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Keratinocytes oxidative damage mechanisms related to airbone particle matter exposure

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ABSTRACT

Epidemiological evidences have correlated airbone particulate matter (PM) to adverse health effects, mainly linking to pulmonary and cardiovascular disease. Nevertheless, only recently, some studies reported detrimental effects of PM on other organs such as skin. In a recent work, we have reported increased oxidative and inflammatory responses in Reconstituted Human Epidermis (RHE) exposed to ambient particles (CAPs) and we also demonstrated the ability of CAPs to penetrate the skin tissue.

The present study was aimed to better understand the cellular mechanisms beyond the oxidative changes induced by CAPs (5–10–25 μg/mL) in human immortalized keratinocytes (HaCaT).

After 24 h of treatment, CAPs were able to enter the cells leading to a decrease in viability, increased levels of 4-hydroxinonenal products (4-HNE) and IL-1α release. Overall these data, suggest lipid and protein oxidative damage, as well as an increase of inflammatory response after being challenged with CAPs. In addition, 3 h after CAPs exposure we found a significant increase in NF-kB and Nrf2 translocation into the nucleus. In contrast, no differences in gene expression and enzymatic activity of Nrf2 target genes were detected. This last finding could be explained by the ability of CAPs to possibly alter the binding of Nrf2 to the ARE DNA sequence.

1. Introduction

Environmental air pollution is mainly related to morbidity and mortality worldwide (Lodovici and Bigagli, 2011). The most recent epidemiological data documented that air pollutants, dramatically increased in the last decades for the parallel expansion of industrialization, has become the primary cause of around 38% of premature death in Europe (Turnock et al., 2016).

Air pollutants encompasses various particulate matters (PM), which, in turn, are a heterogeneous mixture, containing organic and inorganic compounds (Chirino et al., 2010; Kampa and Castanas, 2008). PM composition varies depending on the type of their natural or anthropogenic source, such as vehicle exhaust, road dust, smokestacks, forest fires, windblown soil, volcanic emissions, and sea spray (Nel, 2005). Among this high variety, PM with aerodynamic diameter lower than 10 μm represent the major threat for human health, in virtue of the large reactive surface area and remarkable penetration efficiency after its deposition in the alveoli (and diffusion into pulmonary and systemic circulation) (Chirino et al., 2010).

Skin is the human organ with the greatest surface and the most exposed to the environmental contaminants, acting indeed as the first line of defence against pollution-induced adverse effects (Drakaki et al., 2014). PM are, along with ultraviolet (UV) radiation, ozone, polycyclic aromatic hydrocarbons (PAHs), and cigarette smoke, the stressors that mostly affect skin structure. Exposure to these pollutants, which have different mechanism of action, have been linked to several pathologies such as erythema, edema, psoriasis, hyperplasia atopic dermatitis (Valacchi et al., 2012).

It has been reported that PM influences skin aging by enhancing free radical production (Arimoto et al., 1999) affecting skin functions, including the ability to contrast the entry of pathogens (Nakamura et al., 2015; Vierkötter et al., 2010). A recent study showed that PM exposure increases inflammation through the aryl hydrocarbon receptor (AhR)/Nox2/p47^{phox} pathway which, in turn, promotes the generation of

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endogenous reactive species, mostly represented by ROS (reactive oxygen species); these reactive molecules are able to activate NF-kB and activator protein 1 (AP-1), which can promote the release of pro-inflammatory mediators such as IL-1α and cyclooxygenase 2 (COX-2) (Lee et al., 2016). Findings from our recent study, where reconstituted human epidermis (RHE) were treated with (CAPs), that include particles in the range between 0.1 and 2.5 μm (Ghio and Huang, 2004) are in line with those reported by Lee et al. (Lee et al., 2016) suggesting the ability of CAPs to induce IL-1a release and CYP1A1 increase. Overall, we found that CAPs were able to penetrate the tissue, trigger lipoperoxidative damage and pro-inflammatory responses (Magnani et al., 2016b).

Taken together, the aforementioned evidences clearly point out a role of ROS in mediating the biological damage to skin induced by CAPs. However, the mechanism underlying this observed raise of oxidant species is still elusive. It is well recognized that ROS can become cytotoxic only when their concentration exceeds a certain critical threshold (Zhang et al., 2016). The ability of the cell to prevent/contrast ROS damage mostly lies in the system of enzymatic- and non-enzymatic endogen defensive molecules. Most of the cell capacity to cope with potential insults in redox homeostasis depends on the efficiency of the Nrf2 (Nuclear factor erythroid 2 related factor 2)/ARE (antioxidant response elements) pathway, which governs the expression of fundamental redox defence enzymes, such as glutathione reductase, glutathione peroxidase etc. all involved in quenching the altered oxidative stress status present in the cells (Lu et al., 2016).

In the present study, we examined in human keratinocytes whether the toxic axis oxidative stress-inflammation triggered by CAPs involved the failure of cell redox mechanisms to adequately counteract the insults of these xenobiotics.

2. Materials and methods

2.1. Experimental model

In this study Concentrated Air Particles (CAPs) were used as particles matter (PM) sample. CAPs with size range between 0.1–2.5 μm, provided by B. Gonzalez-Flecha, were collected using a virtual concentrator, the Harvard Ambient Particle Concentrator (HAPC), as previous reported (Magnani et al., 2016b). PM suspension was freshly prepared by re-suspending CAPs particles in culture media at final concentrations of 5, 10 and 25 μg/mL, followed by 10 min incubation in an ultrasonic water bath (Carroll-Ann and Imrich, 1998).

Immortalized human keratinocyte, HaCaT cell line used for the cellular experiments, were cultured at 37 °C in a humidified atmosphere 5% CO_2 with DMEM supplemented with 10% FBS as previously described (Valacchi et al., 2009). HaCaT were exposed to CAPs (5, 10 or 25 μg/mL) for 1, 3, 6 or 24 h based on the marker of interest. Before 4-HNE protein adducts analysis, cells were incubated with CAPs in presence or absence of the iron chelator Deferoxamine (DFO) (400 μM).

2.2. Viability and cytotoxicity determination

Viability was assayed by commercial kit “Count and Viability assay kit” (Millipore Corporation, USA) by Muse Cell Analyzer (Millipore Corporation, USA). The assay was performed followed the recommended protocol as previously reported (Cervellati et al., 2014). Cytotoxicity was determined by lactate dehydrogenase (LDH) release in the media collected after CAPs exposure at different time points (3, 6 and 24 h). LDH was measured by Cytotoxicity kit (Roche, Italy). Briefly, LDH activity is determined in a coupled enzymatic reaction. The reaction produces formazan dye which can be spectrophotometrically read at 500 nm. All tests were performed in triplicate and results expressed as percentage of change relative to not treated cells values (control).

2.3. Morphology analysis

Cells were treated for 24 h with CAPs, scraped and collected in 0.1 M cacodylate buffer (pH 7.4), then centrifuged at 2000 × *g* for 5 min. Pellets were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 h at 4 °C. They were then washed with 0.1 M cacodylate buffer (pH 7.4) three times and post-fixed in 1% osmium tetroxide and 0.1 M cacodylate buffer at pH 7.4 for 1 h at room temperature (RT). The specimens were dehydrated in graded concentrations of ethanol and embedded in epoxide resin (Agar Scientific, U.K.). Cells were then transferred to latex modules filled with resin and subsequently thermally cured at 60 °C for 48 h. Semi-thin sections (0.5–1 μm thickness) were cut using an ultra-microtome (Reichard Ultracut S, Austria) stained with toluidine blue, and blocks were selected for thinning. Ultra-thin sections of about 40–60 nm were cut and mounted onto formvar-coated copper grids. These were then double-stained with 1% uranyl acetate and 0.1% lead citrate for 30 min each and examined under a transmission electron microscope, Hitachi H-800 (Tokyo, Japan), at an accelerating voltage of 100 KV.

2.4. RT-PCR

Quantitative real-time PCR was carried out as described in detail previously (Cervellati et al., 2011). Briefly, total RNA was extracted, using an AURUM total RNA Mini Kit with DNase digestion (Bio-Rad, Italy), from 2×10^5 keratinocytes for each experimental condition, according to the manufacturer’s recommended procedure. First-strand cDNA was generated from 1 μg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Italy). The primer pairs (supplemental material Table S1) capable of hybridization with unique regions of the appropriate gene sequence were obtained from the Real-Time PCR GenBank Primer and Probe Database Primer Bank, RTPrimerDB. Quantitative real-time PCR (qPCR) was performed using SYBR green on the CFX Multicolor real-time PCR detection system (Bio-Rad, Italy). The final reaction mixture (15 μl) contained 300 nM each primer, 1 μl of cDNA, and 7 μl of iQ SYBR Green Supermix (Bio-Rad, Italy). All reactions were run in triplicate. Real-time PCR was initiated with a 3-min hot-start denaturation step at 95 °C and then performed for 40 cycles at 95 °C for 3 s and 60 °C for 5 s. During the reaction, the quantity of PCR products, was continuously monitored as fluorescence by Bio Rad CFX Manager software (Bio-Rad, Italy). In order to calculate the amplification efficiency, each primers pairs were initially used to generate a standard curve over a large dynamic range of starting cDNA quantities. Ribosomal proteins L13a (RPL13a) and L11a (RPL11a) and GAPDH were employed as reference genes. Samples were compared using the relative cycle threshold (CT). After normalization to more stable reference genes, the fold change was determined with respect to control, using the formula $2^{-\Delta\Delta CT}$, where ΔCT is (gene of interest CT)-(reference gene CT), and ΔΔCT is (ΔCT experimental)-(ΔCT control).

2.5. Protein extraction for enzymatic assay

HaCaT treated for 6 or 24 h with 5, 10, 25 μg/mL CAPs were washed with ice-cold PBS, scrapped in PBS and centrifuged at 800 x*g* for 10 min. Pellet was re-suspended in cold lysis buffer 50 mM Hepes, 150 mM NaCl, 1 mM Na_3VO_4, 100 μM NaF, 1%, 0.5 mM EDTA, 1 mM PMSF and protease inhibitor cocktail (Sigma-Aldrich, Italy). The suspension was then incubated at 4 °C for 30 min and centrifuged at 10,000*g* for 30 min (Cervellati et al., 2015). After centrifugation, the protein concentration of the supernatant was measured by Bradford method (Biorad, Italy).

2.6. Glutathione reductase (GR) activity assay

GR activity was detected as increase in absorbance at 412 nm caused by the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB),