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UVA-Induced Modification of Catalase Charge Properties in the Epidermis Is Correlated with the Skin Phototype

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The harmful effects of UVA radiation (320–400 nm) on the skin have been related to the generation of reactive oxygen species. Pheomelanin, the pigment characteristic of fair-skinned individuals, amplifies these effects. *In vitro*, in the presence of photosensitizing agents, UVA light produces singlet oxygen, which reacts with several targets. We have investigated a possible correlation between melanin-type and the antioxidant defense system after UV, focusing on the activities of superoxide dismutase and catalase, which correlated with the phototype of epidermal reconstructs. UVA was more effective than UVB in damaging these enzymatic activities, especially catalase. Furthermore, UVA irradiation induced a free-radical-mediated damage in the cells, leading to an oxidation of cell proteins. On catalase, synthetic pheomelanin amplified this effect on specific targets, such as residues of tryptophan and methionine. UVA irradiation of low phototype reconstructed epidermis and of U937 through synthetic pheomelanin induced a modification in the electrophoretic properties of native catalase, which was counteracted by histidine, a quencher of singlet oxygen. These results demonstrate that pheomelanin could act as a photosensitizing agent, following UVA irradiation, inducing charge modifications of native catalase, by a mechanism involving singlet oxygen or its downstream products.

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INTRODUCTION

UV irradiation induces biological modifications in cells (Afag and Mukhtar, 2001). UVB (290–320 nm) causes direct DNA damage, measurable as pyrimidine dimer formation (Wey *et al.*, 2002), which, if not correctly repaired, can induce an apoptotic program (D'Errico *et al.*, 2003) or transformation processes (Bernerd *et al.*, 2001). Biological effects induced by UVA (320–400 nm) are mainly related to the generation of reactive oxygen species (ROS) (Tobi *et al.*, 2002). ROS react with cellular and extracellular components (Damiani *et al.*, 2002; Tobi *et al.*, 2002; Martinez *et al.*, 2003) and induce cytotoxicity, apoptosis, mutations, and carcinogenesis (Darr and Fridovich, 1994; Damiani *et al.*, 2002; Tobi *et al.*, 2002).

Pigmentation has an important role in reducing UV-induced damage (Kadekaro *et al.*, 2003). Two types of melanin, both capable of absorbing UV light, are physiologically present in human skin. Eumelanin, prevalent in the skin of individuals with black or brown hair, acts as a filter against UV and also possesses scavenger properties (Kadekaro *et al.*, 2003). Pheomelanin, predominantly present in individuals with pale skin and red or fair hair, has been proved to be a photosensitizer, as it increases the generation of ROS after UV (Menon *et al.*, 1983; Ranadive *et al.*, 1986; Prota, 1997). Low phototype individuals are very susceptible to the harmful effects of UV and possess an increased risk of developing skin cancer (Briollais *et al.*, 2000).

Considering that UVA acts mainly through the generation of ROS, physiological antioxidants play a crucial role in the photoprotection of the skin (Briganti and Picardo, 2003). The efficient removal of ROS is accomplished by antioxidant enzymes and small radical trapping molecules. These systems act in a concerted manner to minimize the damage caused by ROS within the cell. The initial free radical scavenging machinery involves superoxide dismutase (SOD), which catalyzes the dismutation of two molecules of the superoxide radical anion into hydrogen peroxide and diatomic oxygen. Then, hydrogen peroxide is converted by catalase (Cat) and

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Abbreviations: Cat, catalase; CDM, cysteinyl-DOPA-melanin; DM, DOPA-melanin; DNPH, 2,4-dinitrophenylhydrazine; His, histidine; H_2O_2, hydrogen peroxide; 5-OH-Trp, L-5-hydroxytryptophan; Met, methionine; Met-SO L-methionine su lfoxide; PBS, phosphate-buffered saline; REK, reconstructed epidermis with only keratinocytes; REMH, reconstructed epidermis with keratinocytes and melanocytes from high phototype skin; REML, reconstructed epidermis with keratinocytes and melanocytes from low phototype skin; ROS, reactive oxygen species; SOD, superoxide dismutase; Trp L-tryptophan

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peroxidases into water (Steenvoorden and van Henegouwen, 1997). Skin, when irradiated with UVA, is depleted of antioxidants (Sanders *et al.*, 2004), among which Cat is the most sensitive. This enzyme is very susceptible to peroxidizing agents, such as its own substrate, and recovers slowly after the damage (Shindo *et al.*, 1994; Shindo and Hashimoto, 1997). Thus, Cat may be monitored as a useful marker of both acute and chronic oxidative stress. We have previously demonstrated that Cat activity, in primary cultures of melanocytes from low phototype individuals, is significantly lower compared to those from high phototype donors (Picardo *et al.*, 1999). Similar results were obtained in *ex vivo* epidermal reconstructs (Bessou-Touya *et al.*, 1998; Cario-André *et al.*, 1999), suggesting that the low Cat activity could represent, in association with the low filter effect exerted by pheomelanin, an adjunctive risk factor for the development of sun-induced damage in subjects with low phototype.

Recently, it has been reported that, after photosensitization reactions and the release of singlet oxygen, the prosthetic group of Cat can be oxidized. The oxidative modification of Cat was also associated with a visible change in the electrophoretic mobility of the protein (Lledías *et al.*, 1998; Lledías and Hansberg, 1999, 2000). Because oxidation of heme *per se* does not change protein charge, the modification of one or a few charged amino acid residues, such as L-tryptophan (Trp), Tyrosine (Tyr) , histidine (His), methionine (Met), and Cystein, which are targets of singlet oxygen, could possibly explain the elecrophoretic shift observed (Díaz *et al.*, 2005). Charge-modified Cat is structurally different from the nonoxidized one, although it retains most of the stability and catalytic efficiency (Lledías *et al.*, 1998; Lledías and Hansberg, 1999, 2000). Accordingly, it has been speculated that preservation of functional properties of Cat has been selected through evolution to assure an active enzyme under disparate oxidative stress conditions (Díaz *et al.*, 2005).

The aim of the present work was to evaluate Cat modifications, induced by UV, as alterations of either the activity or the charge, in correlation to the phototype. *Ex vivo* epidermal reconstructs from low or high phototype subjects and a nonmelanotic cell line irradiated through synthetic melanins were employed. The results confirm the correlation between phototype and antioxidant enzyme activity and demonstrate that, following UVA, pheomelanin could act as a photosensitizing agent in mediating the release of singlet oxygen, responsible for Cat charge modifications.

RESULTS

Antioxidant enzyme activities, in epidermal reconstructs, correlate with the phototype of melanocytes, and UVA is more effective than UVB in impairing their functions

Enzymatic activities of Cat and SOD were evaluated, before and after UV irradiation, in epidermal reconstructs made with keratinocytes alone (REK) or with keratinocytes and melanocytes from low (REML) or high (REMH) phototype skin (Figure 1). Basal activity of Cat, in REML (231.81±30.76 U/mg protein), was significantly lower ($P<0.05$) compared

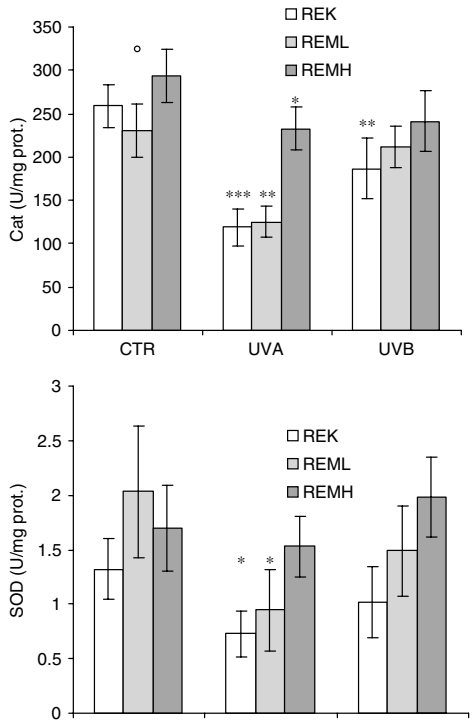

Figure 1. UVA is more effective than UVB in inducing modifications of enzymatic antioxidant activities in reconstructed epidermis and the phototype of melanocytes has a role in the photoprotection. Reconstructed epidermis was irradiated with UVA or UVB. The epidermis was homogenated and enzymatic activities were determined on the supernatants by spectrophotometrical techniques. REK = reconstructed epidermis with only keratinocytes; REML = reconstructed epidermis with melanocytes and keratinocytes from low phototype subjects; REMH = reconstructed epidermis with melanocytes and keratinocytes from high phototype subjects; CTR = not-irradiated samples; UVA = reconstructed epidermis irradiated with 8 J/cm^2 UVA; UVB = reconstructed epidermis irradiated with 0.15 J/cm^2 UVB. Each value is the mean±SD of three determinations in duplicate. °$P<0.05$ in not-irradiated REML with respect to not-irradiated REMH; *$P<0.05$ in UV-treated reconstructed epidermis *versus* not-irradiated ones; **$P<0.005$ in UV-treated reconstructed epidermis *versus* not-irradiated ones; ***$P<0.001$ in UV-treated reconstructed epidermis *versus* non-irradiated ones.

to that in REMH (293.69±30.41 U/mg prot.), but not statistically different from that in REK (258.88±25.4 U/mg prot.). The basal activity of SOD was comparable in all epidermal reconstructs (REK: 1.32±0.27 U/mg prot.; REML: 2.02±1.19 U/mg prot.; REMH: 1.69±0.39 U/mg prot.). UVA had a deleterious effect on Cat, producing a significant decrease (−54%) of the enzymatic activity in REK (118.71±22.15 U/mg prot.; $P<0.001$). Such a decrease was only marginally protected by the presence of low phototype melanocytes (−46%) in REML (125.41±18.64 U/mg prot., $P<0.005$) and significantly shielded by high phototype melanocytes (−21%) in REMH (232.42±25.91 U/mg prot., $P<0.05$). After UVA, a significant decrease in SOD activity was also observed in REK (0.72±0.21 U/mg prot., $P<0.05$) and in REML (0.94±0.37 U/mg prot., $P<0.05$), but not in REMH (1.53±0.28 U/mg prot.), suggesting a protective effect of high phototype melanocytes. After UVB, the decrease of Cat activity was significant in REK (186.35±36.5 U/mg prot.,

$P<0.005$) and this alteration was reduced by the presence of melanocytes both in REML (211.95±23.41 U/mg prot.) and in REMH (240.24±19.97 U/mg prot.). UVB did not modify SOD activity significantly (REK: 1.01±0.31 U/mg prot.; REML: 1.48±0.42 U/mg prot.; REMH: 1.98±0.37 U/mg prot.). These results confirm that the enzymatic antioxidant defense system, in reconstructed epidermis, is correlated with the phototype of melanocytes and demonstrate that UVA is more effective than UVB in impairing this system.

In reconstructed epidermis low phototype melanocytes influence charge properties of Cat after UVA irradiation

Melanocytes from light- or dark-skinned people are characterized by a different concentration of eu- and pheomelanin, with the latter pigment significantly prevalent in melanocytes from light-skinned subjects (Thody *et al.*, 1991). Considering that UVA irradiation, in the presence of other photosensitizing agents, leads to singlet oxygen release (Martinez *et al.*, 2003), which in turn mediates modification of Cat charge (Lledías *et al.*, 1998), we evaluated the role of the melanocyte phototype in the modification of Cat electrophoretic properties in response to UV. For this purpose, a zymographic analysis of tetrameric native Cat was performed in nonirradiated and irradiated REK, REML, and REMH (Figure 2). UVA or UVB treatment did not cause any detectable change in Cat electrophoretic mobility in REK. Even in REMH, neither UVA nor UVB induced Cat charge modifications. By contrast, in REML, UVA irradiation induced a change in Cat electrophoretic mobility, giving rise to a shift corresponding to a more negatively charged protein, whereas UVB exposure did not produce any significant charge modification of the enzyme. These results suggest that pheomelanin, irradiated with UVA, might act as a photosensitizing agent promoting Cat modification to a more acidic isoform.

Cysteinyl-DM (CDM) acts as a photosensitizing agent in the induction of charge modification of Cat after UVA through the release of singlet oxygen

A nonmelanotic cell line (U937) expressing a high Cat activity was irradiated through CDM or DM, separated from the cells by a filter (Figure 3). Irradiation with UVA through CDM produced a charge modification of native Cat not observed in cells treated with UVA alone (Figure 4a). After irradiation through DM, no modification of Cat charge was observed (Figure 4b). In the same model, UVB irradiation, even in the presence of CDM (Figure 4c) or DM (Figure 4d), did not produce any effect on Cat electrophoretic mobility. His in the cell suspension, quenching singlet oxygen, abrogated the effect of UVA through CDM on Cat charge modifications (Figure 4a). In order to investigate whether the Cat charge modification was associated with the alteration of the enzymatic activity, we evaluated this parameter on U937, irradiated or not with UVA, or with UVA through CDM in the presence or not of His (Figure 3). Neither CDM alone placed on the filter (59.27±6.51 U/mg prot.) nor His in the cell suspension (59.23±5.99 U/mg prot.) modified Cat activity compared to the control (60.67±6.02 U/mg prot.). UVA irradiation induced a significant decrease of Cat activity (47.77±3.95 U/mg prot., $P<0.05$) and UVA through CDM did not stress this effect (45.67±4.65 U/mg prot.). The decrease of Cat activity, induced by UVA alone, was not counteracted by the presence of His (46.31±4.01 U/mg prot.). In U937 irradiated with UVA through CDM, His was able to counteract Cat charge modification (Figure 4a) but not its activity decrement (47.24±4.57 U/mg prot.). These results demonstrate that CDM acts as a photosensitizing agent, after UVA, and that the induced Cat charge modification was mediated by singlet oxygen, the only free radical species capable diffusing through air (Dahl *et al.*, 1988). The evidence that singlet oxygen is able to modify the charge

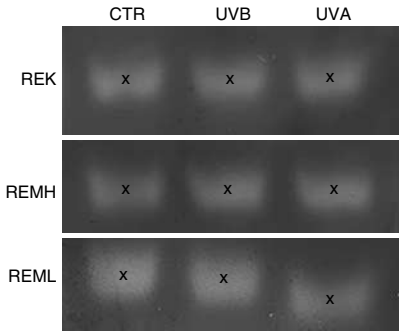

Figure 2. After UVA low phototype melanocytes in reconstructed epidermis influence charge properties of Cat. Reconstructed epidermis, REK, REML, and REMH were irradiated with UVA or UVB. Thereafter, samples were processed for zymography. One unit of Cat activity was loaded in each electrophoretic lane and the run was performed on undenaturing native acrylamide PAGE, as described in Materials and methods. The spots in the gel represent tetrameric Cat in its native three-dimensional folding from differently treated and untreated samples. The "x" symbol identifies the center of each Cat spot. REK = reconstructed epidermis with only keratinocytes; REML = reconstructed epidermis with melanocytes and keratinocytes from low phototype subjects; REMH = reconstructed epidermis with melanocytes and keratinocytes from high phototype subjects; CTR = non-irradiated samples; UVA = reconstructed epidermis irradiated with 8 J/cm^2 UVA; UVB = reconstructed epidermis irradiated with 0.15 J/cm^2 UVB.

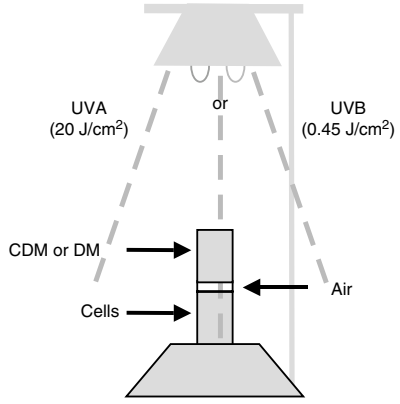

Figure 3. Irradiation of U937 cells was performed through CDM or DM. Synthetic melanins, CDM or DM, were put onto a filter, allowing the cells to be separated from the synthetic pigment by an air space.

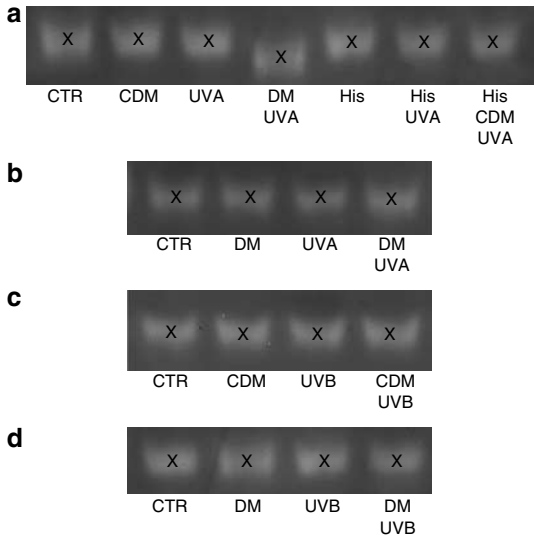

Figure 4. Cysteinyl-DOPA-melanin but not DOPA-melanin acts as a photosensitizing agent in the induction of charge modification of Cat after UVA and His is able to counteract the phenomenon. U937 cells were irradiated with UVA (20 J/cm^2) or UVB (0.45 J/cm^2) through CDM (4 μg/ml) or DM (4 μg/ml) in the presence or absence of His (30 mM) in the cell suspension (see Figure 3). Thereafter, cells were processed for zymographic analysis. (**a**) CDM acts as a photosensitizing agent in the induction of charge modification of Cat in U937 after UVA, and His, a quencher of singlet oxygen, is able to counteract the phenomenon. CTR = non-irradiated cells; CDM = non-irradiated cells with CDM put onto the filter; UVA = cells irradiated with UVA; CDM UVA = cells irradiated with UVA through CDM; His = non-irradiated cells in the presence of His in the cell suspension; His UVA = cells irradiated with UVA in the presence of His in the cell suspension; His CDM UVA = cells irradiated with UVA through CDM in the presence of His in the cell suspension. (**b**) UVA irradiation through DM does not modify charge properties of Cat in U937. CTR = non-irradiated cells; DM = non-irradiated cells with DM put onto the filter; UVA = cells irradiated with UVA; DM UVA = cells irradiated with UVA through DM. (**c**) UVB irradiation through CDM does not modify the charge properties of Cat. CTR = non-irradiated cells; CDM = non-irradiated cells with CDM put onto the filter; UVB = cells irradiated with UVB; CDM UVB = cells irradiated with UVB through CDM. (**d**) UVB irradiation through DM does not modify the charge properties of Cat. CTR = non-irradiated cells; DM = non-irradiated cells with DM put onto the filter; UVB = cells irradiated with UVB; DM UVB = cells irradiated with UVB through DM.

properties of Cat but not its activity suggests that these two alterations are not necessarily associated events.

Oxidative modification of Trp and Met residues could possibly influence charge properties of Cat

Singlet oxygen is able to modify the prosthetic group of Cat (Lledías *et al.*, 1998; Lledías and Hansberg, 1999, 2000), but this modification *per se* is not sufficient to change the protein charge (Díaz *et al.*, 2005). Thus, other oxidative modifications on the backbone of Cat could explain the electrophoretic shift. Singlet oxygen can directly oxidize amino-acid residues and downstream-generated free radicals can propagate the phenomenon (Díaz *et al.*, 2005). In U937, we evaluated the possible production of other ROS from singlet oxygen by flow cytometric analysis, employing dihydroethidium as the fluorescent probe. In comparison with UVA

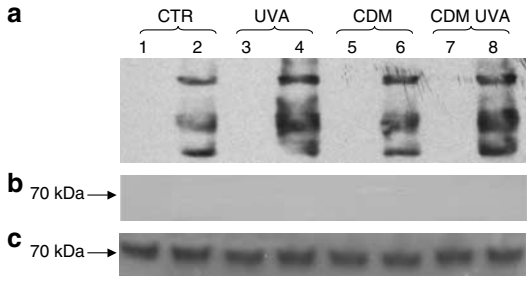

Figure 5. Cat charge alteration, after UVA through CDM, does not seem to be correlated with the introduction of carbonyl groups in the amino-acid residues. The irradiation procedure, with 20 J/cm^2 UVA through 4 μg/ml CDM, was performed as reported in Figure 3. CTR (lanes 1 and 2): non-irradiated cells; UVA (lanes 3 and 4): cells irradiated with UVA; CDM (lanes 5 and 6): not-irradiated cells with CDM put onto the filter; CDM-UVA (lanes 7 and 8): cells irradiated with UVA through CDM. (**a**) Western blot analysis of oxidized proteins from U937 samples, after reaction with a specific antibody that recognizes oxidatively introduced carbonyl groups (aldehydes and ketones) at lysine, arginine, proline, or threonine residues, after derivatization with DNPH (see Materials and Methods): even number lanes (2-4-6-8) represent samples subjected to the derivatization reaction with DNPH; odd number lanes (1-3-5-7) represent negative control of the derivatization reaction (see Materials and Methods). (**b**) Western blot analysis of immunoprecipitated Cat from U937 samples, after reaction with an antibody that recognizes oxidatively introduced carbonyl groups after derivatization with DNPH. (**c**) Western blot analysis of immunoprecipitated Cat from U937 samples, after reaction with an antibody that recognizes the enzyme itself (see Materials and Methods).

irradiation alone (2,206 $\pm$ 58 *vs* 1,474 $\pm$ 28 mean fluorescence) in cells irradiated through CDM, a significant increase of ROS was detected (2,476 $\pm$ 52, $P<0.05$ and $P<0.001$). The pattern of oxidized proteins, evaluated as carbonyl groups introduced in a site-specific manner (Stadtman, 1993), resulted in similar cells irradiated with UVA alone or with UVA through CDM (Figure 5a). This result suggests the absence of any supplementary effect mediated by singlet oxygen or by ROS derived from it. Moreover, to define whether the Cat charge alteration was due to an oxidative introduction of the carbonylic group in the protein backbone, Cat from U937 cells was immunoprecipitated and analyzed by Western blot technique, using an antibody specifically directed to carbonylated residues. In all the samples analyzed, Cat was not carbonylated (Figure 5b and c). On immunoprecipitated Cat, we also evaluated the possible oxidation of Trp residues by fluorimetry and of Met by micro-Raman spectroscopy (Schallreuter *et al.*, 2004). As reported in Figure 6, a decrease in the fluorescence excitation of Trp at 282 nm was observed after UVA, associated with a concomitant increase in fluorescence at 276 nm, which corresponds to L-5-hydroxytryptophan (5-OH-Trp). The oxidation of Trp to 5-OH-Trp was further enhanced by UVA irradiation through CDM and associated with a slight shift of the maximum of absorbance, suggesting a spectral modification due to the occurrence of multiple effects. As regards oxidative Met modifications (Figure 7), the expected oxidation product was L-methionine sulfoxide (Met-SO), which leads to the well-assigned SO stretch at 1,025 cm^{-1}. After UVA irradiation, the spectrum presented the expected stretch, more

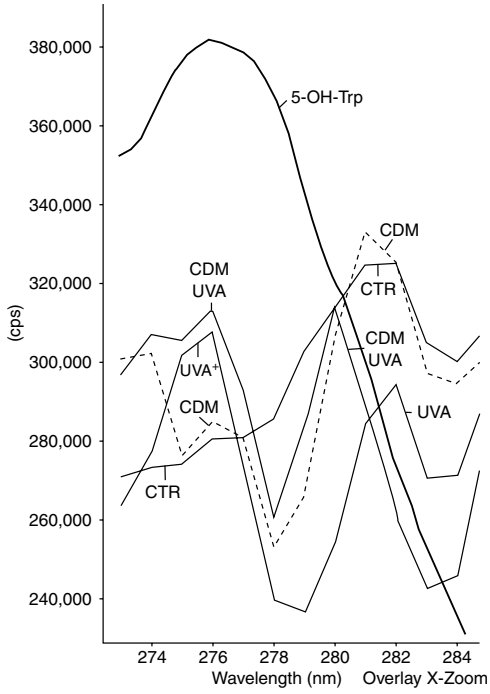

Figure 6. UVA even through CDM modifies Trp residue oxidative status in Cat. The irradiation procedure, with 20 J/cm^2 UVA through CDM (4 μg/ml), was performed as reported in Figure 3. After irradiation with UVA or UVA through CDM, Cat was immunoprecipitated from total cellular lysate, and Cat antibody-conjugated immunocomplexes, associated with GammaBind™ G Sepharose™ beads (Amersham Pharmacia Biotech, Sweden), were analyzed for the detection of 5-OH-Trp by spectrofluorimetric analysis. After UVA irradiation, a decrease of the fluorescence excitation of the Trp (282 nm) was observed, associated with a parallel increase of 5-OH-Trp fluorescence (276 nm). This effect was amplified after irradiation through CDM. CTR: immunoprecipitated Cat from not-irradiated U937 cells; UVA: immunoprecipitated Cat from cells irradiated with UVA; CDM: immunoprecipitated Cat from not-irradiated cells with CDM put onto the filter; CDM-UVA: immunoprecipitated Cat from cells irradiated with UVA through CDM; 5-OH-Trp: standard of oxidized Trp.

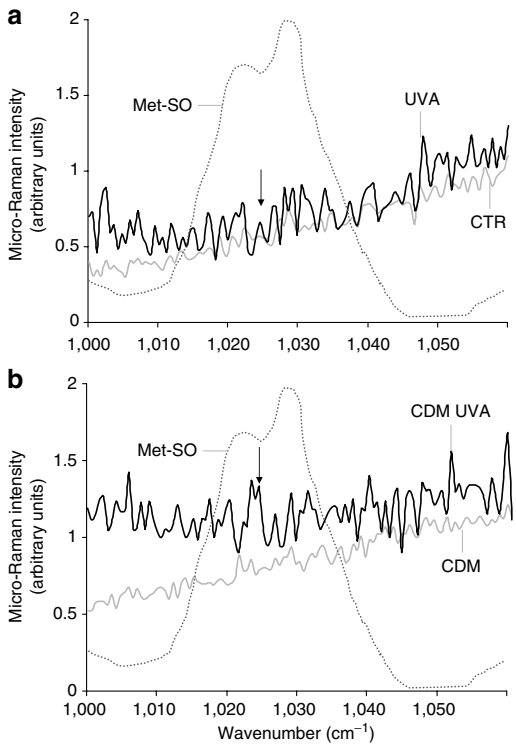

Figure 7. UVA even through CDM modifies Met residue oxidative status in Cat. The irradiation procedure with 20 J/cm^2 UVA through CDM (4 μg/ml), was performed as reported in Figure 3. After irradiation with UVA or UVA through CDM, Cat was immunoprecipitated from total cellular lysate, and Cat antibody-conjugated immunocomplexes, associated with GammaBind™ G Sepharose™ beads (Amersham Pharmacia Biotech, Sweden), were analyzed for the detection of Met-SO by micro-Raman spectroscopy. Met-SO leads to the well-assigned SO stretch at 1,025 cm^{-1}. After UVA irradiation, the spectrum presented the expected stretch, more intense after irradiation with UVA through CDM (see arrows). (**a**) CTR: immunoprecipitated Cat from non-irradiated U937 cells; UVA: immunoprecipitated Cat from cells irradiated with UVA; Met-SO: standard of oxidized Met. (**b**) CDM: immunoprecipitated Cat from non-irradiated cells with CDM put onto the filter; CDM-UVA: immunoprecipitated Cat from cells irradiated with UVA through CDM; Met-SO: standard of oxidized Met.

intense in CDM UVA. These results indicate that UVA irradiation of cells induces free-radical-mediated damage, leading to an oxidation of cell proteins, and that the presence of synthetic pheomelanin, following the generation of singlet oxygen or its downstream products, amplifies these effects on specific targets. In particular, oxidative modification of Trp and Met residues could influence charge properties of Cat.

DISCUSSION

Melanins act as a filter absorbing UV photons as well as a quencher of free radicals generated in the skin after UV exposure. Nevertheless, the photoprotective role of pheomelanin is still controversial. This pigment acts as a photosensitizing agent, amplifying ROS production and increasing DNA damage after UVA (Menon *et al.*, 1983; Ranadive *et al.*, 1986; Prota, 1997; Marrot *et al.*, 1999; Kvam and Dahle, 2004). On a three-dimensional model of epidermal reconstructs, we demonstrated that the phototype of melanocyte influences basal Cat activity. In fact, its value was significantly lower in REML than in REMH. Low levels of Cat activity were previously observed in different cutaneous experimental models and they were always associated with a stress-prone status (Maresca *et al.*, 1997; Bessou-Touya *et al.*, 1998; Grammatico *et al.*, 1998; Picardo *et al.*, 1999). In association with Cat, other antioxidant enzymes participate in the protection against oxidative stress. Thioredoxin reductase together with its electron acceptor thioredoxin, thioredoxin peroxidases, glutathione reductase/glutathione coupled to glutathione peroxidase are involved in the removal of H_2O_2 deriving from enzymatic dismutation of superoxide anion ($O_2^{-\bullet}$) catalyzed by SOD (Nordberg and Arnér, 2001; Schallreuter and Wood, 2001). Nevertheless, in melanocytes, the role of Cat is critical because it is the first enzyme devoted to the neutralization of H_2O_2 (Yohn *et al.*, 1991). When Cat activity is impaired, H_2O_2 accumulates in the cell and damages several structures, including the enzyme itself (Shindo *et al.*, 1994; Shindo and Hashimoto, 1997).

In agreement with previous studies (Zigman *et al.*, 1996; Rhie *et al.*, 2001; Hellemans *et al.*, 2003), our data demonstrate that UVA is more effective than UVB in inducing

an impairment of SOD and Cat activities. Cat oxidative damage is detrimental, because when damaged it recovers slowly (Shindo and Hashimoto, 1997; Rhie *et al.*, 2001). Moreover, in REML with respect to REMH, the decrease of enzymatic activities, induced by UVA, was higher and similar to those observed in the absence of melanins (REK), suggesting the poor filter effect exerted by pheomelanin against UV (Nofsinger *et al.*, 2002; Kadekaro *et al.*, 2003; Hoogduijn *et al.*, 2004).

The decrease of an enzymatic activity by a pro-oxidant agent, such as UVA or H_2O_2, is necessarily associated with structural alterations, which influence the functionality of its active site. Molecular modelling based on the establishment of a three-dimensional structure for actetylcholinesterase (Schallreuter *et al.*, 2004), dihydropteridine reductase (Hasse *et al.*, 2004), and pterin-4a-carbinolamine dehydratase (Schallreuter *et al.*, 2001) supported that H_2O_2-mediated oxidation of specific residues of Trp/Met, in the active site of these enzymes, alters their structure and functionality.

The alteration of Cat observed in human keratinocytes, following UV irradiation, is ascribable not only to ROS production but also to a direct effect of incident light (Punnonen *et al.*, 1991). It has been well established that millimolar concentrations of H_2O_2 can overwhelm the detoxifying capacity of Cat and deactivate it (Aronoff, 1965; Schallreuter *et al.*, 1995, 2001), and that the decrease of Cat activity mediated by near UV (99% UVA, 1% UVB) was associated with an alteration in the spectral absorbance of Trp (Zigman *et al.*, 1996). In U937, we show that UVA-mediated decrease of Cat activity was associated with an oxidation of Trp to 5-OH-Trp. Considering that in the Cat a Trp residue is localized in the channel leading to its active site (Putnam *et al.*, 2000), it is conceivable that the oxidation of this residue could influence the functionality of the enzyme. Moreover, near UV also promoted a modification in the charge properties of some monomers of Cat, and this effect was increased when irradiation of the enzyme was carried out in the presence of riboflavin as a photosensitizing agent (Zigman *et al.*, 1996). After irradiation with UVA, we did not see any charge modification of native Cat neither in U937 nor in REK or REMH. We saw this effect in REML and in U937 irradiated through CDM, demonstrating the possible role of pheomelanin as a photosensitizing agent in mediating the charge modification of Cat in response to UVA. The irradiation modality of U937 maintaining cells and CDM in separate compartments (see Figure 3) permitted us to establish, in agreement with previous studies, that the charge modification of Cat was due to singlet oxygen (Lledías *et al.*, 1998; Lledías and Hansberg, 1999, 2000; Díaz *et al.*, 2005), the only ROS able to diffuse through air (Dahl *et al.*, 1988). The key role of singlet oxygen in mediating charge modification was strengthened by the observation that a quencher of this species was able to counteract the charge modification of Cat. Furthermore, the results obtained on U937 irradiated with UVA demonstrate that the decrease of activity and the alteration in the charge properties of Cat are two distinct alterations, mediated by different free radical species. This assumption arises from the following observations: in U937 irradiated with UVA through CDM, (a) the decrease in enzymatic activity was similar to that observed in cells irradiated with UVA alone, and (b) the decrease of Cat activity was not counteracted by His. Other studies show the independence of the two types of Cat alterations (Lledías *et al.*, 1998; Lledías and Hansberg, 1999, 2000; Díaz *et al.*, 2005) underlying that the oxidized Cat is structurally different from the nonoxidized one, although it retains the stability and the catalytic efficiency proper of the nonoxidized enzyme. Nevertheless, the present work is the first one that attributes a possible role to pheomelanin in mediating a charge modification of Cat in response to UVA.

As described previously, singlet oxygen is able to mediate the oxidation of the prosthetic group of Cat (Lledías *et al.*, 1998; Lledías and Hansberg, 1999, 2000), and this alteration, *per se*, is unable to provide the enzyme with a modification in the electrophoretic properties (Díaz *et al.*, 2005). Considering that, in addition to the prosthetic ring, singlet oxygen also reacts quickly with amino-acid residues, particularly Trp, Tyr, His, Met, and Cys, damage to these residues in the protein also likely occurs (Díaz *et al.*, 2005). Furthermore, considering that the decrease of activity and the modification in the charge properties of Cat are two distinct alterations, biochemical modifications of amino-acid residues in the enzyme could probably concern those ones that contribute to the total superficial charge of the protein. In order to focus on the possible alterations of specific amino-acid residues, in oxidized Cat, we evaluated, in particular, the oxidative modifications of Trp and Met. The results obtained show an oxidation of these two residues in response to UVA through CDM. At this moment, it is not possible to recognize exactly the sites in which these specific amino acids resulted modified on oxidized Cat and we can only supply general information on the oxidation of Trp and Met. The exact localization and the possible modifications of other amino-acid residues by singlet oxygen could be carried out in a subsequent study of structural analysis of this enzyme in photosensitization experiments.

In conclusion, our data indicate that in low phototype pigmented epidermal reconstructs, a peculiar pattern of antioxidants exists and that pheomelanin, following UVA exposure, induces epidermal Cat charge modifications mediated by singlet oxygen. This alteration could be considered as an early and specific marker for acute UVA-mediated oxidative damage affecting fair skin. How this finding relates to an enhanced skin carcinogenesis in low phototype individuals needs further investigation using longer-term irradiation procedures.

MATERIALS AND METHODS

Epidermal biopsies and epidermal reconstruction *ex vivo*

Skin samples were obtained, following informed consent, from the skin of adult donors undergoing plastic surgery (breast reconstruction and abdominoplasty), using a protocol approved by University Victor Ségalen of Bordeaux Institutional Review Board in accordance with the Declaration of Helsinki. The phototype, from I to VI, was determined according to Fitzpatrick's classification (Fitzpatrick, 1988). Reconstructed epidermis was performed by seeding kerati-

nocytes and melanocytes onto the surface of dead de-epidermized dermis. Keratinocytes and melanocytes derived from nonphoto-exposed skin areas of low (II, REML) or high phototype (V–VI) (REMH) subjects. Keratinocytes from phototype III subjects were employed for reconstructed epidermis without melanocytes (REK). The correct three-dimensional architecture of the skin reconstruction was confirmed by a morphological analysis performed as described previously (Cario-Andre *et al.*, 2000).

U937 culture

Myeloid U937 cells were cultured in RPMI-1640 medium (Euroclone Ltd, Wetherby, Yorkshire, UK) supplemented with FBS (10%), penicillin (100 μg/ml)–streptomycin (100 μg/ml), and L-glutamine (2 mM) (all from Gibco Life Technologies S.r.l., Milan, Italy) at 37°C in a 5% CO_2/95% air atmosphere in a humidified incubator. For the irradiation experiments, cells (5×10^6) were washed three times with phosphate-buffered saline solution (PBS, Euroclone Ltd, Wetherby Yorkshire, UK), re-suspended in PBS with or without 30 mM His (Sigma-Aldrich S.r.l., Milan, Italy), a quencher of singlet oxygen, and then irradiated as described below.

Synthetic melanins

DOPA-melanin (DM) and cysteinyl-DOPA-melanin (CDM), synthetic homologs of eumelanin and pheomelanin, respectively, were synthesized (Ozeki *et al.*, 1996). Briefly, a mixture of 1 mM L-DOPA (Merck, Dermstadt, Germany) without (for DM) or with 1.25 mM L-cysteine (Sigma-Aldrich S.r.l., Milan, Italy) (for CDM), in 1 ml of 10 mM sodium phosphate buffer (pH 6), was oxidized by 200 μg of mushroom tyrosinase (Sigma-Aldrich, S.r.l., Milan, Italy). After incubation for 4 hours at 37°C, 100 μl of 1 M acetic acid (Merck, Dermstadt, Germany) was added to stop the oxidation and to precipitate the melanin produced. Melanins analogs were collected by centrifugation. The resulting precipitate was washed twice with water, acetone (Merck, Dermstadt, Germany), and dried over P_2O_5.

Irradiation procedure of samples

Reconstructed epidermis and U937 cells were irradiated with UVB or UVA by a Biotronic device (Vilber Lourmat, Marne La Vallée, France). The spectral distribution of the UVB lamp showed a major peak at 312 nm and a minor peak at 365 nm. The UVA lamp delivered UV in the range of 312–400 nm with a peak at 365 nm and a minor peak at 312 nm, 11 times smaller. The irradiance was regularly quantified by a calibrated radiometer equipped with an SCS 280 photodetector (International Light, Inc., Newburyport, MA). UV doses were as follows: UVB 0.15 J/cm^2 for reconstructed epidermis and 0.45 J/cm^2 for U937; UVA 8 J/cm^2 for reconstructed epidermis and 20 J/cm^2 for U937. U937 were irradiated with higher doses than those used for reconstructed epidermis because they were found to have high levels of Cat activity and to be very resistant to oxidative stress (Lledías *et al.*, 1999). Experiments were performed irradiating U937 through synthetic CDM (4 μg/ml) or DM (4 μg/ml), with or without His. The synthetic melanins were separated from the cells by a filter (Millicell-PC 0.4 μM PIHT 012 50, Millipore S.p.a., Milan, Italy) with a selective permeability to gases and small molecules. This system allowed us to study the effect of the singlet oxygen only, produced in photosensitization reactions on Cat charge properties. Non-irradiated U937 and reconstructed epidermis were used as controls.

Enzymatic activities

In reconstructed epidermis, the epidermis was separated from the dermis after incubation overnight in a solution of cold trypsin (0.25%) (Euroclone Ltd, Wetherby, Yorkshire, UK) and homogenized in PBS with a Potter–Elvejeim homogenizer in the presence of a protease inhibitors cocktail (Sigma-Aldrich S.r.l., Milan, Italy). U937 were lysed in PBS by repeated freezing in liquid nitrogen and thawing, in the presence of a protease inhibitors cocktail. Cell lysates (4×10^6) and epidermal homogenates were centrifuged at $10{,}000 \times g$ for 10 minutes at 4°C. Enzymatic activities were determined, on the supernatants, by a Lambda 25 UV/Vis spectrophotometer (Perkin-Elmer, London, UK). SOD activity was evaluated according to Spitz and Oberley (1989). Superoxide, generated by a xanthine/xanthine oxidase system, was detected by monitoring the reduction of nitroblue tetrazolium (Sigma-Aldrich S.r.l., Milan, Italy) at 560 nm, at pH 7.8 in phosphate buffer. One unit of SOD activity was defined as the amount of protein that yields 50% of maximal inhibition of nitroblue tetrazolium reduction. Cat activity was determined spectrophotometrically by the disappearance of hydrogen peroxide (10 mM) (Claiborne, 1985). After setting the baseline at 240 nm, against air, 2 ml of a solution of 10 mM H_2O_2 (Merck, Dermstadt, Germany) in 0.2 M phosphate buffer (pH 7.4) was placed in a quartz cuvette. Thereafter, 10–50 μl of the supernatant was gently mixed with the buffer for 10 seconds with a tip. The kinetics of H_2O_2 consumption by Cat was then started and monitored at 240 nm for 2 minutes at 25°C. The H_2O_2 consumption/minute in the buffer was converted to units of enzymatic activity on the basis of a standard curve obtained testing scalar units of bovine Cat (Sigma-Aldrich S.r.l., Milan, Italy). Units were normalized for the content of protein in the supernatant of cell lysate. Protein concentration was determined on the supernatants of cell lysates by Bradford reagent (Sigma-Aldrich S.r.l., Milan, Italy). Three tests were performed on each supernatant and experiments were repeated twice.

Charge properties of Cat on polyacrylamide gels

Discontinuous native minigels (8 × 9 cm and 0.75 nm thick) of 8% polyacrylamide (Bio-Rad Laboratories S.r.l., Milan, Italy) and 0.2% bis-acrylamide (Bio-Rad Laboratories S.r.l., Milan, Italy) were loaded with cell lysates containing 1 U of Cat activity in each lane. Gels were run at 200 V for 2 hours 45 minutes at 4°C on a Miniprotean II Biorad apparatus (Bio-Rad Laboratories S.r.l., Milan, Italy). Native enzyme was detected by incubating the gels for 5 minutes in 5% aqueous methanol (Merck, Dermstadt, Germany) and after rinsing in 10 mM H_2O_2 (Merck, Dermstadt, Germany). The gels were rinsed with tap water and then incubated in 1:1 mixture of freshly prepared 2% potassium ferric cyanide and 2% ferric chloride (both from Sigma-Aldrich S.r.l., Milan, Italy). Blue color developed in the gel, except where H_2O_2 was decomposed by Cat. Staining was stopped by soaking the gel in a 10% acetic acid (Merck, Dermstadt, Germany) and 5% methanol solution (Lledías *et al.*, 1999).

Immunoprecipitation

Cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% NP-40; 0.25% sodium deoxycholate) supplemented with protease inhibitors (1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin) and phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM NaF) (all from Sigma-Aldrich S.r.l., Milan, Italy).

Total proteins (500 μg) were immunoprecipitated overnight at 4°C with a sheep anti-human Cat antibody (1:300) (Biodesign International, Industrial Park Saco, Maine). Immunocomplexes, aggregated with 100 μl of GammaBind™ G Sepharose™ (Amersham Biosciences Europe, Gmbh, Cologno Monzese, Milan, Italy), were collected by centrifugation and washed three times with lysis buffer. For the detection of oxidation of the protein moiety (Stadtman, 1993), immunoprecipitated Cat was resolved under reducing conditions by 10% SDS-PAGE and processed for immunoblotting as described below. For the fluorimetric and micro-Raman spectroscopic analyses of Cat, the immunocomplexes aggregated with GammaBind™ G Sepharose™ were directly analyzed (see below) and GammaBind™ G Sepharose™ conjugated with the anti-human Cat antibody was used as a blank, in order to set the baseline.

Carbonylated protein detection

Cells were lysed in radioimmunoprecipitation assay buffer plus protease inhibitors. Total proteins (10 μg) or immunoprecipitated Cat were subjected to 2,4-dinitriphenylhydrazine (DNPH) derivatization (Thiele *et al.*, 1999) according to the manufacturer's instructions (Intergen Company, Purchase, NY). Incubation of equal aliquots with a control solution lacking DNPH served as a negative control. The DNPH-derivatized protein samples and the controls were separated by 10% SDS-PAGE and transferred to nitrocellulose (Amersham Biosciences Europe Gmbh, Cologno Monzese, Milan, Italy). The membranes were blocked with 5% non-fat dry milk (Bio-Rad Laboratories S.r.l., Milan, Italy) in PBS containing 0.1% Tween-20 (Sigma-Aldrich S.r.l., Milan, Italy) and incubated with rabbit anti-dinitrophenylhydrazone antibody (1:150) (Intergen Company, Purchase, NY) for 1 hour at room temeperature. After washing the membrane, a secondary goat anti-rabbit IgG horseradish peroxidase-conjugated antibody (1:300) (Intergen Company, Purchase, NY) was added for 1 hour at room temperature. The membrane was washed and antibody reactivity was visualized by an enhanced chemiluminescence reagent (Santa Cruz Biotechnology Inc., Italy).

ROS detection

The production of ROS was assessed by flow cytometric analysis. Cells were incubated for 15 minutes at 37°C in 5 mM glucose in PBS with 2 μM dihydroethidium (Molecular Probes, Eugene, OR) and analyzed by a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA; 1×10^4 events/sample) (Kannan and Jain, 2004).

Fluorescence spectroscopic analysis of Cat

For the *in vitro* fluorescence measurements, a Fluoromax-3 spectrofluorimeter (Horiba Jobin Yvon S.r.l., Milan, Italy) was used, equipped with a xenon lamp as light source and subtractive monochromators (reflection gratings, 1,200 grooves/minute) for both excitation and emission. For the excitation spectra measurements of Trp residues in Cat (Putnam *et al.*, 2000). the fluorescence intensity was followed at a fixed emission wavelength of 340 nm with varying excitation wavelengths ranging from 250 to 325 nm, 15 nm below the emission wavelength to avoid the Rayleigh scatter peak. The spectra were corrected for the spectral output (wavelength and voltage changes) of the xenon lamp using the reference photodiode. 5-OH-Trp (Sigma-Aldrich S.r.l., Milan, Italy) was employed as a standard.

Raman measurements

The micro-Raman measurements of Met-SO were performed using a Dilor XY triple-spectrograph equipped with a liquid-nitrogen-cooled CCD detector and a modified Olympus microscope in confocal configuration. The spectral resolution was 0.5 cm^{-1}. The 514.5 nm line of an Ar^+ ion laser was used as the exciting source. The diameter of the laser spot onto the sample was 2 μm, with a laser power around 2 mW. The spectra were collected in back-scattering geometry focusing the laser on the dried beads deposited from a water solution on a polished face of a silicon plate. The polarization of the scattered light was not analyzed. The frequencies were calibrated using an Hg/Ne lamp. The SO stretch of Met-SO was visualized as a peak at 1,025 cm^{-1}. This peak was confirmed with Met-SO (Sigma-Aldrich S.r.l., Italy) as a standard.

Statistical analysis

Statistical differences were evaluated using Student's *t*-test.

CONFLICT OF INTEREST

The author states no conflict of interest.

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