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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.