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Modulation of paraoxonase-2 in human dermal fibroblasts by UVA-induced oxidative stress: A new potential marker of skin photodamage

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ABSTRACT

Paraoxonase-2 (PON2) is an intracellular protein, that exerts a protective role against cell oxidative stress and apoptosis. Genetic and environmental factors (i.e. dietary factors, cigarette smoke, drugs) are able to modulate cellular PON2 levels. The effect of ultraviolet A radiation (UVA), the oxidizing component of sunlight, on PON2 in human dermal fibroblasts (HuDe) has not been previously explored. Excessive UVA radiation is known to cause direct and indirect skin damage by influencing intracellular signalling pathways through oxidative stress mediated by reactive oxygen species (ROS) that modulate the expression of downstream genes involved in different processes, e.g. skin photoaging and cancer. The aim of this study was, therefore, to investigate the modulation of PON2 in terms of protein expression and enzyme activity in HuDe exposed to UVA (270 kJ/m^2). Our results show that PON2 is up-regulated immediately after UVA exposure and that its levels and activity decrease in the post-exposure phase, in a time-dependent manner (2–24 h). The trend in PON2 levels mirror the time-course study of UVA-induced ROS. To confirm this, experiments were also performed in the presence of a SPF30 sunscreen used as shielding agent to revert modulation of PON2 at 0 and 2 h post-UVA exposure where other markers of photo-oxidative stress were also examined (NF-KB, γH2AX, advanced glycation end products). Overall, our results show that the upregulation of PON2 might be related to the increase in intracellular ROS and may play an important role in mitigation of UVA-mediated damage and in the prevention of the consequences of UV exposure, thus representing a new marker of early-response to UVA-induced damage in skin fibroblasts.

1. Introduction

Paraoxonase-2 (PON2) is an intracellular protein belonging to the multigene family of paraoxonases (PONs) which comprises three genes (PON1, PON2 and PON3) endowed with hydrolase and lactonase activities [1]. Although the physiological functions of PON proteins are still poorly understood, their antioxidant, anti-apoptotic and anti-atherogenic roles have been demonstrated [2,3]. PON2 is widely expressed in many cell types and tissues and is mainly localized within cells at the level of the lipid bilayer of the endoplasmic reticulum, the perinuclear region, mitochondria and the plasma membrane [4]. The predominant localization of PON2 in mitochondria supports its role in the prevention of oxidative mitochondrial damage by interacting with Coenzyme Q10 [5,6]. In fact, by using different experimental models, it has been demonstrated that PON2 modulates cell oxidative stress and apoptosis and that genetic and environmental factors (i.e. dietary factors, cigarette smoke, drugs) are able to modulate cellular PON2 levels [1,3]. As example, its role against oxidative stress generated by various sources has been demonstrated in human vascular endothelial cells [7–9], intestinal cells [10–13] and in macrophages [6,14–16]. Furthermore, alterations in PON2 levels have been observed in several diseases where oxidative stress represents a pathogenetic factor [3,17–19] including skin diseases [20]. Indeed, it has recently been shown to be involved in human skin cancer, where PON2 overexpression in both melanoma and basal cell carcinoma was correlated with its aggressiveness [21,22]. Interestingly, PON2 gene expression was also shown to be significantly up-regulated 3-fold in keratinocytes (HaCaTs) in response to a single dose of long UVA light (385 nm), indicating its susceptibility

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to regulation upon UV exposure, most likely as a defensive measure of skin against UV-induced oxidative stress [23].

It is well-known that of the UV rays reaching the earth's surface, the UVA ones spanning between 320 and 400 nm are the most abundant (~95%) and most penetrating within the skin layers, reaching the dermis where resident fibroblasts reside [24]. Within the skin dermal layer, UVA rays trigger the generation of reactive oxygen species (ROS) *via* photosensitization reactions mediated by skin chromophores such as riboflavin, urocanic acid and porphyrins [24]. This results in oxidative damage to major biological macromolecules comprising DNA, with the formation of oxidized bases and cyclobutane pyrimidine dimers (CPDs) that can lead to skin cancer [25]. Moreover, excessive UVA exposure causes the breakdown of elastic fibres and denaturation of collagen fibres, responsible for skin photoaging [26]. Consequently, to defend itself against UVA-induced damage, the skin responds with several lines of protection, including the increase in thickness of the stratum corneum layer, increase in the amount of melanin [27], induction of DNA repair enzymes and antioxidant defence enzymes [28]. Among the antioxidant defence enzymes, superoxide dismutase (SOD1), catalase (CAT), thioredoxin, and glutathione peroxidase (GPx) are those most well-known and studied for their role in detoxifying ROS [29]. However, besides the aforementioned study on HaCaTs exposed to UVA light where the only endpoint studied concerning PON2 was gene expression, not much else is known about the involvement of PON2 as a skin antioxidant enzyme for mitigating UVA-induced damage. Therefore, the present study was carried out to evaluate the modulation of PON2 in terms of protein expression and enzyme activity, in cultured human dermal fibroblasts exposed to UVA light, with the aim of proposing PON2 as a novel potential marker of skin photodamage. To confirm this, experiments were also performed in the presence of a sunscreen used as shielding agent to revert modulation of PON2 by UVA exposure.

2. Materials and methods

2.1. Reagents

Cell culture reagents were obtained from Euroclone (Euroclone S. p. A., Pero, MI, Italy) and Corning (Fisher Scientific Italia, Segrate,MI, Italy). All chemical reagents unless otherwise stated were purchased from Merck (Merck KGaA, Darmstadt, Germany). The fluorescent chloromethyl derivative of 2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) and GuavaVia Count solution was from Invitrogen (Invitrogen, Carlsbad, CA, USA) and Cytek (Cytek, Fremont, CA, USA) respectively.

2.2. Cell culture

Human dermal fibroblast cells (HuDe) were purchased from the Istituto Zooprofilattico Sperimentale (Brescia, Italy) and derived from pooled samples of female donors (40 years). HuDe were grown at 37 °C in a CO_2 Heraeus BB15 incubator (ThermoFisher Scientific, Schwerte, Germany) under humidified atmosphere. Minimum Essential Medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM stable glutamine, 1% penicillin (100 U/mL), 1% streptomycin (100 μg/mL) was used for cell culture, and it was refreshed every 2 days before sub-culturing when cells reached 80% confluence by trypsinization. For the experiments, cells were seeded in 6-well plates at an optimal density of 10×10^3 cells/cm^2 unless otherwise stated.

2.3. UVA exposure

After reaching the right confluence, HuDe cells were irradiated from above at a distance of 20 cm from the light source consisting of a Philips Home Solarium sun lamp (model HB 406/A; Philips, Groningen, Holland) equipped with a 400 W ozone-free Philips HPA Lamp, UV type 3, delivering a flux of 45 mW/cm^2 between 300 and 400 nm. The dose of UVA and emission spectrum were measured as reported previously [30]. For all experiments unless otherwise stated, cells were exposed to 10 min UVA (~270 kJ/m^2) based on preliminary time-course experiments. Before UVA exposure, cells were firstly washed and covered with a thin layer of PBS before placing a 2 mm-thick quartz slab of the same dimension as the cell-culture plate on top. The plate was then placed on a brass block embedded on ice to limit evaporation during UVA irradiation.

When sunscreen (Hallstar Sunscreen) with Blue Light Protection and with a Sun Protection Factor (SPF) 30 was used, a quantity equivalent to 2 mg/cm^2 was spread with a gloved finger on custom-made quartz discs of exactly the same dimension as the wells of a 6-well culture plate. After 15 min in the dark, the discs were placed over each well containing HuDe before exposure. As positive control, a disc with no sunscreen was used, while for the negative control, the cells were not exposed to UVA.

2.4. Cell viability and intracellular ROS detection

After UVA exposure, cells were washed with PBS and a 10 μM CM-H_2DCFDA solution in MEM (with 1% FBS) was added to each sample and incubated in the dark for 15 min at 37 °C. After trypsinization, cells were harvested and centrifuged at 600 *g* for 5 min and the resulting cell pellet was resuspended in approximately 50 μL of culture medium. An aliquot of 20 μL from each sample was then added to 180 μL Guava Via-count solution as previously reported [31]. This counterstaining was used to detect viable, apoptotic and dead cells.

The analyses for cell viability and intracellular ROS production were conducted simultaneously on a Guava Easycite flow cytometer (Luminex, Austin, Texas, USA) using an excitation wavelength of 488 nm. Emissions were recorded using the green channel for CM-H_2DCFDA and the red and yellow channels for the Via-count dye, using the following gain settings: FSC 29.3; SSC 23.6; G18.2; Y49.4; R22.6 and a threshold of 1000 on FSC. The fluorescence intensity was recorded on an average of 5000 cells from each sample.

Oxidation of the ROS-sensitive probe results in a large shift in green fluorescence, proportional to ROS formation. For analyzing the production of intracellular ROS, one region or gate relative to cells with high levels of green fluorescence (% of cells with high ROS) was arbitrarily set around 10% in non-irradiated cells. This setting was then maintained for all experiments. The results were analyzed using In-cyte software (Luminex, Austin, Texas, USA).

2.5. Western immunoblotting

Total cell lysates for Western immunoblotting were obtained using RIPA Buffer (1% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate) containing protease (Roche, Switzerland) and phosphatase inhibitor cocktail. Proteins were then quantified using the BCA protein assay. Proteins were resuspended in Laemmli buffer, denatured at 90 °C for 5 min and loaded (20 μg) onto 12% sodium dodecyl sulphate polyacrylamide gel for separation following SDS-PAGE. Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes overnight at 4 °C for Western blotting analysis. Following blocking and washing, the membranes were incubated with specific primary antibodies for immunoblotting analysis. The antibodies used were as follows: rabbit polyclonal PON2 (1:500) (Sab1303623, Merck KGaA, Darmstadt, Germany), rabbit monoclonal Vinculin (1:1000) (A2752, Abclonal, Woburn, MA, USA), mouse monoclonal NFκB p65 (1:500) (Sc-8008, Santa Cruz, TX, USA), rabbit monoclonal Phospho–NF–κB p65 (Ser536) (1:500) (3033, Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal Histone H2AX (1:500) (ab124781, Abcam, Cambridge, UK) and rabbit monoclonal H2AX phospho S139 (1:500) (AB81299, Abcam, Cambridge, UK), Anti-AGE (AB9890, Merck KGaA, Darmstadt, Germany). Goat anti-mouse IgG-HRP (1:5000) (sc-2005, Santa Cruz Biotechnology, Dallas, TX, US) and goat anti-rabbit IgG-HRP (1:8000) (12–348, Merck KGaA, Darmstadt, Germany) were used as secondary antibodies. Protein bands were developed using Clarity Max Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA). The chemiluminescent signal was acquired using ChemiDoc XRS + System (Bio-Rad Laboratories, Hercules, CA, USA) and protein levels were analyzed using Image J software (Version 1.50i, National Institute of Health, Bethesda, MD, USA).

2.6. PON2 activity assay

PON2 lactonase activity was evaluated using dihydrocoumarin (DHC), as substrate as described by Simonetti et al. [20] with some modifications. Briefly, total cell lysates were obtained in RIPA buffer containing protease and phosphatase inhibitors. Protein concentration was determined using the BCA assay. A stock solution of 100 mM DHC was freshly prepared in methanol. The reaction buffer (50 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$) containing 3 mM DHC was incubated with cell lysate (30 μg of protein) in 96 multiwell plates. DHC hydrolysis was measured by monitoring the increase in absorbance at 270 nm for 10 min at room temperature with readings every 15 s. Non-enzymatic hydrolysis of DHC was subtracted from the total rate of hydrolysis. PON2 lactonase activity was expressed as U/mg protein; one unit of lactonase activity is equal to μmol of DHC hydrolyzed per minute.

2.7. Statistical analysis

All experiments were performed at least three times in duplicate and conducted in different experimental sessions. The data are shown as the mean ± SD. One-way analysis of variance (ANOVA) was carried out using GraphPad PRISM 8.2 software to evaluate any statistical differences among more than two different samples. A value of $p < 0.05$ was considered statistically significant (Tukey's post-hoc multiple-comparison test).

3. Results

3.1. Effect of UVA exposure on HuDe cell viability and intracellular ROS levels

The effect of 10 min UVA exposure (270 kJ/m^2) on HuDe cells was assessed both at time 0 h (T0), i.e. right after exposure, and at 2 h, 4 h and 24 h post-UVA exposure. As shown in Fig. 1A, UVA exposure induced a significant decrease in cell viability at T0 along with a concomitant increase in apoptotic and dead cells compared to the non-irradiated ones (Ctrl). However, a significant increase in cell viability was observed at 2 h post-exposure with the percentage of live cells returning to those of the Ctrl cells; the percentage of apoptotic cells 2 h post-exposure was about half of that found in UVA-exposed cells at time 0 h and this percentage did not return to baseline values, even after 24 h post-exposure (Fig. 1A). As shown in Fig. 1B, a significant increase in percentage of cells with high ROS was observed in UVA-exposed cells (73.1 ± 6.6%), compared to the non-irradiated ones (11.9 ± 1.0%). In the post-irradiation phase, a time-dependent decrease in the percentage of cells with high ROS levels was observed; the percentage of cells with high ROS levels 2 h post-exposure was almost halved compared to T0 (43.7 ± 6.2%); a slight decrease was observed also at 4 h and 24 h post-exposure (Fig. 1B).

3.2. Effect of UVA exposure on modulation of PON2 protein levels and enzyme activity in HuDe

PON2 levels in irradiated HuDe were almost 2-fold higher compared to the unexposed cells (Ctrl). After 2 h post-exposure, UVA-induced upregulation of PON2 started to decline becoming significant from 4 h onwards, reaching those of the unexposed Ctrl cells at 24 h (Fig. 2). To determine whether the UVA-induced modulation of PON2 protein results in an active protein, we measured lactonase activity. A significant increase in PON2 activity was observed in UVA-exposed cells (0.39 ± 0.08 U/mg) compared to the Ctrl (0.18 ± 0.03 U/mg) ($p < 0.001$). At 2 h post-exposure, this activity (0.19 ± 0.02 U/mg) returned similar to that of the Ctrl cells (data from Fig. 4C without sunscreen (−), see later).

3.3. Immunoblotting of markers of photo-oxidative stress

Based on the above results, 2 h was selected as post-exposure time to carry out a series of subsequent experiments, using other markers of photo-oxidative stress to further support our findings regarding PON2 modulation by UVA in HuDe. Literature data reports that ultraviolet rays increase the content in skin of advanced glycation end products (AGEs) [32]. Therefore, to better characterize our experimental model, we investigated the formation of AGE-proteins in UVA-exposed HuDe. Higher levels of advanced glycation end products (AGE)-modified proteins were observed in UVA-exposed cells compared to Ctrl cells; 2 h post-UVA exposure the levels were not significantly different compared to Ctrl (data from Fig. 5B without sunscreen (−), see later).

γH2AX is considered a sensitive marker of UVA-induced DNA damage [33], as also observed in the present study. We found that UVA exposure induced the phosphorylation of H2AX at Ser139 (γH2AX) with a significant increase in γH2AX/H2AX ratio. The ratio in UVA-exposed cells at T0 (2.26 ± 0.45) was about twice compared to the control one (1.03 ± 0.04) ($p < 0.001$), whereas at 2 h post-exposure, the levels returned close to those of the unexposed control cells (0.62 ± 0.30) (data from Fig. 6 A/B without sunscreen (−), see later).

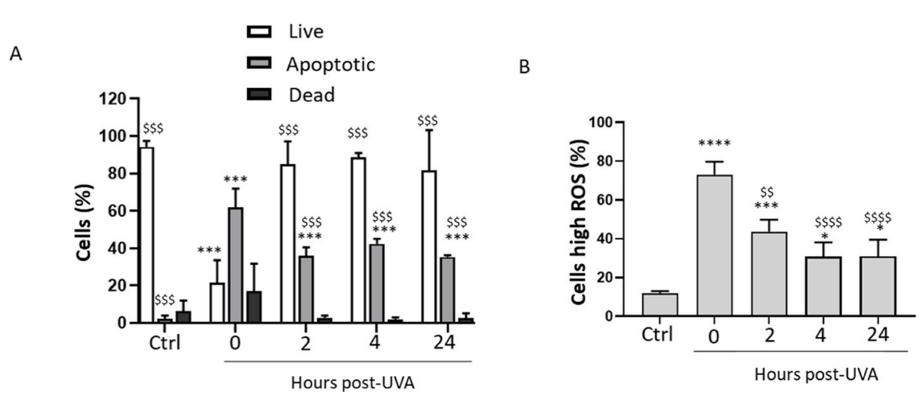

Fig. 1. Flow cytometric analysis of cell viability (A) and intracellular levels of high ROS (B) in HuDe not exposed (Ctrl) or exposed to 10 min UVA (270 kJ/m^2) at time 0 h, i.e. right after exposure, and 2 h, 4 h and 24 h post-UVA exposure. Data are reported as percentage of live, apoptotic or dead cells determined using Guava® ViaCount™ reagent (A), and as percentage of cells presenting high intracellular ROS levels determined using the ROS-sensitive probe, CM-H_2DCFDA (B). Error bars represent ± S.D. *$p < 0.05$, ***$p < 0.0001$ vs Ctrl; $$ $p < 0.001$, $$$ $p < 0.0001$, $$$$ $p < 0.00001$ vs UVA-exposed cells at 0 h.

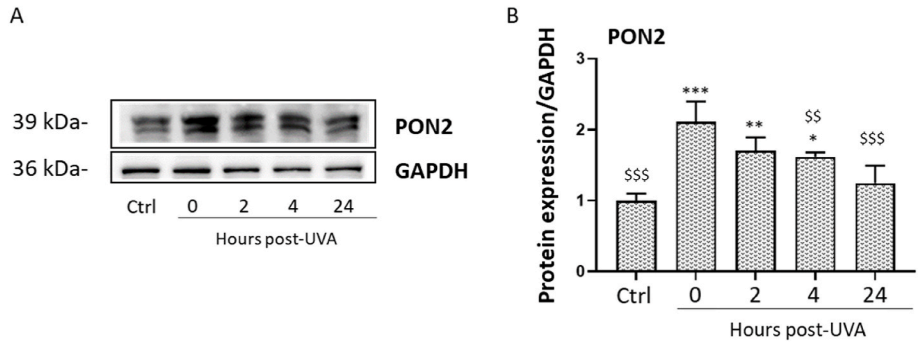

Fig. 2. PON2 levels measured by Western immunoblotting in HuDe not exposed (Ctrl) or exposed to 10 min UVA (270 kJ/m^2) at 0 h, i.e. right after exposure, and 2 h, 4 h and 24 h post-UVA exposure. Representative Western blots of PON2 expression (A); densitometry data normalized on GAPDH (B). Error bars represent ± S.D. *p < 0.05, **p < 0.001, ***p < 0.0001 vs Ctrl; $$ p < 0.001, $$$ p < 0.0001 vs UVA-exposed cells at 0 h.

The transcription nuclear factor-Kb (NF-KB) is known to be activated by oxidative stress induced by UVA exposure in skin fibroblasts leading to immunological, inflammatory and apoptotic responses in cells through transcription activation [34,35]. Indeed, in UVA-exposed cells at T0, an increase in pNF-KB/NF-KB ratio (1.69 ± 0.33) was observed compared to Ctrl cells (1.02 ± 0.04) and that at 2 h post-exposure, the pNF-KB/NF-KB ratio (1.01 ± 0.28) dropped back down to those of the unexposed cells (data from Fig. 6C/D without sunscreen (−), see later). Table 1 summarizes the main effects of UVA exposure in HuDe cells right after exposure and 2 h post-UVA exposure.

3.4. Effect of sunscreens on UVA-induced damage in HuDe

The above characterized cellular model of UVA-induced damage on HuDe (summarized in Table 1) was used to investigate the shielding effect of sunscreens to revert PON2 expression and the other markers of photo-oxidative stress. As shown in Fig. 3, no significant modification in cell viability was observed in screened cells at 0 h and at 2 h post-UVA exposure with respect to non-irradiated cells (Ctrl) (Fig. 3A (+)). An increase in the percentage of high ROS cells was observed in screened cells (18.1 ± 5.2) with respect to Ctrl ($p < 0.05$) at time 0 h; 2 h post-UVA exposure the levels were similar to the Ctrl. At both time-points, the percentage of high ROS in the presence of sunscreen were significantly lower compared to UVA exposed cells in the absence of cream (Fig. 3B).

Concerning modulation of PON2, the data reported in Fig. 4B shows that after UVA exposure, an increase in PON2 levels was observed in screened cells (+) compared to the non-irradiated Ctrl; no significant modification in PON2 levels was observed 2 h post-exposure compared to Ctrl. At both time points, PON2 levels observed in screened samples were significantly lower compared to UVA exposed cells in the absence of sunscreen (Fig. 4B (+)). No significant modification in PON2 activity was observed in UVA exposed cells in the presence of sunscreen compared to Ctrl (Fig. 4C (+)) and, at T0, PON2 activity in screened cells was significantly lower compared to UVA-exposed cells in the absence of sunscreen.

A lower increase in AGE-modified proteins levels was observed in screened cells (+) compared to cells irradiated without sunscreen at both times (Fig. 5B). At 2 h post-UVA exposure, the levels of AGE-modified proteins returned close to those of the Ctrl (Fig. 5B).

Concerning γH2AX/H2AX and pNF-KB/NF-KB ratios (Fig. 6B/D), these were reduced in the presence of sunscreen compared to the UVA exposed samples in the absence of sunscreen at T0. The ratios reached were similar to those found in the unirradiated control cells.

Table 1
Scheme summarizing the main effects of UVA exposure in HuDe cells right after exposure, 0 h and 2 h post-exposure. (↑) increase, (↓) decrease, (=) no change compared to non-irradiated cells.

Markers	Hours post-UVA exposure	
	0 h	2 h
Live cells (%)	↓↓↓↓	=
Apoptotic cells (%)	↑↑↑↑	↑↑
Dead cells (%)	↑	=
Cells High ROS (%)	↑↑↑↑	↑↑
PON2 protein	↑↑	↑
PON2 activity (U/mg)	↑↑	=
AGE-modified proteins	↑↑	=
γH2AX/H2AX	↑↑	=
pNF-KB/NF-KB	↑↑	=

4. Discussion

In this study we demonstrated for the first time that the enzyme PON2 is modulated by UVA exposure in dermal fibroblasts. We observed an up-regulation of PON2 by UVA exposure with a time-dependent decrease in the post-exposure phase; the enzyme level and activity decreased until they reached the basal levels at 2 h and 24 h, respectively. This increase in PON2 levels mirrored the time-course study of UVA-induced ROS and cellular viability in these dermal cells, where a rapid increase in intracellular ROS production and a rapid decline in viability straight after exposure were observed, followed by a decrease in the post-irradiation phase, until the ROS and viability levels reached the basal ones at about 24 h. These data confirm that UVA exposure induces oxidative stress in fibroblasts [30,31,36,37]. Besides fibroblasts, other dermal cell types have also been shown to be differently susceptible to UVA toxicity, suggesting a complex and unelucidated relationship between cell type, pigmentation, oxidative stress and DNA repair [38,39]. Higher levels of AGE-modified proteins were also observed in UVA-exposed fibroblasts and the levels returned close to those of the unexposed control at 2 h post-UVA exposure. The UVA-induced formation of AGE-modified proteins has not been previously investigated in HuDe cells; however the data are in agreement with previous studies where it was shown that UV rays increase the content of skin AGEs and that UV light-induced accumulation of AGEs such as carboxymethyllysine and pentosides in the skin promotes oxidative stress [32,40].

As previously mentioned, NF-KB signalling plays a key role in several cellular responses and in cellular survival under stress, including UVA exposure [34,35]. NF-KB activation requires its dissociation from its inhibitory protein IκB and subsequent p65 phosphorylation at serine residues 276, 529, and 536 to facilitate nuclear translocation [41]. Previous studies have reported that ROS are able to activate upstream kinases (IKK, NIL, Akt) that can influence the degradation of IκB and the nuclear translocation of NF-KB [42]. We observed a transient activation of NF-KB (through serine 536 phosphorylation) with an increase in the pNF-KB/NF-KB ratio in UVA-exposed HuDe that returned to basal levels 2 h after exposure, demonstrating that indeed, HuDe undergo oxidative

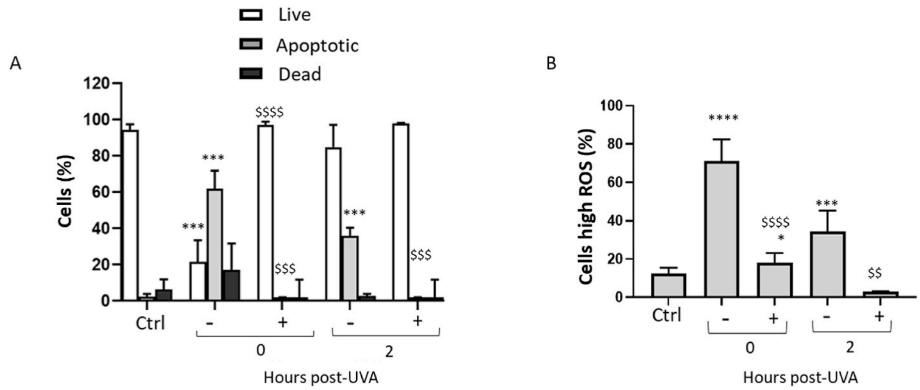

Fig. 3. Flow cytometric analysis of cell viability (A) and intracellular levels of ROS (B) in HuDe exposed to 10 min UVA (270 kJ/m^2), in the presence (+) or in the absence (−) of sunscreen at time 0 h, i.e. right after exposure, and 2 h post-UVA exposure. Data are reported as percentage of live, apoptotic or dead cells determined using Guava® ViaCount™ reagent (A) and as percentage of cells presenting high intracellular levels of ROS determined using the ROS-sensitive probe, CM-DCFH-DA (B). Error bars represent ± S.D. *p < 0.05, ***p < 0.0001, ****p < 0.00001 vs Ctrl; $$ p < 0.001, $$$ p < 0.0001, $$$$ p < 0.00001 vs UVA-exposed cells without sunscreen at their respective times.

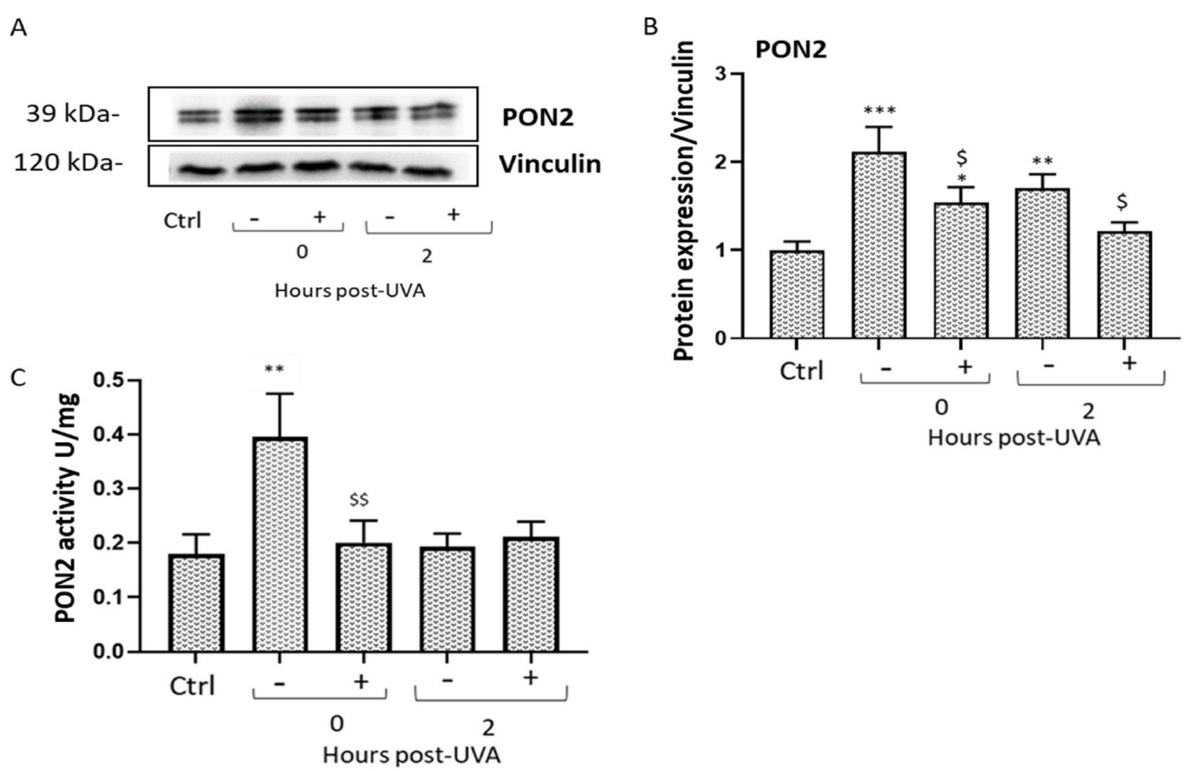

Fig. 4. PON2 levels measured by Western immunoblotting (A, B) and PON2 lactonase activity (C) in HuDe exposed to 10 min UVA (270 kJ/m^2) in the presence (+) or in the absence (−) of sunscreen, at time 0 h, i.e. right after exposure, and 2 h post-UVA exposure. Representative Western blots of PON2 expression (A); densitometry data normalized on Vinculin (B). Error bars represent ± S.D. *p < 0.05, **p < 0.001, ***p < 0.0001 vs Ctrl; $ p < 0.05, $$ p < 0.001 vs UVA-exposed cells without sunscreen at their respective times.

stress upon UVA exposure.

In our experimental model, we also observed a concomitant increase in serine 139-phosphorylated γ-histone H2AX (γH2AX) levels and increase in γH2AX/H2AX ratio in UVA-exposed cells, confirming that UVA treatment is associated with DNA damage. This is in accordance with Greinert et al. who demonstrated that γH2AX is induced upon UVA irradiation of primary HuDe as observed using the comet assay and markers of DNA double strand breaks [33]. We observed that the γH2AX/H2AX ratio returned to basal levels 2 h after exposure. This trend in γH2AX activation is also in agreement with data from Valente et al. (2002) who reported that in HuDe treated with 0.2 Gy of X-ray irradiation, γH2AX reached the maximum peak in a very short time (about 30 min after irradiation), after which, the levels decrease until they reach the basal levels at about 24 h after irradiation [43].

For children older than 6 months as well as adults, regular sunscreen use with an SPF of 30 or higher for people of all skin types is recommended for any extended outdoor activity by dermatologists worldwide. An SPF30 sunscreen is considered a high SPF factor and is the most popular one used. Our study also demonstrates that the presence of an SPF30 sunscreen was able to protect HuDe cells from UVA damage at the cellular and molecular level by reverting the markers of photo-oxidative stress and PON2 expression. In fact, PON2 levels and activity in screened samples were significantly lower compared to UVA exposed cells in the absence of sunscreen and are similar to those of non-irradiated cells.

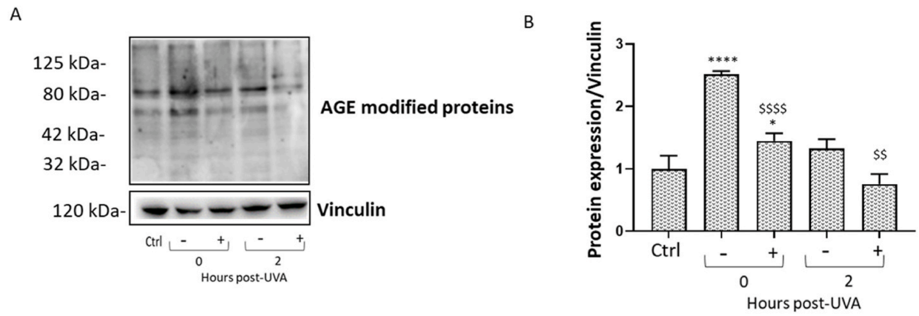

Fig. 5. AGE-modified proteins measured by Western immunoblotting in HuDe exposed to 10 min UVA (270 kJ/m^2) in the presence (+) or in the absence (−) of sunscreen, at time 0 h, i.e. right after exposure, and 2 h post-UVA exposure. Representative Western blots of AGE-modified protein expression (A); densitometry data normalized on Vinculin (B). Error bars represent ± S.D. *p < 0.05, ****p < 0.00001 vs Ctrl; $$ p < 0.001, $$$$ p < 0.00001 vs UVA-exposed cells without sunscreen at their respective times.

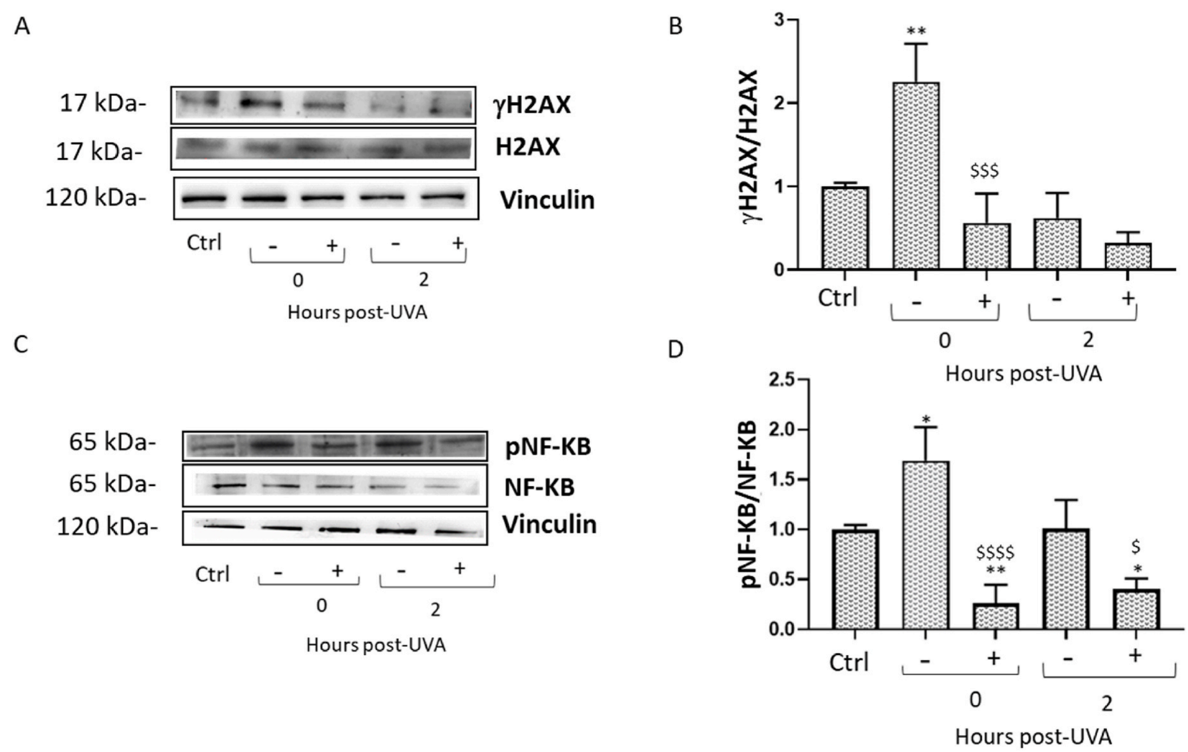

Fig. 6. γH2AX, H2AX, pNF-KB/NF-KB proteins measured by Western immunoblotting in HuDe exposed to 10 min (270 kJ/m^2) with UVA in the presence (+) or in the absence (−) of sunscreen, at time 0 h, i.e. right after exposure and 2 h post-UVA exposure. Representative Western blots of γH2AX and H2AX (A) and of pNF-KB and NF-KB (C); densitometric ratios of γH2AX/H2AX (B) and pNF-KB/NF-KB (D). Error bars represent ± S.D. *p < 0.05, **p < 0.001 vs Ctrl; $ p < 0.05, $$$ p < 0.0001, $$$$ p < 0.00001 vs UVA-exposed cells without sunscreen at their respective times.

Overall, these data suggest that PON2 upregulation could represent a marker of very-early response to UVA-induced damage in skin fibroblasts. DNA damage and the activation of NF-KB may contribute to the apoptotic process observed in UVA-stimulated cells and may be involved in the UVA-mediated regulation of PON2 levels. In fact, previous studies have reported that NF-KB is involved in the activation of antioxidant targets by regulating the expression of different genes involved in the regulation of ROS and the sensitivity of cells against oxidative stress, including mitochondrial antioxidants (i.e manganese superoxide dismutase) [44] and of paraoxonase-1 (PON1) [45,46]. PON1 and PON2 are both members of the paraoxonases gene family and PON2 displays a 66% sequence identity and 81% similarity with PON1, suggestive of a similar regulation [1,47]. Whatever are the mechanisms involved in the UVA-induced regulation of PON2 expression and activity beyond transcription in HuDe cells, several hypotheses can be advanced concerning the physiological consequences.

We can hypothesize that UVA-induced increase in PON2 expression and activity may represent a mechanism to compensate the enhancement in oxidative stress in order to minimize the damaging effects of ROS in HuDe cells. This hypothesis is supported by the evidence in the literature on *in vitro* and *in ex-vivo* models. High cellular oxidative stress showed a stimulatory effect on PON2 activity and protein expression in different cell models, including macrophages during differentiation [15] or under treatment with different oxidants (AAPH, or $CuSO_4$, or Fe-ascorbate) [16] and bladder cancer cells (T24) treated with caper extract [48]. In addition, mouse peritoneal macrophages (MPMs) isolated from atherosclerotic apolipoprotein E deficient mice undergoing oxidative stress, showed higher PON2 mRNA levels compared with MPMs from control C57BL6 mice [14]. A reduction in ROS formation by vitamin E was associated with a parallel decrease in PON2 expression and activity both *in vitro* in THP-1 cells and *ex-vivo* in MPM isolated from Balb/C mice after supplementation with vitamin E [15].

The high expression level of PON2 in skin cells such as fibroblasts and keratinocytes, suggests a potential role of this enzyme in skin protection and in the development of skin diseases, which is still poorly investigated [21–23]. In this context, it is important to underline that the UVA dose used in this study (270 kJ/m^2) is a relevant dose of sun exposure considering that a dose of 600 kJ/m^2 can be easily reached during the summer months at midday at a latitude of 50–55° N [49].

UV exposure is one of the most important environmental health hazards, clearly causative for age-related skin changes such as wrinkling, pigmentary changes, thinning and carcinogenesis. UV-induced oxidative stress activates signaling pathways, interferes with genome maintenance and affects apoptosis. In conclusion, our results show that the upregulation of the antioxidant and anti-apoptotic enzyme PON2 might be related to the increase in intracellular ROS and may play an important role in mitigation of UVA-mediated damage and in the prevention of the consequences of UV exposure, thus representing a new marker of early-response to UVA-induced damage in skin fibroblasts. Finding new molecular markers of early response to protect skin cells against UVA insults may have clinical relevance [50]. The induction and regulation of endogenous antioxidant enzymes, such as PON2, and the better understanding of the regulatory mechanisms involved, may offer a good strategy for the treatment and prevention of aging and photoaging in human skin [1,3]. Indeed, the effective transdermal delivery of antioxidant enzymes, such as GPx, CAT and SOD1 to prevent UV oxidative stress-induced pathologic changes in skin has been contemplated [51]. Finally, this work may have consequences in the photoprotection field since alterations in PON2 could potentially be used to determine the efficacy of photoprotective agents at the cellular level, especially during the early phases of research and development of new sunscreen agents.

Author contributions

Conceptualization, T.B. and E.D.; methodology, C.M., A.L., F.M, T.B, E.D.; formal analysis, C.M., A.L., F.M,.; investigation, T.B., E. D,G.F; resources, T.B., E.D., G.F., R.G.; data curation, T.B., E.D.,C.M,A.L,F.M. writing—original draft preparation, T.B., E.D.; writing—review and editing, T.B., E.D., F.G.; supervision, T.B., E.D. All authors have read and agreed to the published version of the manuscript. They confirm that there are no other persons who satisfied the criteria for authorship but are not listed. They further confirm that the order of authors listed in the manuscript has been approved by all authors. They understand that the Corresponding Authors are the sole contact for the Editorial process.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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