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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),

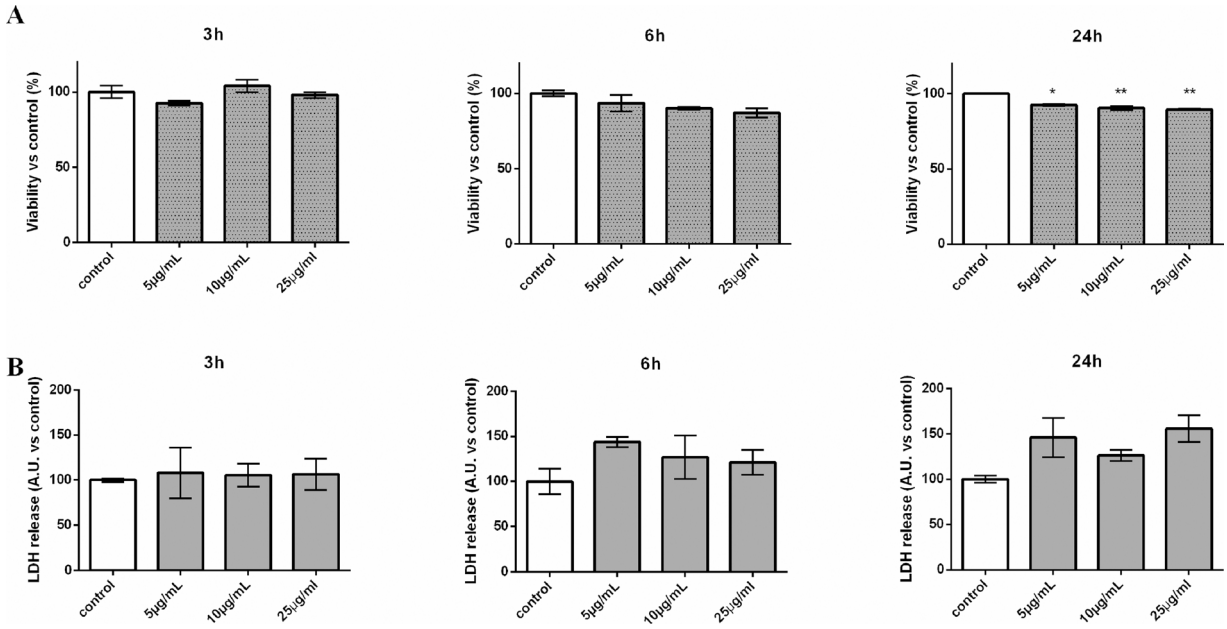

Fig. 1. Viability assay (A) and LDH release (B) in HaCaT maintenance media after 3 h; 6 h; 24 h exposure to different concentrations of CAPs. Data presents as mean ± SEM of four different experiments. Data are expressed as percentage compared to not treated cells (control). *p < 0.05 vs control; **p < 0.01 vs control.

as previously reported (Cervellati et al., 2015), according to the method of Smith et al. (Smith et al., 1988). GR activity was calculated using extinction coefficient 14,150 M^{-1} cm^{-1} and expressed as U/mg protein.

2.7. Glutathione peroxidase (Gpx) activity assay

Gpx was assayed by a commercially available kit (Cayman Chemical, USA). Enzymatic activity was evaluated as NADPH consumption, which led to decrease in absorbance monitored at 340 nm at over 5 min. Gpx activity was calculated using extinction coefficient 6220 M^{-1} cm^{-1} and expressed as U/mg protein.

2.8. NAD(P)H-quinone oxidoreductase (NPQO1)

NPQO1 assay was based on the enzyme ability to reduce menadione to menadiol by NADPH, coupled with non-enzymatically reduction of MTT carried by derived menadiol. Briefly, 10 µl of cell lysate were added to 200 µl reaction mix (25 mM TRIS-HCl buffer, pH 7.4; 0.67 mg/mL BSA, 0.01% tween 20; 5 µM flavin adenine dinucleotide (FAD), 1 mM Glucose-6-phosphate; 30 µM NADPH; 2U/L Glucose 6-phospho-dehydrogenase; 0.3 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT); 0.3 mg/mL; 50 µM menadione). The enzyme activity was spectrophotometrically monitored at 610 nm for 5 min. NPQO1 activity was calculated using extinction coefficient 11.3 mM^{-1} cm^{-1} and expressed as U/mg protein.

2.9. Immunocytochemistry

Human keratinocytes were grown on coverslips at a density of 1×10^5 cells/mL, and after treatment fixed in 4% paraformaldehyde for 30 min at room temperature as previously described (Valacchi et al., 2015). Cells were permeabilized for 5 min at RT with PBS containing 0.2% Triton X-100, blocked with 1% BSA in PBS at RT for 1 h and then incubated with primary antibody (Nf-kB-p65, 1:100, sc372 Santa Cruz; Nrf2 1:200 ABE413 Millipore) in PBS containing 0.5% BSA at 4 °C overnight. After washing, coverslips were incubated with appropriate secondary antibody for 1 h at RT. Nuclei were stained with 1 µg/mL DAPI (Sigma- Aldrich, Italy) for 1 min. Coverslips were mounted onto glass slides using anti-fade mounting medium 1,4 diazabicyclooctane (DABCO) in glycerin. Negative controls for the immunostaining experiments were performed by omitting primary antibodies. Images were acquired and analyzed with Leica AF CTR6500HS (Microsystems) and analyzed with Cell Profiler software.

2.10. IL-1α levels

IL-1α content was assayed by commercially available ELISA kit (Thermo Scientific, Italy) in cell culture media collected at different time points (3, 6 and 24 h). The optical absorbance was measured with a spectrophotometer microplate reader at 450 nm and correction at 530 nm. Final concentrations were calculated by interpolation with a standard curve of Recombinant Human IL-1α provided by the kit and results expressed as pmol IL-1α/mL,

2.11. Nrf-2 nuclear translocation

Nrf2 binding activity to DNA antioxidant response elements (ARE) were evaluated using “TransAM Nrf2” ELISA kit (Active Motif, USA). Nrf2 protein presented in cellular extract were incubated with oligonucleotides containing ARE sequencing, immobilized on 96-well plate. A secondary antibody conjugated with a horseradish peroxidase provides a colorimetric output spetrophotrometrically detected at 450 nm.

2.12. Statistical analysis

Results were expressed as mean value ± SEM and represent the mean of triplicate determinations obtained in four separate experiments. ANOVA followed by Bonferroni post-hoc test were performed by GraphPad Prism (version 6.00 for Windows, GraphPad Software, USA) and statistical significance was considered at $p < 0.05$.

3. Results

3.1. Viability and cytotoxicity determination

The first step of the study was to analyze cellular viability in HaCaT cells treated with different concentrations of CAPs (5, 10 and 25 µg/mL) at different time points (3, 6 and 24 h). As showed in Fig. 1A, CAPs exposure did not dramatically affect cellular viability over the detected

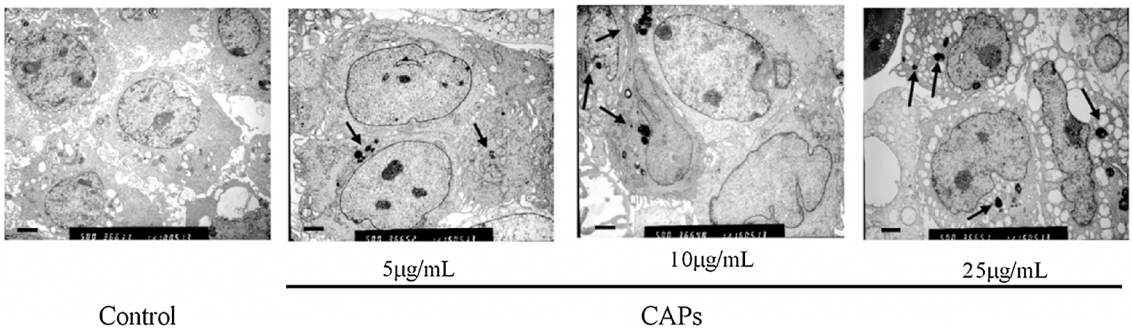

Fig. 2. Not treated cells (Control) cells and cells after 24 h CAPs exposure. Black arrows shown particles compatible with CAPs structures. Representative pictures of four different experiments are taken at 5000 x magnifications. Scale Bar 1 μm.

time points but reached significance after 24 h with a decrease of 7.5%; 9.5% and 10.5% for 5 μg/mL; 10 μg/mL and 25 μg/mL respectively (Fig. 1A).

The viability results were in line with the LDH release (marker of cellular damage), as it is shown in Fig. 1B where it is possible to observe that the increased levels of LDH was noticeable at the later time points (6 and 24 h).

3.2. Ultrastructure analysis

The electron microscopy analysis of keratinocytes after 24 h exposure with different CAPs concentrations clearly showed the presence of CAPs inside the cells. CAPs were diffuse in the cellular space, from the membrane to the cytoplasm (Fig. 2).

3.3. NF-kB activation and inflammatory markers

NF-kB is redox sensitive transcription factor and its activation by nucleus translocation promotes transcription of genes involved in the inflammatory response. As showed in Fig. 3 after 1 and 3 h of CAPs treatment, there was a time and dose dependent significant increase in NF-kB translocation (p65) from cytoplasm to the nucleus (1 h: 5 μg/mL, 63%; 10 μg/mL, 121%; 25 μg/mL, 106%; 3 h: 5 μg/mL: 254%; 10 μg/mL: 240%; 25 μg/mL: 300%; $p < 0.01$).

The activation of the inflammatory response was confirmed by the increasing levels of IL-1α. As it is depicted in Fig. 4, this cytokine while remained unvaried at 3 and 6 h time point, increased sharply at the later time point (24 h), and this was noticeable for all the concentrations of CAPs analyzed (5 μg/mL: 215.2%; 10 μg/mL: 243.7%; 25 μg/mL: 198.6%; $p < 0.05$) (Fig. 4).

3.4. Oxidative damage markers

The activation of NF-kB suggests the presence of a cellular redox state alteration and since a consequence of increased oxidative stress is the oxidation of lipids, the next step was to evaluate the presence of 4-hydroxynonenal (4-HNE) protein adducts in HaCaT cells exposed to CAPs. As shown in Fig. 5A, CAPs treatments induce a dose and time dependent increase of 4-HNE protein adducts in keratinocytes for 24 h (Fig. 5A) which were significantly attenuated by DFO treatment (Fig. 5B), suggesting an essential role of iron, in CAPs induced cellular oxidative damage.

3.5. Exploration of the possible interaction between CAPs and NRF-2/ARE pathway

The finding of increased levels of lipid peroxidative damage might reflect the failure of cellular defensive mechanism to cope with pro-oxidative perturbation. In this light, we explored the activation of Nrf2 pathway, which plays a prominent role in modulating the expression of phase II enzymes such as gluthatione peroxidase (Gpx), Glutathione reductase (GR), NADP(H) quinone oxidoreductase (NQO1) and superoxide dismutase.

As displayed in Fig. 6 after CAPs treatment there was an evident increase in nuclear translocation of and Nrf2 suggesting a possible up-regulation of the above mentioned defensive enzymes. By contrast, neither gene expression (Fig. 7) nor enzymatic activity (Fig. 8) of Gpx, GR and NPQO1 resulted significantly changed after 6 or 24 h of CAPs exposure and these results parallel with the decreased DNA-Nrf2 binding (Fig. 9).

These unexpected result could be possibly explained by the failure of Nrf2 to interact with the correspondent coding sequence. To address this hypothesis, we determined the Nrf2/ARE binding levels. As it is shown in Fig. 10, CAPs treatment decreased the efficiency of Nrf2 to bind the ARE sequence in a dose dependent manner.

4. Discussion

The aim of our study was to better identify possible mechanisms involved in the axis PM-oxidative damage-inflammation that has been previously suggested from us and other groups (Lodovici and Bigagli, 2011; Magnani et al., 2016b)

Several studies reported implication of particulate matter on cardiovascular and lung disorder, in particular, due to the presence of transition metals, they are proposed as pro-oxidant agents (Li et al., 2017; Marchini et al., 2015). In addition, a fairly recent work has shown that particles exposure is able to induce NADPH oxidase (NOX) activation (Kampfrath et al., 2011) which is among the main endogenous sources of O2-. This confirmed our previous study in where NOX activation was also observed in keratinocytes exposed to CS (which is composed of both, air and particulate phase) (Sticozzi et al., 2012). Therefore, beside the ability of the mere particles to induce OxS due to the presence of transition metals, PM are also able to induce the production of endogenous ROS, contributing to alter the homeostasis redox of the cells. Oxidative stress can trigger redox sensitive pathways leading to inflammation and eventually to cell damage (Shi et al., 2017). Considering that the skin is the first and the major target for air pollutants (Drakaki et al., 2014; Valacchi et al., 2012) it is possible to hypothesize a similar mechanism of action observed in other organs such as lung and cardiovascular system.

Since in our previous work we were able to demonstrate that particles are able to penetrate the skin and reach the epidermis layer (Magnani et al., 2016b), in the current work we wanted to evaluate the keratinocytes responses to CAPs exposure. Our findings clearly indicated that the exposure of keratinocytes to CAPs leads to cellular damage as detected by LDH release.

This toxic effect appeared not to stem from mere contact with external biocomponents of the cells, but rather with a direct interaction between CAPs and intracellular environment. Indeed, we observed that ultrafine particles, which have been already shown to penetrate the

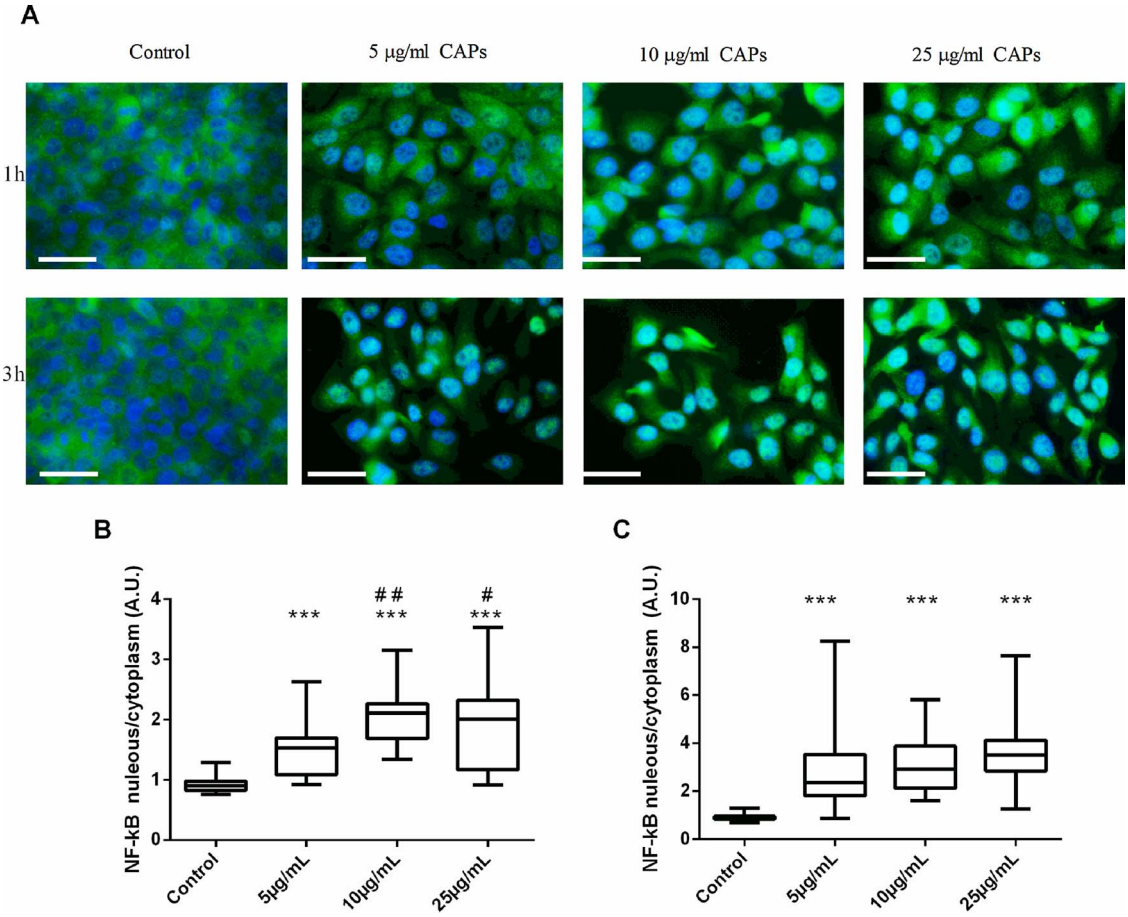

Fig. 3. (A)Immunofluorescence for NF-kB in HaCaT treated with CAPs at different concentrations for 1 or 3 h. Representative pictures of four different experiments are presented. Nuclei (blue) were stained with DAPI. Fluorescence quantification is reported for 1 h (B) and 3 h (C) treatment. The horizontal line represents the mean, the box shows the 25°–75° percentile and vertical lines issuing from the box extend to the minimum and maximum values of the analysis variable. *** $p < 0.001$ vs control; ## $p < 0.001$ vs 5ug/mL; # $p < 0.05$ vs 5ug/mL Scale bar = 50 µm. Original magnification × 630. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stratum corneum and diffuse until the deeper epidermis by using a 3 dimensional skin model (Magnani et al., 2016b), are also able to enter into the cells.

Noteworthy, this penetrative capacity of CAPs has been detected at concentration up to 20 times lower than those used in the previous experiments with RHE (Magnani et al., 2016b). The ability of PM to enter the skin tissues has been documented although the exact mechanism is still under investigation (Rothen-Rutishauser, 2008). It has been proposed that PM are able to enter the cutaneous tissues via the hair follicle or even using the different kind of glands present in the epidermis (Rothen-Rutishauser, 2008). It is not clear how particles can enter the skin cells, however it is tempting to hypothesize similar mechanisms to those reported by Brandenberger et al. in epithelial lung cell line, in which has been shown that the majority of 1 µm particles are taken up by macropinocytosis or phagocytosis leading to apical plasma membrane enlargement (Brandenberger et al., 2009; Rothen-Rutishauser, 2008).

The effect induced by the particles on the skin depends not only by the particles size by mainly by their composition, which is different based on their origin (Nel, 2005).

As postulated elsewhere, tissue adsorption of CAPs can even occur via PM-bound polyciclic aromatic hydrocarbons (PAHs) (Li et al., 2008). Low-molecular-weight PAHs occur in the atmosphere predominantly in the vapor phase, whereas heavier multi-ringed PAHs are

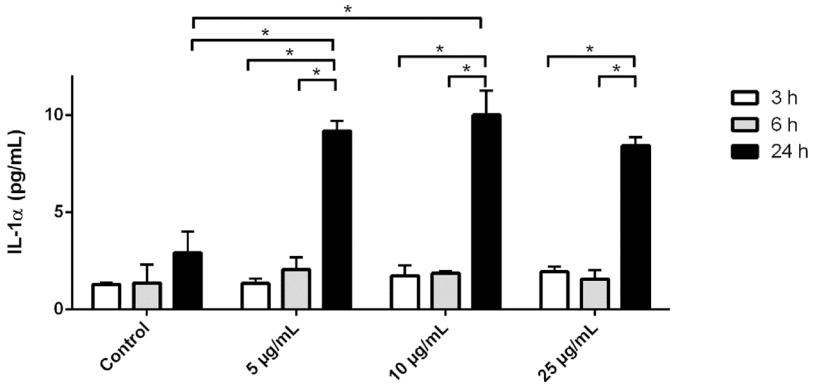

Fig. 4. IL-1α levels in HaCaT treated with CAPs at different concentration for 3;6 and 24 h. Data are presented as mean ± SEM of four different experiments *$p < 0.05$.

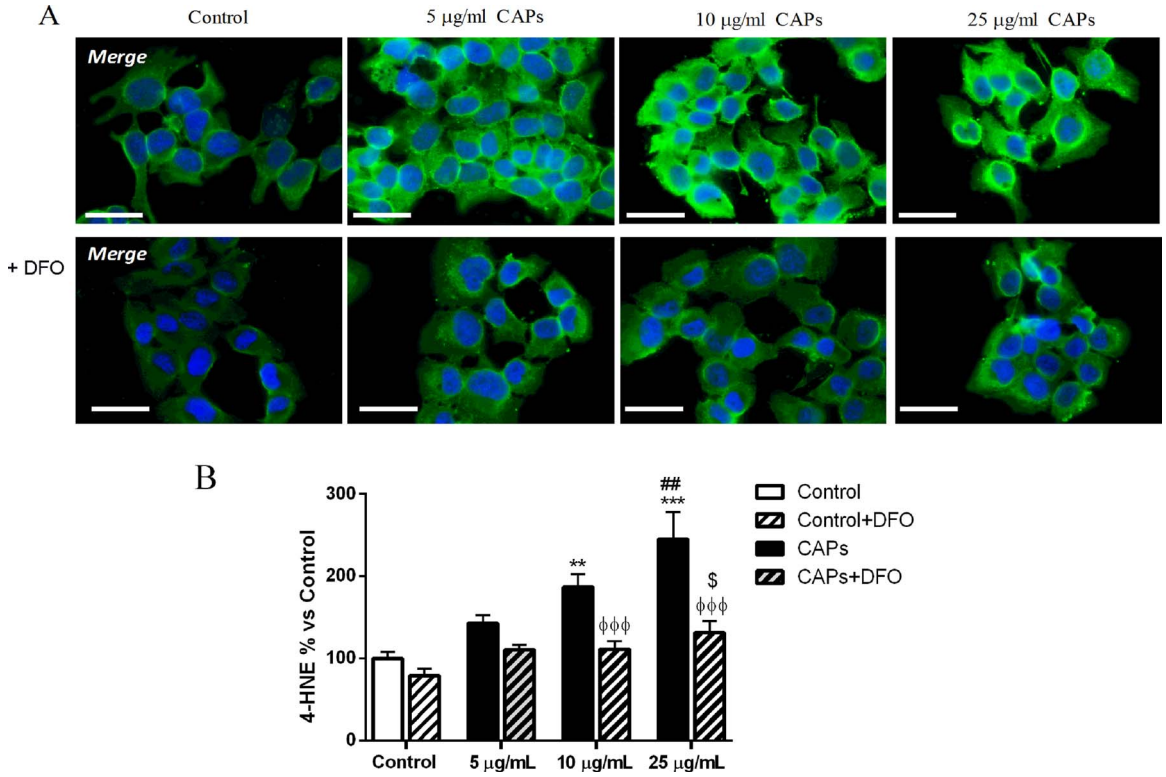

Fig. 5. (A) Immunofluorescence for 4-HNE in HaCaT treated with different concentrations of CAPs in the presence or not of DFO for 24 h. Representative pictures of four different experiments are presented. Nuclei (blue) were stained with DAPI. Scale bar = 50 μm. Original magnification × 630. (B) Quantification and statistical analysis. Data are presents as mean ± SEM, ** $p < 0.01$ vs control, *** $p < 0.001$ vs control, ## $p < 0.01$ vs 5 mg/mL CAPs; $ $p < 0.05$ vs control + DFO; φφφ $p < 0.001$ vs CAPs of corresponding dose. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

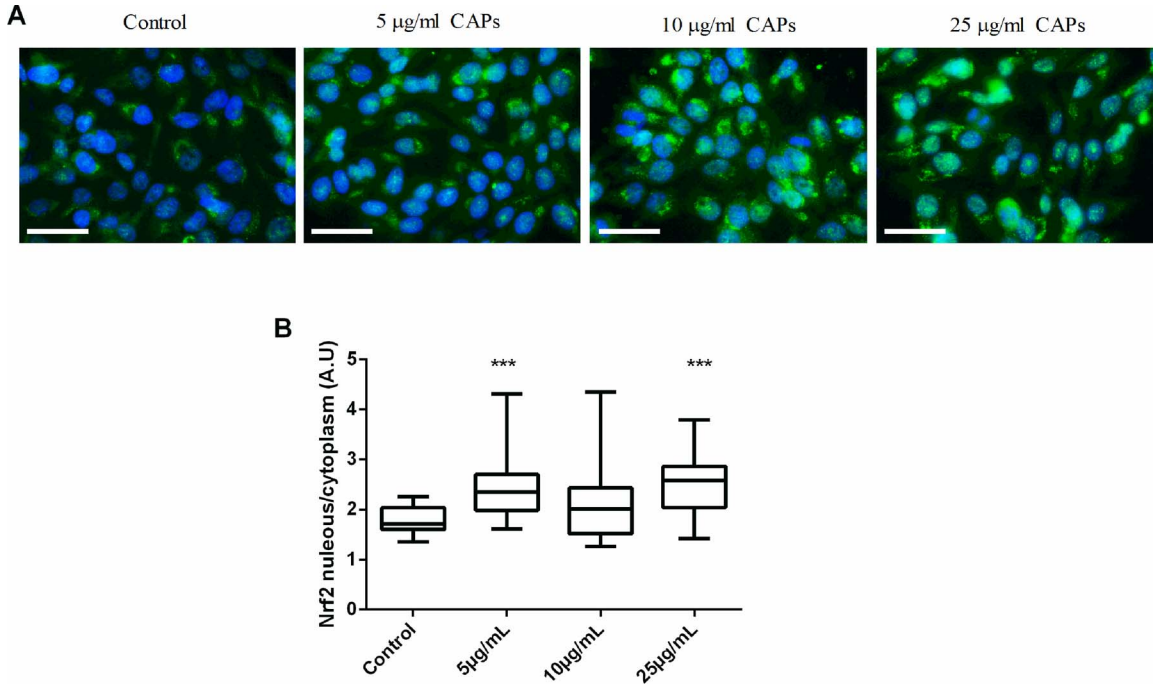

Fig. 6. A) Immunofluorescence for Nrf2 in HaCat treated with CAPs at different concentration for 3 h. Representative pictures of four different experiments are presented. Nuclei (blue) were stained with DAPI. Statistical analysis is reported in (B). The horizontal line represents the mean, the box shows the 25°–75° percentile and vertical lines issuing from the box extend to the minimum and maximum values of the analysis variable *** $p < 0.001$ vs control; Scale bar = 50 μm. Original magnification × 630. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

largely bound to particles, especially PM with diameter lower than 2.5 μm ($PM_{2.5}$) (Hassanvand et al., 2015). The major route of exposure to these compounds is through the lungs, respiratory tract, and, although less efficiently, skin (Gerde, 2001; Sanders et al., 1984). Thus, seemingly, thank to this lipophilic carriers, particles can reach the intracellular environment and causing the following, interconnected,

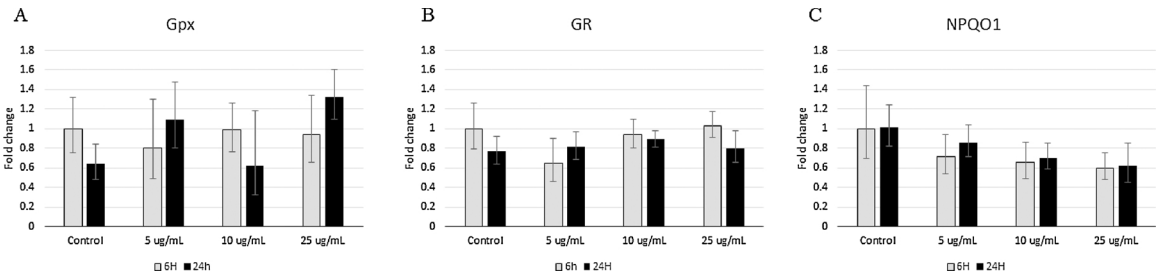

Fig. 7. Gene expression levels of glutathione peroxidase (Gpx) (A), glutathione reductase (GR) (B) and NADPH quinone oxidoreductase (NQO1) (C) in HaCaT treated for 6 or 24 h with CAPs at different concentrations. Data are presents as mean ± SEM of four different experiments in triplicate. No significant changes where found with statistical analysis.

observed cytotoxic events: 1) increase of oxidative stress-induced damage as shown by the significant increase of 4-HNE levels and this could be a consequence of the metals present in the particles 2) inflammatory responses, as demonstrated by the activation of redox-sensitive transcription factor NF-kB, and the related increase in IL-1α production.

Inflammation has already been shown as primary protective events in lungs and cardiovascular cells under PM exposure with macrophage recruitment, ROS production, and induction of antioxidant genes (Marchini et al., 2016). In a second phase, protective response fails, leading to cytotoxic effects. As reported by Magnani et al., in lungs from mice, PM exposure lead to loss of GSH/GSSG ratio in association with increase in ROS production by NADP(H) oxidase (Lodovici and Bigagli, 2011; Magnani et al., 2016a). On contrary, taking into account the severe damage detected in CAPs-exposed keratinocytes, it is tempting to hypothesize that the insult should occur in an "explosive" manner so to overwhelm the attempts of the cells' self-defense systems.

In line with previous observations, the increase in reactive species responsible of oxidative injury might be the transition metals, such as Fe, that we and others found on the surface of the PM (Magnani et al., 2016b). This divalent metal, when not "quenched" by molecules present in the protein envelope, participates to Fenton-like reactions, which is a major intracellular (and plasmatic) source of hydroxyl radical, i.e. the main initiator of lipid oxidative injury mechanisms (Lodovici and Bigagli, 2011). This hypothesis is supported by the finding that, iron chelators such as desferoxamine (DFO) abrogated the pro-oxidative effect of CAPs (Magnani et al., 2016b).

Redox homeostasis accounts for a healthy physiological steady state where the levels of oxidants are maintained within a physiological range by means of preventive and repairing mechanisms (Zhang et al., 2016). Once presented with a pro-oxidative challenge, various complex and finely tuned pathways are activated with the scope of re-stablishing pre-challenge equilibrium. When this feedback reaction is inadequate, biological damage occur due to the combination of oxidative stress/inflammation also called oxinflammation (Valacchi et al., 2017). Since, this cytotoxic dichotomy also emerged to affect keratinocytes exposed to CAPs, we have pointed our attention to Nrf2 pathway, which plays a key role in preserving cellular homeostasis, upon chemical or oxidative challenges in all human cells, including keratinocytes (Gęgotek and Skrzydlewska, 2015; Lu et al., 2016). The literature reports that the exposure of airbone PM, i.e. diesel exhaust particles (DEPs), the major component of these mixture, induce raise of electrophiles which can affect cellular redox balance reactions (Gęgotek and Skrzydlewska, 2015).

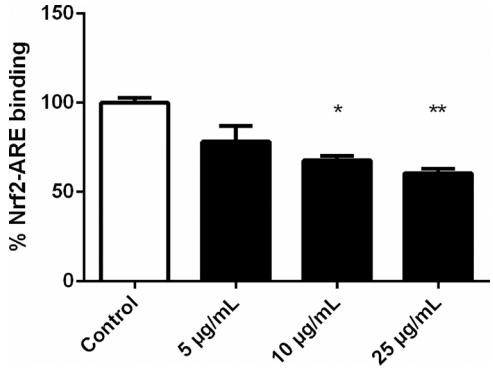

Fig. 9. Nrf2 binding to DNA antioxidant response element (ARE) in HaCat not treated (control) or treated with CAPs at different concentration for 3 h. Data are presents as mean ± SEM of four independent experiment in triplicate.*$p < 0.05$ vs control; **$p < 0.01$ vs control.

. Briefly, under homeostatic conditions, Kelch-like erythroid CNC homologue (ECH)-associated protein 1 (Keap1) anchor Nrf2 in cytoskeleton. Oxidation of cysteine residues of Keap-1, facilitate dissociation of Nrf2 and its translocation to the nucleus. Here, it forms a complex with a small MAF proteins and, subsequently, then is bound to the DNA in a characteristic sequence 5′-TGACnnnGCA-3′ labeled (ARE) and in consequence initiates the transcription of cytoprotective genes encoding for detoxifying enzymes and low-weight molecules involved in redox reactions (Gęgotek and Skrzydlewska, 2015; Kimura et al., 2007).

The activation of Nrf2 in response to PMs and other pollutants has been extensively demonstrated to occur in epithelial cells of respiratory tract or alveolar resident macrophages (Rubio et al., 2010) and (Li et al., 2013). In contrast, the present is the first investigation of the implication of this transcription factor in pro-oxidative processes induced by airborne ultrafine particles in keratinocytes. We found that exposure of keratinocytes to CAPs, even at the lowest dose, led to a

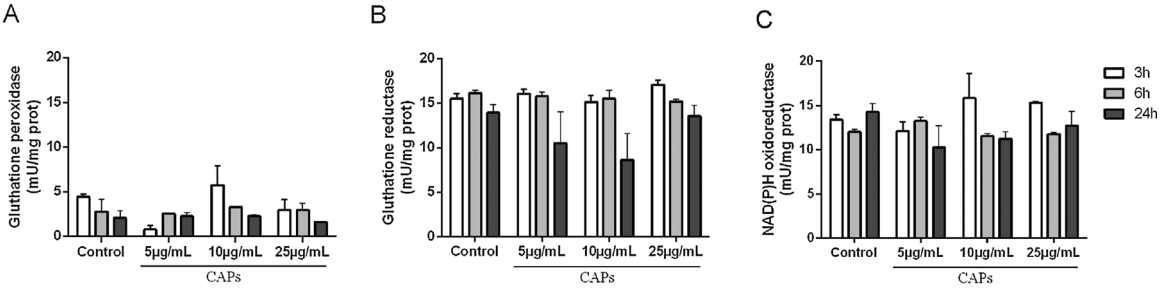

Fig. 8. Enzymatic activity in HaCat treated with CAPs at different concentration for 3; 6; and 24 h. Data are presents as mean ± SEM of four different experiments in triplicate. No significant changes where found with statistical analysis.

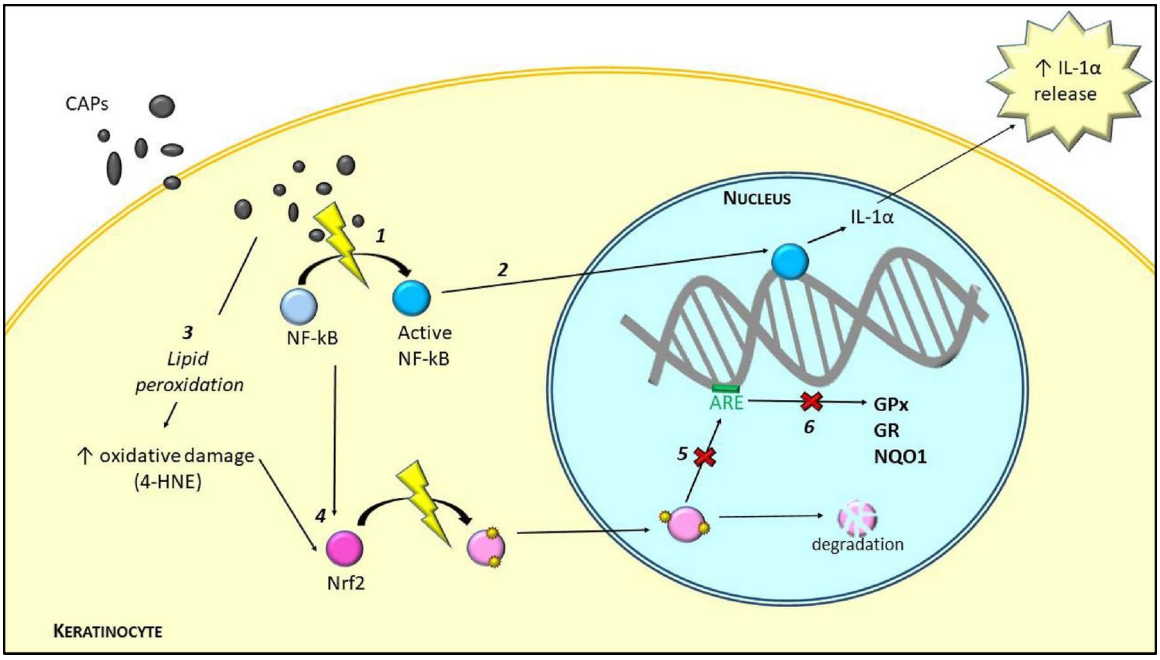

Fig. 10. Proposed model of CAPs-induced oxi-inflammation in keratinocytes (HaCaT). CAPs internalization induce activation of NF-kB (1) followed by (2) translocation into the nucleus and induction of inflammatory genes like IL-1α. Moreover, CAPs cause lipid peroxidation with increase of oxidative by-products (4-HNE) (3). Oxidative stress and NF-kB activation might promote Nrf2 activation (4). We hypothesize that Nrf2 underwent oxidative modifications (for instance 4-HNE adducts) that allow the translocation into the nucleus but prevent the binding to ARE sequences (5). Nrf2 do not promote transcription of antioxidant genes (6).

rapid translocation of Nrf2 to nucleus. This result suggests that the increased levels of ROS, or electrophiles, due to interaction of particles with cell structures freed Nrf2 from Keap1 bond, spared it from ubiquitination and subsequent proteasome-driven degradation. Considering previous reports, it is plausible to hypothesize that activation of Nrf2 could be the effect of the stimulation by 4-HNE (Chen et al., 2005; Ishikado et al., 2010). This unsaturated aldehyde, formed by the reaction of reactive species with membrane enriched arachidonic acid, can act as a signaling molecule, depending on the dose (Poli et al., 2008), and induce Nrf2 dependent signaling pathway as adaptive response (Chen et al., 2005). Nonetheless, we did not find increase in either expression or activity of Nrf2 sensitive defensive enzymes such as glutathione peroxidase, glutathione reductase and NADP(H) quinine oxidoreductase. Nrf2 translocation are an early event (increase observed 3 h after CAPs treatment), unable to promote transcription of genes tested even with prolonged CAPs exposure (6 and 24 h). On the other hand, antioxidant enzymes activity resulted as well unaltered al all time point considered (3, 6, 24 h). Taking into account the presence of oxidative by-products after 24 h exposure, it is noteworthy that oxidative stress, at time and concentration tested do not affect enzyme activity (no oxidative enzyme inactivation) but it is lack in adaptive transcription that lead to cytotoxic effect.

An ineffective Nrf2-driven response of keratinocytes to oxinflammation mediated damage, could be very detrimental for those cells. This transcription machinery seems, indeed, fundamental for preventing deformation during differentiation (Piao et al., 2011), malignant transformation (auf dem Keller et al., 2006), the onset of typical skin diseases, such as atopic dermatitis (Tsuji et al., 2012). Besides, it has been shown that Nrf2 has a key-role in skin protection from sensitizing xenobiotics, including arsenic, formaldehyde and other harmful molecules (Gęgotek and Skrzydlewska, 2015; Pi et al., 2008).

This lack of adaptive response is the result of the observed inability of nucleus translocated Nrf2 to bind DNA, and thus ARE region. Owing to the still incomplete knowledge of these process and the factors co-regulating the binding and transcription of specific consensus cis-element, it is only possible to hypothesize possible mechanism responsible to this result (Fig. 10). Among the recognized transcriptional cofactors of the Keap1–Nrf2 regulatory pathway, small MAF proteins stand out for their possible implication in keratinocytes differentiation and skin aging (Motohashi et al., 2004). Noteworthy, it has been shown that Nrf2 can bind DNA and initiate transcription of genes only after MAF-Nrf2 heterodimeric complex formation. The expression and activity of these multifaceted molecules is finely regulated and still not fully understood (Kannan et al., 2012). Intriguingly, recent data have shown the p65 subunit of NF-kB represses the Nrf2–ARE pathway by various mechanisms, among which through promoting MAF-associated HDAC recruitment to the ARE locus of the hemeoxygenase-1(HMOX1) enhancer (Liu et al., 2008). This enhanced HDAC recruitment inhibits MAF co-transcriptional activity. In our view, the widely-suggested cross-talk between Nrf2 and NF-kB, might contribute to the short-circuit of defensive mechanisms observed in keratinocytes after CAPs exposure.

In addition, it has been shown that Nrf2 can be degraded also once in the nucleus independently to Keap1 since it is a target for SUMO2/3 leading to the polysumolynation and then ubiquitination of Nrf2 (Malloy et al., 2013).

In conclusion, the present work suggests that CAPs ability to penetrate into keratinocytes do not interfere with the nuclear translocation of Nrf2, but prevents, somehow, its ability to properly transcribe for several defensive enzymes.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mad.2017.11.007.

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