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ORIGINAL CONTRIBUTION

Protective effect of ferulic acid ethyl ester against oxidative stress mediated by UVB irradiation in human epidermal melanocytes

F. DI DOMENICO[1]*, M. PERLUIGI[1]*, C. FOPPOLI[2], C. BLARZINO[1], R. COCCIA[1], F. DE MARCO[3], D. A. BUTTERFIELD[4–6], & C. CINI[1,2]

[1]*Department of Biochemical Sciences, University of Rome 'La Sapienza', P.le Aldo Moro, 5-00185 Rome, Italy,* [2]*CNR Institute of Molecular Biology and Pathology, P.le Aldo Moro, 5-00185 Rome, Italy,* [3]*Laboratory of Virology, 'Regina Elena Institute for Cancer Research', Via delle Messi d'oro, 156-00156 Rome, Italy,* [4]*Department of Chemistry,* [5]*Sanders-Brown Center on Aging, and* [6]*Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506, USA*

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Abstract
UV solar radiation is the major environmental risk factor for malignant melanoma. A great effort is currently posed on the search of new compounds able to prevent or reduce UV-mediated cell damage. Ferulic acid is a natural compound recently included in the formulation of solar protecting dermatological products. The purpose of the present work was to assess whether its ethyl ester derivative, FAEE, could protect skin melanocytes from UV-induced oxidative stress and cell damage. Experiments on human melanocytes irradiated with UVB showed that FAEE treatment reduced the generation of ROS, with a net decrease of protein oxidation. FAEE treatment was accompanied by an induction of HSP70 and heme oxygenase, by a marked suppression of PARP activation and a significant suppression of apoptosis. Moreover FAEE prevented iNOS induction, thus suppressing the secondary generation of NO-derived oxidizing agents. FAEE may represent a potentially effective pharmacological approach to reduce UV radiation-induced skin damage.

Keywords: *UVB, oxidative stress, melanocytes, heme oxygenase-1*

Abbreviations: *3-NT, 3-nitrotyrosine; DCF, dichlorodihydrofluorescein; DCFH-DA, 2′,7′-dichloro dihydrofluorescein diacetate; DNPH, 2,4-dinitrophenylhydrazine; FA, ferulic acid; FAEE, ferulic acid ethyl ester; HNE, 4-hydroxy nonenal; HO-1, heme oxygenase 1; HSP70, heat shock protein 70; iNOS, inducible nitric oxide synthase; MGM, melanocytes growth medium; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NHEM, normal human epidermal melanocytes; PARP, poly(ADP-ribose) polymerase; RNS, reactive nitrogen species; ROS, reactive oxygen species.*

Introduction

The whole body surface is covered by the skin, a complex organ that acts as a defensive shield with the primary purpose of protecting the organism against the outer environment. As a screening barrier, skin is directly exposed to solar UV light and thus is the organ most sensitive to UV-induced injury. UV light, the most described physical attack, is well-known to cause skin damage, resulting in both pre-cancerous and cancerous skin lesions and acceleration of skin ageing [1] and so far is the only identified external risk factor for the development of malignant melanoma, the most severe tumour of the skin arising from epidermal melanocytes [2,3]. UV radiation causes massive production of reactive oxygen species (ROS), including hydrogen peroxide, superoxide anion, singlet oxygen and hydroxyl radical, in

*These authors contributed equally to this work.

Correspondence: Dr Marzia Perluigi, Department of Biochemical Sciences, 'Sapienza' University of Rome–P.le Aldo Moro, 5-00185 Rome, Italy. Tel: +390649910900. Fax: +39064440062. Email: permarzia@hotmail.com

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exposed cells. ROS are short-lived molecules that are capable of causing oxidative damage in DNA, as well as in cellular protein and lipids, leading to permanent genetic changes [4] and activating signal transduction pathways that promote skin carcinogenesis [5]. Although some ROS perform useful functions, the production of ROS exceeding the ability of the organism to mount an antioxidant defence results in oxidative stress and the ensuing tissue damage could be involved in neoplastic diseases.

The damaging effects of an increased ROS generation can be attenuated by antioxidants, which can reverse many of the events that contribute to epidermal toxicity and disease. During the past decade there has been an increased research emphasis about antioxidant molecules able to exert effective protection against the damage provoked by UV-induced ROS generation. Currently, the UV protective properties of various plant extracts are being intensively studied.

Ferulic acid (FA) is a hydroxycinnamic acid largely present in plants and also in vegetable foods, such as olives and olive oil. Its biological properties and especially its antioxidant activity are well recognized [6–9]. In particular, this compound is employed as a photoprotective ingredient in many skin lotions and sunscreens because of its capacity to scavenge free radicals induced by UV radiation [10,11]. Furthermore, FA was shown to be a strong UV absorber [12]. These findings represent an interesting background supporting a potentially successful employment of hydroxycinnamic acids as topical protective agents against UV radiation-induced skin damage. In addition, some authors showed that the naturally occurring ethyl ester of FA, named FAEE (ethyl 4-hydroxy-3-methoxycinnamate) (Figure 1), has a stronger antioxidant potential when compared to FA [8]. Recently, we demonstrated the protective effect of FAEE against oxidative stress induced by radical producers or amyloid-beta peptide *in vitro* in rat primary neuronal cell cultures and *in vivo* in synaptosomes isolated from FAEE-treated rodents [13–16].

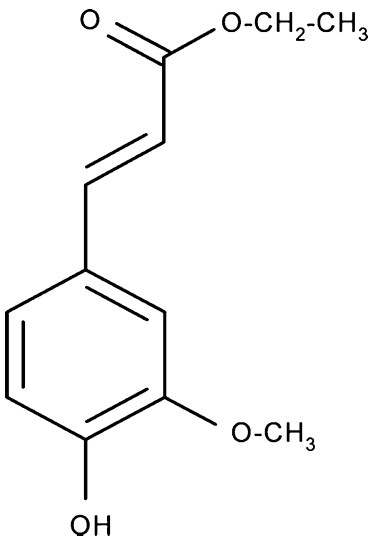

Figure 1. Ethyl 4-hydroxy-3-methoxycinnamate or ferulic acid ethyl ester (FAEE).

Taking into account that epithelial cells, being at the interfaces between the organism and the environment, are heavily exposed to stimuli able to determine oxidative stress, we assayed whether epidermal melanocytes could be protected by FAEE treatment from UV-induced oxidative stress. For this purpose, human primary epidermal melanocytes were irradiated with UVB—the UV radiation considered to be the aetiological wavelength for melanoma—and the protective capacity of FAEE was evaluated. We focused on the FAEE effect on UVB-induced cellular oxidative stress, particularly on ROS generation, protein oxidation and lipid peroxidation. The possible mechanisms through which this protective effect is exerted have also been investigated.

Materials and methods

Materials

Ferulic acid (FA); ferulic acid ethyl ester (ethyl 4-hydroxy-3-methoxycinnamate) (FAEE), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), Sigma-Fast Chromogen, alkaline phosphatase-conjugated and peroxidase-conjugated secondary antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Fresh FAEE solutions (50 μM) were prepared in DMSO. Melanocytes Growth Medium (MGM) was purchased from PromoCell GmbH (Heidelberg, Germany). The Oxyblot oxidized protein Kit and primary antibodies for 4-hydroxynonenal (HNE) and 3-nitrotyrosine (3-NT) were obtained from Chemicon International (Temecula, CA). Primary antibodies for heat-shock protein 70 (HSP70), heme oxygenase (HO-1), inducible nitric oxide synthase (iNOS) and Poly(ADP-ribose) polymerase (PARP) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All other reagents were analytical grade products purchased from current laboratory suppliers.

Cell lines and culture conditions

Primary normal human epidermal melanocytes (NHEM) were isolated from infant human foreskin obtained from patients attending the paediatric section of the Department of Surgery of Ospedale 'Sandro Pertini', Rome. To be eligible patients were to be aged below 12, to be free of any chronicle or metabolic disease and with no reason to seek medical advice apart from the need for phimosis surgical correction. Among those matching the above criteria, a total of six boys

(range 2–12 years), whose parents had given written consent, were selected to participate. The study design and enrolment criteria had been approved by both the 'Regina Elena' Cancer Institute and the Ospedale 'Sandro Pertini' local Ethical committees. Cells were cultivated in MGM supplemented with 0.4% bovine pituitary extract, 1 ng/ml bFGF, 5 μg/ml insuline (porcine), 0.5 μg/ml hydrocortisone, 10 ng/ml phorbol myristate acetate, 50 ng/ml amphotericin B and 50 μg/ml gentamicin. Cells were grown in a 5% CO_2 humidified atmosphere with medium renewal every 48 h. At sub-confluence (roughly 50×10^3 cells/cm^2) cell monolayer was dissolved with trypsin/EDTA, washed with PBS plus soybean trypsin inhibitor and the cell suspension re-plated at a 10^4/cm^2. To avoid senescence bias modification of cell metabolism, the experiments were conducted between the third and ninth passage.

UV treatment

Immediately before irradiation, incubation medium was removed, attached cells were washed twice with PBS and then UVB-irradiated in a home-made irradiation hood in the absence of any medium or PBS. The UVB source was provided by a bank of Sankyo Denki G15T8E fluorescent tubes emitting 270–320 nm wavelength radiation with a maximum at 313 nm. The administered dose was calculated based on the tubes adsorbed power times exposure length per surface unit and expressed in J/m^2 (a 1 s time exposure corresponding to a 5 J/m^2 dose). UVB dosage was chosen to induce intermediate cell damage. After UVB exposure, fresh medium was added and the cultures were further incubated. Sham irradiated cell cultures were used as negative control in each study point.

Cell viability determination

Cell viability was measured by the MTT reduction assay [17], as previously described [18]. Briefly, NHEM were plated in 96-well microplates at 1.5×10^4/well, eight replicas per condition. After overnight incubation, the medium was replaced with fresh medium containing FAEE at concentrations ranging from 1–50 μM. After 1 h, medium was removed, the cells were submitted to UVB irradiation and further incubated in the standard conditions for 24 h. Then, MTT was added at a final concentration of 1.25 mg/ml. After 2 h of incubation at 37°C, medium was discarded and the reduced insoluble dye was extracted with 0.04 N HCl/isopropanol. Cell viability was evaluated by the absorbance ($A_{540–750}$) measured in a microplate reader (Labsystem Multiscan MS).

ROS measurement

ROS levels were detected by the DCF method [19] with minor modifications. Briefly, cells were plated in 6-well plates at 3×10^4 cells/cm^2, allowed to attach by overnight incubation and then treated with FAEE at the indicated concentration. After a further 1 h incubation, cells were challenged with 10 μM DCFH-DA for 30 min and then submitted to UVB irradiation, as described above. Two hours post-irradiation, the medium was discarded, cells washed twice with PBS and finally gently scraped into 1 ml of PBS. The cellular suspension was then transferred into a fluorescence cuvette where the ROS-driven conversion of the non-fluorescent DCFH into the highly fluorescent dichlorofluorescein (DCF) [20] was monitored by fluorescence intensity measured in a FP 6300 Jasco Spectrofluorometer with excitation wavelength at 502 nm (bandwidth 5 nm) and emission wavelength at 520 nm (bandwidth 5 nm).

Sample preparation for Western blot analyses

For Western blot analyses, cells were plated at 3×10^4 cells/cm^2 in Petri plates and after overnight incubation were treated with FAEE at indicated concentration. The media was removed 1 h later and the cells were submitted to UVB irradiation. Cell monolayers of sub-confluent cultures were scraped with a rubber policeman 5 h after irradiation, washed twice in PBS and collected as pellets by centrifugation at 250 × g for 10 min. Pellets were then lysed by the addition of 50 μl of Media 1 (0.32 M sucrose, 10 mM Tris-HCl, pH 8.0, 0.1 mM $MgCl_2$, 0.1 mM EDTA) containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mg/ml aprotinine. After cooling in ice for 30 min, samples were sonicated (10 s three times) and then centrifuged at 18 000 × g for 10 min. Protein determination was performed on the supernatant using the Coomassie Plus Pierce Protein Assay (Rockford, IL).

Western blot analysis

The evaluations of protein carbonyls, HNE, 3-NT, HSP70, HO-1, PARP and iNOS were performed by Western blot analysis. Aliquots of cell lysates containing 30 μg protein were added to sample buffer, denaturated for 5 min at 100°C, loaded on 10% SDS-polyacrylamide gels and separated by electrophoresis at 80 mA for 2 h. Following electrotransfer to PVDF membranes, the membranes were then blocked for 2 h in 3% BSA in TBS-T and next probed for 2 h at room temperature with primary antibodies of interest: anti-DNPH (1:200) for carbonyl groups, anti-HNE (1:100), anti-3-NT (1:200), anti-HSP70 (1:500), anti HO-1 (1:500), anti-PARP

(1:200) and anti-iNOS (1:500). After three washes with TBS-T, membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (or alkaline phosphatase-conjugated, only for 3-NT detection) and developed using a chemiluminescence kit (Amersham, Piscataway, NJ) (while 3-NT blots were developed with Sigma Fast chromogen). Blots were scanned into Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA) and quantized with Scion Image (PC version of Macintosh-compatible NIH Image).

Apoptosis determination

Apoptosis was evaluated by means of cytoplasmic nucleosomes determination; the cytoplasmic histone-bound DNA fragments (mono- and oligonucleosomes) were measured by the Cell Death Detection ELISA^{PLUS} Kit (Roche Diagnostics S.p.A, Monza, Italy), used according to the manufacturer's instructions. Briefly, aliquots of cell lysates corresponding to 1.0×10^4 cells were incubated with a mixture of Anti-histone biotin-labelled and Anti-DNA peroxidase-labelled monoclonal antibodies in a streptavidin-coated microplate. After 30 min incubation at room temperature, unbound components were washed away and nucleosomes concentration was revealed by the peroxidase activity measured by the difference between the absorbance at 490 nm and that at 450 nm.

Statistical analysis

Results were expressed as means $\pm$ SD from six independent samples (six donors). Each experiment was repeated at least three times to confirm the reproducibility of findings. Multiple groups were analysed by one-way analysis of variance (ANOVA) followed by a post-hoc Student-Newman-Keuls test. $p < 0.05$ was considered significant

Results

Protective effect of FAEE against UVB-induced damage to cell viability

In order to evaluate the dose response effect of UVB irradiation, NHEM cell cultures were exposed to increasing UVB doses ranging from 20–100 J/m^2. As a preliminary step, the UVB sub-toxic dose was evaluated. As can be seen in Figure 2A, a dose of 50 J/m^2 determined a partial decrease of viability and it was chosen to exploit all the experiments.

Viability determination of UVB-irradiated cells grown in the presence of various FAEE concentrations (Figure 2B) indicated a FAEE dose-dependent protective effect. A dose of 25 μM was able to completely prevent UVB toxicity. Analogous experiments performed using FA as antioxidant molecule indicated that the most effective non-toxic dose of FA

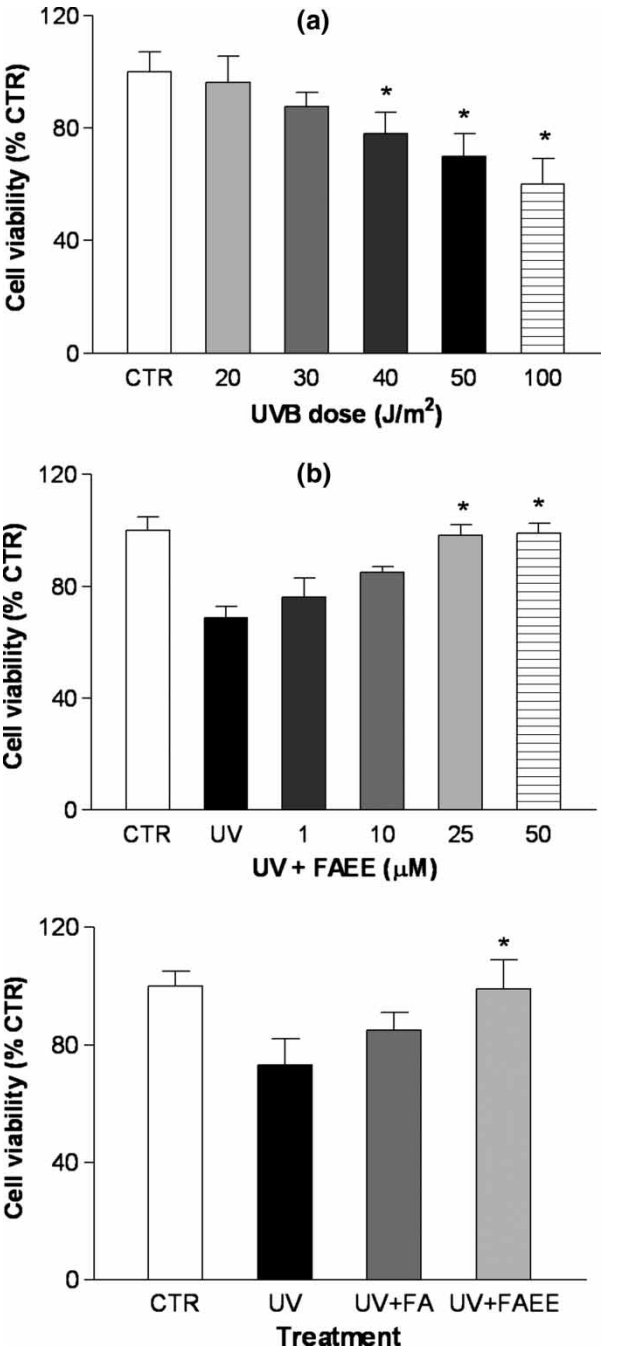

Figure 2. (A) Effect of UVB dose on cell viability. NHEM were exposed to UVB irradiation for variable times (from 4–20 s, corresponding to radiation doses from 20–100 J/m^2). After 24 h incubation, viability was measured by MTT method. (B) Dose-dependent protective effect of FAEE on UVB-exposed NHEM. Cells growing in medium containing FAEE at the indicated concentrations (1–50 μM) were submitted to UVB irradiation (50 J/m^2). After 24 h incubation, viability was measured by MTT method. (C) Comparison between protective effect of FAEE and FA treatments on UVB-exposed NHEM. Cells growing in medium containing 25 μM FAEE or 100 μM FA were submitted to UVB irradiation (50 J/m^2). After 24 h incubation, viability was measured by MTT method. CTR = not irradiated cells; UV = irradiated untreated cells. Results are given as percentage of CTR values ($n = 6$) and represent the mean $\pm$ SEM of eight independent replicas of a representative experiment in a set of six. *$p < 0.05$ vs CTR (A and B); *$p < 0.05$ vs UV (C).

was 100 μM (data not shown). The comparison between the protective effects of FAEE and its corresponding acid FA is shown in Figure 2C. FAEE was much more active than FA; in fact, 25 μM FAEE completely restored the cell viability of irradiated samples, while 100 μM FA-treated cells achieved a viability of 85% of control.

Protective effect of FAEE against UVB-induced ROS production and protein oxidation

The levels of ROS generated by UVB were measured by using the DCF assay. Figure 3 shows that UVB irradiation determined a marked increase of fluorescence, more than 2-fold compared with control cells. Conversely, in the samples that received FAEE treatment the UV-induced increment of fluorescence was lower (1.3-fold), indicating that FAEE significantly modulated ROS accumulation.

A major feature of protein UVB damage is protein oxidation. To assess the extent of oxidation and the potential protection exerted by FAEE, various kinds of oxidative adducts were evaluated. Protein carbonylation, as reported in Figure 4A, shows a significant increase (~3.5-fold above control) in carbonyl level induced by UVB irradiation This level was found to be significantly lower (1.6-fold) in FAEE-treated cells.

The antioxidant properties of FAEE were further confirmed by measuring the levels of 3-nitrotyrosine (3-NT), another marker of protein oxidation formed by the reaction of tyrosine residues with peroxynitrite, a strong oxidant compound formed by the reaction of NO with superoxide anion [21,22]. Figure 4B shows that the large increase in 3-NT levels in cells submitted to UVB irradiation (~2-fold compared to control) was markedly reduced by FAEE treatment.

A further source of oxidative damage of protein function is provided by lipid peroxidation products. ROS attack on arachidonic acid produces reactive alkenals, such as 4-hydroxynonenal (HNE), that bind to proteins by Michael addition [23,24], altering their

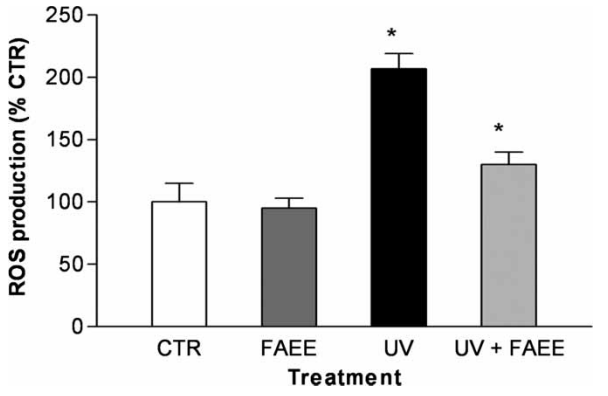

Figure 3. Protective effect of FAEE treatment against UVB-induced ROS production. NHEM growing in medium containing 25 μM FAEE were submitted to UVB irradiation (50 J/m^2). After 2 h incubation, ROS levels were determined by the DCF fluorescence assay. Results are given as percentage of CTR values ($n=6$) and represent the mean ± SEM of six independent experiments. *$p<0.05$. CTR = not irradiated untreated cells; FAEE = not irradiated treated cells; UV = irradiated untreated cells; UV + FAEE = irradiated treated cells.

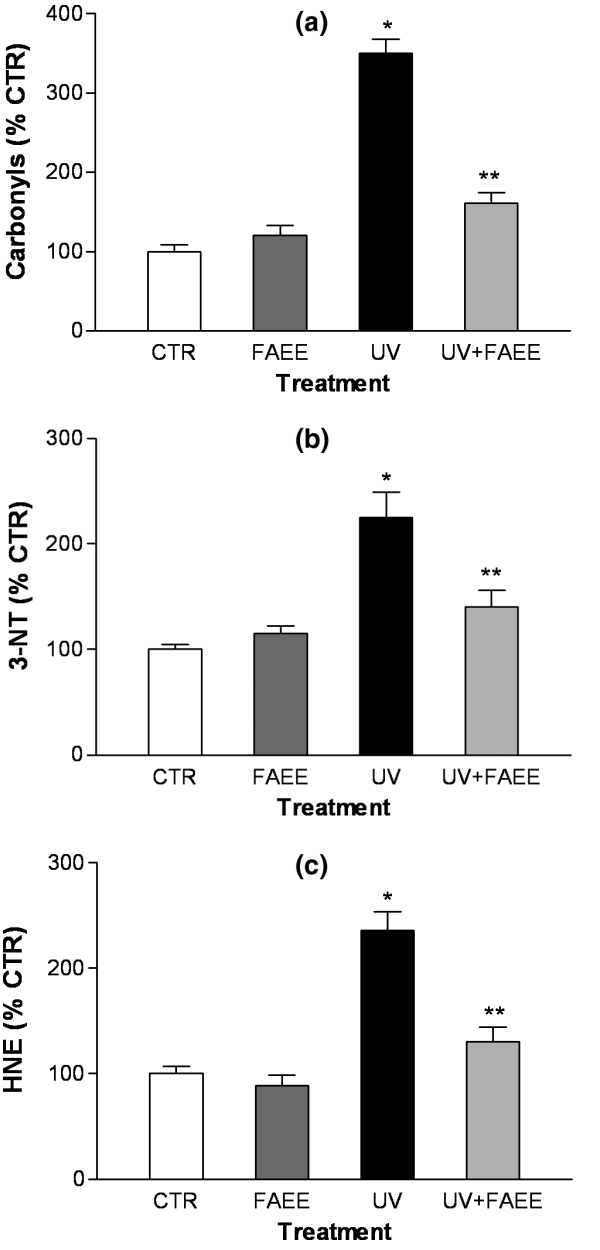

Figure 4. Protective effect of FAEE treatment against UVB-induced protein oxidation, calculated as carbonylation (A), 3-NT formation (B) or HNE-protein bound formation (C). NHEM growing in medium containing 25 μM FAEE were submitted to UVB irradiation (50 J/m^2). After 5 h incubation, the levels of carbonyls, 3-NT and protein-bound HNE were determined by western blot. Results are given as percentage of CTR values ($n=6$) and represent the mean ± SEM of six independent experiments. *$p<0.05$ vs CTR; **$p<0.02$ vs UVB. CTR = not irradiated untreated cells; FAEE = not irradiated treated cells; UV = irradiated untreated cells; UV + FAEE = irradiated treated cells.

conformation and thereby changing their activity [25]. To evaluate this pathway, we measured the protein-bound HNE levels in UVB irradiated melanocytes. As expected (Figure 4C), HNE-protein adducts were sharply increased following UVB, while, once again, FAEE treatment almost completely suppressed their generation, indicating that the lipid peroxidation pathways were clearly reduced.

Modulation of HSP70, HO-1 and iNOS expression by FAEE

Oxidative cell damage occurs whenever oxidative stresses overcome the cell control capacity. This latter relies on the concurrent action of two different activities: (i) the surveillance on the oxidant species level and (ii) the modulation of mechanisms able to counteract their effects or repair cells damages. To evaluate the effect of FAEE on this second line of action, three different proteins were evaluated: HSP70, heme oxygenase 1 (HO-1) and inducible nitric oxide synthase (iNOS).

HSP70 and HO-1 (also called HSP32), originally identified as 'heat shock proteins', are well known cellular proteins [26] with chaperone activity and involvement in protein folding and cell response during stress conditions. Figure 5A shows that both FAEE treatment or UVB irradiation alone determined an elevation of HSP70 above the levels seen in control cells. FAEE treatment of irradiated cells determined an increase of HSP70 level compared to irradiated cells that did not receive FAEE, indicating that FAEE treatment provided a cellular phenotype well suited for standing the UVB-related stress. In Figure 5B the effects of FAEE and UVB on the expression of HO-1 are depicted. Also in this case, NHEM that received FAEE treatment or UVB irradiation showed protein levels higher than control cells. No significant difference was observed in cells pre-treated with FAEE and subsequently exposed to UVB irradiation when compared with UVB-treated cells.

UVB irradiation is associated with an increased expression of iNOS and the following generation of

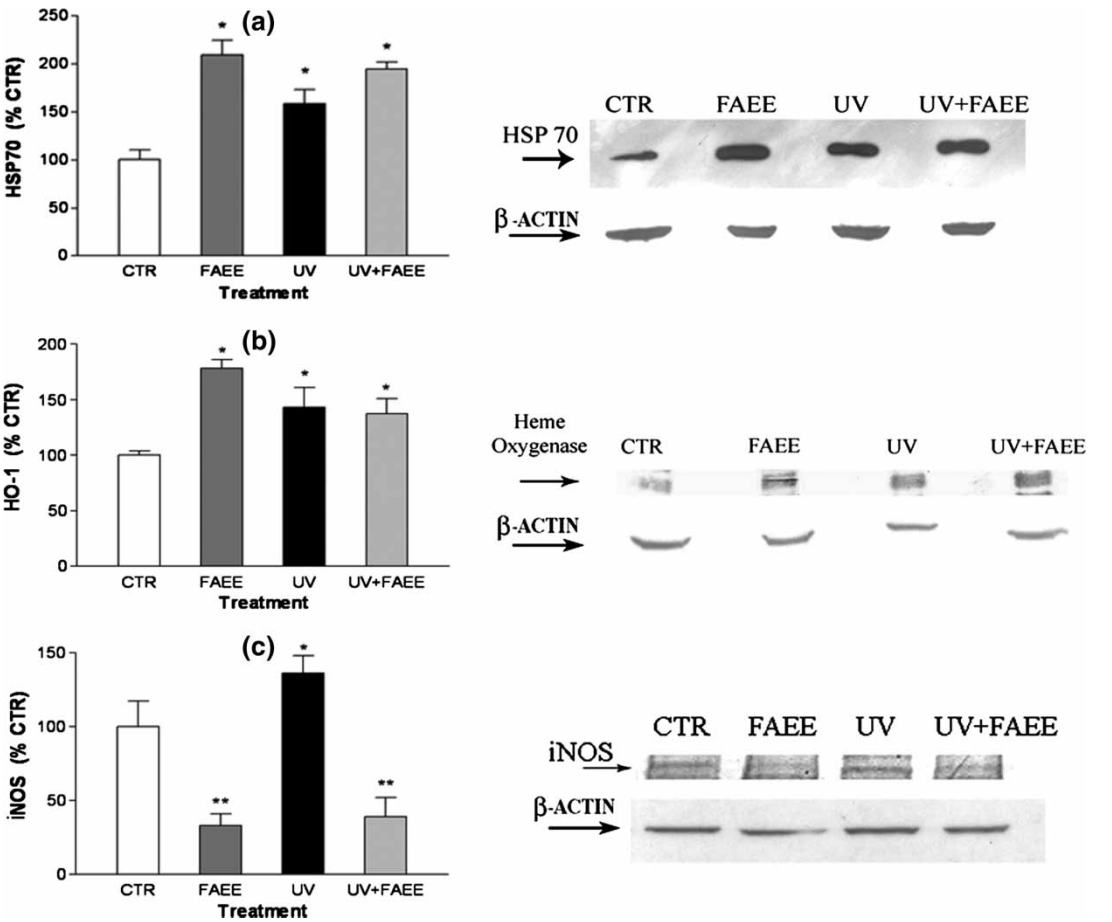

Figure 5. Induction of HSP70 (A) and HO-1 (B) and depletion of iNOS expression (C) by FAEE. NHEM growing in medium containing 25 μM FAEE were submitted to UVB irradiation (50 J/m^2). After 5 h incubation, HSP70, HO-1 and iNOS levels were measured by Western blot analysis using specific antibodies. Immunoblots were scanned by densitometry and all values were normalized to β-actin. Densitometric values are given as percentage of CTR values ($n=6$) and represent the mean $\pm$ SEM of six independent experiments. $^{*}p<0.05$ vs CTR; $^{**}p<0.01$ vs CTR. CTR = not irradiated untreated cells; FAEE = not irradiated treated cells; UV = irradiated untreated cells; UV + FAEE = irradiated treated cells.

nitric oxide (NO) [27]. The NO level is a crucial factor in the regulation of many homeostatic mechanisms. However, NO, beside this regulatory function, through the reaction with superoxide anion and the production of the strong oxidant species peroxynitrite, may be responsible for severe oxidative damage to the cell structures. In the current study, we show that FAEE treatment markedly decreased the basal level of iNOS expression and that following UVB irradiation its level was only slightly elevated, remaining largely below the level of control cells (Figure 5). Conversely, in FAEE untreated NHEM UVB irradiation induced a sharp elevation of iNOS levels.

Protective effect of FAEE against UVB-induced DNA damage and apoptosis

To assess the functional effects of FAEE treatment on cell survival, the expression level of PARP and the accumulation of cytoplasmic nucleosomes were measured. PARP is an enzyme involved in DNA repair whose levels are elevated during the early steps of apoptosis induction. As can be seen in Figure 6A, UVB irradiation induced a sharp elevation of PARP expression, which was completely suppressed in FAEE-treated cells. The same kind of data is observed for cytosolic nucleosomes levels, a late stage marker of apoptosis activation (Figure 6B). A sharp elevation of apoptosis was seen in UVB-treated cells. FAEE treatment completely counterbalanced the UVB stimulus, as indicated by the level of nucleosomes, which consistently remained at the basal level.

Discussion

Recreational exposure to solar radiation is unanimously recognized as the main cause of the increasingly growing incidence of malignant melanoma. Indeed, the UV component of solar light and mostly the UVB band has proved to be a powerful carcinogen, with both initiating and promoting activities. These carcinogenic activities are generated by two distinct mechanisms of genetic damage: (i) the induction of structural alteration of DNA (and of other macromolecules) by direct physical interaction of incident radiation with hydrogen bonds within the genetic material and (ii) the generation of the highly aggressive ROS ($O_2^{\bullet -}$; H_2O_2; $OH^{\bullet}$) and reactive nitrogen species (RNS). These in turn activate a cascade of reactions that directly and indirectly generate various alterations and derivatizations of the genetic material as well as of other macromolecules [29,30]. Accordingly, a variety of antioxidant agents have been widely used in the formulation of skin care products and great attention has been posed on the identification and characterization of new molecules able to prevent, reduce or repair the UV-mediated cell damage. Ferulic acid (FA) is an antioxidant agent widely distributed in the plant kingdom. Biochemical studies have shown that FA possesses interesting antioxidant and scavenging activities and because of these properties it has been included into the formulation of skin protecting creams. In recent works, we demonstrated the *in vitro* and *in vivo* antioxidant properties and cytoprotective effects exerted by FAEE, the ethyl ester of FA, on neuronal cells and synaptosomal systems under chemical and oxidative stresses [13–16].

In the present study we evaluated the protective effects of FAEE against the UVB- induced cell damage on human melanocytes, the specialized skin cells whose elective function is radiation shielding. Our findings showed that FAEE treatment completely prevented the impairment of cell viability

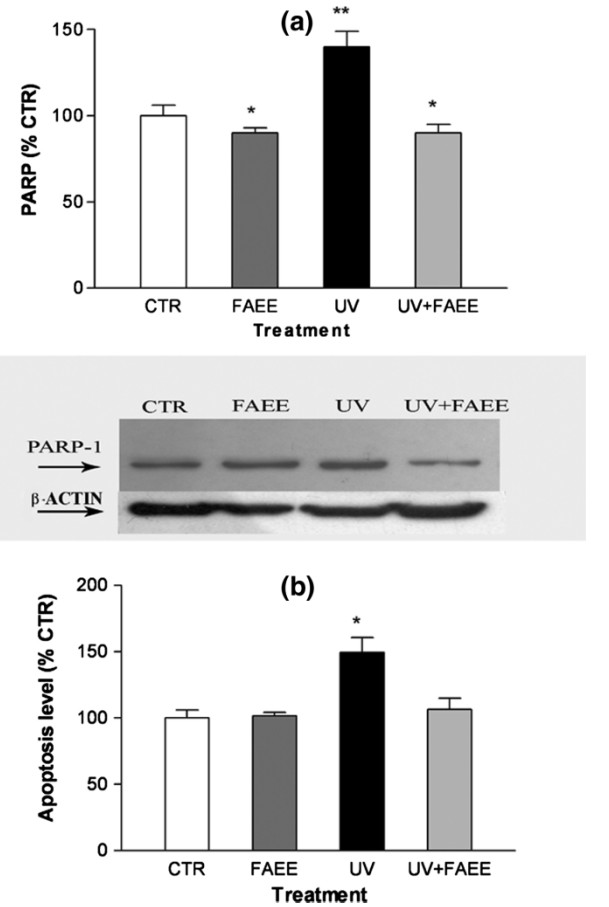

Figure 6. Protective effect of FAEE against UVB-induced DNA damage (A) and apoptosis (B). Estimation of DNA damage was performed by determining PARP expression levels. NHEM growing in medium containing 25 μM FAEE were submitted to UVB irradiation (50 J/m^2). After 5 h incubation, PARP levels were measured by Western blot analysis using specific antibodies. Immunoblots were scanned by densitometry and all values were normalized to β-actin. Apoptosis was evaluated by means of cytoplasmic nucleosomes determination. Results are given as percentage of CTR values ($n=6$) and represent the mean ±SEM of six independent experiments. $^{\star}p<0.05$; $^{\star\star}p<0.01$. CTR = not irradiated untreated cells; FAEE = not irradiated treated cells; UV = irradiated untreated cells; UV+FAEE = irradiated treated cells.

induced by exposure to UVB radiation. This protection was achieved with a concentration much lower than the one needed to induce a similar effect using the corresponding free acid. This result confirms the reports of other authors [8,31] and lends support to the hypothesis that, because of its increased hydrophobicity and its three dimensional structure, the ethyl ester (but not the free acid) can establish an interaction with cell membranes, rendering them less prone to the structural perturbation of oxidative stress [14]. Because membranes are particularly vulnerable to oxidative stress, representing a primary target of radiation toxicity, this ability to stabilize membranes represents an important feature of the FAEE pharmacological profile.

Another immediate effect produced by UVB interaction with the aqueous environment of living systems is the generation of ROS. Through the interaction with the abundant O_2 and e^- species the superoxide ion is generated. This relative mild radical fuelling the Fenton reaction generates the much more reactive, non-charged highly diffusible H_2O_2 species and the mostly reactive $OH^{\bullet}$.

FAEE treatment markedly suppressed intracellular ROS generation following UV irradiation. This improved efficiency may be related to the FAEE structure: through the phenolic moiety and the extended side chain, free radical species, such as hydroxyl and peroxyl radicals, are readily trapped, generating resonance-stabilized phenoxy radicals [7,16].

One of the mechanisms linking ROS generation to cell damage is the structural and functional oxidative modification of macromolecules. Essentially, oxidatively modified proteins are either functionally inactive or deregulated. Accordingly, often they are not repaired and removed from the cellular pool by proteolytic degradation. These modified molecules can be quantitatively measured and their content has been shown to increase in a number of diseases and processes [32].

In order to confirm that in FAEE-treated cells the reported suppression of ROS generation was indeed associated with a lack of oxidative damage, we evaluated the extent of cell protein oxidation. Our results have clearly demonstrated that carbonylated proteins in melanocytes submitted to UVB irradiation and FAEE treatment were significantly lower than those seen in cells that did not receive FAEE treatment. Superimposable results were also obtained measuring HNE and 3-nitrotyrosine (3-NT) adducts on proteins. HNE is a reactive alkenal generated by the attack of ROS to arachidonic acid that binds to proteins, altering their conformation [23,25], 3-NT is formed by reaction of RNS with proteins and is another index of oxidative damage [22,33]. Both adducts were found significantly lower in irradiated melanocytes submitted to FAEE treatment compared to irradiated control samples.

The generation of oxidizing species is a constant issue for living systems. Accordingly a wide number of cellular mechanisms have been evolved to contrast the generation of oxidizing species and to reduce their impact. Thus, oxidative stress actually occurs whenever the burden of oxidizing potential overwhelms the cell total antioxidant capacity. In response to oxidative stress a wide set of response proteins are induced in the recipient cell, including the heat shock proteins (HSPs). These proteins, initially characterized in the context of cellular stress responses [34], were shown to be induced by oxidative stress [35–37].

HSP70 is the major heat-inducible protein [26] and it has been reported to exert a cytoprotective effect under a number of conditions [38]. The inducible isoform of HSP 32, named HO-1, is considered the most sensitive and reliable indicator of cellular oxidative stress. The proposed antioxidant role for HO-1 has been attributed to increased generation of the antioxidant bilirubin during heme degradation catalysed by the enzyme [39] and it has been proposed as an effective system to counteract oxidant-induced cell injury [40–42].

Therefore, to evaluate the effect of FAEE treatment on antioxidant-related cellular mechanisms the expression of HSP70 and HO-1 were examined. Interestingly, FAEE treatment was able to induce an elevation of both protein expression levels, mimicking the effects of naturally occurring stimuli such as UVB or hyperthermia. This finding suggests that FAEE can act as an indirect antioxidant by inducing stress responce proteins, such as HSPs [43]. Dinkova-Kostova et al. [44] demonstrated that, similarly to curcumin, ferulic acid derivatives also contain Michael acceptor functionalities and have been shown to react with sulphydryl groups of Keap 1 that, once inhibited, do not repress transcription factor Nrf2 which then binds to the antioxidant response element (ARE) and activates transcription of stress responce proteins [45].

On the other hand, the cell response mechanisms elicited by oxidative stress may even contribute to amplify its pathological effects. Indeed skin UVB irradiation is associated with an increased expression of cytosolic inducible nitric oxide synthase (iNOS) and generation of nitric oxide [26]. This gas can react with superoxide anion, forming peroxynitrite, a reactive and strong oxidant substance able to induce protein nitration and nitrosation, which alters the function of different key enzymes, therefore inducing a marked cellular cytotoxicity [46]. Accordingly, it

has also been reported that iNOS expression is increased in oxidative stress conditions [27] and suppressed by antioxidant compounds [14,47]. Consonant with these reports, our results indicate that UVB irradiation in control untreated cells resulted in significant iNOS induction, along with a sustained condition of nitrosative stress, as demonstrated by the formation of 3-NT. FAEE pretreatment prominently suppressed iNOS induction, indicating that FAEE could reduce the direct generation of RNS-induced secondary damage. However, FAEE alone was able to modulate the basal levels of iNOS, as we have previously demonstrated in a synaptosomal system [14]. We suggest that, besides the effect of FAEE alone on iNOS, the protective effect of FAEE relies on its ability to block the activation of iNOS induced by UVB irradiation, thus modulating the inflammatory process and the oxidative burden cascade activated by nitric oxide. Further studies are needed to elucidate the effects of FAEE on iNOS protein expression levels by investigating the intracellular pathways possibly responsible for this molecular response.

Considering the importance of NO in mediating immune and inflammatory responses, this effect may contribute to quenching the inflammation component of UVB-mediated tissue damage. We speculate that such an effect of FAEE may be related to the reported induction of HSP70 [15,16] and observed in the present study. Indeed, an interaction between HSP70 and iNOS has been reported and it has been hypothesized that the complex formed between the two enzymes might decrease the enzymatic activity of iNOS and subsequently decrease NO production [48].

At the cellular level, a major consequence of UVB damage is the induction of apoptosis. This indicates that the genotoxic damage is too great to be efficiently repaired and therefore the deeply deregulated cell, potentially able to originate a neoplastic clone, has to be suppressed. Therefore, to evaluate at the cellular level the impact of the reported molecular effects of FAEE, we measured the level of cellular apoptosis. In accordance with the bio-molecular results reported above, in UVB-irradiated, FAEE-treated cells a clear reduction of apoptosis induction was seen when compared with that of untreated cells. This protection involved both PARP activation and cytoplasmic nucleosomes formation, namely early and late steps in the apoptotic pathway, indicating that FAEE treatment is associated with a global protection from UVB damage.

Once again, this anti-apoptotic effect, as far as PARP activation is concerned, might be mediated by the HSP70 induction by FAEE. A reduced apoptosis in cells that expressed HSP70, caused by a decreased caspase-3 activity [49,50] has been reported. Further, cells which contained elevated levels of HSPs were more resistant to stress conditions and spared from the apoptotic pathway [51].

In summary, through experiments with UVB—the most relevant skin oxidative stress—on its elective cell target—human melanocytes—data have been obtained indicating the antioxidant and cell protection power of FAEE. This polyphenol proved able to reduce the generation of ROS and to elevate cell protective mechanisms (HSPs), resulting in a marked reduction of oxidative adducts on cell proteins. Moreover, FAEE treatment was able to suppress the secondary generation of NO-derivate oxidizing agents due to iNOS induction. These biochemical activities integrate at the cellular level in a significant suppression of cell apoptosis, the ultimate effect of oxidative genotoxic damage. Thus, FAEE is strongly implicated as a potentially valuable pharmacological agent to reduce UVB-related damage and to potentiate protective cell responses in human melanocytes.

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