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UVB irradiation-induced impairment of keratinocytes and adaptive responses to oxidative stress

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Abstract
UVB irradiation of human skin is known to induce pathophysiological processes as oxidative stress and inflammation. HaCaT keratinocytes represent a well-established *in vitro* model system to investigate the influence of UVB irradiation on cell cultures. It was the aim of these investigations to study the effects of moderate UVB doses on cellular and mitochondrial integrity of HaCaT keratinocytes, biomarkers of oxidative stress and antioxidant protection by superoxide dismutases. F_2-isoprostane concentrations were UVB dose-dependently enhanced reaching a plateau at 50 mJ/cm^2. Cell viability was reduced and apoptosis was enhanced with increasing UVB doses. The activities of the respiratory chain complexes were practically not altered at lower UVB doses, up to 50 mJ/cm^2, whereas remarkable decreases, also for the levels of cardiolipin species, were seen at 100 mJ/cm^2. As an adaptive response to the enhanced oxidative stress, protein levels of MnSOD increased about 3-fold at 50 mJ/cm^2 and decreased at higher doses. From the data it can be concluded that keratinocytes are sufficiently protected at low UVB doses, whereas higher doses lead to irreversible cell damage.

Keywords: *UVB, HaCaT cells, F_2-isoprostanes, MnSOD, Cu/ZnSOD, mitochondria*

Abbreviations: *NICI-GC-MS, negative ion chemical ionization-gas chromatography mass spectrometry; mNOS, mitochondrial nitric oxide synthase; NO, nitric oxide; PBS, phosphate buffered saline; ROS, reactive oxygen species*

Introduction

As the outermost barrier of the body, the skin is directly and frequently exposed to pro-oxidant environment including UV light. UVB irradiation (290–320 nm) of the skin is associated with the onset of various physiological and pathophysiological processes including oxidative stress and acute inflammation, characterized by the development of erythema, oedema and skin cancer [1]. Keratinocytes represent the major cell population in the skin [2] and are the major targets of UVB effects. They have to protect from UV light not only themselves, but also the underlying organism and are equipped with a strong antioxidant potential consisting of low-molecular weight antioxidants and cellular antioxidant enzymes [3]. For many years it has been known that UVB

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irradiation induces oxidative stress in keratinocytes [4] and the release of inflammatory mediators, as cytokines, nitric oxide (NO) and arachidonic acid-derived lipids as prostaglandins [5,6]. Reactive oxygen species (ROS) as cause of oxidative stress are considered as an important factor of UVB-induced keratinocyte injury. Besides NADPH oxidase and cyclo-oxygenase [7], it is generally accepted that the mitochondrial respiratory chain is an important source of ROS production [8] and, hence, a potent contributor to UVB-induced injury. Otherwise, the enzyme complexes of the respiratory chain and cardiolipin, the only specific mitochondrial phospholipid providing stability and functional integrity to the respiratory chain complexes and cytochrome c [9], are particularly susceptible to ROS attack. Cardiolipin and its oxidation products are important participants and signalling molecules in the mitochondrially mediated apoptotic cell death programme [10]. UVB-induced apoptosis of keratinocytes is described as a natural protective mechanism against UVB-mediated DNA damage [11]. Damaged keratinocytes are removed and the risk of malignant transformation can be reduced. Using DNA arrays, Li et al. [12] observed that at least 198 genes in human epidermal keratinocytes were regulated by UV light. They include DNA repair building blocks, energy producing enzymes, transcription factors and other signal transducing proteins, cytokines, growth factors and antioxidant proteins. Among the antioxidant constituents, MnSOD (SOD-2, EC 1.15.1.1) is the most active scavenger of superoxide anion radicals in mitochondria, which may play a primary protective role against UVB-induced injury and may be supported by the mainly cytosolically localized Cu/ZnSOD (SOD-1, EC 1.15.1.1).

The aims of this study were (1) to investigate the ability of moderate UVB irradiation to induce the generation of reactive oxygen species by measuring F_2-isoprostanes in HaCaT keratinocytes as biomarkers of oxidative stress in dependence on increasing UVB doses, (2) to evaluate UVB-induced alterations of vitality of cells as well as mitochondrial enzyme activities (respiratory chain complexes and mNOS) and phospholipids (cardiolipin species) and (3) to examine the possible role of endogenous MnSOD and Cu/ZnSOD as mitochondrial and cellular defence against dose-dependent UVB-induced keratinocyte injury *in vitro*.

Materials and methods

Cell culture and UVB irradiation

HaCaT keratinocytes (human adult low calcium high temperature) were obtained from the German Cancer Research Centre (Heidelberg, Germany). They were routinely grown in Dulbecco's Medium, supplemented with 10% foetal calf serum, 2% L-glutamine and 1% antibiotic-antimycotic solution (all chemicals by GIBCO/Invitrogen, Karlsruhe, Germany) under a humidified atmosphere of 5% CO_2 at 37°C [13].

For the purpose of UVB irradiation, 1 day before the start of the experiment, sub-confluent cultures were harvested (0.25% trypsin-EDTA), and 1×10^5 cells/cm^2 were sub-cultured in flat bottom tissue culture plates (nunc, Wiesbaden, Germany). HaCaT keratinocytes were irradiated with 10, 30, 50 and 100 mJ/cm^2 UVB (solar UV simulator, Solar Light, Philadelphia). Controls underwent identical manipulations without irradiation. After an additional incubation for 24 h, supernatants were collected, centrifuged (2000 g, 5 min) and kept cell-free frozen at −80°C until measurements of metabolites. HaCaT keratinocytes were harvested in phosphate buffered saline (PBS). A cell aliquot was counted by means of a Neubauer cell counting chamber using trypan blue staining. Immediately, the cells were directed to further analysis or frozen in liquid nitrogen and stored in deep freeze (−80°C) until further analysis (e.g. for quantification of F_2-isoprostanes and cardiolipin). Protein content of HaCaT keratinocytes was determined according to Lowry et al. [14].

Determination of cell viability

Vital staining. The irradiation-dependent viability of HaCaT keratinocytes was assessed by double-labelling with propidium iodide and fluorescein diacetate. The technique is based on the phenomenon that living cells are able to hydrolyse fluorescein diacetate (10 μg/ml PBS) by intracellular esterases resulting in a green-yellow fluorescence. Dead cells were labelled by propidium iodide (4 μg/ml PBS), which interacts with DNA to yield a red fluorescence of cell nuclei. Evaluation was carried out on a fluorescence microscope (Axiophot, Zeiss, Jena, Germany) equipped with phase-contrast, rhodamine and fluorescein optics).

TUNEL-staining. The technique of terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labelling (TUNEL) was used to detect apoptotic DNA fragmentation in the cultured HaCaT keratinocytes. Therefore, HaCaT cultures were washed, permeabilized with methanol:chloroform:acetic acid (66:33:1, 10 min) and incubated in a humidified box (37°C, 90 min) using 50 μl reaction solution: 0.4 μl terminal deoxynucleotidyl transferase (TdT, 0.5 U/μl), 2 μl cobalt chloride (2.5 mM), 4 μl TdT reaction buffer, 0.8 μl fluorescein-dUTP (2 nM), 12.8 μl MilliQ (Roche Diagnostics, Germany). Incubation was stopped by washing cultures with saline sodium citrate (3 × 5 min) and PBS (2 × 5 min). Again, cells were examined on an Axiophot fluorescence microscope.

Determination of biomarkers of oxidative stress and inflammation

F_2-isoprostanes (sum of esterified and free F_2-isoprostanes) were quantified by gas chromatography mass spectrometry/negative ion chemical ionization (selected ion monitoring mode) described in detail elsewhere [15–18]. The share of free F_2-isoprostanes was measured in the same way, but by omitting alkaline splitting.

Mitochondria and sub-mitochondrial membranes

Keratinocyte mitochondria were isolated from the HaCaT cells according to Navarro et al. [19]. Briefly, HaCaT keratinocytes were homogenized in 0. 23 mol/L mannitol, 0.07 M sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4) at a ratio of 1 ml of homogenization medium/25×10^6 cells in a Potter homogenizer with a Teflon pestle. The homogenate was centrifuged at 700 *g* for 10 min and the supernatant at 8000 *g* for 10 min to precipitate mitochondria that were washed in the same conditions. Mitochondrial samples, twice thawed and frozen, were homogenized by passage through a 15/10 tuberculin needle; the resulting sub-mitochondrial membranes were used for the determination of enzyme activities. Protein content of samples was determined using Folin's reagent [14] and bovine serum albumin as a standard.

Mitochondrial electron transfer activities

The electron transfer activities of the complexes I–III, II–III and IV were determined spectrophotometrically at 30°C in 100 mM phosphate buffer (pH 7.4) [20]. For NADH-cytochrome c reductase (complexes I–III) and succinate-cytochrome c reductase (complexes II–III) activities, samples were added with 0.2 mM NADH or 20 mM succinate as substrates, 0.1 mM cytochrome c^{3+} and 1 mM KCN and the enzymatic activities were determined at 550 nm ($\varepsilon = 19\ mM^{-1} \times cm^{-1}$) and expressed as nmol cytochrome c reduced/mg protein. Cytochrome oxidase (complex IV) activity was determined in the same phosphate buffer added with 0.1 mM cytochrome c^{2+} which was prepared by reduction with $NaBH_4$ and HCl. The rate of cytochrome c oxidation was calculated as the first order reaction constant/mg protein and expressed as nmol cytochrome c oxidized at 20 μM cytochrome c and at 1 mg protein/ml, which gives rates of the order of physiological activities.

Mitochondrial nitric oxide synthase (mNOS) activity

Mitochondrial nitric oxide (NO) production was determined by the oxyhaemoglobin (HbO_2) oxidation assay as described by Boveris et al. [21]. The reaction medium consisted of 0.1 mM NADPH, 0.2 mM arginine, 1 mM $CaCl_2$, 4 μM Cu/ZnSOD, 0.1 μM catalase and 25 μM HbO_2 heme, in 50 mM phosphate buffer at pH 7.2. A diode array sensitive spectrophotometer (Agilent Corp., model 8453) was used to follow the absorbance change at 577 nm with a reference wavelength at the isosbestic point of 591 $mM^{-1} \times cm^{-1}$ ($\varepsilon_{577-591} = 11.2$ mM/cm). NO production was calculated from the absorbance change that was inhibited by 2 mM N^G-methyl-L-arginine, usually 86–94%, and expressed in nmol NO/min × mg protein.

Quantification of cardiolipin species

Cardiolipin species were analysed as described in detail by Schlame et al. [22]. Tetrastearoyl-cardiolipin, the internal standard, was generated by complete hydrogenation of commercial cardiolipin. Lipids were extracted from cells according to Bligh and Dyer [23]. Samples were treated with diazomethane for derivatization and then marked for HPLC fluorescence detection with 1-naphtylacetic anhydride dissolved in anhydrous pyridine. Following solid phase extraction on Supelclean columns (Supelco LC/Si SPE; Bellefonte, PA), cardiolipin-containing samples were dried, redissolved in n-hexane-ethanol 1:1 (by volume) and separated by HPLC using a LiChroCart Supersphere column (250 × 4 mm, 4 μm). A solvent gradient was run from acetonitrile-2-propanol 8:2 (by volume) to acetonitrile-2-propanol 5:5 (by volume) in 50 min. The HPLC was a Waters system consisting of a 616 pump (total flow rate 1.0 ml/min), a 600 S controller, a 717plus Autosampler and a 2475 Multi λ fluorescence detector (excitation wavelength 280 nm, emission wavelength 360 nm). Data were collected and processed by the Waters Milllennium 32 Software. Three cardiolipin peaks were calculated representing three different molecular species. The fourth peak was the internal standard, a hydrogenated cardiolipin that does not occur in nature. The cardiolipin concentrations were calculated in reference to the internal standard. Recovery during sample work-up procedure determined by analysis of phosphate [24] was 78% ($n = 6$).

RT-PCR analysis of Mn-SOD and Cu/Zn-SOD

Total RNA was isolated from cultures of HaCaT keratinocytes using guanidinium isothiocyanate/phenol/chloroform (peqGOLD TriFast, peqlab, Erlangen, Germany). Each experiment was repeated at least 3-fold. To remove DNA contamination, 5 μg of total cell RNA were treated with Turbo DNA-free (Ambion, Austin) according to the manufacturer's instructions; 4.5 μl RNA (2.25 μg input RNA) were reverse transcribed using the RevertAid™ H Minus First strand cDNA Synthesis Kit primed with Oligo$(dT)_{18}$ primers (Fermentas, St. Leon-Rot, Germany). cDNA (2 μl) was then PCR amplified with

Taq-DNA-polymerase (peqlab) and the respective primers: MnSOD, forward 5′-AAGCGTGCTCCC ACACATC-3′, reverse 5′-GGCACAAGCACAGC CTCCC-3′ (497 bp; gene bank No Y00497); CuZn-SOD, forward 5′-GCAGAAGGAAAGTAATGGA CCAG-3′, reverse 5′-TTATTGGGCGATCCCA ATTACAC-3′ (400 bp, gene bank No X02317); GAPDH, forward 5′-TTAGCACCCCTGGCCA AGG-3′, reverse 5′-CTTACTCCTTGGAGGCCAT G-3′ (house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase, 531 bp, gene bank No NM_017008). The optimum number of amplification cycles were determined for each gene to be in the exponential phase. With one-tenth of each reaction product electrophoresis has been performed on a 1% agarose gel. The PCR product bands were quantified by densitometric analysis using a Biometra BioDoc-Analyzer and the ratio of their expression to the housekeeping gene expression was calculated.

Western blot analysis

The procedure according to Lorenz et al. [25] was used. Again, at least three series of experiments were done. Cultured HaCaT keratinocytes were washed twice with PBS and lysed with sodium dodecyl sulphate (SDS)-phosphate buffer (50 mM Na_2HPO_4, 50 mM NaH_2PO_4 and 0.1% SDS, pH 7.4). Lysates were collected in microcentrifuge tubes and homogenized using a Potter homogenizer. Protein contents of homogenates were determined using the bicinchoninic acid (BCA) 'Protein Assay Kit'. Bovine serum albumin was used as standard and absorption was measured at 562 nm [26]. Material was mixed with protein loading buffer (roti-Load 1, Carl Roth GmbH, Karlsruhe, Germany) according to the manufacturer's procedure and placed in a heating bath (95°C) for 5 min. Proteins were separated using SDS-PAGE (gradient gels from 5–25%). The protein amount loaded per lane was 20 µg. After separation, the proteins were stained with Coomassie Brilliant Blue or transferred to nitrocellulose paper and unspecific protein binding sites were blocked with blocking buffer (Chemicon International, Hofheim, Germany). The blots were incubated overnight with antibodies against MnSOD (polyclonal, Biomol, Hamburg, Germany, 1:5000) or Cu/ZnSOD (polyclonal, Biomol, 1:800), followed by incubation with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit and anti-mouse IgG^+ peroxidase, Boehringer Mannheim GmbH, Germany, 1:10 000). Immunoreactivity was visualized using the ECL detection system (Amersham Pharmacia Biotech Lim., Buckinghamshire, UK) and quantified by densitometric analysis using a Biometra BioDoc Analyzer. Additionally, β-actin (monoclonal, Sigma, Germany, 1:2500) detection was used to show equal sample loading.

Immunohistochemistry

Cultured keratinocytes were fixed in 4% buffered paraformaldehyde for 30 min and then incubated with antibodies against MnSOD (polyclonal, Biomol, 1:1000) or Cu/ZnSOD (polyclonal, Biomol, 1:500), diluted in PBS with 0.3% Triton X-100 and 1% foetal calf serum (FCS) overnight. Following incubation with primary antibodies, cultures were washed in PBS (3×5 min), incubated overnight with a secondary anti-rabbit-IgG Alexa Fluor 546 antibody (Molecular Probes, Göttingen, Germany, 1:500) and examined on a fluorescence microscope (Axiophot) equipped with phase-contrast and rhodamine optics. The specificity of the immunoreactions was controlled by the application of buffer instead of the primary anti-serum. All control sections were free of any immunostaining.

Statistical analysis

The data were expressed as means $\pm$ SD or $\pm$ SEM, as indicated in the figure legends and analysed by Student's *t*-test.

In Table I, mean values $\pm$ SEM were given. Data were analysed by one-way ANOVA. A *p*-value of < 0.05 was considered to be indicative for biologically significant differences. Statistical analyses were carried out using a statistical package (SPSS 11.5 for Windows).

Results

UVB-induced oxidative stress and inflammation

Concentrations of F_2-isoprostanes as highly specific *in vivo* biomarkers of oxidative stress-mediated lipid

Table I. Effect of UVB irradiation on the mitochondrial inner membrane enzyme activities from HaCaT keratinocytes.

Keratinocytes/enzymes	Complexes I–III	Complexes II–III	Complex IV	mNOS
Non-irradiated cells	171 ± 7	84 ± 5	90 ± 6	13.8 ± 0.7
30 mJ/cm^2 irradiated cells	168 ± 7	79 ± 5	88 ± 6	10.4 ± 0.5*
50 mJ/cm^2 irradiated cells	157 ± 5*	77 ± 5	82 ± 6	10.2 ± 0.5*
100 mJ/cm^2 irradiated cells	136 ± 5*	64 ± 5*	69 ± 5*	8.8 ± 0.3*

Mitochondrial enzymatic activities NADH-cytochrome c reductase (complexes I–III), succinate-cytochrome c reductase (complexes II–III) and cytochrome oxidase (complex IV) are expressed in nmol cytochrome c reduced or oxidized/min × mg protein; and mitochondrial nitric oxide synthase (mNOS) is expressed in nmol NO/min × mg protein. * $p < 0.05$ (vs non-irradiated cells).

peroxidation and biologically potent prostaglandin-like mediators of inflammation, dose-dependently increased in HaCaT keratinocytes to until more than 2- (8-iso-$PGF_{2\alpha}$) and 5-fold (9α,11α-$PGF_{2\alpha}$) higher levels 24 h after 50 mJ/cm^2 UVB irradiation (Figure 1). At 100 mJ/cm^2, 8-iso-$PGF_{2\alpha}$ concentrations were not significantly higher than those at 50 mJ/cm^2, whereas concentrations of 9α,11α-$PGF_{2\alpha}$ significantly declined to about half-maximum values. It must be mentioned, however, that only the '8-iso-$PGF_{2\alpha}$' peak of the NICI-GC-MS chromatogram represents pure F_2-isoprostanes, whereas the '9α,11α-$PGF_{2\alpha}$' peak represents both, F_2-isoprostanes and prostaglandins. The non-enzymatic origin of 9α,11α-$PGF_{2\alpha}$ is compatible with its detection in HaCaT keratinocytes in contrast to PGE_2, which is completely released in the supernatant and the partial occurrence in the esterified phospholipid-bound form (not shown). The enzymatic origin corresponds with the predominant occurrence in the free form ($\sim$75%) and an additional release in the cell-free supernatant (not shown).

Vital testing (phase contrast, vital staining, TUNEL staining)

With increased UVB-irradiation intensity, the state of cultured HaCaT keratinocytes changed. Conspicuously is the enhanced number of vacuoles (arrows) as demonstrated in the phase contrast microscope (Figure 2C). Moreover, irradiation with 100 mJ/cm^2 (Figure 2E) led to a significant cell loss. The double-labelling technique reflected that the viability of HaCaT keratinocytes is reduced with increasing intensity of UVB-irradiation. In control cultures (Figure 2F) and cultures irradiated with 10 mJ/cm^2 (Figure 2G), red nuclei, indicating dying or dead cells, were seen, if at all, only sporadically. Starting with 30 mJ/cm^2 (Figure 2H), the number of red cell nuclei increased significantly and reached the highest level ($\sim$85%) at 100 mJ/cm^2 (Figure 2J). The increased cell death rate was, at least in part, a result of an enhanced apoptosis as demonstrated by the TUNEL technique [(green fluorescence) (Figure 2K–O)]. The number of apoptotic cells (demonstrated by DNA fragments) was steadily increased in dependence on the UVB-irradiation intensity ($\sim$7% in cultures irradiated with 30 mJ/cm^2, $\sim$15% in cultures irradiated with 50 mJ/cm^2, $\sim$75% in cultures irradiated with 100 mJ/cm^2).

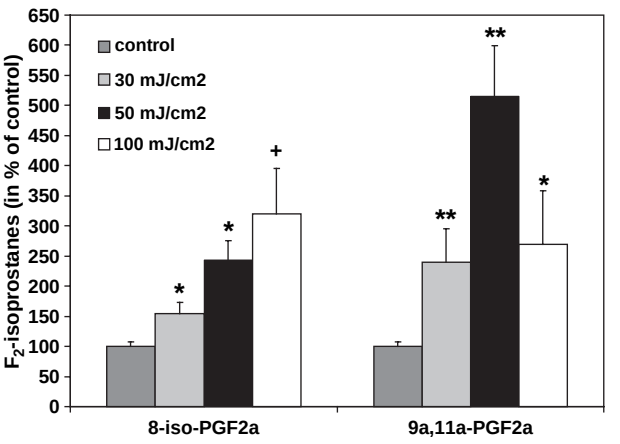

Figure 1. Dose dependent enhancement of F_2-isoprostanes in HaCaT keratinocytes following UVB irradiation. Values are means $\pm$SEM. Endogenous concentrations (controls without UVB irradiation) were 85.1$\pm$7.1 pg/mg protein for 8-iso-$PGF_{2\alpha}$ (n=28) and 177.3$\pm$14.2 for 9α,11α-$PGF_{2\alpha}$ (n=28), 30 mJ/cm^2 (n=20) and 50 and 100 mJ/cm^2 (n=6). Significance between different doses (each vs the corresponding lower dose) was calculated by Student's t-test. * p <0.05 and ** p <0.01. +100 vs 30 mJ/cm^2.

By all techniques used, it can be seen that irradiation with 100 mJ/cm^2 led to significant cell damage and cell loss as a result of detachment of dead cells from the culture dish.

Impairment of mitochondrial electron transfer complexes, mNOS and cardiolipin

Activities of mitochondrial electron transfer complexes were not significantly inhibited after mild UVB irradiation with 30 mJ/cm^2 in comparison to control incubations without irradiation (Table I); 50 mJ/cm^2 UVB irradiation inhibits only the NADH-cytochrome c-reductase by $\sim$10%, whereas 100 mJ/cm^2 leads to a significant inhibition of all three respiratory chain complexes by 20.5% (complex I–III), 23.8% (complex II–III) and 23.3% (complex IV).

The activity of the mNOS, an integral protein of the inner mitochondrial membrane [21], was measured for the first time in HaCaT keratinocytes (Table I). UVB irradiation with 30 and 50 mJ/cm^2 significantly reduced the activities by $\sim$25% and 100 mJ/cm^2 leads to a stronger inhibition than seen for the respiratory chain complexes (36.2%).

The content of cardiolipin, a phospholipid rich in unsaturated fatty acids, mainly linoleic acid, and localized in the inner mitochondrial membrane, decreased by 45–56.8% (cardiolipin species I–III) 24 h after UVB irradiation of HaCaT cells with 100 mJ/cm^2 (Figure 3). Concerning the three different cardiolipin species, no significant differences in the UVB-induced loss were seen for tetralinoleoyl-cardiolipin and trilinoleoyl-oleoyl-cardiolipin, but an $\sim$10% higher stability for dilinoleoyl-dioleoyl-cardio lipin (p < 0.05).

MnSOD and Cu/ZnSOD; RT-PCR and Western blot analysis

Superoxide dismutases are among the most active scavengers of ROS, providing defence against the cellular and in particular the mitochondrial oxidative stress. In control HaCaT cultures, mRNA transcripts of MnSOD and Cu/ZnSOD were expressed at basic levels. Following UVB irradiation we observed, in

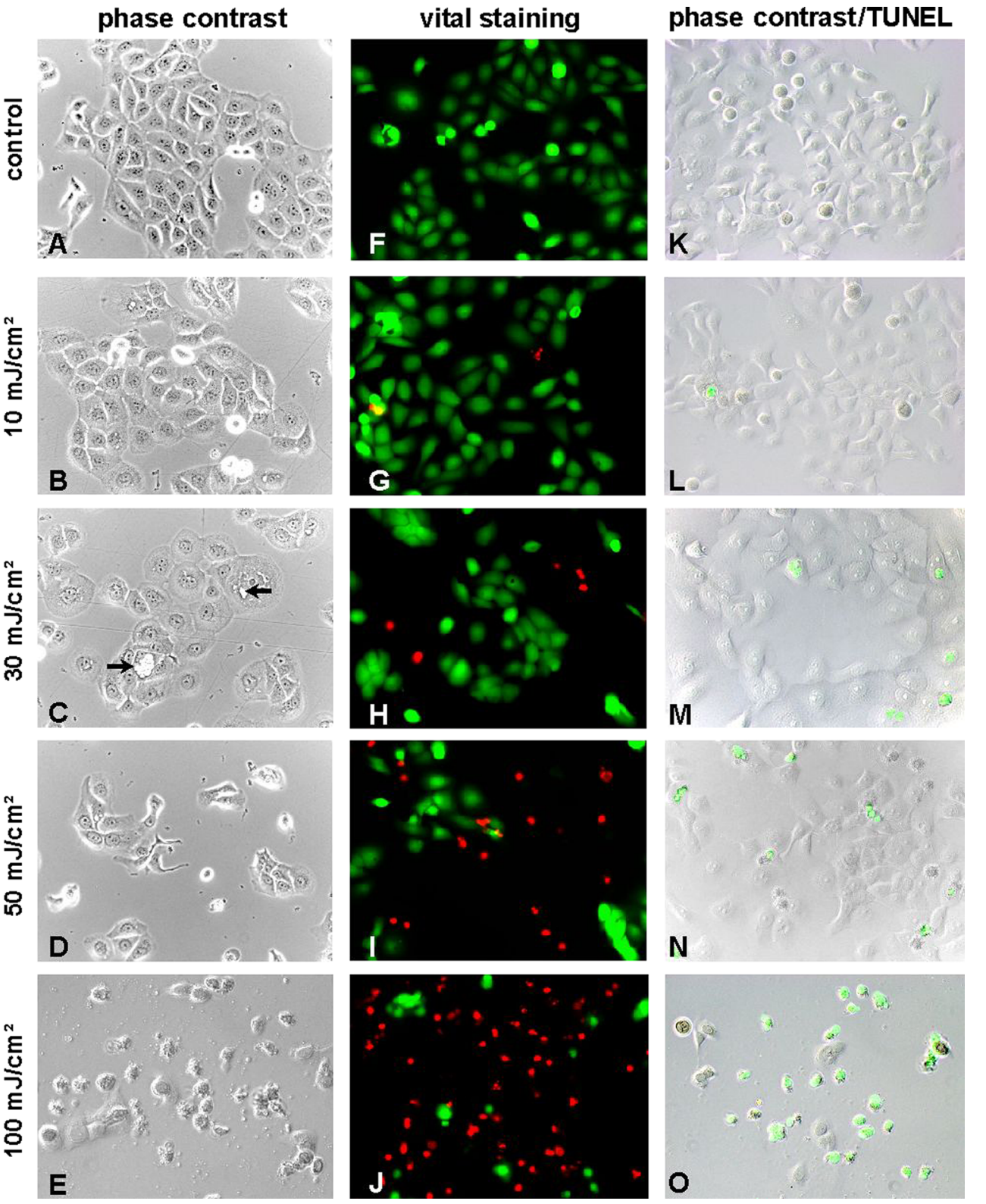

Figure 2. Determination of cell viability following UVB irradiation. Phase contrast microscopy (A–E): Phase contrast microscopy, carried out on a fluorescence microscope with phase contrast optics, allows a general estimation of the respective cell cultures. The dependency on UVB irradiation intensity of cell stress and, subsequently, cell death can be demonstrated by vacuoles (arrows in C) and an enhanced number of degenerated cells (E). Vital staining (F–J): The influence of UVB-irradiation on the viability of keratinocytes was assessed by double-labelling fluorescence vital staining technique. Living cells generate a green-yellow fluorescence, whereas nuclei of dead cells are labelled by propidium iodide, which interacts with DNA resulting in a red fluorescence. TUNEL staining (K–O): The TUNEL technique (green fluorescence) was used to detect apoptotic DNA fragmentation in cultured HaCaT keratinocytes.

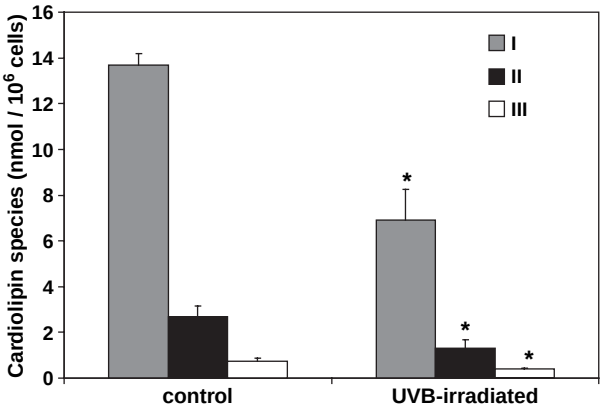

Figure 3. Degradation of cardiolipin species in HaCaT keratinocytes 24 h following UVB irradiation (100 mJ/cm^2). I: tetralinoleoylcardiolipin (L_4); II: trilinoleoyl-oleoyl-cardiolipin (L_3O); III: dilinoleoyl-dioleoyl-cardiolipin (L_2O_2). Values are means ± SEM ($n = 3$). * $p < 0.05$ (vs the respective control values without UVB irradiation) was considered to be significant.

particular for Cu/ZnSOD, a tendency to increased expression with a peak at 30 mJ/cm^2 (Figure 4).

To obtain a semi-quantitative measure of the changes at the protein level, Western blots of total HaCaT keratinocyte lysates were performed (Figure 4). As suggested by PCR findings, the intensity of the immunoreactive bands of MnSOD and Cu/ZnSOD were increased in result of UVB irradiation, but here the expression peaks were found at 50 mJ/cm^2. Cu/ZnSOD was enhanced ~1.5-fold, whereas MnSOD was enhanced more pronounced, beginning at 10 mJ/cm^2 and reaching 3-fold higher maximum levels at 50 mJ/cm^2. The reduced intensity at 100 mJ/cm^2 for both enzymes reflected the enhanced cell death rate.

Immunohistochemistry

Immunocytochemistry also demonstrated that MnSOD and Cu/ZnSOD were expressed in untreated HaCaT keratinocytes at basic levels (Figure 5A and B). UVB-irradiation with 50 mJ/cm^2 strongly enhanced the expression intensity (Figure 5C and D). Irradiation with 100 mJ/cm^2 led to a significant cell loss as dead cells detached from the culture dish (Figure 5E and F). Thus, immunohistochemistry parallelled the findings of RT-PCR, Western blot analysis and vital staining.

Discussion

It was the purpose of this study to investigate the critical role of moderate UVB doses between 10–100 mJ/cm^2 measuring in HaCaT keratinocytes the levels of indicators of oxidative stress and inflammation, ROS-induced damage to cells and mitochondria and the adaptive responses of primary antioxidant enzymes 24 h post-irradiation. The results suggest an increasing toxicity of UVB to cells and mitochondria,

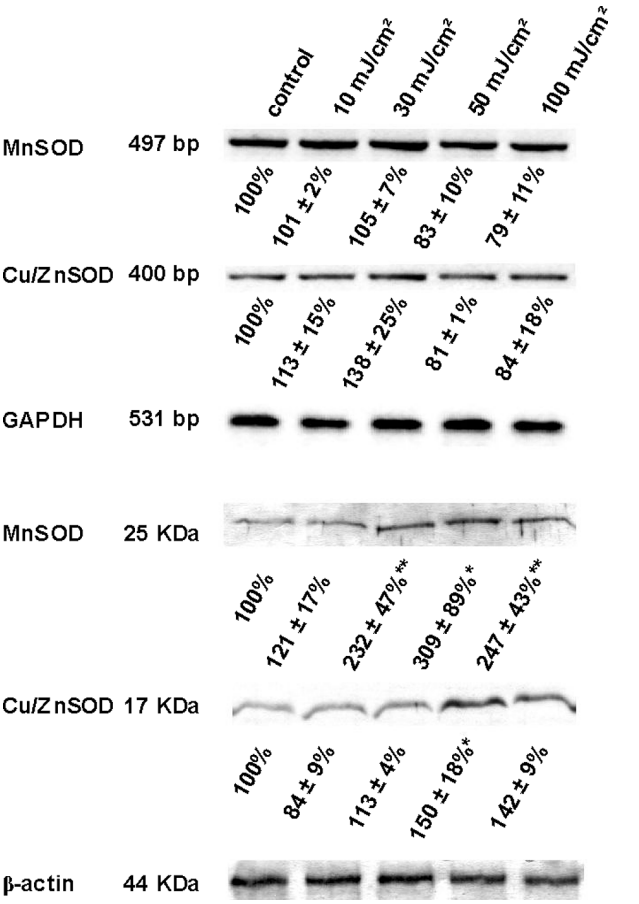

Figure 4. RT-PCR/Western blot analysis of MnSOD and Cu/Zn-SOD from HaCaT keratinocytes. Quantification of PCR product bonds and Western blots were done by densitometric analysis (Biometra BioDoc Analyser) after normalizing the PCR-values with GAPDH and the values of immunoreactivity with β-actin in the same blot. At least three series of experiments were performed. The differences to the respective control values are indicated: * $p < 0.05$, ** $p < 0.005$.

obviously caused by enhanced oxidative stress, which seems to be irreversible at ~100 mJ/cm^2. UVB exposure resulted in reduced cell viability, increased apoptosis, inhibition of mitochondrial respiratory chain enzyme and mNOS activities and cardiolipin degradation. Furthermore, we demonstrated a dose-dependent large UVB-mediated increase of the mitochondrial MnSOD protein and a smaller increase of the Cu/ZnSOD with maximum levels at 50 mJ/cm^2 using Western blot analysis. Significant increases in SOD protein levels may reflect the UVB-induced expression of both enzymes as a cutaneous antioxidant defence mechanism that protects against UVB-mediated cytotoxicity. At 100 mJ/cm^2, protein levels of MnSOD and Cu/ZnSOD decreased, strongly indicating a serious damage to HaCaT keratinocytes and a reduced ability to resist against UVB-induced injury.

UVB-induced oxidative stress

UV irradiation on the skin results in the formation of reactive oxygen species that interact with proteins,

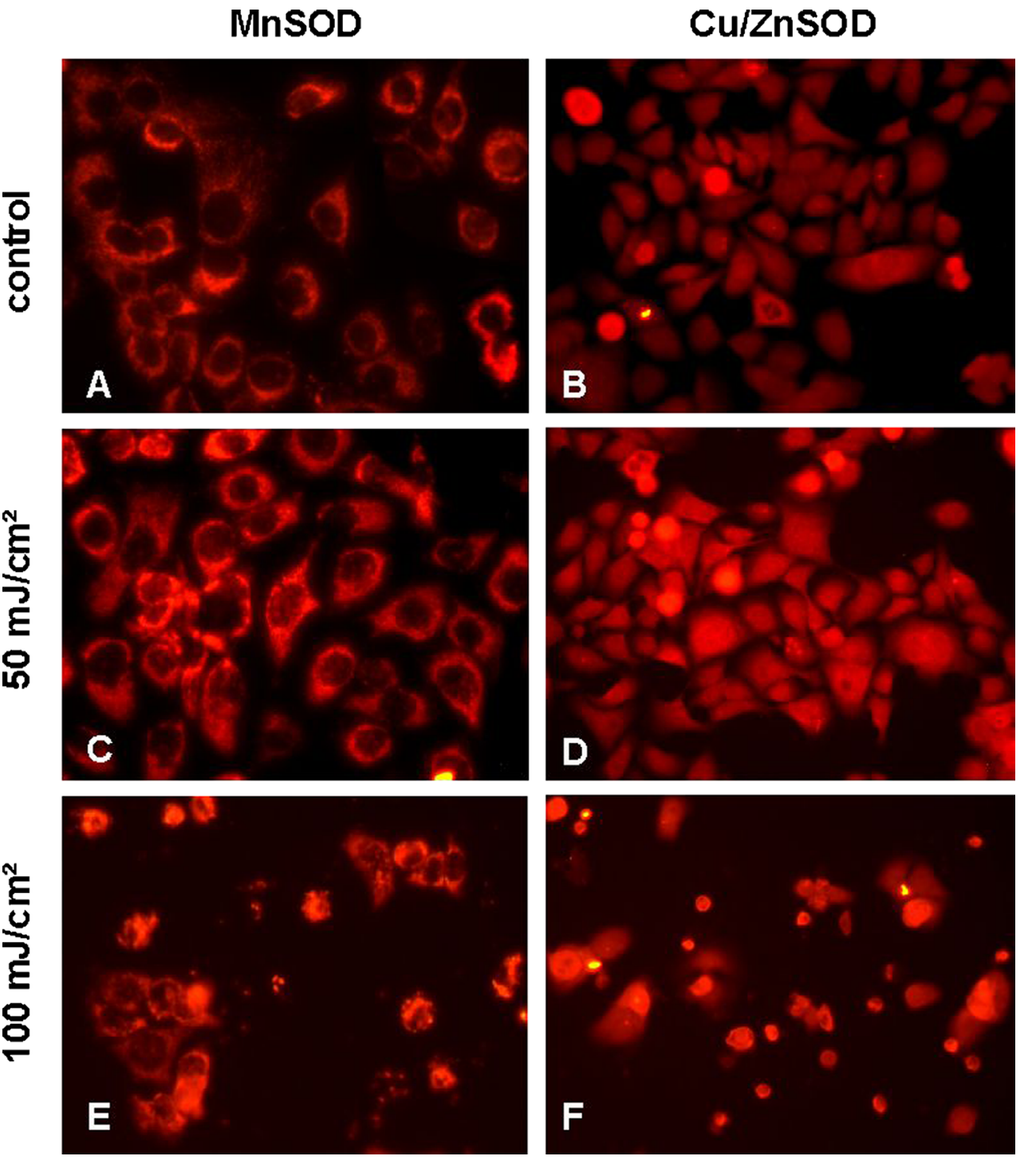

Figure 5. Immunohistochemical expression pattern of MnSOD (A,C,E) and Cu/Zn-SOD (B,D,F). HaCaTs were fixed in 4% paraformaldehyde and then incubated with antibodies against MnSOD and Cu/ZnSOD. Following incubation with a second anti-rabbit-IgG Alexa Fluor 546 antibody, they were examined on a fluorescence microscope equipped with phase contrast and rhodamine optics.

lipids and DNA, thus altering cellular functions [27]. As markers of UVB-induced oxidative stress, conju gated double bonds, malonic dialdehyde, 4-hydroxynonenal, carbonyls and 8-hydroxydesoxy-guanosin were measured [27]. Rezvani et al. [28] reported that UVB irradiation of human keratinocytes induced an increase in ROS levels at two distinct stages: immediately following irradiation and 3 h after irradiation. Catalase over-expression inhibited only the later increase in ROS levels [28]. Whereas high concentrations of ROS, generated following strong UVB irradiation, caused direct damage to cellular macromolecules, physiological doses of UVB induced small amounts of ROS modulating signalling

transduction pathways [29], the expression of redox-sensitive genes, transcription factors and mediators [30,31].

For ~10 years, F_2-isoprostanes have been well-known as reliable indicators of ROS generation and lipid peroxidation [32]. In HaCaT keratinocytes their levels were found to be relatively small, but the highly specific and sensitive GC-MS technique allowed one to follow changes of their concentrations in relation to different doses of moderate UVB exposure. The reason that F_2-isoprostane levels at 50 mJ/cm^2 reached a plateau and do not increase further may be due to the UVB-induced damage of cells and the enhanced release of isoprostanes in the supernatant.

Cell viability, apoptosis and mitochondrial damage

UVB irradiation that generates ROS also induces apoptosis and formation of so-called 'sunburn cells' (apoptotic keratinocytes) as a protective mechanism against carcinogenic effects of UVB irradiation [33]. UVB-induced apoptosis was suppressed by the treatment with antioxidants, e.g. catalase, glutathione and α-tocopherol [34] and may therefore be at least partially ROS mediated. In HaCaT keratinocytes the mitochondrial pathway of apoptotic cell death may play an important role [35] and the respiratory chain and the acidic phospholipid cardiolipin are involved. The activities of the respiratory chain complexes, as reported in this paper, were relatively unchanged until 50 mJ/cm^2. This is in correspondence with the data of Li et al. [12] who reported that among the most strongly induced genes after UVB irradiation in human epidermal keratinocytes were several mitochondrial proteins: cytochrome c-1, cyto chrome c oxidase sub-unit VIIb and cytochrome b light chain. It seems that energy-producing enzymes were upregulated or expressed, whereas energy-requiring processes, as transport, gluconeogenesis and lipogenesis were shut down [12]. So it may be important for saving the energy of the UV-damaged cell.

Following a critical UVB dose higher than 50 mJ/cm^2 mitochondrial damage by UVB may have serious pathological consequences. Cell viability strongly decreased, the number of apoptotic cells increased, respiratory enzyme activities decreased and mitochondrial cardiolipin species were reduced. Cardiolipin, an exclusively mitochondrially localized phospholipid, has a high share of unsaturated fatty acids (mainly linoleic and oleic acid), which are sensitive against UVB-induced free radical mediated damage. It seems that the UVB-induced injury to HaCaT keratinocytes may be more pronounced to phospholipids than to proteins.

The mitochondrial nitric oxide synthase (mNOS) is described as an integral protein of the inner mitochondrial membrane [36], that is regulable and adapted to physiological conditions such as environmental hypoxia [37] and hormonal regulation [38]. It is worth noting that the mNOS activity in keratinocytes is extremely high as compared with other cell types [36–38], which can be interpreted as an adaptive response of keratinocytes to produce NO as superoxide ($O_2^{\cdot -}$) and free radical scavenger [39] for the conditions of normal exposure to UVB irradiation.

Changes in antioxidants after acute UVB irradiation; SOD activities, protein and RNA levels

UVB irradiation has been shown to decrease the activities of antioxidants, such as catalase, glutathione reductase, α-tocopherol, β-carotene, ubiquinol, ubiquinone and glutathione due to either direct damage to or ROS-mediated depletion of the reducing system [40–42].

The important role of MnSOD and Cu/ZnSOD in the antioxidant defence of skin and skin derived cells was described several times in the literature. Punnonen et al. [43] demonstrated decreased activities of SOD and also of catalase in human keratinocytes following UVB irradiation and an impairment of the cellular defence system.

Takahashi et al. [34] reported that UVB irradiation of human keratinocytes decreased transiently Cu/ZnSOD and MnSOD activities and their protein levels, with subsequent recovery to the basal levels by 24 h. We did not follow SOD activities and analysed SOD protein and RNA levels as well as all the other different parameters 24 h after one single UVB exposure. So we are not able to comment on the time course of UVB-mediated alterations. UVB-induced increases in SOD protein levels were dose-dependent and the maximal effect was obtained at 50 mJ/cm^2. Sasaki et al. [44] described that Cu/ZnSOD and MnSOD protein levels changed in a different manner after UVB irradiation. Cu/ZnSOD increased immediately after UVB exposure and then gradually declined, whereas MnSOD decreased and recovered 24 h after irradiation. The recovery of MnSOD 24 h after UVB irradiation was found to be mediated through cytokines (IL-1α, TNFα) secreted from keratinocytes, while these cytokines had no effects on Cu/ZnSOD. From further investigations [45] using inhibitors of Cu/ZnSOD and MnSOD, it was derived that Cu/ZnSOD may play a primary protective role against UVB-induced injury of the human keratinocyte cell line HaCaT. Our results suggest that 24 h after an UVB dose of 50 mJ/cm^2 the protection against cytotoxity as a result of UVB irradiation may be more attributed to MnSOD than to Cu/ZnSOD.

Naderi-Hachtroudi et al. [46] and Lontz et al. [47] investigated the role of MnSOD as a primary antioxidant enzyme that crucially contributes to the

homeostasis of oxygen radicals within the mitochondria and participates in the control of ageing and tumour generation. They investigated MnSOD activities and messenger RNA levels in cultured human dermal fibroblasts, HaCaTs and primary keratinocytes at different times after repetitive UVB exposure and did not detect any increase in MnSOD activity in fibroblasts, HaCaTs and primary keratinocytes until 24 h after UVB irradiation. However, fibroblasts, incubated with supernatants from UVB-irradiated keratinocytes showed a significant increase in specific MnSOD messenger RNA and activity. Removal of cytokines, as IL-1α, IL-1β and TNFα from the supernatants led to a significant reduction of MnSOD mRNA in fibroblasts. It was concluded from these results that irradiation of keratinocytes with UVB induced a release of cytokines that amplified MnSOD activity in fibroblasts via a paracrine mechanism.

Lontz et al. [47] reported that the level of MnSOD mRNA expression was consistently higher in lesional psoriatic skin as compared to adjacent uninvolved skin and healthy control skin. Furthermore, they observed an increased m-RNA expression of IL-1β, TNFα and GM-CSF in lesional psoriatic skin. It seems more likely that cutaneous over-expression of MnSOD in psoriatic epidermis represents a protective cellular response evoked by cytokines released from inflammatory cells invading the diseased skin. In our studies we did not include investigations concerning cytokines, but we found strong indications for a primary role of MnSOD as adaptive antioxidant response to UVB-induced keratinocyte impairment.

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