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Modulation of cutaneous scavenger receptor B1 levels by exogenous stressors impairs “in vitro” wound closure

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ABSTRACT

Scavenger receptor B1 (SR-B1) is a trans-membrane protein, involved in tissue reverse cholesterol transport. Several studies have demonstrated that SR-B1 is also implicated in other physiological processes, such as bacteria and apoptotic cells recognition and regulation of intracellular tocopherol and carotenoids levels. Among the tissues where it is localized, SR-B1 has been shown to be significantly expressed in human epidermis. Our group has demonstrated that SR-B1 levels are down-regulated in human cultured keratinocytes by environmental stressors, such as cigarette smoke, via cellular redox imbalance. Our present study aimed to investigate whether such down-regulation was confirmed in a 3D skin model and under other environmental challengers such as particulate matter and ozone. We also investigated the association between oxidation-induced SR-B1 modulation and impaired wound closure. The data obtained showed that not only cigarette, but also the other environmental stressors reduced SR-B1 expression in epidermal cutaneous tissues and that this effect might be involved in impaired wound healing.

1. Introduction

Scavenger receptor B1 (SR-B1) is a trans-membrane glycoprotein identified as the main physiological receptor for HDLs (Acton et al., 1996) and thus it has been studied mostly for its role in cholesterol transport and uptake from HDLs to liver and steroidogenic tissues, influencing cholesterol plasma levels as well as cholesterol distribution into peripheral body compartments (Krieger, 2001). Further studies have demonstrated that SR-B1 exerts several other functions, such as recognition of viruses and bacteria (Bartosch et al., 2005; Vishnyakova et al., 2006), uptake of lipophilic vitamins among which α-tocopherol and carotenoids (Borel et al., 2007) and identification of apoptotic cells (Cao et al., 2004). Recently, the involvement of SR-B1 in cellular exosomes trafficking has been reported as well (Angeloni et al., 2016). In addition to being a multifunctional receptor (Valacchi et al., 2011a,b), SR-B1 is also ubiquitous, being expressed in multiple cells and organs not only related to liver and steroidogenic tissues (Rhainds and Brissette, 2004). Indeed, this receptor was found expressed in human skin, with higher expression in epidermis (Sticozzi et al., 2012) and sebaceous glands (Crivellari et al., 2017).

Skin acts as a shield towards the external environment, therefore its physical integrity is crucial in order to accomplish its functions. Dermal and epidermal injuries, which compromise cutaneous physical barrier, lead to the activation of several cellular mechanisms that intent to restore the structural and functional integrity of the skin, process known as wound healing (Gurtner et al., 2008). During the wound-healing process, keratinocytes are stimulated to migrate over a provisional extracellular matrix, in order to form a cell monolayer over the denuded dermis. Wound healing is crucial and impairment of tissue repair is often associated with pathological conditions (Eming et al., 2014).

Due to its location, cutaneous surface and especially epidermal lipids are constantly subjected to oxidative challenges that can lead to generation of bioactive by-products and eventually affect the equilibrium of the deeper cutaneous layers (Weber et al., 2001). Oxidative processes can be induced by environmental stressors, and air pollution is one of the most studied types characterized by an heterogeneous mixture of gaseous and particles of which composition, size and toxicity depend on the origin microenvironment (Nel, 2005). Cigarette smoke (CS), ozone (O_3) and particulate matter (PM) represent well-known escalating outdoor pollutants and have been associated to delayed

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wound repair and even to several skin disorders, due to their ability to induce oxidative stress and inflammation (Sticozzi et al., 2012; Valacchi et al., 2016; Magnani et al., 2016).

Several studies reported that SR-B1 is sensitive to oxidative stress (Valacchi et al., 2011a,b; Sticozzi et al., 2013). It has been shown that CS-induced oxidative damage led to SR-B1 post-translational modifications and subsequent degradation in human keratinocytes (Sticozzi et al., 2012). As a follow up of our previous work, we want to evaluate the response of cutaneous SR-B1 in a more complex cutaneous model and evaluate its expression in a three-dimensional skin model after exposure not only to CS but also to O_3 and PM. Furthermore, we evaluated SR-B1 as a possible player in wound healing impairment related to oxidative damage.

2. Materials and methods

2.1. Reconstructed human epidermis

Reconstructed human epidermis (RHE, EpiDerm™ Tissue) model was purchased from MatTek (MatTek In Vitro Life Science Laboratories, Bratislava, Slovak Republic). RHE were kept at liquid/air interface in a humidified 5% CO_2 atmosphere at 37 °C in a maintenance medium provided by manufacturer.

2.2. Cell culture

Spontaneously immortalized human keratinocyte HaCaT cells (American Type Culture Collection (ATCC) -P.O. Box 1549 Manassas, VA 20108 USA) were cultured at 37 °C in humidified 5% CO_2 atmosphere in high glucose Dulbecco's Modified Eagle's Medium (EuroClone, Milan, Italy) supplemented with 10% FBS (EuroClone), 2 mM L-Glutamine (EuroClone), 100 U/ml penicillin and 100 µg/ml streptomycin (EuroClone).

2.3. CS exposure

RHE or HaCaT cells were exposed for 30 min to CS generated by burning one research cigarette (12 mg tar, 1.1 mg nicotine) using a vacuum pump to draw air through the burning cigarette and leading the smoke stream into the exposure chamber as described by Valacchi et al. (2011a,b). Control RHE and cells were exposed to filtered air. After exposure, RHE were incubated in fresh media at 37 °C in a humidified 5% CO_2 atmosphere for 30 min and 24 h, while cells were incubated in fresh media at 37 °C in humidified 5% CO_2 atmosphere for different time points from 0 to 24 h.

2.4. Air particles exposure

Concentrated air particles (CAPs) were used as air particulate matter model. CAPs are recognized ambient PM and were generously provided by B. Gonzalez-Flecha. They were collected using a virtual concentrator, the Harvard Ambient Particle Concentrator (HAPC), which concentrates ambient air particles for subsequent exposure in different animal models (Harvard School of Public Health, Boston, Massachusetts). For the exposure of RHE, CAPs suspension in PBS (25 µg/ml) was topically applied on the tissue surface, while for HaCaT cells, CAPs were suspended in culture media (10 µg/ml). Control tissues and cells were exposed to the vehicle (PBS and culture media, respectively). After exposure, tissues were kept at 37 °C in a humidified 5% CO_2 atmosphere in maintenance medium for 24 and 48 h, while cells were incubated at 37 °C in humidified 5% CO_2 atmosphere for different time points going from 0 to 24 h.

2.5. O_3 exposure

O_3 exposure was performed in a Teflon-lined chamber where O_3 was generated from O_2 by electrical corona arc discharge (ECO3 model CUV-01, Torino, Italy) as previously described (Valacchi et al., 2016). The O_2–O_3 mixture (95% O_2, 5% O_3) was combined with ambient air and allowed to flow into the exposure chamber, with the O_3 concentration in chamber adjusted to 0.1 or 0.8 ppm output and continuously monitored by an O_3 detector. RHE were exposed to 0.8 ppm O_3 for 1 and 4 h. HaCaT cells were exposed to 0.1 ppm O_3 for 30 min. Control RHE and cells were exposed to filtered air. RHE were processed immediately after exposure, while cells were incubated in fresh media at 37 °C in a humidified 5% CO_2 atmosphere for time periods going from 0 to 24 h. Doses and timing were chosen based on our previous work using the same described models (Valacchi et al., 2015a,b; Valacchi et al., 2016).

2.6. Protein extraction

At each time point, skin tissues were washed with PBS and freezed in liquid nitrogen. Lysates from liquid nitrogen-freezed RHE were extracted in ice-cold T-PER buffer (Thermo Fisher Scientific, MA, USA) added of protease and phosphatase inhibitor cocktails (Sigma, Milan, Italy). Lysates were cleared by centrifugation (13,500 rpm) for 15 min at 4 °C and protein concentration was measured by Bradford method (BioRad, CA, USA).

2.7. Protein carbonyls

The levels of proteins carbonyl groups in RHE were determined by OxyBlot (Chemicon, USA). Briefly, after derivatization of carbonyl groups to dinitrophenylhydrazone (DNP-hydrazone) by reaction with dinitrophenylhydrazine (DNPH), the DNP-derivatized protein samples of each experimental condition were separated by polyacrylamide gel electrophoresis followed by Western blotting. Control conditions were pooled together and analyzed as Control group.

2.8. Western blot analysis

Equivalent amounts of proteins were subjected to 10% SDS-PAGE, electro-transferred onto nitrocellulose membrane, which was then blocked in Tris-buffered saline, pH 7.5, containing 0.5% Tween 20 and 5% not-fat milk (BioRad). Membranes were incubated overnight at 4 °C with rabbit anti-SR-B1 antibody (Novus Biologicals, Milan, Italy). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (Millipore, Darmstadt, Germany) for 1 h at RT, and the bound antibodies were detected in a chemiluminescent reaction (ECL, BioRad). Control conditions for each stressor exposure were pooled together and analyzed as Control group. Images of the bands were digitized and the densitometry of the bands was performed using ImageJ software.

2.9. Immunohistochemistry

RHE were immersion-fixed in 10% NBF (neutral-buffered formalin) for 24 h at RT, then dehydrated in alcohol gradients and embedded in paraffin. Sections (4 µm) were deparaffinized in xylene and rehydrated in alcohol gradients. After dewaxing, sections were incubated overnight at 4 °C with rabbit anti-SR-B1 antibody (Novus Biologicals), followed by 1 h with goat anti-rabbit Alexa Fluor 488 antibody (Thermo Fisher Scientific) at RT. Nuclei were stained with 1 µg/ml DAPI (Molecular Probes, Oregon, USA) for 30 min after removal of secondary antibody. Sections were mounted onto glass slides using anti-fade mounting medium 1,4-diazabicyclooctane in glycerine (DABCO) and examined by the Zeiss Axioplan2 light microscope equipped with epifluorescence at different magnifications. Negative controls were performed by omitting primary antibody. Images were acquired with Axio Vision Release 4.6.3 software.

2.10. Wound healing assay

HaCaT cells were grown to complete confluence and the adherent cell layer was scratched with a sterile pipette tip (Valacchi et al., 2009). Cellular debris were removed by washing off with PBS and fresh media was added. Cells were immediately exposed to stressors under the above mentioned conditions, followed by incubation at 37 °C. Images of the scratches for each sample were recorded in three different areas, using a built-in camera in an inverted Nikon Eclipse microscope at time points going from 0 to 24 h. Same time points were considered when scratch assay was performed on SR-B1 knockdown cells. The width of the scratches was analyzed with ImageJ software.

2.11. SR-B1 knockdown

HaCaT cells of 50–60% confluence were transfected using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. 300 µl Opti-MEM medium (Gibco, NY, USA) were mixed with 6 µl of Lipofectamine 2000 and 2 µl of a 100 µM siRNA solution (Ambion, Cambridgeshire, UK) or scrambled control RNA solution (Invitrogen). After 10 min incubation at RT, 250 µl of the mixture were added to 2 ml DMEM supplemented with 2 mM L-Glutamine and transferred to the cells. Cells were then incubated at 37 °C for 24 h.

2.12. Immunocytochemistry

HaCaT cells were grown to complete confluence on coverslips at a density of 1×10^5 cells/ml and scratched with pipette tip. Cells were immediately exposed to the above-mentioned stressors. At each post-exposure time point, cells were fixed in 4% paraformaldehyde (PFA) in PBS for 30 min at RT. Cells were permeabilized and then blocked in PBS containing 1% BSA at RT for 1 h. Coverslips were then incubated for 1 h with rabbit anti-SR-B1 antibody (Novus Biologicals), followed by 1 h with goat anti-rabbit Alexa Fluor 488 antibody (Thermo Fisher Scientific). Nuclei were stained with 1 µg/ml DAPI (Molecular Probes) for 5 min after removal of secondary antibody. Coverslips were mounted onto glass slides using anti-fade mounting medium 1,4-diazabicyclooctane (DABCO) in glycerine and examined by the Zeiss Axioplan2 light microscope equipped with epifluorescence. Negative controls were performed by omitting primary antibody. Images were acquired with Axio Vision Release 4.6.3 software.

2.13. Statistical analysis

For each of the variables tested, analysis of variance (ANOVA), followed by Bonferroni post-hoc test, or Kruskal-Wallis, followed by Dunn's post-hoc test, was used. Statistical significance was considered at $p < 0.05$. Data are expressed as mean ± SD of triplicate determinations obtained in three independent experiments.

3. Results

3.1. Environmental stressors induced oxidative damage in cutaneous tissue

Multiple in vitro and in vivo studies have demonstrated the ability of outdoor stressors, such as CS, PM and O_3, to promote oxidative-related cellular modifications to the skin (Valacchi et al., 2012). We evaluated the effect of these 3 different pollutants (CS, CAPs, as PM model, and O_3) on tridimensional reconstructed human epidermis (RHE). RHE contains all differentiated epidermal layers (Font et al., 1994), therefore represents an efficient in vitro skin model. As depicted in Fig. 1, all stressors analyzed were able to significantly increase oxidative stress levels in the epidermis, as indicated by protein carbonyls levels. After 30 min of CS exposure the levels of carbonyls reached a pick (28 AU) that persisted for the following 24 h (left upper panel). A different trend was noticeable for CAPs and O_3 were the level of protein carbonyls increased over the exposure time. Indeed, there was an increase of carbonyls in CAPs-exposed tissues of *circa* 50% between the 24 and the 48 h and 25% between 1 and 4 h in O_3 exposed tissues (central and right panels, respectively).

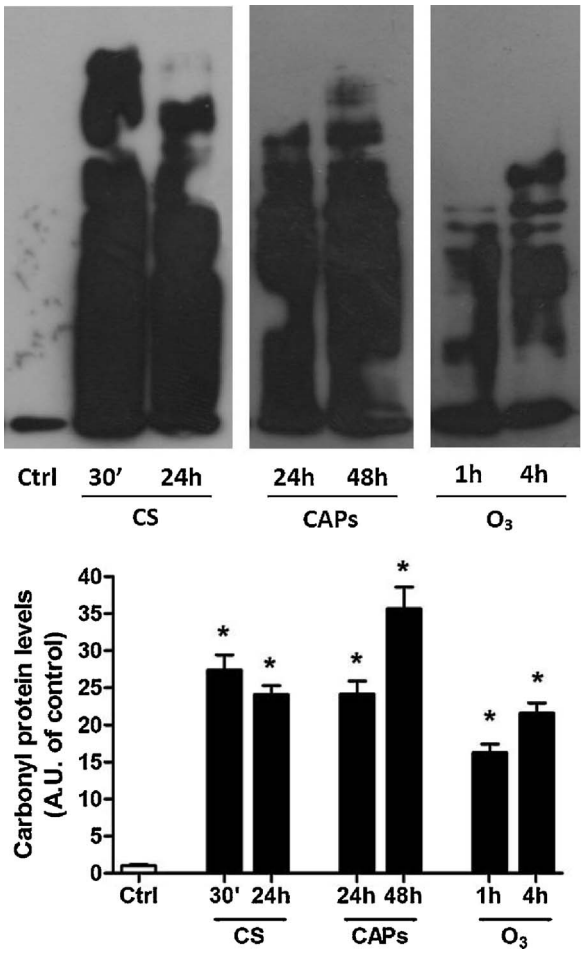

Fig. 1. Stressors-induced protein oxidation in RHE. RHE were exposed to CS, CAPs and O_3. WB shown in the top are representative of three experiments. Quantification of protein carbonyl bands is shown in the panel bellow. Data are expressed as arbitrary units (averages of three different experiments, *$p < 0.05$ by ANOVA).

3.2. CS exposure reduced SR-B1 protein expression in human epidermis

As illustrated by in Fig. 2A, upon CS exposure, SR-B1 protein expression decreased of 40% already after 30 min and the decrease was even more evident after 24 h (50%). Furthermore, immunofluorescence analysis not only confirmed the decrease of SR-B1 (green signal) after CS exposure but let us appreciate the even distribution of the receptor thoroughly the epidermis. (Fig. 2B).

3.3. SR-B1 levels in RHE were reduced upon particles exposure

As depicted in Fig. 3A, SR-B1 protein levels decreased in time-dependent manner after CAPs exposure, reducing the levels of the receptor of circa 50% and 40% after 24 h and 48 h respectively. This effect was also appreciated by immunofluorescence, where SR-B1 green signal appeared clearly down regulated in tissues exposed to CAPs at the different time points. This down regulation was more accentuated in the upper epidermal layers after 24 h, while it appeared more evenly distributed throughout all layers at 48 h (Fig. 3B).

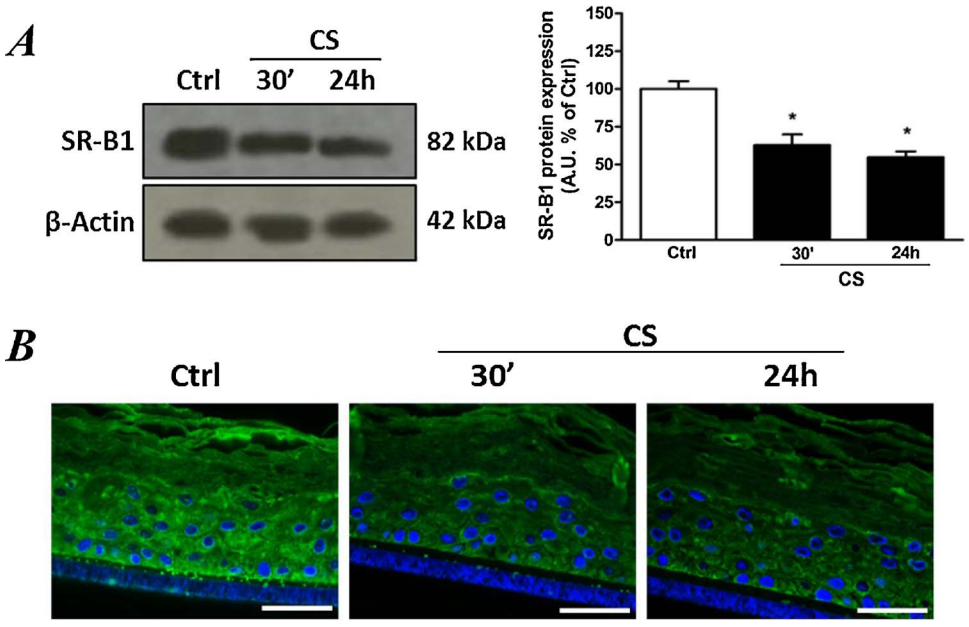

Fig. 2. Exposure to CS decreased SR-B1 protein levels in RHE. RHE were exposed to CS for 30 min and tissues were harvested after 30 min and 24 h. WB shown in the top is representative of three independent experiments. Quantification of SR-B1 bands is shown in the right panel. Data are expressed as arbitrary units (averages of three experiments, *p < 0.05 by ANOVA). β-Actin was used as loading control (A). Data were confirmed by IHC for SR-B1; green staining represents SR-B1 and blue staining (DAPI) represents nuclei; scale bar 50 μm (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Ozone affected SR-B1 epidermal levels

Finally, we exposed RHE to O_3 for 1 and 4 h and, as shown in Fig. 4A, O_3 induced a clear and significant decrease of SR-B1 expression at the different time points (40% after 1 h and of 50% after 4 h). Again, the immune blot results could be appreciated also by immunofluorescence (Fig. 4B).

3.5. Outdoor stressors impaired keratinocytes repair ability possibly through SR-B1 regulation

CS, O_3 and air particles are pollutants that have in common the ability to induce oxidative stress and have been associated with poor skin repair ability. Keratinocytes play a crucial role in cutaneous healing, in order to cover the wound and protect it from external environment. Therefore, we exposed human keratinocytes to CS, O_3 and CAPs and investigated the wound closure process. As shown in Fig. 5A, exposure to CS significantly impaired the ability of the cells to recover from the induced scratch-wound after 24 h. Similar pattern, although less dramatic, was observed when keratinocytes were exposed to CAPs and O_3. Wound closure reached circa only 50% upon CAPs exposure (Fig. 5B) and approximately 40% after O_3 exposure (Fig. 5C).

As expected, SR-B1 expression was down regulated by all three pollutants also in cultured keratinocytes, as indicated by immunostaining performed during scratch closure (Fig. 5D). Surprisingly, in control condition we observed an evident increase of SR-B1 expression (green signal) in the cells present at the edge of the scratch 6 and 12 h after wounding. Such increase, together with the association between SR-B1 down regulation and delayed scratch closure upon stressors exposure, suggests that this receptor could have an active role in cutaneous wound healing process.

3.6. SR-B1 played a key role in keratinocytes wound healing process

The use of siRNA for SR-B1 was applied to confirm the role of SR-B1 in keratinocytes wound closure. As depicted in Fig. 6, SR-B1-silenced

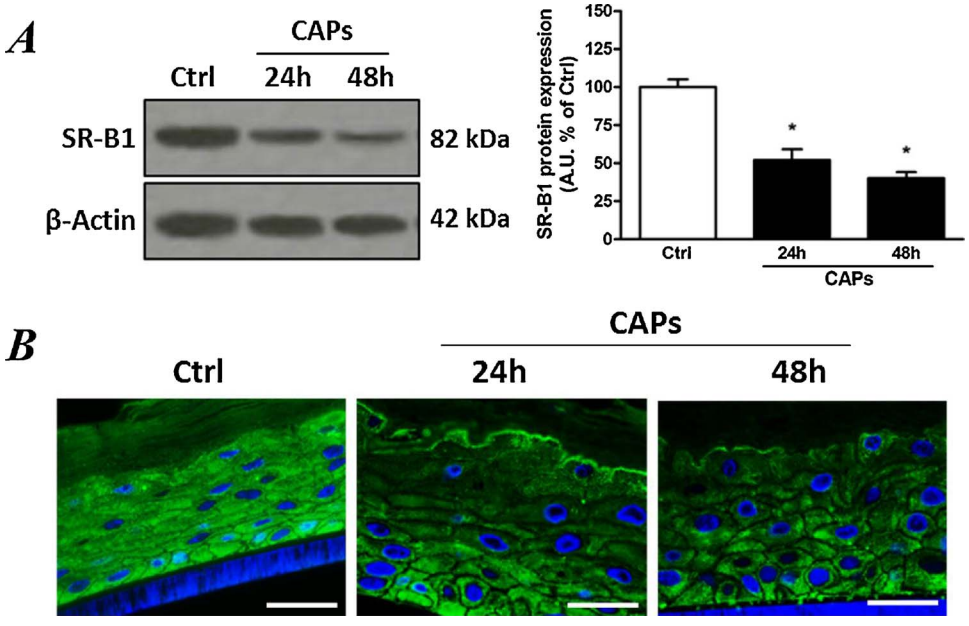

Fig. 3. Exposure to CAPs decreased SR-B1 expression in RHE. RHE were exposed to 25 μg/ml CAPs for 24 and 48 h and tissues were harvested forthwith. WB shown in the top is representative of three experiments. Quantification of SR-B1 bands is shown in the panel on the right. Data are expressed as arbitrary units (averages of three different experiments, *p < 0.05 by ANOVA). β-Actin was used as loading control (A). Data were confirmed by IHC for SR-B1; green staining represents SR-B1 and blue staining (DAPI) represents nuclei; scale bar 25 μm (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

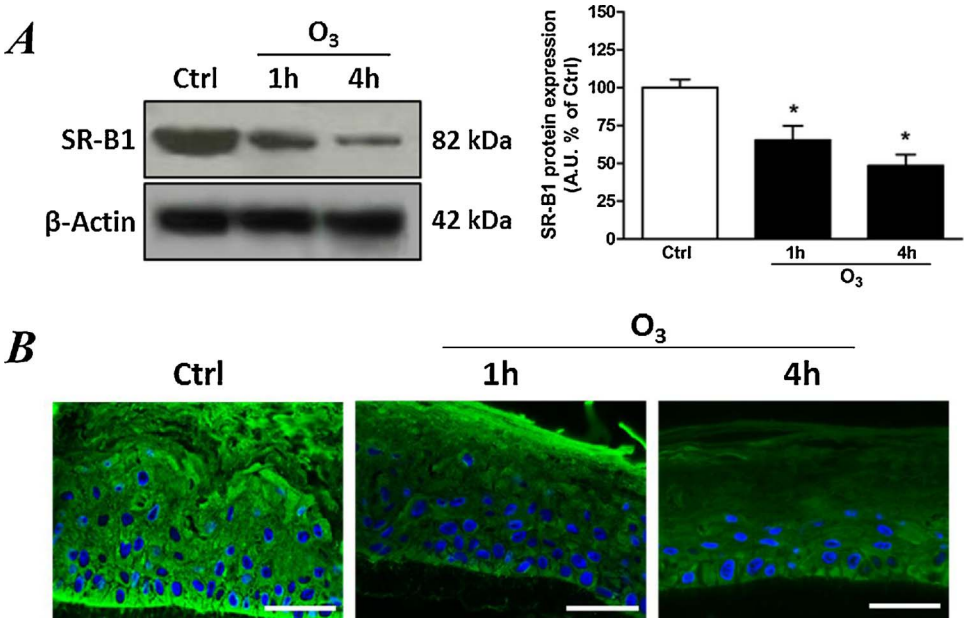

Fig. 4. Exposure to O_3 decreased SR-B1 expression in RHE. RHE were exposed to 0.8 ppm O_3 for 1 and 4 h and tissues were harvested forthwith. WB shown in the top panel is representative of three independent experiments. Quantification of SR-B1 bands is shown in right panel. Data are expressed as arbitrary units (averages of three experiments, *$p < 0.05$ by ANOVA). β-Actin was used as loading control (A). Data were confirmed by IHC for SR-B1; green staining represents SR-B1 and blue staining (DAPI) represents nuclei; scale bar 50 μm (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cells had a reduced ability to recover the scratch wound compared to control condition. Indeed, a delayed wound closure of circa 50% after 24 h was determined in SR-B1 KO cells.

4. Discussion

Environmental pollution is a challenge to modern society, especially in developing countries. Today, nations such as China are making huge efforts to reduce air pollution, by promoting energy conservation and emissions reduction (Chen et al., 2013). International initiatives, such as the recent Conference on Climate Change 2015 (COP 21) in Paris, are leading global efforts to address recovery from current serious environmental pollution. It has now been estimated that more than 90% of the urban population lives with pollutant levels over the WHO standard limits, and by 2020, over 5.5 million of people will die due to pollution (www.WHO.int).

Human skin represents the largest organ in human body and it is among the main routes by which environmental chemicals can enter our body (Goldsmith, 1996; Valacchi et al., 2012). Several are the pollutants that have been shown to be noxious to cutaneous tissues and, among them, the most toxic are represented by CS, O_3 and PM. Indeed, all three pollutants have been associated to skin diseases as well as skin aging (Vierkötter et al., 2010; Lee et al., 2011; Xu et al., 2011) and their ability to induce oxidative stress has been associated to their toxic effect. Cutaneous tissue is equipped with a wide array of defensive systems (Packer and Valacchi, 2002) both enzymatic and non enzymatic, therefore, when the exposure to environmental stressors is not excessive, human skin is able to counteract the oxidative damage (Thiele et al., 2002). Thanks to its ability to play a role in tissue uptake of redox quencher such as tocopherol (Mardones et al., 2002), SR-B1 has been investigated also as a receptor involved in the tissue defensive system against oxidative stress and its role in skin has been recently highlighted (Sticozzi et al., 2012). Moreover, SR-B1 might be essential in human skin also for the other physiological processes in which it is involved, such as lipids metabolism and maintenance of skin lipid barrier. Indeed, a study by Tsuruoka and colleagues, has demonstrated that SR-B1 expression is up regulated after mechanic lipid barrier disruption (Tsuruoka et al., 2002) and this can be interpreted as a tissue feedback to keep SR-B1 baseline levels. Furthermore, SR-B1 is relevant also in maintaining the cholesterol reservoir in human sebocytes (Crivellari et al., 2017), which represent an important source of epidermal lipids. In addition SR-B1 is involved in pathogens recognition (Vishnyakova et al., 2006), which might appear significant for receptor's cutaneous expression, since several skin diseases, such as acne (Jahns et al., 2012) and cellulitis (Valacchi et al., 2015a,b), are connected with pathogens intrusion. All these functions underline that the expression of SR-B1 in human cutaneous tissues may have an important role in maintaining skin integrity and homeostasis.

A previous study of our group reported that environmental oxidants, such as CS, induced a striking loss of SR-B1 expression in cultured human keratinocytes due to oxidative post-translational modifications as a consequence of lipid peroxidation products formation (Sticozzi et al., 2012). Cell culture system is more susceptible to CS exposure since it lacks the protective physiological cutaneous structure; therefore, the use of a model closer to skin real morphology is recommended to better understand the effect that environmental pollutants have on human skin. Reconstructed human epidermis (RHE), which presents all functional differentiated epidermal layers (Font et al., 1994) is one of the most reliable in vitro models to investigate cutaneous stress responses.

As previously shown in a 2D keratinocytes culture (Sticozzi et al., 2012), also in a 3D model CS reduced SR-B1 levels and this paralleled with the increased protein carbonyls formation, confirming the involvement of oxidative stress in SR-B1 loss. It is not clear whether the effect of CS on RHE is due to the gas or particulate phase present in CS, but it is possible to speculate that both phases have a role in this process. Indeed, we were able to notice similar effect also by CAPs and O_3 exposure, which represent particulate and gas contaminants.

Previous studies have suggested that small air particles might overpass skin surface due to the adsorbed molecules, which may originate more lipophilic particles (Vierkötter et al., 2010). Another suggested mechanism is the infiltration of the particles through the air follicles (Lademann et al., 2005). Recently, it has been demonstrated the ability of air particles to diffuse through the cornified layer and penetrate into the epidermis, inducing oxidative reactions and lipid peroxidation (Magnani et al., 2016), which might be able to promote SR-B1 degradation. Furthermore, the present study demonstrated that O_3, a strong environmental oxidant, is capable to down-regulate SR-B1 levels within RHE. Although O_3 does not penetrate the skin, it reacts with the superficial lipid envelope, inducing a cascade of oxidative events that affect deeper cutaneous layers (Valacchi et al., 2003; Valacchi et al., 2016). These events, together with the formation of lipid peroxidation-induced reactive aldehydes, might explain the loss of SR-B1 expression also in the inner layers of the epidermis. Studies revealed

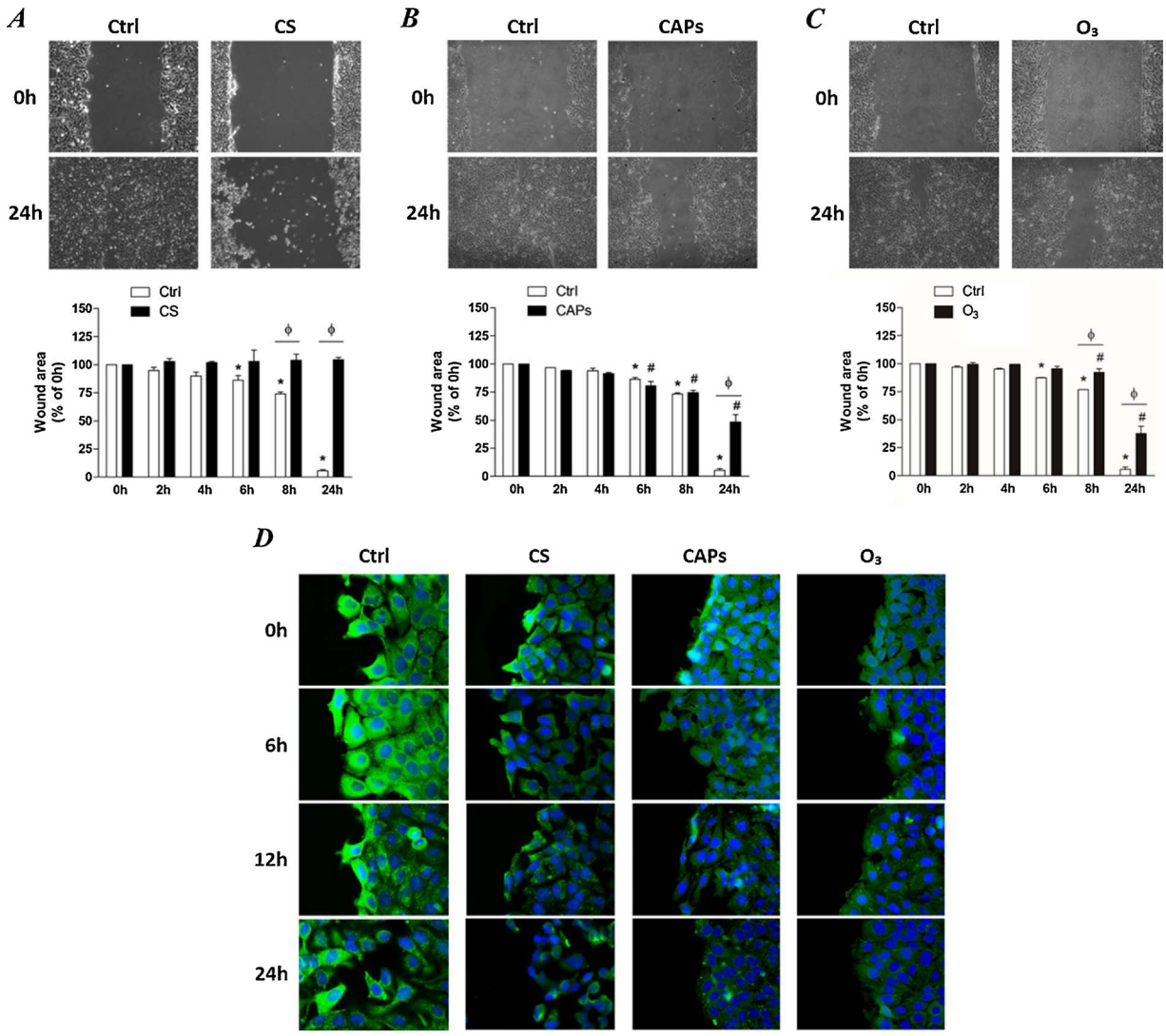

Fig. 5. Wound healing impairment by environmental stressors was associated with SR-B1 decrease. Scratch was performed on confluent HaCaT cells layer, followed by exposure to CS (A), CAPs (B) and O_3 (C). For all stressors, wound area was measured at different time-points (0–24 h). Pictures in the top show scratch closure after 0 and 24 h. Quantification of wound area below pictures is representative of three experiments. Data are shown as % of 0 h; $p < 0.05$ by Kruskal-Wallis; * vs Ctrl 0 h, # vs stressor 0 h, φ vs Ctrl of same time point. SR-B1 expression was determined by IF on scratched cell monolayer exposed to stressors at time points of 0, 6, 12 and 24 h. Green staining represents SR-B1; blue staining (DAPI) represents nuclei; pictures taken at magnification 40× (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that all environmental oxidants used in the present study are able to induce lipid peroxidation (Sticozzi et al., 2012; Valacchi et al., 2016; Magnani et al., 2016); and formation of 4HNE that itself can bind to SR-B1 and then modify its structure and be then the consequence of its lost as previously demonstrated (Sticozzi et al., 2012).

Therefore, it is possible that SR-B1 decrease in cutaneous tissues is a consequence of two correlated episodes that we have defined as "OxInflammation" (Valacchi et al., 2017) consisting with the alteration of the cellular redox homeostasis by the generation of Reactive Oxygen Species (ROS) (endogenous and exogenous) that can not only damage the proteins, in our case SR-B1, but also activate an inflammatory response able itself to further generate ROS leading to an

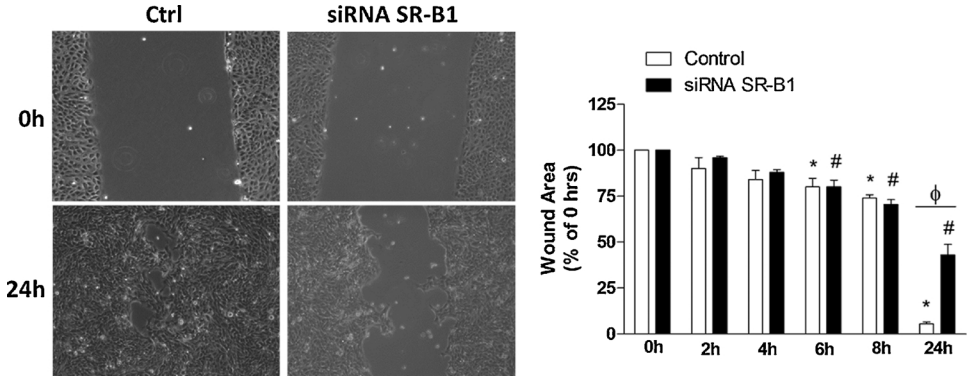

Fig. 6. SR-B1 knockdown delayed keratinocytes scratch closure. Scratch was performed on confluent HaCaT cells monolayer and wound area was measured at different time points (0–24 h). Pictures on the left show scratch closure after 0 and 24 h. Quantification of wound area on the right is representative of three experiments. Data are shown as % of 0 h; $p < 0.05$ by Kruskal-Wallis; * vs Ctrl 0 h, # vs siRNA SR-B1 0 h, φ vs Ctrl 24 h.

"OxInflammatory" vicious cycle.

Among the damaging effects that chemical pollutants have on human skin, wound repair disruption has been also reported. Different studies have associated CS to impaired wound healing, due to events among which delayed angiogenesis and vasoconstriction (Ejaz and Lim, 2006; Martins-Green et al., 2014). Also O_3 has been reported to affect wound healing, in an in vivo study. In fact, it has been found a strong association between O_3, oxidative stress-dependent NF-kB status, TGF-β signaling and impaired wound repair in aged mice (Lim et al., 2006). Furthermore, airborne PM as well has been demonstrated to have negative effects on cutaneous wound healing, due to disruption of wound contraction and re-epithelialization (Ejaz et al., 2009). In the present study, we confirmed the detrimental effects of all the above-mentioned stressors on wound repair, by the use of an in vitro scratch healing assay on human keratinocytes. The scratch healing assay is characterized by both migration and proliferation and since SR-B1 is involved in cholesterol intracellular uptake and cholesterol is essential for cell proliferation (Singh et al., 2013), we evaluated SR-B1 expression in wounded cells at different time points. Surprisingly, we observed a noticeable increase of SR-B1 in the cells at the wound edges, which are directly involved in wound closure. Such response suggested an active implication of SR-B1 in healing, as well as a relationship between SR-B1 down-regulation and wound repair delay induced by outdoor oxidants. As a proof of concept, we performed wound healing assay on transiently SR-B1 knockdown keratinocytes and noticed that knockdown cells presented reduced ability of scratch closure, confirming our hypothesis on SR-B1 implication in wound healing process. This result is supported by other in vitro studies that reported an involvement of SR-B1 in cell proliferation (Danilo et al., 2013) and cellular migration (Zhu et al., 2008).

Altogether, the data presented in this study showed that stressors, such as CS, O_3 and PM are able to induce oxidative damage in a 3D skin model, as indicated by the increase of protein carbonyl levels, and down-regulate SR-B1 protein expression in human epidermis. Moreover, our results indicate SR-B1 as a new player in pollutants-related delayed cutaneous wound healing.

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