Photoprotection, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany

3,3′-Dihydroxyisorenieratene (DHIR) is a structurally unusual carotenoid exhibiting bifunctional antioxidant properties. It is synthesized by *Brevibacterium linens*, used in dairy industry for the production of red smear cheeses. The compound protects cellular structures against photo-oxidative damage and inhibits the UV-dependent formation of thymidine dimers. Here we show that DHIR prevents a UV-induced intracellular release of zinc ions from proteins in human dermal fibroblasts. The effect is correlated with a decreased formation of intracellular reactive oxygen species. In contrast, zinc release from cellular proteins induced by hyperthermia is not affected by pretreatment of cells with the antioxidant DHIR. It is suggested that the intracellular zinc release upon UV irradiation is due to oxidative modifications of the zinc ligands in proteins (*e.g.* cysteine) and that protection by DHIR is due to intracellular scavenging of reactive oxygen species generated in photo-oxidation.

Received: January 30, 2009
Revised: April 16, 2009
Accepted: April 22, 2009

Keywords:
3,3′-Dihydroxyisorenieratene / Carotenoids / Photoprotection / UV / Zinc

1 Introduction

Carotenoids are natural colorants present in various fruits and vegetables. They efficiently scavenge reactive oxygen species (ROS) and their antioxidant activity is thought to be responsible for photoprotective effects attributed to this class of compounds. Human intervention studies with β-carotene, lycopene or carotenoid-rich products have shown that long-term intake is correlated with a decreased UV-sensitivity of the skin as determined by UV-evoked erythema [1, 2]. 3,3′-Dihydroxyisorenieratene (DHIR) is a carotenoid with an exceptional structure (Fig. 1), carrying phenolic end groups that affect the geometry and physicochemical properties of the compound [3]. It is biosynthesized from several bacteria including *Brevibacterium linens,* used in dairy industry for the production of various cheeses, such as Munster, Limburger, Brick, Tilsiter or Romadur [4, 5]. DHIR is a major pigment and relevant for the characteristic red-orange color covering the rind of such smear cheeses. Recently it has been shown that DHIR acts as a bifunctional antioxidant due to the presence of both a polyenic and a phenolic substructure [6]. As a radical scavenger the compound is superior to other dietary carotenoids such as lutein, zeaxanthin or astaxanthin.

Its singlet oxygen quenching activity is typical for carotenoids carrying nine conjugate double bonds in the polyene core. Studies in model systems and in cell culture revealed that DHIR prevents direct and indirect photo damage. In

Correspondence: Dr. Wilhelm Stahl, Institute of Biochemistry and Molecular Biology I, Heinrich-Heine-University Düsseldorf, P.O. Box 101007, D-40001 Düsseldorf, Germany
E-mail: wilhelm.stahl@uni-duesseldorf.de
Fax: +49-211-8113029

Abbreviations: DCF, 2′,7′-dichlorofluorescein; **DHIR,** 3,3′-Dihydroxyisorenieratene; **DPBS,** Dulbecco's PBS; **FBS,** fetal bovine serum; **HBSS,** Hank's buffered saline solution; **ROS,** reactive oxygen species; **UV,** ultraviolet

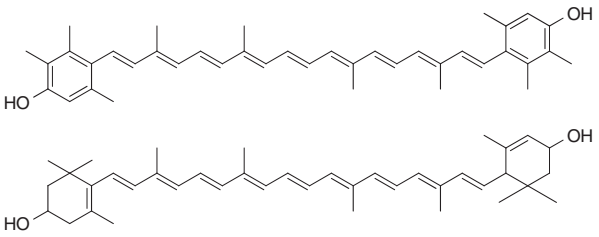

Figure 1. Chemical structures of Dihydroxyisorenieratene (top) and lutein (bottom).

the presence of DHIR, hemeoxygenase-1 expression as a measure for oxidative damage following UV exposure was diminished. The compound prevented the formation of thymidine dimers in human dermal fibroblasts irradiated with UV-B light. The latter effect has been attributed to UV-absorbing properties associated with the presence of aromatic substituents.

Zinc is an essential mineral required for numerous biological processes including immune function, protein synthesis, wound healing and cell growth. Among other tissues the skin is directly affected by zinc deficiency. Major dietary sources of zinc are meat, poultry and seafood [7]. Numerous zinc proteins have been characterized up till now and it has been proposed that about 2800 human proteins are potentially zinc-binding [8]. Among them are the so-called zinc-finger proteins that represent a specific type of transcription factors involved in the regulation of gene expression probably also acting as redox-sensitive molecular switches controlling several crucial cellular processes [9]. Complexation of the ion in zinc proteins is most common with either four cysteine residues (cys_4) or two cysteines and two histidines (cys_2his_2) as ligands. Intracellular Zn^{2+} levels are efficiently controlled and are in the range of about 200 μM. Under physiological conditions, almost all of the intracellular zinc is protein bound and only very minor amounts occur as ''free'' zinc. However, zinc homeostasis is disturbed under pro-oxidative conditions and the level of ''free'' zinc increases in situations of oxidative or nitrosative stress [10]. It has been suggested that modifications of zinc-complexing cysteine residues results in the ejection of zinc accompanied by a loss of protein function. However, tightly controlled fluctuation of ''free'' zinc in the picomolar range triggers cellular signaling pathways [11]. Recently, we demonstrated that UV-A irradiation of cells with sublethal doses induces a transient cytoplasmic and nuclear increase of intracellular ''free'' zinc ions [12]. UV irradiation results in photo-oxidative and phototoxic reactions and UV-dependent destruction of zinc-proteins or disturbance of the intracellular zinc homeostasis may be important in this context.

In the present study, we investigated whether photoprotective carotenoids are able to protect cells from UV-dependent intracellular zinc release.

2 Materials and methods

2.1 Chemicals

DHIR and lutein were provided by Hansgeorg Ernst (BASF, Ludwigshafen, Germany). Other chemicals were obtained from Sigma (Deisenhofen, Germany).

2.2 Cell culture and irradiation with UV-A light

Human dermal fibroblasts CCD-1064Sk were from ATCC (CRL-2076) and grown in DMEM without phenol red, supplemented with 10% v/v heat inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine (Glutamax, Invitrogen, Karlsruhe, Germany) and penicillin/streptomycin (20 mg/mL).

Cells were kept at 37°C in a humidified atmosphere containing 5% CO_2 and used at a confluence of 80–90%. For the experiments, medium was removed and fresh medium without FBS was added. Cells were incubated with DHIR and lutein (10 μmol/L medium) for 24 h. Carotenoid stock solutions (10 mmol/L) prepared in THF were diluted 1:1000 with DMEM (0% FBS); solvent controls were with THF only.

Before irradiation, cells were washed twice with PBS, once with Hank's buffered saline solution (HBSS) and then covered with HBSS. Irradiation was performed with various doses of UV-A light (0–20 J/cm^2). UV source was a dose and temperature controlled irradiation system suitable for culture dishes (BioSun, Vilbert Lourmat, France). Lamp intensity was approximately 4.5 mW/cm^2. Thermal stress (hyperthermia) was induced by incubating the cells for 30 min at 43°C.

Cytotoxicity was determined with the Sulforhodamine B-assay [13]. Cells were grown in 24-well plates and treated with DHIR or lutein. After irradiation, HBSS was replaced by fresh DMEM and cells were postincubated for 24 h. Absorption was registered photometrically and values at 620 nm subtracted from those at 490 nm; evaluation was in comparison to the control. Within each experiment a set of four measurements was performed at each concentration; each experiment was repeated independently three times.

2.3 Analyses of DHIR and lutein

After 24 h of incubation with DHIR or lutein, cells were harvested, suspended in 4 mL HBSS and snap frozen at −80°C until further processing. For analyses, cells were destroyed by sonication. An aliquot of the obtained suspension was centrifuged and proteins were determined in the supernatant with the Bradford protein assay. For HPLC, 0.5 mL of THF and 0.2 nmol of β-apo 8′-carotenale (internal standard) were added to 2.5 mL of the suspension

and sonicated. Carotenoids were extracted with 3 mL of n-hexane, sonicated for 5 min and vortexed. About 2 mL of the organic layer was withdrawn and the solvent was evaporated under a gentle stream of nitrogen. The residue was dissolved in 200 μL of mobile phase and 50 μL were injected for HPLC analyses. The HPLC system consisted of a Merck-Hitachi L-7100 pump connected with a Merck-Hitachi UV/Vis detector and a data registration system. Analyses was performed isocratically with a mobile phase composed of 52% methanol, 42% ACN, 4% water and 2% v/v/v/v propanol at a flow rate of 1 mL/min and a reversed phase column (pKb-100, 250 × 4.6 mm, Supelco, Bellefonte, PA, USA) protected by a guard column (4.6 × 4.6 mm) with the same stationary phase. Detection wavelength was 450 nm. Carotenoid levels were calculated from a calibration curve and normalized for protein content.

2.4 Determination of intracellular ROS formation

Formation of ROS was determined with 2′,7′-dichlorodihydrofluorescein diacetate as a probe [14]. The compound is absorbed by the cells and enzymatically cleaved to yield membrane-unpermeable 2′,7′-dichlorodihydrofluorescein, which is oxidized to the fluorescent 2′,7′-dichlorofluorescein (DCF). Fluorescence intensity is correlated with ROS generation.

For the evaluation of ROS generation by fluorescence microscopy, cells were grown on coverslip-bottomed 30 mm Petri dishes and treated as described in Section 2.2. Immediately after irradiation, fresh HBSS containing 1 μM 2′,7′-dichlorodihydrofluorescein diacetate was added and cells were incubated for 1 h at 37°C in the dark. Adjacent coverslips were fixed in an Attafluor cell chamber (Invitrogen) and covered with 0.5 mL HBSS.

Images were taken with a fluorescence microscope (Axiovert 100 TV, Zeiss, Oberkochen, Germany). Excitation wavelength was 485 nm and emission wavelength 520 nm.

Quantitative evaluation of DCF generation was performed in a separate set of experiments. Cells were irradiated in HBSS on 24-well plates and directly after irradiation medium was replaced with HBSS containing 100 μmol/L 2′,7′-dichlorodihydrofluorescein diacetate. Plates were immediately placed into the spectrometer (FluoStar Optima-Spectrometer, BMG, Offenburg, Germany) at 37°C and DCF formation was followed for 60 min at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

2.5 Determination of intracellular ''free'' zinc ions

Zinquin ethyl ester was used as a probe for intracellular ''free'' Zn^{2+} [15]. The lipophilic, zinc-sensitive fluorophore penetrates cell membranes and is retained in living cells after cleavage of the ethyl ester by cytosolic esterases. Human dermal fibroblasts were grown on coverslip-bottomed 30 mm Petri dishes to a confluence of 80–90% and treated as described in Section 2.2. Directly after treatment, cells were washed once with Dulbecco's PBS (DPBS) and cultured with 10 μmol/L Zinquin ethyl ester in DPBS for 20 min at 37°C in the dark. Subsequently, cells were washed once with DPBS, glass slides were fixed in Attafluor cell chambers and covered with 0.5 mL DPBS. Formation of fluorescent Zinquin–Zn^{2+} complexes was imaged with a fluorescence microscope using an excitation wavelength of 364 nm and an emission wavelength of 485 nm.

3 Results

3.1 Cell viability and carotenoid uptake

The sulforhodamine B-assay provides a measure for cell viability and was applied to investigate cytotoxic effects of UV-A irradiation in the presence or absence of DHIR and lutein (Table 1). Compared with the unirradiated solvent control (THF) none of the compounds was toxic without irradiation or at UV doses of 5 or 10 J/cm^2. Exposure of cells to 20 J/cm^2 decreased cell viability by about 25%. The cytotoxic effect at the highest UV dose was even more pronounced in the presence of lutein and DHIR and is likely due to pro-oxidative activities of the carotenoids.

Fibroblasts were incubated for 24 h with the carotenoids (10 μM in DMEM) and cellular uptake of DHIR and lutein was determined by HPLC (Table 2). The level of carotenoids in the cells is given as percentage of the total amount present at the start of the experiment. In unirradiated cells, about 45% of the carotenoids were accumulated. Less of the parent compounds was found in the cells irradiated with UV-A light; however, the amount of DHIR was higher than that of lutein. At 20 J/cm^2 only 10% of lutein was recovered in the cells whereas more than 25% of DHIR was found in fibroblasts.

Table 1. Cell viability of human skin fibroblasts 24 h after irradiation with UV-A light given as percentage of unirradiated control

UV-A dose (J/cm^2)	Substance		
	Control (THF)	Lutein	DHIR
0	100	107 ± 7	113 ± 5
5	99 ± 10	104 ± 13	107 ± 5
10	95 ± 5	100 ± 7	105 ± 5
20	76 ± 11	59 ± 10	56 ± 5

Cells were preincubated with 10 μM carotenoid.

Table 2. Carotenoid levels in cells under UV-A exposure given as percentage of total carotenoids present in the incubation mixture

UV-A dose (J/cm^2)	Substance	
	Lutein	DHIR
0	45.3±2.9	47.1±3.9
10	15.4±2.1	29.3±7.2
20	10.0±2.2	25.7±7.8

3.2 UV-A-dependent generation of ROS

The fluorescent dye DCF is formed by the oxidation of 2′,7′-dichlorodihydrofluorescein and this reaction was used to assess UV-A-dependent generation of ROS in fibroblasts. Fluorescence microscopy (Fig. 2) showed that only small amounts of DCF are generated in cells that were not exposed to UV light either in the absence or presence of lutein and DHIR (upper row).

Table 3. Effects of DHIR and lutein on ROS formation in human skin fibroblasts 1 h after irradiation with UV-A light

UV-A dose (J/cm^2)	Substance		
	Control (THF)	Lutein	DHIR
0	1540±83	1379±28	1337±44
5	1989±39	2058±73	1688±37
10	2321±205	2368±97	1947±144
20	2629±87	2846±114	2182±94
(−) Hyperthermia	1570±58	1411±166	1488±78
(+) Hyperthermia	1618±153	1533±143	1583±111

Fluorescence intensity of DCF (relative fluorescence units) is correlated with ROS generation.

With increasing doses of UV-A, cellular DCF levels were elevated (left lane) indicating photo-oxidation. Preincubation of the cells with 10 μM lutein did not affect ROS-dependent DCF formation (middle lane) whereas it was decreased in cells preloaded with DHIR (right lane).

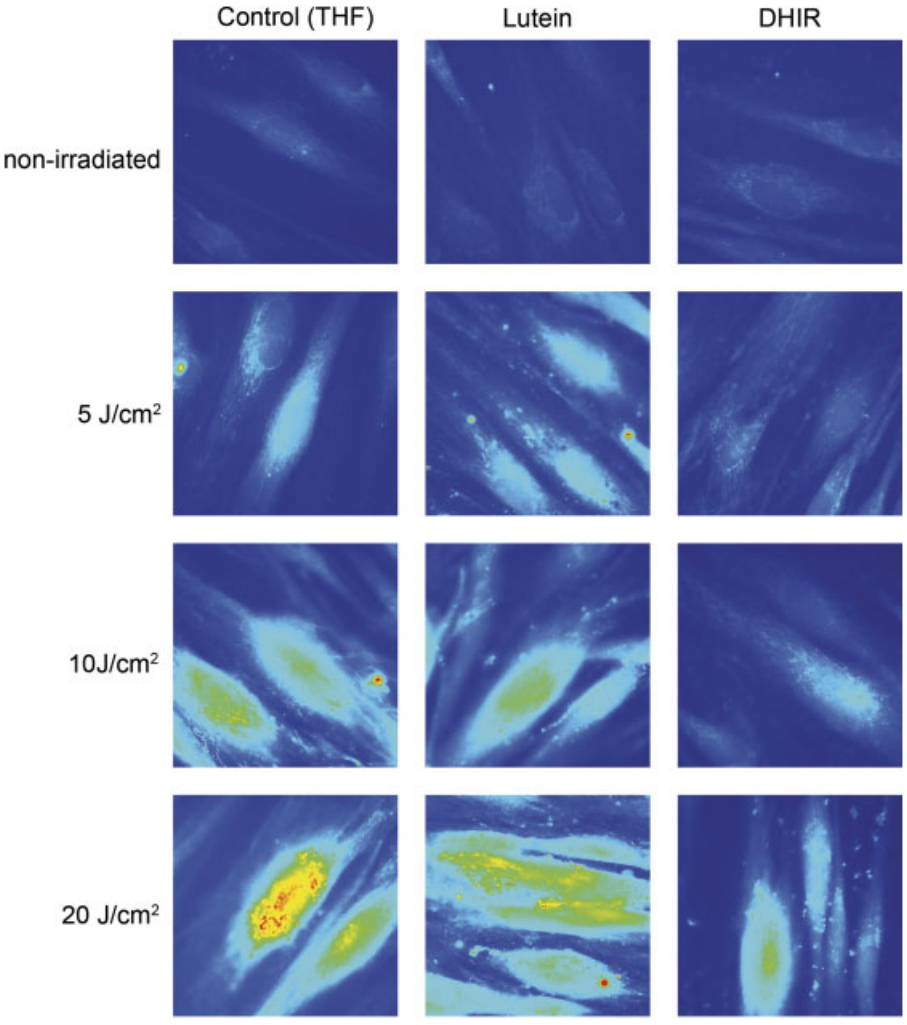

Figure 2. Effects of DHIR and lutein on ROS formation in human skin fibroblasts1 h after irradiation with UV-A light. Fluorescence intensity of DCF is correlated with ROS generation.

DCF generation was quantitatively measured in a separate set of experiments with cells grown under the same conditions on 24-well plates applying fluorescence spectroscopy (Table 3). With increasing UV doses, ROS-dependent formation of DCF was elevated (control). The data obtained for lutein were similar to the solvent control indicating no protective effect of this carotenoid in the applied system. However, upon preincubation of cells with DHIR, fluorescence was lower compared with that of controls at all UV dose levels.

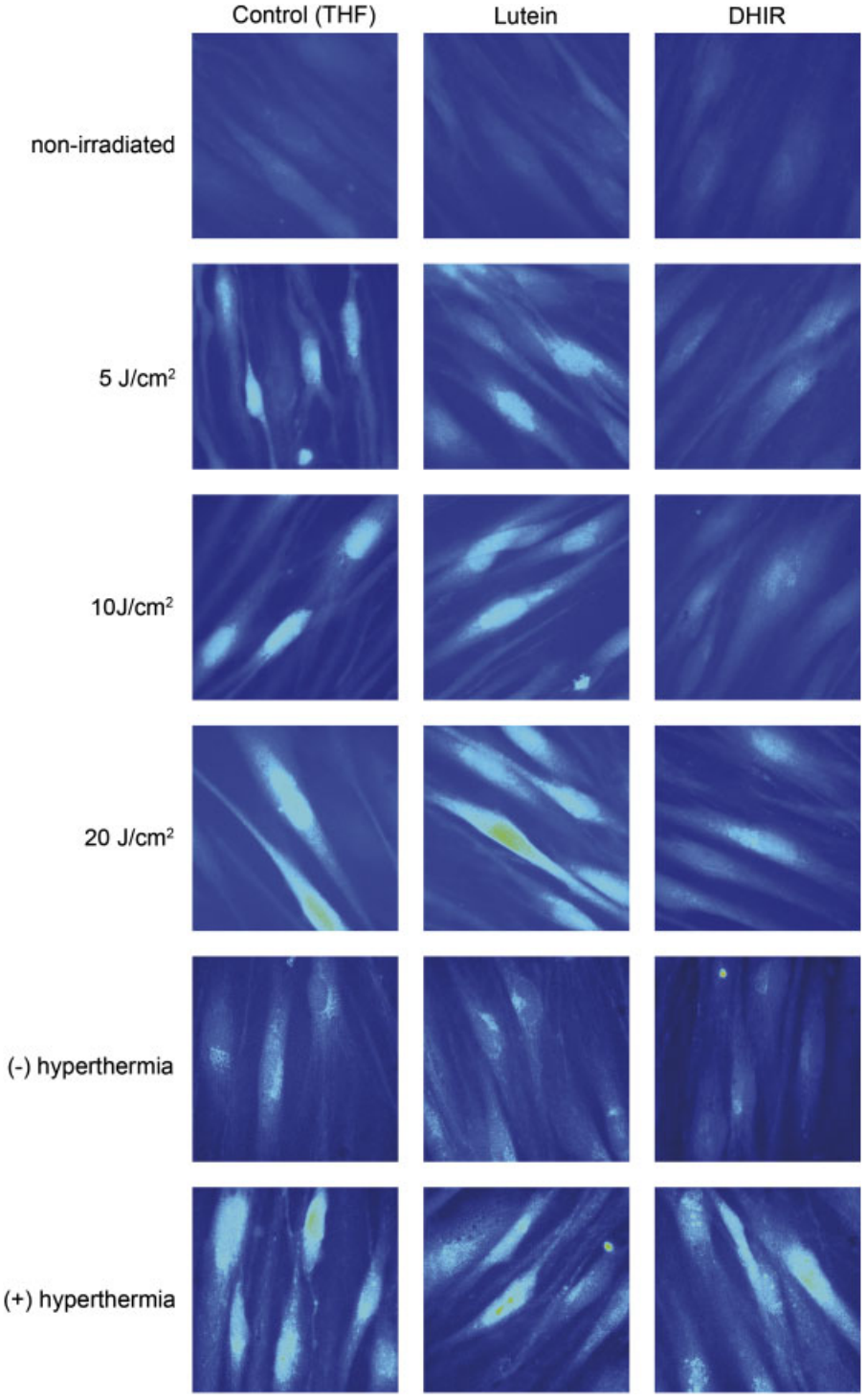

Figure 3. Effects of DHIR and lutein on the intracellular release of Zn^{2+} from zinc proteins after irradiation of human skin fibroblasts with UV-A light or exposure to hyperthermia. Formation of fluorescent Zinquin–Zn^{2+} complexes is correlated with the intracellular level of ''free'' Zn^{2+} ions.

3.3 UV-A induced release of intracellular Zn^{2+}

UV-A irradiation induces the release of intracellular Zn^{2+} from zinc proteins, which can be followed by the formation of fluorescent Zn^{2+}–zinquin complexes (Fig. 3). With increasing UV doses the fluorescent probe indicated elevated concentrations of ''free'' Zn^{2+} within the fibroblasts (left lane, control). Without irradiation, the amount of intracellular ''free'' zinc ions was low and not affected by the presence of either DHIR or lutein (upper row). No changes in UV-dependent intracellular zinc release were determined in cells preincubated with lutein (middle lane). However, in the presence of DHIR, the UV-induced formation of intracellular ''free'' zinc was lower than control (right lane), indicating a protective effect of the compound.

3.4 Hyperthermia (thermal stress)

Exposure of human dermal fibroblasts to hyperthermia (43°C for 30 min) results in a release of Zn^{2+} from intracellular proteins (Fig. 3). Zinquin fluorescence was more pronounced in thermally stressed cells (+hyperthermia) than in cells kept at 37°C (−hyperthermia). Under the conditions applied, neither DHIR nor lutein counteracted thermal stress-induced zinc release. Hyperthermia did not lead to a significant increase of intracellular ROS (Table 3). Thus, it is concluded that thermal stress-induced intracellular Zn^{2+} release is not due to ROS formation and that the preventive effects of DHIR against UV-dependent zinc release are related to the antioxidant properties of the compound.

4 Discussion

Carotenoids constitute a group of phytochemicals that are suggested to play a role in the prevention of degenerative diseases. They are potent antioxidants and some carotenoids such as β-carotene significantly contribute to dietary vitamin A supply [16]. In plants and micro-organisms, carotenoids participate in photoprotective systems and there is evidence that they also protect human tissues against photo-oxidative damage [17]. Among all carotenoids known up till now, only few contain aromatic or phenolic groups in their structure. DHIR represents such a phenolic carotenoid synthesized by bacteria that are used in dairy industry for the production of smear cheese specialties [4, 18].

In the present paper, we show that in human dermal fibroblasts this structurally unusual compound prevents UV-induced ROS formation as well as intracellular zinc release. Protective effects correlate with the antioxidant properties of DHIR since the release of zinc from intracellular proteins induced by hyperthermia *via* a non-oxidant mechanism is not inhibited in the presence of the compound.

Zinc, which in biological systems is generally present as Zn^{2+}, is an essential cofactor required for the accurate structure and function of many intracellular proteins [19]. Under physiological conditions almost all intracellular Zn^{2+} is co-ordinately bound in proteins by cysteine, histidine, glutamate or aspartate side chains. Protein binding sites for Zn^{2+} providing four ligands that are either cysteine or histidine residues are termed zinc fingers. Under various conditions of stress the intracellular levels of ''free'' (non-protein bound) zinc is elevated [10]. We recently demonstrated that irradiation of cells with sublethal doses of UV-A induces a transient increase of intracellular ''free'' zinc. Exposure to non-toxic concentrations of pro-oxidant tert-butyl hydroperoxide or singlet oxygen mimics the effects of UV-A irradiation [12]. Shifting the intracellular redox balance by depleting cells of their main intracellular thiol glutathione also results in an increase of intracellular ''free'' zinc [9]. Thus, it is suggested that after UV-A irradiation first and foremost oxidation of cysteine thiols is the primary cause of the disturbed intracellular zinc homeostasis. As zinc fingers are a major DNA-binding motive in many transcription factors, disruption of these protein domains may interfere with transcription and gene expression. However, other zinc finger containing proteins may also be affected, such as chaperones, DNA repair proteins and proteins involved in the regulation of cell differentiation or replication.

Intact zinc fingers are essential for the proper function of cells and antioxidants may protect cells from UV-A-induced intracellular zinc release associated with, for example, dysfunction of gene expression. The antioxidant properties of DHIR have been demonstrated in various *in vitro* assays [6]. Apart from radical scavenging and singlet oxygen quenching UV absorption mechanisms are also suggested to contribute to the superior antioxidant and UV-protective effects of this compound. Both properties of DHIR are likely responsible for the protection against UV-induced disturbance of cellular zinc homeostasis.

The present work has been performed as a project of the SFB 663 (B1) at the Heinrich-Heine-University, Düsseldorf, and is printed at its instigation with financial support provided by the Deutsche Forschungsgemeinschaft.

The authors have declared no conflict of interest.

5 References

[1] Aust, O., Stahl, W., Sies, H., Tronnier, H., Heinrich, U., Supplementation with tomato-based products increases lycopene, phytofluene, and phytoene levels in human serum and protects against UV-light-induced erythema. *Internat. J. Vit. Nutr. Res.* 2005, *75*, 54–60.

[2] Stahl, W., Sies, H., Carotenoids and flavonoids contribute to nutritional protection against skin damage from sunlight. *Mol. Biotechnol.* 2007, *37*, 26–30.

[3] Marian, C. M., Kock, S. C., Hundsdörfer, C., Martin, H. D. *et al.*, Spectroscopic properties of phenolic and quinoid carotenoids: a combined theoretical and experimental study. *Photochem. Photobiol. Sci.* 2009, *8*, 270–278.

[4] Kohl, W., Achenbach, H., Reichenbach, H., The pigments of *Brevibacterium linens*: aromatic carotenoids. *Phytochemistry* 1983, *22*, 207–210.

[5] Rattray, F. P., Fox, P. F., Aspects of the enzymology and biochemical properties of *Brevibacterium linens* relevant to cheese ripening: a review. *J. Dairy Sci.* 1999, *82*, 891–909.

[6] Martin, H. D., Kock, S., Scherrers, R., Lutter, K. *et al.*, 3,3′-Dihydroxyisorenieratene, a natural carotenoid with superior antioxidant and photoprotective properties. *Angew. Chem. Int. Ed.* 2009, *48*, 400–403.

[7] Hambidge, M., Human zinc deficiency. *J. Nutr.* 2000, *130*, 1344S–1349S.

[8] Andreini, C., Banci, L., Bertini, I., Rosato, A., Counting the zinc-proteins encoded in the human genome. *J. Proteome Res.* 2006, *5*, 196–201.

[9] Kröncke, K. D., Klotz, L. O., Zinc fingers as biological redox switches? *Antioxid. Redox Signal.* 2009, *11*, 1015–1027.

[10] Kröncke, K.D., Cellular stress and intracellular zinc dyshomeostasis 2007. *Arch. Biochem. Biophys.* 2007, *463*, 183–187.

[11] Krezel, A., Hao, Q., Maret, W., The zinc/thiolate redox biochemistry of metallothionein and the control of zinc ion fluctuations in cell signaling. *Arch. Biochem. Biophys.* 2007, *463*, 188–200.

[12] Pirev, E., Calles, C., Schroeder, P., Sies, H., Kröncke, K.D., Ultraviolet-A irradiation but not ultraviolet-B or infrared-A irradiation leads to a disturbed zinc homeostasis in cells. *Free Radic. Biol. Med.* 2008, *45*, 86–91.

[13] Skehan, P., Storeng, R., Scudiero, D., Monks, A. *et al.*, New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* 1990, *82*, 1107–1112.

[14] Wardman, P., Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects. *Free Radic. Biol. Med.* 2007, *43*, 995–1022.

[15] Zalewski, P. D., Forbes, I. J., Betts, W. H., Correlation of apoptosis with change in intracellular labile Zn(II) using zinquin [(2-methyl-8-p-toluenesulphonamido-6-quinolyloxy)acetic acid], a new specific fluorescent probe for Zn(II). *Biochem. J.* 1993, *296*, 403–408.

[16] Rao, A. V., Rao, L. G., Carotenoids and human health. *Pharmacol. Res.* 2007, *55*, 207–216.

[17] Demmig-Adams, B., Adams, W. W., Antioxidants in photosynthesis and human nutrition. *Science.* 2002, *298*, 2149–2153.

[18] Bockelmann, W., Willems, K. P., Neve, H., Heller, K. H., Cultures for the ripening of smear cheeses. *Internat. Dairy J.* 2005, *15*, 719–732.

[19] Tapiero, H., Tew, K. D., Trace elements in human physiology and pathology: zinc and metallothioneins. *Biomed. Pharmacother.* 2003, *57*, 399–411.

