Photoaging is Associated with Protein Oxidation in Human Skin *In Vivo*

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There is increasing evidence for the generation of reactive oxygen species in skin upon ultraviolet exposure, but little is known about their pathophysiologic relevance in human skin *in vivo*. We hypothesized that chronic and acute photodamage is mediated by depleted antioxidant enzyme expression and increased oxidative protein modifications. Biopsies from patients with histologically confirmed solar elastosis, from non-ultraviolet-exposed sites of age-matched controls, and from young subjects were analyzed. To evaluate the influence of acute ultraviolet exposures, buttock skin of 12 healthy subjects was irradiated repetitively on 10 d with a solar simulator and compared intraindividually to non-ultraviolet-treated contralateral sites. The antioxidant enzymes catalase, copper-zinc superoxide dismutase, and manganese superoxide dismutase were investigated by immunohistochemistry. Protein carbonyls were analyzed by immunohistochemical and immunoblotting techniques in human skin and in cell models. Whereas overall expression of antioxidant enzymes was very high in the epidermis, low baseline levels were found in the dermis. In photoaged skin, a significant depletion of antioxidant enzyme expression was observed within the stratum corneum and in the epidermis. Importantly, an accumulation of oxidatively modified proteins was found specifically within the upper dermis of photoaged skin. Upon acute ultraviolet exposure of healthy subjects, depleted catalase expression and increased protein oxidation were detected. Exposures of keratinocytes and fibroblasts to ultraviolet B, ultraviolet A, and H_2O_2 led to dose-dependent protein oxidation and thus confirmed *in vivo* results. In conclusion, the correlation between photodamage and protein oxidation was demonstrated for the first time, which hence may be a relevant pathophysiologic factor in photoaging.

Key words: catalase/oxidative stress/photoaging/photodamage/superoxide dismutase. J Invest Dermatol 118:618–625, 2002

Exposure of human skin to ultraviolet (UV) irradiation, the major environmental factor that affects the structure and function of skin, induces a spectrum of well-documented acute and chronic responses. Upon acute UV irradiation erythema, hyperproliferation, and desquamation are observed. Solar elastosis, the most prominent histologic feature of photoaging, is characterized by the degradation of collagen and accumulation of abnormal elastin in the superficial dermis. The mechanisms that lead to these acute and chronic effects are not yet fully understood, however (Yaar and Gilchrest, 1998; Wenk *et al*, 2001). Oxidative stress is discussed as one of the major contributors, but convincing evidence is still lacking.

As it is well known that UVB and UVA irradiation induce the formation of reactive oxygen species (ROS) in cutaneous tissues (Kitazawa *et al*, 1997; Scharffetter-Kochanek *et al*, 1997), numerous studies have focused on establishing baseline levels of enzymatic and nonenzymatic antioxidants in dermis, epidermis, and stratum corneum (SC) (Shindo *et al*, 1993, 1994a; Thiele *et al*, 1998a). The skin's enzymatic antioxidant defense includes copper-zinc superoxide dismutase (CuZnSOD), manganese SOD (MnSOD) and catalase (CAT). SOD converts superoxide anion into hydrogen peroxide, whereas CAT degrades hydrogen peroxide into water. These enzymes, which maintain a redox balance within cells, have been shown to be modulated by UVB and UVA irradiation *in vitro* (Shindo *et al*, 1994b; Sasaki *et al*, 1997; Leccia *et al*, 1998, 2001; Poswig *et al*, 1999) and *in vivo* in murine skin (Shindo *et al*, 1993; Okada *et al*, 1994). Recently, antioxidant enzyme activities and nonenzymic antioxidants were investigated in human aged and photoaged skin revealing a complex regulation of the antioxidant defense system during intrinsic aging and photoaging processes (Rhie *et al*, 2001).

In cases of increased ROS generation, antioxidant enzymes may be overwhelmed, resulting in oxidative stress and oxidative protein damage (Thiele *et al*, 1999a; Wenk *et al*, 2001).

Recently, photoaging and solar elastosis were correlated with different markers of oxidative stress including the accumulation of lipid peroxidation and glycation products (Jeanmaire *et al*, 2001; Tanaka *et al*, 2001). To date, however, there is a lack of knowledge on the involvement of protein oxidation in photoaging.

Proteins are known to be important targets for oxidative modifications. Oxygen radicals and other activated oxygen species cause modifications of the amino acids of proteins that frequently result in functional changes of structural or enzymatic proteins (Stadtman, 1992). Protein carbonyls may be formed either by

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Abbreviations: CAT, catalase; DNPH, 2,4-dinitrophenylhydrazine; SC, stratum corneum; SOD, superoxide dismutase.

oxidative cleavage of proteins or by direct oxidation of lysine, arginine, proline, and threonine residues (Levine *et al*, 1994). In addition, carbonyl groups may be introduced into proteins by reactions with aldehydes (4-hydroxy-2-nonenal, malondialdehyde) produced during lipid peroxidation or with reactive carbonyl derivatives generated as a consequence of the reaction of reducing sugars or their oxidation products with lysine residues of proteins (Berlett and Stadtman, 1997). Carbonyl residues can be readily detected by reaction with 2,4-dinitrophenylhydrazine (DNPH) generating dinitrophenylhydrazones. The determination of hydrazones by immunohistochemistry or immunoblotting can, in turn, be used as a measure of the oxidative state of cells and tissues (Levine *et al*, 1994; Smith *et al*, 1996). The detection of carbonyl groups in skin has therefore been used as a marker of reactive-oxygen-mediated protein oxidation (Thiele *et al*, 1998b, 1999a; Dimon-Gadal *et al*, 2000).

We hypothesized that chronic and acute UVA/UVB exposures deplete antioxidant enzymes and induce protein oxidation in human skin.

MATERIAL AND METHODS

Tissue samples Tissue samples from patients with photodamaged skin, defined as chronically UV-exposed sites with histologically confirmed solar elastosis, were obtained by biopsy from the face, neck, and back of the hand (age 69.9 ± 3.2 y, mean ± SD; $n = 12$; hereafter called ''photoaged skin''). Elastosis was evaluated using conventional histology as well as immunohistochemical stainings with an anti-elastin antibody (Linaris, Wertheim-Bettingen, Germany). Control biopsies were taken from non-UV-exposed sites of abdominal and lower back skin of age-matched subjects (age 68.8 ± 4.9 y, mean ± SD; $n = 12$; hereafter called ''intrinsically aged skin'') and from young controls without current or prior skin disease and aged less than 30 y ($n = 12$; hereafter called ''young control skin'').

To mimic typical vacation UV exposure behavior with extensive sun exposure within a few days, buttock skin of 12 healthy subjects (all caucasian, skin type II, female, 20–35 y) was irradiated with a solar simulator (''dermalight vario'' with filter h2, Dr Hoehnle, UV Technology, Planegg, Germany) on 10 d with daily increments. The initial irradiation dose was 0.5 minimal erythema dose, which was determined individually prior to treatment and ranged from 60 to 120 mJ per cm^2 UVB. The cumulative total dose reached after 10 d of irradiation was 2046 ± 574 mJ per cm^2 UVB (mean ± SD). The UV spectrum of the solar simulator was composed of < 0.2% UVC, 8.5% UVB, and 91.5% UVA. Non-UV-treated contralateral sites served as internal controls.

The human subject study was approved by the ethics committee of the Friedrich Schiller University Jena, and all subjects provided written informed consent.

Immunohistochemistry Skin biopsy samples were rapidly frozen and stored at –80°C. Tissue sections (6 μm) were cut with a cryostat at –25°C and stored at –80°C.

Expression of the antioxidant enzymes CuZnSOD, MnSOD, and CAT was analyzed using the streptavidin–biotin complex method. Briefly, tissue sections were washed with phosphate-buffered saline (PBS) for 5 min followed by incubation with blocking solution (TissueGnost, Merck, Darmstadt, Germany) for 30 min. Sections were incubated with primary antibody solution (concentration for anti-MnSOD and anti-CuZnSOD 1:250, and for anti-CAT 1:500) for 1 h at 37°C in humidified chambers. CuZnSOD and CAT antibodies were obtained from Calbiochem-Novabiochem, Bad Soden, Germany. MnSOD antibody was from Binding Site, Birmingham, U.K. Slides were then incubated with biotinylated secondary antibody, followed by avidin–peroxidase complex treatment according to the manufacturer's instructions (TissueGnost). Slides were submerged in 3-amino-9-ethylcarbazole substrate solution in 0.1 M acetate buffer (pH 5.2). Subsequently, slides were counterstained with hematoxylin and covered with mounting medium (Aquatex, Merck).

As a marker of ROS-mediated protein oxidation and oxidative stress, protein carbonyls were detected by the DNPH method using Oxyblot Protein Oxidation Detection Kit (Qbiogene, Heidelberg, Germany). After blocking treatment sections were incubated with DNPH solution (1:100) or control derivatization solution (1:100) for 1 h at 21°C. Sections were washed three times for 2 min each with PBS, and incubated with rabbit anti-dinitrophenylhydrazone antibody in PBS (1:250) for 1 h at 37°C. The following staining procedure was carried out as described for antioxidant enzyme expression.

Immunodetection of carbonylated proteins

Tissue extracts All biopsies were stored at –80°C. For separation of epidermis and dermis, adjacent subcutaneous tissue was removed and biopsies were incubated in 0.25% trypsin per 1 mM ethylenediamine tetraacetic acid (EDTA) solution for 18 h at 4°C. Tissue was washed three times for 2 min each with PBS. Then the epidermis was carefully removed from the dermis with tweezers and placed into a tube with the following lysis buffer: 50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 1% Triton X-100; pH 7.5; containing the protease inhibitors (purchased from Promega, Mannheim, Germany) vanadate (1 mM), phenylmethylsulfonyl fluoride (0.1 mM), benzamidine (1 mM), leupeptin (0.5 μg per ml), pepstatin (0.7 μg per ml), and aprotinin (0.4 μg per ml).

The dermis was placed upper side down on a plane surface and frozen at –20°C. Horizontal layers of the whole papillary dermis were cut with a cryostat and placed into a tube containing lysis buffer. Subsequently, epidermis and papillary dermis were homogenized separately in a dounce homogenizer while kept on ice. Tissue extracts were centrifuged at 16,000 × *g* for 20 min at 4°C and supernatants were harvested for protein determination (BCA assay, Pierce, Rockford, IL) and immunoblotting.

Isolation of human keratinocyte proteins after UVB treatment Human immortalized keratinocytes (HaCaTs) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U per ml penicillin and streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Prior to UVB irradiation experiments, cell culture medium was replaced by PBS. Confluent cells were exposed to single doses of 10, 20, and 30 mJ per cm^2 UVB (UVB medical bulb, absorption maximum 313 nm; Philips-Medicine, Hamburg, Germany). After irradiation, cells were incubated again in cell culture medium for 24 h. Cells were harvested by removing medium, washing three times with PBS, and freezing the cells at –80°C. Cells were lyzed with lysis buffer as described above. Extracts were centrifuged and protein determination was performed prior to immunoblotting to ensure equal loading.

Isolation of human fibroblast proteins after UVA treatment WS1 human embryogenic skin fibroblasts, American Type Culture Collection CRL 1502 obtained from Cell Lines Service (Heidelberg, Germany), were cultured in DMEM/HAM's F-12 medium supplemented with 10% FBS and 100 U per ml penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. For the UVA irradiation experiments, cell culture medium was replaced by PBS. Confluent cells were exposed to single doses of 10, 25, and 50 J per cm^2 UVA_1 (spectrum 340–440 nm, sunbank ''dermalight ultra A1''; Dr Hoehnle). After irradiation, cells were treated as described above for keratinocytes.

Oxidative treatment of human fibroblasts and keratinocytes Confluent cells of either HaCaTs or human WS1 fibroblasts were washed three times with PBS and subsequently cultured in serum-free medium with 1 mM H_2O_2 for 12 h. Harvesting was carried out as described for the UV irradiation experiments.

Western blotting Samples of 10 μg protein were subjected to DNPH derivatization as described elsewhere (Thiele *et al*, 1999a). Incubation of equal aliquots with a control solution lacking DNPH served as negative control. The dinitrophenylhydrazone-derivatized protein samples and controls were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto nitrocellulose membranes. The membranes were blocked with 5% dry milk in TBS-Tween and incubated with a rabbit anti-dinitrophenylhydrazone antibody in 1% dry milk in TBS-Tween (1:150) for 18 h at 4°C, and then with a peroxidase-coupled goat antirabbit IgG antibody (1:300) for 1 h at room temperature. The membranes were then treated with chemiluminescence reagents (ECL, Amersham-Pharmacia, Freiburg, Germany). Protein carbonylation was determined by densitometry of the bands on the autoradiograph. After developing, blots were stained with Coomassie Blue to confirm equal loading of proteins.

Statistical analysis After immunohistochemical staining sections were analyzed using densitometric image analysis. Three areas from SC, epidermis, and dermis of each section were selected and the staining for a total area was measured and quantified by Analysis 3.0 Software (Soft Imaging System, Muenster, Germany). Statistical analysis was carried out using Instat (Graphpad, San Diego, CA). ANOVA was performed to determine differences between groups. For immunodetection of oxidized

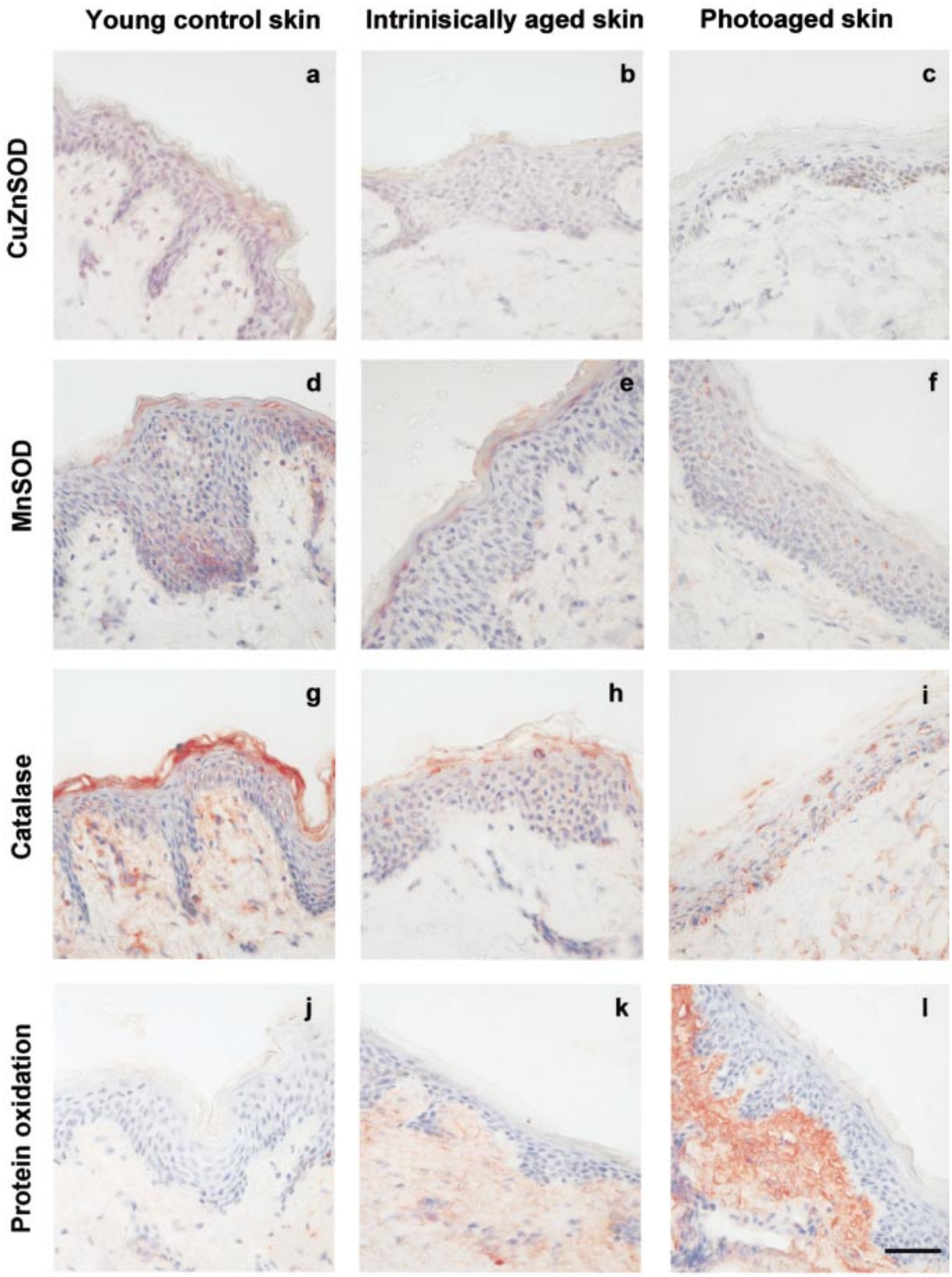

Figure 1. Immunohistochemical localization of antioxidant enzymes and protein oxidation in human skin. Immunostaining of CuZnSOD (*a–c*), MnSOD (*d–f*), and CAT (*g–i*) in frozen human skin sections. Identification of protein oxidation (*j–l*) by incubating the sections with DNPH and, subsequently, with an anti-dinitrophenylhydrazone antibody. Sections were prepared from young control skin (*a*, *d*, *g*, *j*), intrinsically aged skin (*b*, *e*, *h*, *k*), and photoaged skin (*c*, *f*, *i*, *l*). *Scale bar*: 50 μm.

proteins, Western blots were analyzed densitometrically using an ImageScanner and ImageMaster Totallab software (Amersham Pharmacia, Freiburg, Germany). All data are expressed as means ± standard error of the mean; *n* is given in each experiment.

RESULTS

Antioxidant enzyme expression is depleted in chronically UV-exposed human skin In all cases, the epidermis displayed immunoreactivity for the three investigated antioxidant enzymes within the cytosol of keratinocytes, whereas only weak expression was found in the dermis (**Fig 1*a–i***). This confirms earlier reported work on the level of enzyme activity (Shindo *et al*, 1994a). Intriguingly, the SC showed clearly enhanced CuZnSOD, MnSOD, and CAT immunostaining. Photoaged skin revealed significantly lower expression for CuZnSOD in the epidermis ($p < 0.01$) and SC ($p < 0.001$) compared to control skin (**Fig 2*a***).

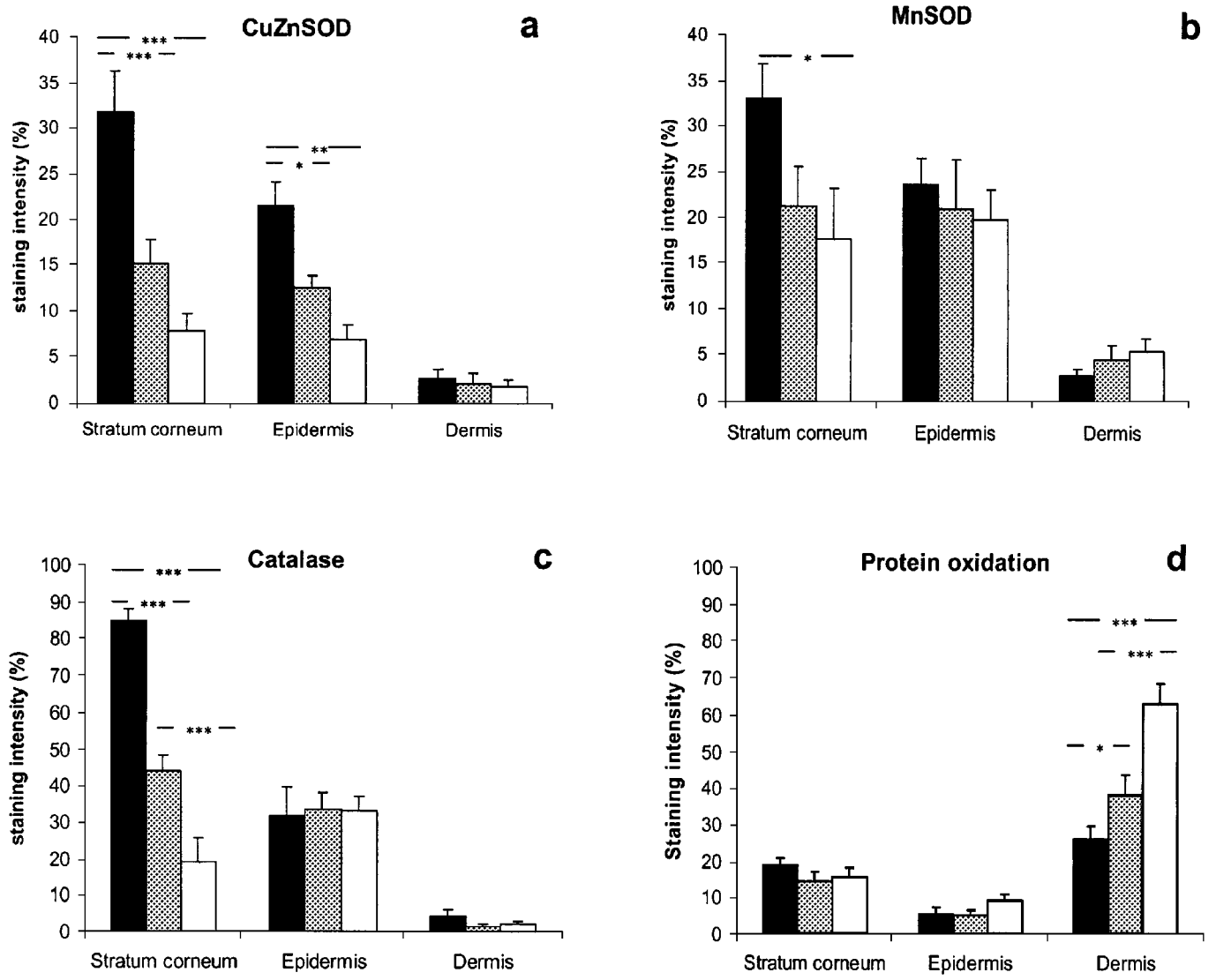

Figure 2. Depleted antioxidant enzyme expression and accumulated protein oxidation in photoaged human skin. Densitometric analysis of staining intensity by image analysis software. Scanning of sections stained for CuZnSOD (*a*), MnSOD (*b*), CAT (*c*), and protein oxidation (*d*). *Black bars*, young control skin; *grey bars*, intrinsically aged skin; *white bars*, photoaged skin. *p < 0.05, **p < 0.01, ***p < 0.001. Mean ± SEM, $n = 12$.

MnSOD was markedly depleted in the SC of photoaged skin ($p < 0.05$; **Fig 2*b***). For CAT a significant reduction of staining intensity was found within the SC compared to young healthy controls ($p < 0.001$) and age-matched controls ($p < 0.001$; **Fig 2*c***).

Oxidatively modified proteins accumulate in photoaged skin Most remarkably, a significant increase of protein carbonyls was detected specifically within the upper dermis of chronically UV-exposed biopsies compared to intrinsically aged skin ($p < 0.001$) and young controls ($p < 0.001$; **Figs 1*j–l*, 2*d***), indicating oxidative protein damage in this area. The epidermis and SC of all subjects revealed weak staining intensity.

CAT is depleted in the SC of acutely UV-exposed human skin After repetitive UV exposures for 10 d in young healthy subjects, CAT was strongly depleted within the SC ($p < 0.001$; **Figs 3*a*, *b*, 4*a***). CAT expression within epidermis and dermis was lowered as well, but not at significant levels.

Acute UV exposure induces protein oxidation within SC and dermis Intriguingly, following acute UV exposures, there was a significantly higher level of protein oxidation within the SC, especially in the outermost layers, as well as within the upper part of the dermis (**Figs 3*c*, *d*, 4*b***). Epidermal tissue was less affected by protein carbonylation, however, suggesting more effective antioxidative protection mechanisms.

Chronic UV exposure induces accumulation of protein oxidation in human skin – investigation by immunoblotting In tissue extracts prepared from separated epidermal and dermal human cutaneous tissue dramatically higher levels of protein oxidation were detectable in the dermis according to the immunohistochemical results. The epidermis of all groups contained lower levels of protein carbonyls (**Fig 5*a***). Photoaged dermis revealed the highest levels of protein carbonyls ($p < 0.01$; **Fig 5*b***). Experiments were carried out using tissue samples of at least four donor samples for each group; densitometric analysis is presented in **Fig 5(*b*)**.

Protein oxidation increases in human fibroblasts and keratinocytes after UVA/UVB exposure and hydrogen peroxide treatment Human fibroblasts revealed susceptibility to UVA-induced protein oxidation. Subcytotoxic doses from 10 to 50 J per cm^2 induced a dose-dependent increase of protein carbonyls (**Fig 6*a***). H_2O_2, a ROS generated upon UVA and UVB exposure, caused significant protein carbonylation (**Fig 6*b***), indicating induceability of protein oxidation by ROS. Similarly,

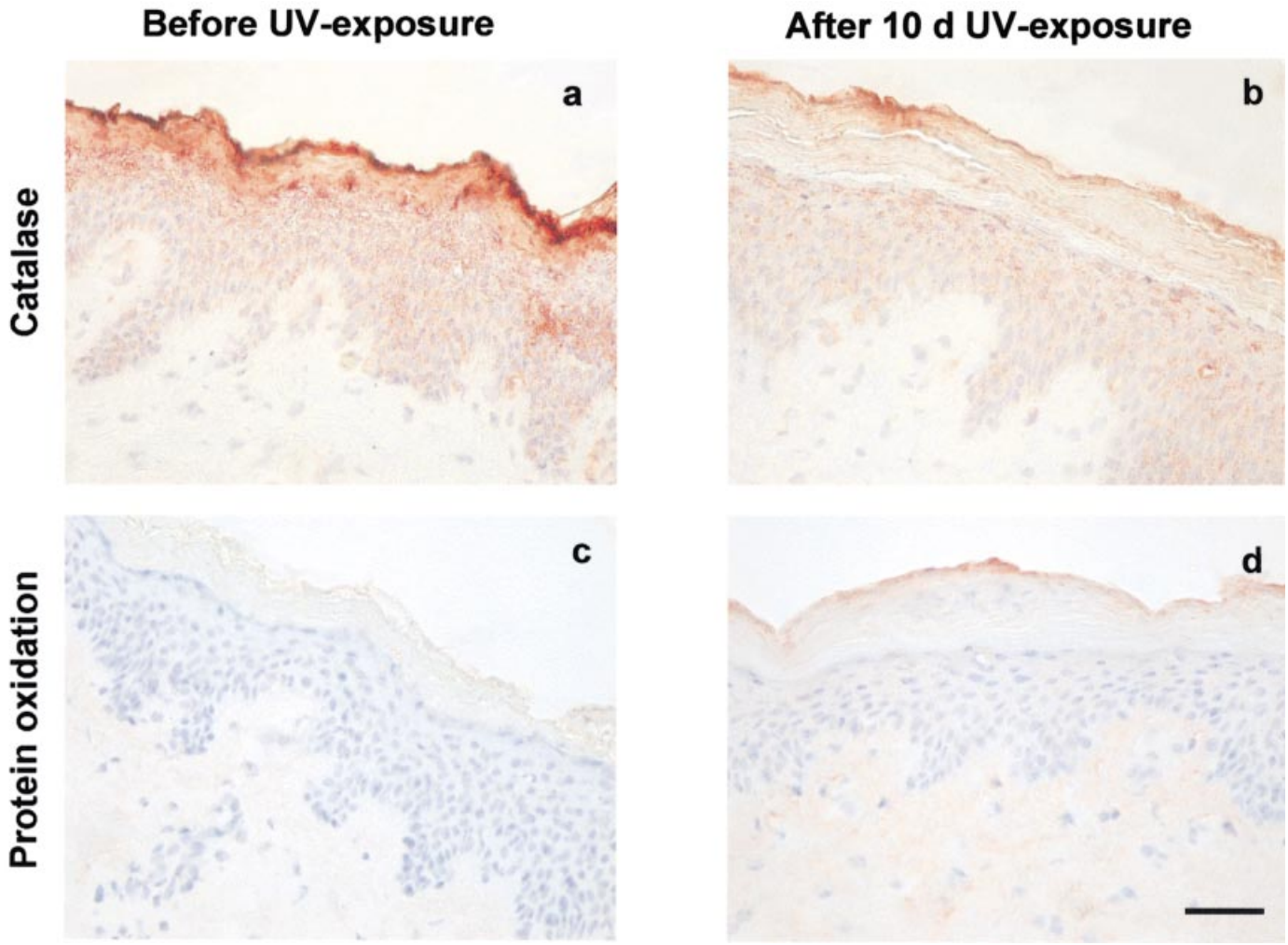

Figure 3. Immunohistochemical localization of CAT and protein oxidation in human skin upon repetitive solar simulated UV exposures. Immunostaining of CAT (*a*, *b*) in frozen human skin sections. Identification of protein oxidation (*c*, *d*) by incubating the sections with DNPH and, subsequently, with an anti-dinitrophenylhydrazone antibody. Sections were prepared from buttock skin of human subjects (*a*, *c*) before UV exposure and (*b*, *d*) after a 10 d repetitive UV irradiation protocol using a solar simulator (''dermalight vario'' with filter h2, Dr Hoehnle). *Scale bar*: 50 μm.

protein oxidation occurs in keratinocytes upon exposure to UVB (**Fig 7*a***) and H_2O_2 (**Fig 7*b***).

DISCUSSION

In this study, we provide direct evidence that (i) human dermal skin is poorly equipped with antioxidant enzymes, (ii) antioxidant enzyme levels in epidermis and SC are depleted by chronic and acute UV exposures, and (iii) UV exposure induces oxidative protein damage. Lowered antioxidant enzyme expression in photodamaged human skin compared to age-matched and young controls is associated with higher levels of protein carbonylation and oxidative stress. Importantly, the correlation between photoaging and protein oxidation was demonstrated for the first time *in vivo*.

Previous studies focusing on the activity of antioxidant enzymes in human skin have found many-fold higher activities within the epidermis than in the dermis (Shindo *et al*, 1994a; Thiele *et al*, 2000). Accordingly, in this study, highest protein levels were found for all investigated enzymes within the epidermis. Strong expression of CAT, CuZnSOD, and MnSOD was found within the SC of healthy subjects (**Fig 1*a***, ***d***, ***g***). Whereas nonenzymatic antioxidants have been studied extensively in the SC and skin surface lipids (Thiele *et al*, 1998a, 1999b; Weber *et al*, 1999), little information is available on SC antioxidant enzymes; recently, high activities of CAT and SOD have been reported.[1] Alterations of antioxidant enzymes have been reported to be involved in aging and carcinogenesis (Kobayashi *et al*, 1991; Yan *et al*, 1998; Davies, 2000). SOD protects human keratinocytes against UVB-induced injury (Sasaki *et al*, 1997, 2000). Fibroblasts develop an adaptive antioxidant response of antioxidant enzymes following UVA exposure (Shindo and Hashimoto, 1997; Poswig *et al*, 1999; Meewes *et al*, 2001). Previously, it was proposed by Leccia *et al* that an upregulation of the antioxidant defense system may function as a SOS-like response in irradiated human skin (Leccia *et al*, 2001). It was demonstrated in human skin that single exposures of solar-simulated UV resulted in a transient reduction of SOD activity, which was followed by increased amounts of conjugated diene double bonds, an index for lipid peroxidation (Punnonen *et al*, 1991a). After chronic UVB irradiation, however, an induction of epidermal SOD activity was observed (Punnonen *et al*, 1995). This study demonstrates a significant depletion of antioxidant enzymes

[1]Declercq L, Hellemans L, Corstjens H, Pelle E, Mammone T, Marenus K, Maes D: Both direct photodeactivation and oxidative damage can contribute to the seasonal loss of catalase activity in human stratum corneum. *J Invest Dermatol* 117: 500, 2001 (abstr.)

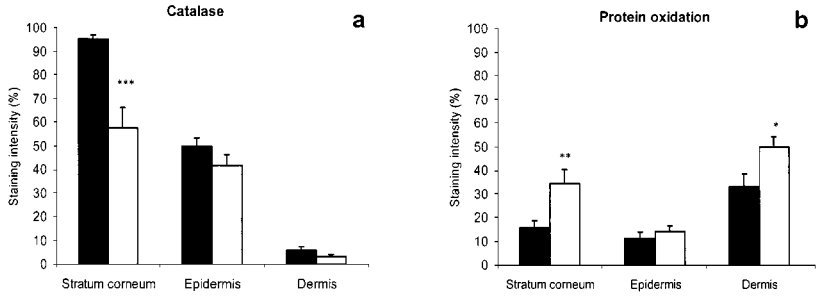

Figure 4. Depleted CAT expression and accumulated protein oxidation in human skin upon repetitive solar simulated UV exposures. Densitometric analysis of staining intensity by image analysis software. Scanning of sections stained for CAT (*a*) and protein oxidation (*b*). *Black bars*, before UV exposure; *white bars*, after 10 d UV exposure. *p < 0.05, **p < 0.01, ***p < 0.001. Mean ± SEM, *n* = 12.

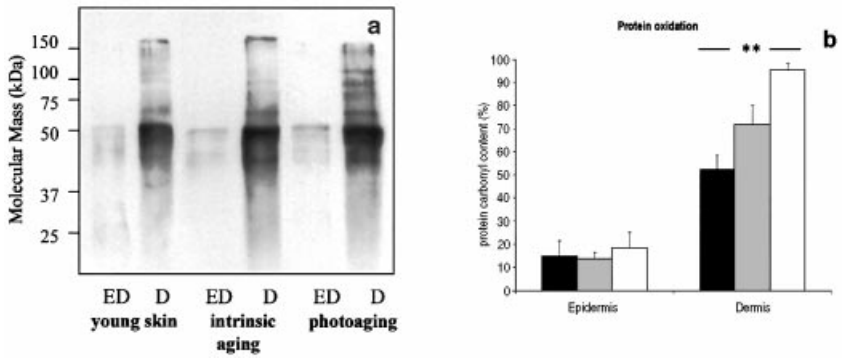

Figure 5. Increased protein oxidation in photoaged human skin. Immunoblot analysis of carbonyl residues in protein extracts from epidermal (ED) and dermal (D) tissue of young control skin, intrinsically aged skin, and photaged skin (*a*). 10 μg of protein extracts were incubated with DNPH, subsequently electrophoresed by SDS-PAGE, blotted onto a nitrocellulose membrane, and incubated with polyclonal rabbit anti-dinitrophenylhydrazone antibody. Densitometric analysis of total carbonylated proteins was performed (*b*). *Black bars*, young control skin; *grey bars*, intrinsically aged skin; *white bars*, photoaged skin. **p < 0.01. Mean ± SEM, *n* = 4.

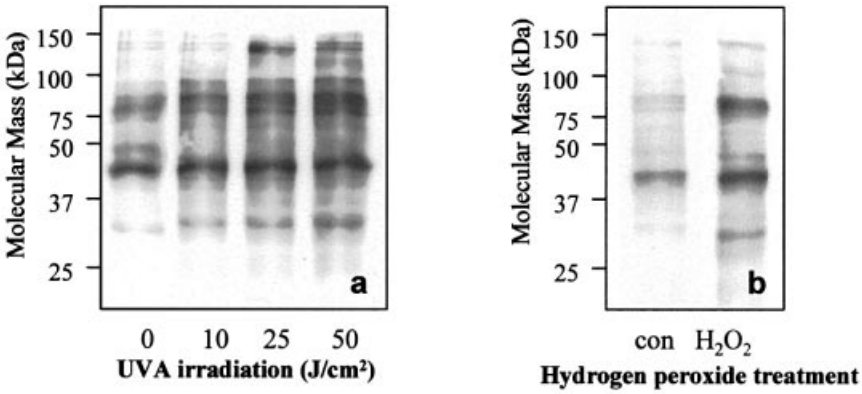

Figure 6. UVA irradiation and H_2O_2 treatment increased protein oxidation in human fibroblasts. Immunoblot analysis of carbonyl residues in protein extracts from (*a*) UVA-irradiated (spectrum 340–440 nm, UVA_1 sunbank "dermalight ultra A1", Dr Hoehnle) and (*b*) hydrogen-peroxide-treated (1 mM for 12 h in serum-free medium) human fibroblasts (human embryogenic skin fibroblasts, American Type Culture Collection, CRL 1502). 10 μg of protein extracts were incubated with DNPH, subsequently electrophoresed by SDS-PAGE, blotted onto a nitrocellulose membrane, and incubated with polyclonal rabbit anti-dinitrophenylhydrazone antibody.

depending on age and solar exposure (**Figs 1*a–i*, 2*a–c***). In chronically UV-exposed human skin revealing typical features of photoaging, a significantly depleted expression was found for all antioxidant enzymes within the SC compared to intrinsically aged skin as well as to young controls. Importantly, for CuZnSOD a significant depletion was observed in the epidermis. Dermal levels of antioxidant enzymes were not significantly reduced; however, baseline expressions were already up to 10-fold lower than in epidermal layers (**Fig 2*a–c***). The low ratio of cells to extracellular tissue within the dermis may be responsible for the low expression of antioxidant enzymes and thus contribute to the high susceptibility of dermal proteins to oxidative damage. Not only in photoaged skin but, to a lesser extent, also in intrinsically aged skin a significant depletion of CuZnSOD and CAT was found within the SC and epidermis. Generally, aging is believed to be associated with impaired antioxidant capacity of tissues (Ames *et al*, 1993; Yan *et al*, 1999).

Furthermore, after a 10 d repetitive UV exposure protocol in young healthy human subjects, a significant depletion of CAT was observed within the SC (**Figs 3*a*, *b*, 4*a***). Earlier studies have demonstrated a photo-susceptibility of CAT in various cell and animal experiments (Punnonen *et al*, 1991a, b; Shindo *et al*, 1994b; Shindo and Hashimoto, 1997).

Oxidative stress has been related to photoaging as well as to photocarcinogenesis (Scharffetter-Kochanek *et al*, 1997). Recently, photoaging and actinic elastosis were correlated with the accumulation of lipid peroxidation and glycation products (Jeanmaire *et al*, 2001; Tanaka *et al*, 2001). A recent study has found increased levels of protein oxidation in keratinocytes from aged donors (Petropoulos *et al*, 2000).

In this study, protein oxidation was shown to be significantly increased in human photoaged skin. Protein carbonyl formation was most pronounced within the papillary dermis, where major pathologic changes of photoaging occur (**Figs 1*j–l*, 2*d***). It was demonstrated in previous histologic and biochemical studies on the nature of the accumulated fibers that altered elastin is the primary component of solar elastosis (Chen *et al*, 1986; Hirose and Kligman, 1988). The mechanism of the formation of solar elastosis in photodamaged skin is still unclear, however. Oxidatively modified proteins colocalizing with solar elastosis in chronically UV-exposed

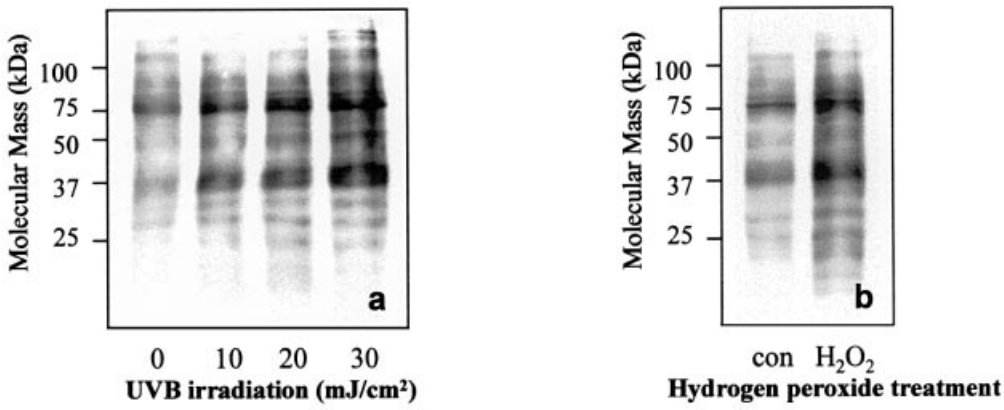

Figure 7. UVB irradiation and H_2O_2 treatment increased protein oxidation in human keratinocytes. Immunoblot analysis of carbonyl residues in protein extracts from (*a*) UVB-irradiated (UVB medical bulb, absorption maximum 313 nm; Philips-Medicine) and (*b*) hydrogen-peroxide-treated (1 mM for 12 h in serum-free medium) human immortalized keratinocytes (HaCaT). 10 μg of protein extracts were incubated with DNPH, subsequently electrophoresed by SDS-PAGE, blotted onto a nitrocellulose membrane, and incubated with polyclonal rabbit anti-dinitrophenylhydrazone antibody.

skin could contribute to the accumulation of structurally and functionally impaired proteins such as elastin, collagen, and matrix metalloproteinases (Tanaka *et al*, 2001).

Protein carbonyls are considered chemically stable compounds making them a suitable target for laboratory measurement (Levine *et al*, 2000). An increase in the amount of oxidized protein can be explained by either a stress- or an age-dependent increase in the rate of protein oxidation or a decrease in the ability to degrade oxidized proteins (Stadtman, 1992). Protein carbonyls are readily generated under experimental conditions. We have previously demonstrated UV-induced protein oxidation in human skin as early as 30 min after exposure (Thiele *et al*, 1999a). The fact that a dramatic increase of protein oxidation was found in photoaged skin suggests that severely oxidized proteins accumulate in human dermis, as the latter are extensively cross-linked and aggregated, and thus are poor substrates for proteasome degradation (Grune *et al*, 1997). To investigate whether UV exposure is indeed capable of inducing protein oxidation *in vivo*, a human subject study mimicking extensive solar holiday exposure was performed. Intriguingly, we observed a significant increase of oxidatively modified proteins within the papillary dermis as well as in the outermost layers of the SC (**Fig 3*c*, *d***), the latter corresponding to earlier reported findings with a DNPH immunoblot assay (Thiele *et al*, 1999a). In addition, our results indicate that living epidermal layers are less affected by protein oxidation than dermal layers (**Fig 3*a*, *b***), which is probably due to the far greater antioxidant capacity of the epidermis (Thiele *et al*, 2000). The high susceptibility of dermal tissue to protein oxidation suggests that cumulative exposures may lead to long-term carbonylation of dermal proteins and, subsequently, contribute to the development of solar elastosis and premature aging.

To confirm our immunohistochemistry results and to investigate whether protein oxidation can be induced *in vitro* in cutaneous cells by UVA/UVB irradiation we employed an immunoblot assay in tissue extracts from human skin as well as in UVA-irradiated fibroblasts and UVB-irradiated keratinocytes (**Figs 5–7**). Protein oxidation was significantly higher in dermal than in epidermal tissue of chronically UV-exposed subjects (**Fig 5**), confirming immunohistochemical results. It remains the subject of further investigation, however, what the exact nature of these protein bands is. We have previously reported increased keratin oxidation in UV-exposed human SC using the DNPH method (Thiele *et al*, 1999a). Thus, the increased 50 kDa bands in epidermal extracts may also point to an involvement of keratins. The even more pronounced bands of oxidized proteins in dermal tissue extracts from photoaged skin (**Fig 5*a***) may involve collagen and other extracellular matrix proteins, which represent the most altered proteins in photoaging (Wenk *et al*, 2001). This needs to be confirmed, however, by future studies involving protein analysis, e.g., MALDI-MS.

UVA irradiation increased protein oxidation in human fibroblasts in a dose-dependent manner (**Fig 6*a***). Similarly, UVB irradiation of human keratinocytes at subcytotoxic doses also led to protein oxidation (**Fig 7*a***). Moreover, hydrogen peroxide, one of the major ROS accounting for the deleterious effects of UVA irradation (Vile and Tyrrell, 1995; Hockberger *et al*, 1999), and which was found to be generated intracellularly in keratinocytes after UVB irradiation (Peus *et al*, 1998), was able to induce protein oxidation in fibroblasts (**Fig 6*b***) and keratinocytes (**Fig 7*b***). Thus, both UVB and UVA exposures may lead to oxidative protein damage in human skin via formation of H_2O_2. This process, however, may also involve other ROS generated upon exposure to UVA and UVB such as singlet oxygen (Berneburg *et al*, 1999).

In conclusion, it was demonstrated for the first time *in vivo* that depleted antioxidant enzyme expression in photodamaged skin is associated with higher levels of protein oxidation. Conclusive evidence is presented for a link between solar elastosis and protein oxidative damage in human skin. Furthermore, oxidative stress is likely to be involved in the perturbation of the skin barrier following acute UV exposures. These results provide a rationale for the development of efficient antioxidant strategies to prevent photoaging and acute photodamage in skin. Although sunscreens are indispensable in the prevention of skin photodamage, antioxidants in combination with sunscreens seem to be highly effective adjuncts increasing the safety and the efficacy of photoprotective products.

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