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The skin Oxi-Proteome as a molecular signature of exposome stress

KEYWORDS: Skin aging, exposome, oxidative stress, proteomics, carbonylated proteins, human skin models.

ABSTRACT

Exposure to environmental stressors (pollution, UV and HEVL irradiation) inflicts oxidative damage and premature skin aging. The impact of stress on the skin proteome is of special interest since proteins are key players to assure skin function and homeostasis. One of the most detrimental consequences of skin oxidation is protein carbonylation. Their accumulation leads to impaired skin function as decreased hydration and skin barrier disruption. However, the nature of these modifications and the identity of those targeted proteins remain elusive. By using state-of-the art targeted proteomics approaches we have evidenced that urban pollutants and irradiation induce oxidative damage of our skin proteins in keratinocytes, fibroblasts and melanocytes. Ex vivo studies in human skin explants showed that different environmental stressors target distinct skin anatomical compartments. Interestingly, oxidative damage targets only a restricted set of proteins upon the whole proteome, which we defined as the skin "Oxi-proteome". Further studies should underline the benefits from increased protection against carbonylation of such specific proteins and/or the stimulation of their elimination.

INTRODUCTION

Compelling evidence indicate that exposure to environmental insults (indoor or outdoor pollution, UV or high energy visible light (HEVL) irradiation) is a major global concern for public health (1). The skin maintains a major interface between the body and the environment offering a functional biological barrier against external aggressions. Nevertheless, environmental stressors may overcome the skin protective potential. This imbalance can modify the skin structure, resulting in skin diseases, as well as premature aging (2).

As an early event, environmental stressors catalyse the generation of reactive oxygen species (ROS) and they in turn will impact and damage skin macromolecules (proteins, lipids and nucleic acids) leading to oxidative stress and impaired skin cellular functions, resulting in accelerated skin aging and disease (3). Although ROS are generated physiologically in our skin contributing to chronological aging, in the case of exposition to xenobiotic and pollutants, ROS are also generated from the surface of particulate matter (PM) where polycyclic aromatic hydrocarbons (PAH) and benzo(a)pyrene(s) are adsorbed, other than transition metals (iron, copper, etc.) that catalysing Fenton's reaction generate the highly reactive hydroxyl radical able to induce oxidative damage to skin macromolecules (2). In addition, UV irradiation damages skin indirectly through photosensitized production of ROS mainly driven by UV-A, in the range of 320–400 nm, that are able to penetrate to the deeper sections of the epidermis up to reaching the dermis (4).

ROS originating from environmental stressors will impact first our skin proteins, due to their abundance and chemical reactivity (5). Importantly, skin functions are mainly regulated and executed by proteins, though proteomics analyses hold great promise to provide exposome-associated fingerprints as a molecular reflection of genetics, but also environmental factors (e.g., life style). If environmental factors can be disentangled from genetic ones, such fingerprints might even provide a basis for targeted personal care approaches (Figure 1).

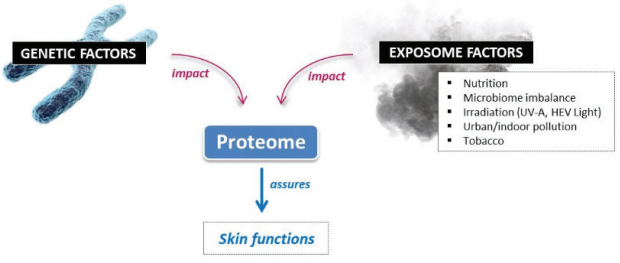

Figure 1. Genetic background and environmental stressors modulate the proteome which is directly related to skin biofunctions.

Upon oxidative stress, increased protein oxidation, leading to the formation of carbonyl derivatives (aldehyde, ketones and lactams) in amino-acid side chains, is particularly detrimental as the resulting damages can render carbonylated proteins inactive and lead to cellular functional abnormalities (6,7), or even toxicity (15). Protein carbonylation is induced either directly by ROS or indirectly by reactions with secondary products of oxidative stress such as reactive aldehydes as 4-hydroxy-2-nonenal and malondialdehyde (8). These aldehydes are produced by the peroxidation of polyunsaturated fatty acids of membrane lipids and can react with protein to form covalent Michael adducts. Proteins can also be carbonylated through the reaction with reducing sugars or reactive dicarbonyl compounds such as glyoxal and methylglyoxal (6).

The negative impact of carbonylated proteins on skin conditions has been demonstrated in previous epidemiological surveys such skin color changes to yellow-dark with aging (9). The skin color changes are induced by the accumulation of oxidized proteins including carbonylated and glycated proteins, which have brownish optical properties in the skin. Further, carbonylated proteins accumulation in the *Stratum*

Corneum (SC) has a negative correlation with skin moisture functions consisting of the water content and *trans*-epidermal water loss (TEWL) (10). It has also been reported that carbonylated proteins in the SC of dry skin in the winter season can be seen at a higher frequency compared with the summer season, indicating that carbonylation extensively affect skin conditions. In order to further understand the mechanisms involved in exposome-induced skin oxidative stress and aging, in this study we have performed targeted-proteomics analyses of carbonylated proteins upon exposome stress on *in vitro* and *ex vivo* human skin models.

RESULTS AND DISCUSSION

Different sources of environmental stress induces carbonylation on distinct anatomic skin compartments

For the first time, we have used *in situ* fluorescent carbonyl detection on human skin explants in order to unravel specific mechanisms of damage upon different environmental stressors. In the past, carbonyls were only detected indirectly by antibodies, upon derivatization with 2,4-Dinitrophenylhydrazine (DNPH) (11). DNPH-based methods have strong limitations: DNPH is a non-selective reactive substance often resulting in over-estimation and non-reproducibility in quantification of carbonylation level. Such indirect detection may complicate and bias quantification (12). More recently, DNPH has been progressively replaced by amino-oxy or hydrazide functionalized fluorophores (13,16). Compared with DNPH, the use of functionalized fluorophore probes increased the accuracy and sensitivity of carbonyl detection.

We have optimized the *in situ* labelling of carbonyls by using amino-oxy functionalized fluorophores to ensure an optimal signal to noise ratio. This innovative approach allows visualizing oxidative damage not only at the molecular level, but also its spatial distribution along different anatomical skin compartments (Figure 2). Human skin explants were irradiated with UV-A or incubated with PM *ex vivo*. Irradiation and PM stress on human skin explants resulted both in increased carbonyl levels. Interestingly, UV-A specifically induces carbonylation in the SC and the dermis, while PM exposition, impacts only in the outermost anatomical compartments of the skin (SC and epidermis). Furthermore, both intracellular and extracellular proteins are impacted by carbonylation (Figure 2). The differential pattern of carbonylated proteins observed suggests the occurrence of different responses of the skin depending on the different type of stress. These differences may pave the road for the rational development of specific protective actives compounds targeting specific sources of environmental stress.

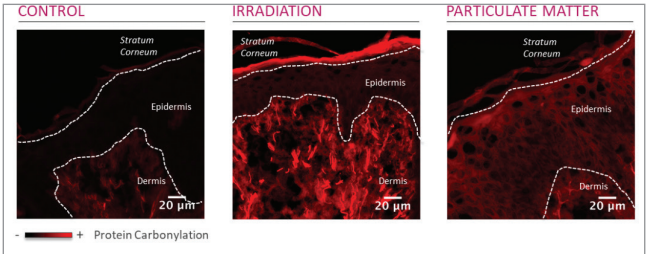

Figure 2. Exposome stress targets oxidative damage on distinct skin compartments. Human skin explants were irradiated with UV-A (center panel) or topically treated with a solution containing particulate matter (right panel). Carbonylated proteins (red) were labelled with specific fluorescent probes and evidenced by epi-fluorescence microscopy (63X). Representative images from at least 10 different donors.

Skin keratinocytes, fibroblasts and melanocytes proteome is oxidized by environmental stress

The occurrence of protein carbonylation in different skin cell types has been studied *in vitro*. Recently, particular attention has been raised to the potential negative effects of overexposure to high energy visible light, and more in particular blue light (400-510nm) emitted by solar irradiation but also from artificial sources, like electronic screens (computers, smartphones, tablets). Recent studies have shown that blue light irradiation induces molecular and cellular changes in normal human fibroblasts impairing cellular functions (14).

Carbonylated proteins are generated in human keratinocytes, fibroblasts and melanocytes upon blue light irradiation and PM exposure (Figure 3). Absolute quantification of carbonylated proteins upon extraction and their electrophoretic separation showed a dose-dependent increase, supporting carbonylated proteins as a sensitive and reliable quantitative readout for exposome-induced skin damage.

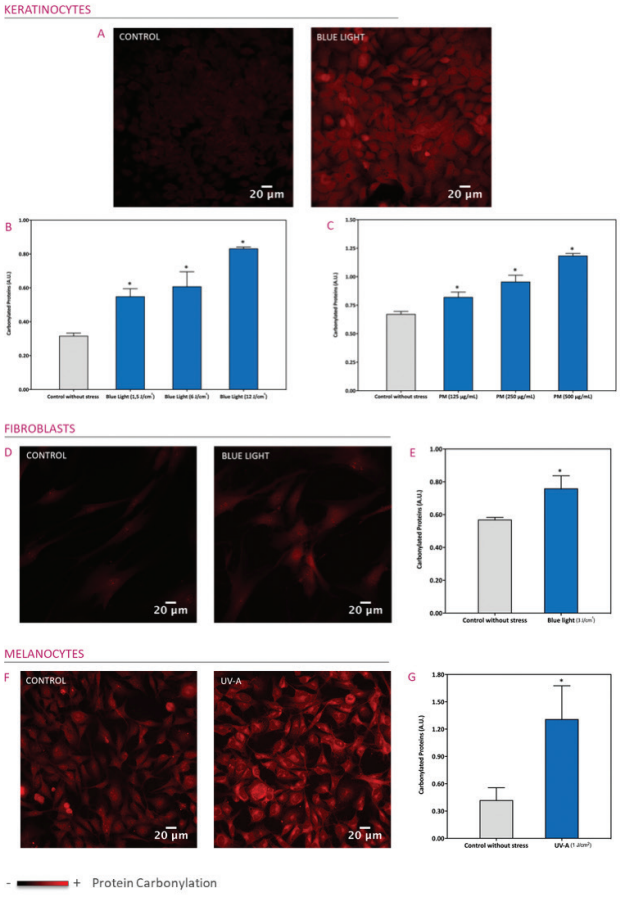

Figure 3. Protein carbonylation on skin cells upon exposome stress. Human keratinocytes (A, B & C), fibroblasts (D&E), or melanocytes (F&G) were exposed *in vitro* to PM, UV-A (365 nm) or blue light (460 nm) irradiation and carbonylated proteins were labelled (A, D & F) and evidenced by epifluorecence microscopy. Absolute quantification was performed upon their extraction and separation by SDS-PAGE. Quantification was performed by densitometric analysis of protein bands. Histograms are presented as mean±S.D (n=5) and analyzed using ANOVA or Student's t-test; *p<0.05 (B, C, E & G).

Different sources of exposome stress target distinct proteins for carbonylation

Carbonylated proteins from skin explants were separated by 2D high-resolution electrophoresis. Interestingly, only a restricted set of skin proteins are target of oxidation and

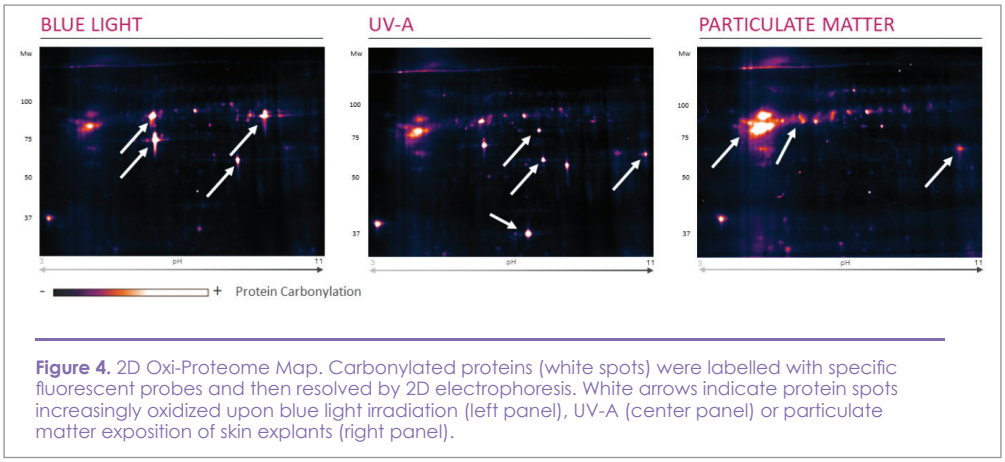

Figure 4. 2D Oxi-Proteome Map. Carbonylated proteins (white spots) were labelled with specific fluorescent probes and then resolved by 2D electrophoresis. White arrows indicate protein spots increasingly oxidized upon blue light irradiation (left panel), UV-A (center panel) or particulate matter exposition of skin explants (right panel).

distinct proteins are damaged upon PM, blue light or UV-A irradiation (Figure 4), suggesting that not all proteins have the same susceptibility for oxidation and they represent a molecular signature of skin damage. Most carbonylated protein spots are in the range of 37 to 100 kDa. Although some proteins spots are targeted by all type of stress, we evidenced that some others are distinct from different sources of stress (Figure 4) Protein spots are now being identified by mass spectrometry approaches. Further studies are focused on the benefits to be gained from increased protection against oxidative modification of such specific proteins and/or the stimulation of their elimination.

MATERIALS AND METHODS

Human organotypic skin explants culture

Skin explants were obtained from abdominal surgery of female Caucasian donors with their informed consent (30-40 years old, phototype II/III). After surgery, they were kept alive by culturing on metal grids into standard 12-well plates in contact with DMEM medium, supplemented with Fetal Bovine Serum (FBS 10%) and penicillin-streptomycin (1%) at 37°C in 5% CO2. Medium was renewed every 24h. Experiments have been performed on at least from 10 different donors.

Human skin cells culture

Immortalized human keratinocytes (HaCat) and human dermal fibroblast (HDF) were routinely cultivated on DMEM, supplemented with FBS 2% or 10% and penicillin streptomycin (1%) at 37°C in 5% CO2. Medium was renewed every other day and cells were passaged at 90% of confluence. hIPSCs derived melanocytes were cultivated on fibronectin coated plates in 254 medium supplemented with human melanocytes growth supplement (HMGS) and FBS 10% at 37°C in 5% CO2. Medium was renewed every other day and cells were passaged at 80% of confluence.

Exposome stress

Skin explants or cells were irradiated with a specific UV-A (365 nm) source at 1 J/cm^2 (melanocytes), 6 J/cm^2 (skin explants) or blue light (460 nm) source at 3 J/cm^2 (fibroblasts), at 1,5 J/cm^2, 6 J/cm^2, 12 J/cm^2 (keratinocytes) and 14 J/cm^2 (explants) using the OxiProteomics® irradiation system in HBSS medium. After irradiation, HBSS as replaced by DMEM for cell recuperation and further analyses. To model urban pollution, keratinocytes were incubated with PM_{10} (Sigma-Aldrich, ERCZM100) at 125, 250, 500 µg/mL or 30 µl of aqueous solutions containing PM_{10} (300 µg/cm^2) were applied topically on explants and incubated at 37°C in 5% CO_2 humidified air for 24h.

Carbonylated proteins *in situ* visualization (Oxi-Proteome View)

Explant sections of 4 µm thickness or cells were fixed with a solution containing ethanol (95% v-v) and acetic acid (5% v-v). Carbonylated proteins were labelled using a fluorescent probe ((Ex = 480 nm / Em = 530 nm) functionalized with an amino-oxy group (13) and DAPI (4',6-diamidino-2-phénylindole) for nuclear staining. Fluorescent images were collected with an epi-fluorescent microscope. Image comparisons were achieved using identical conditions of acquisition (100ms and 63X objective for explants or 40X for cells).

Carbonylated proteins absolute quantification (Carbonyl Score)

Skin explants or cellular pellets were homogenized using a lysis buffer and clarified by centrifugation. Carbonylated proteins were labelled with the above-mentioned fluorescent probe and separated by SDS-PAGE (4-20%). Total proteins were post-stained with Coomassie Blue. Fluorescent scanning was performed using Ettan DIGE (Imager) system. Quantification of carbonylated proteins was performed on digitalized images by densitometric analysis using total protein staining as loading control.

$$\textbf{Carbonylated proteins}\ (\text{sample } X)\ (\text{A. U}) = \frac{\text{carbonylated prot. fluorescent signal (sample X)}}{\text{total prot. fluorescent signal (sample X)}}$$

Statistical analyses.

Two-tailed Student's t-test (Figure 3E, 3G) or one-way analysis of variance (ANOVA) (Figure 3B and 3C) were performed to compare statistical differences between groups. Alpha (α) of 0.05 was used as the cut-off for significance.
All statistical analyses were performed using Graph pad prism software (Graphpad software Inc., San Diego, CA, USA).

Carbonylated proteins 2D electrophoresis resolution (Oxi-Proteome Map)

Samples labelled for carbonylated proteins were separated onto 18 cm immobilized pH gradient strips (pH 3-11, non-linear (NL)). After rehydration, isoelectric focusing was performed using the Ettan IPGphor3 system. After focusing and equilibration, strips were applied to 8-18% (w/v) gradient SDS-PAGE gels and the second dimension was performed. Gels were co-migrated in parallel (1 gel per sample). Data Analysis Preliminary image analyses, including spot detection, background subtraction, normalization, and spot matching were performed using Progenesis Same Spots software (Nonlinear Dynamics, Durham, NC). Protein spots exhibiting Carbonylated proteins ratio consistently two-fold higher than control without stress were considered as increasingly modified.

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