Stimulating Nrf2 and Inhibiting NF-kB to Help Skin Combating Pollution

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abstract

The skin is routinely exposed to stresses from environmental pollution. These repeated and regular exposures can profoundly affect skin physiology, and may lead to irreversible consequences. We studied the potential of a *Schisandra chinensis* (S.C.) extract, particularly enriched in lignans, to enhance skin defense mechanisms involved in pollution stress response, with the main pathways involved in skin being Nuclear Factor Kappa B (NF-kB), DJ-1, the nuclear factor erythroid 2-related factor 2 (Nrf2) and aryl hydrocarbon receptor (AhR) signaling pathways. Using an *in vitro* model, we showed that co-treatment with Urban dust and S.C. extract was able to stimulate the expression of Nrf2 and DJ-1, as well as the decrease of NF-kB and AhR, suggesting that this active extract may provide wide protection for skin against daily environmental insults. In a skin equivalent model stressed by Urban dust, the extract provided a global protection of skin components as shown by key proteins involved in the epidermal barrier. Even if further investigations are needed, we speculate that S.C. extract can be used to buffer the harmful effects of free radicals and enhance the skin barrier,due to air pollutant interactions with the skin, allowing a global preservation of cutaneous structures.

Introduction

Environmental air pollution, which contains a quantity of microscopic suspended particulate matter (PM) carrying various toxic chemical molecules, including polycyclic aromatic hydrocarbons (PAHs), has to be considered nowadays as one of the main characteristics of areas where, worldwide, human population density is at a high level [1]. It has been recognized by the World Health Organization as the most important environmental health issue in the world. In fact, much evidence exists on the relationship between air pollution and the development or exacerbation of cardiovascular and respiratory diseases; however, fewer studies concern the impact of air pollution and PM on skin integrity, even if it has been shown that they are significantly associated with weakened barrier function, and oxidative stress, skin diseases, and skin aging [2,3]. Generally speaking, PM penetrates skin either through hair follicles or transdermally, and exerts its detrimental effects through the generation of reactive oxygen species (ROS), which contributes to extrinsic skin aging, characterized particularly by pigment spots on the face and nasolabial folds, and less so by wrinkles. ROS activate the mitogen-activated protein kinase (MAPK) signaling pathway including ERK1/2, JNK, and p38 MAPK, and then the activated MAPK induces various transcription factors, such as Nuclear Factor Kappa B (NF-kB) and activator Protein-1 (AP-1) (**Fig. 1**). As a result of translocation of the activated transcription factors, inflammatory cytokines, which are closely related to inflammatory skin diseases and skin aging, are generated [4].

PAHs and PM are also well-established to activate the aryl hydrocarbon receptor (AhR) pathway, increasing ROS production [5]. Upon ligation by PAHs and PM, the activated AhRtranslocates from the cytoplasm into the nucleus (**Fig. 2**). This trans-

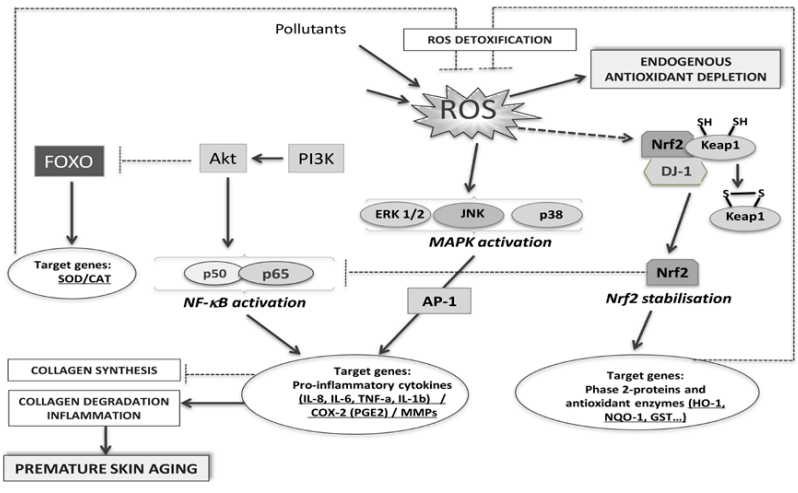

Fig. 1 Figure 1 summarizes the molecular mechanisms for pollutant-induced ROS generation and pro-inflammatory cytokines such as IL-1α, IL-6, IL-8, and TNF-α, resulting in increased skin aging. Mitogen-activated protein kinases (MAPK); NF-kB: nuclear factor kB; AP-1: activator protein-1; PI3K: phosphoinositide 3-kinase.

located AhR binds to its specific DNA recognition site, namely, xenobiotic-responsive element, and upregulates the transcription of responsive genes, such as cytochrome P450 family 1, member A1 (CYP1A1), CYP1A1 being a member of a multigene family of xenobiotic-metabolizing enzymes. Besides its physiological role in the detoxification of dioxins, the activity of CYP1A1 can be deleterious as it generates mutagenic metabolites and ROS [6].

Endogenous defense mechanisms including two fundamental biochemical pathways (nuclear factor erythroid 2-related factor 2 (Nrf2) and AhR pathways) are activated in order to fight the deleterious effects of all pollutants on skin. They are able to help eliminate and inactivate exogenous toxic agents by fundamental biological pathways closely interconnected. The involvement of the Nrf2 pathway in skin is of high importance; playing a role in skin homeostasis and skin renovation [7]. Nrf2 regulates not only a variety of antioxidant enzymes, such as NAD(P)H: quinoneoxidoreductase (NQO1), thioredoxin, or heme oxygenase-1, but also several phase I and phase II drug metabolizing enzymes, for example, glutathione S-transferase. Phase-II protective enzymes are responsible for the antioxidant response, xenobiotic disposition, inflammatory response, metabolic programming cell proliferation and survival, through the antioxidant-response element (ARE) (**Fig. 1**). Activity of Nrf2 is regulated by various mechanisms. Under homeostatic conditions, Nrf2 is generally localized in the cytoplasm, where it is sequestered by its inhibitor, Kelch- like ECH-associated protein 1 (Keap1). In response to ROS, Keap1 acts as a molecular sensor and undergoes chemical modifications in a series of reactive cysteine residues, allowing the release of Nrf2, which escapes from degradation and translocates to the nucleus, where it recruits the small Maf (sMaf) protein and binds to the ARE, an acting DNA regulatory element that activates the promoter region of several genes encoding phase II detoxification enzymes and antioxidants. Additionally, Nrf2 is stabilized by the Parkinson's-associated protein, (DJ-1), a multifunctional protein expressed in almost all tissues involved in various physiological processes such as transcriptional regulation, anti-oxidative stress reaction, mitochondrial regulation, and signal transduction [8] (**Fig. 1**). More precisely, DJ-1 promotes Nrf2 binding to antioxidant response elements by which Nrf2 can regulate the expression of several endogenous antioxidative enzymes and reduce ROS

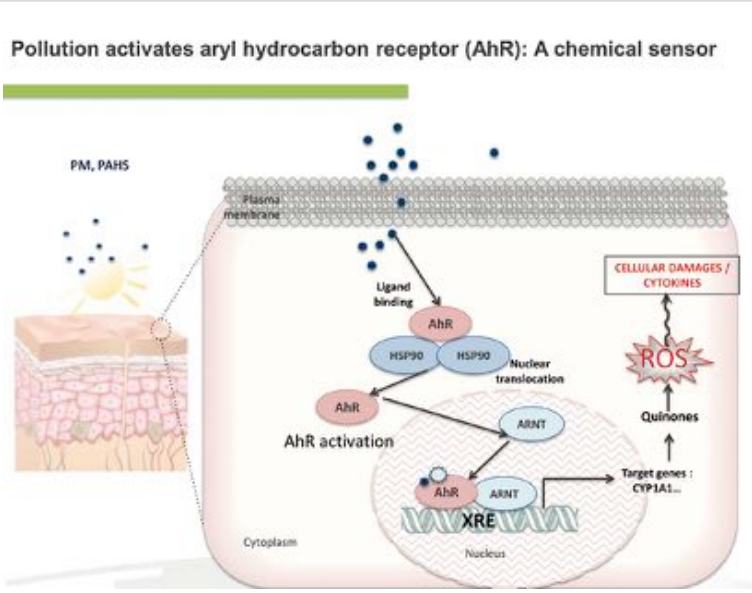

Fig. 2 Polycyclic aromatic hydrocarbons (PAHs) and Particulate matter (PM) induce ROS generation and pro-inflammatory cytokines. Upon ligation by PAHs and PM, the activated AhR translocates from the cytoplasm into the nucleus. This translocated AhR binds with ANRT, resulting in the activation of Cytochrome P450, family 1, member A1 (CYP1A1) transcription. ROS generated by CYP1A1 stimulates the production of TNF-α and IL-8.

production to protect mitochondria and can also respond to oxidative stress. Under oxidative stress, DJ-1 plays critical antioxidant defense roles by several molecular processes. In addition to directly regulating some antioxidant gene expression, DJ-1 functions as an atypical ROS scavenger peroxiredoxin-like peroxidase through the oxidization of conserved cysteine residue (Cys106). Additionally, it protects mitochondria by directly maintaining mitochondrial complex I activity [9] and translocating into mitochondria as an endogenous antioxidant.

The second line of defense involved in the cytoplasm is the AhR pathway, which is activated by dioxins, PAHs, or polyphenols and induces gene encoding expression for detoxification enzymes such as CYP1A1 (Phase I) and glutathione S-transferase (Phase II). Besides its physiological role in the detoxification of dioxins, the activity of CYP1A1 can be deleterious because it generates mutagenic metabolites and ROS [6]. A balance between phase I and phase II enzymes and other antioxidants is therefore crucial for maintaining cellular integrity. It also appears that AhR regulated Nrf2 [10].

A possible approach to attack ROS-mediated disorders for both preventive and treatment means is based on the use of substances, which can be found in plants as secondary metabolites. Various phytochemicals and herbal extracts exert their antioxidant properties by activating the NRF2 system in an AhR-dependent or AhR-independent manner in human epidermal keratinocytes [6]. *Schisandra chinensis* (S.C.) is a traditional Chinese herbal medicine that has been used for the treatment in Asia for thousands of years. The lignans as the main active ingredients in S.C. have various pharmacological effects such as antioxidative, anti-inflammatory, antitumor, and hepatoprotective activities [11]. Thus, it possesses various biological effects, which may activate the endogenous mechanisms of defense and biological pathways to fight against the pollutants. Schisandra extract also seems to inhibit IkB activation, thereby suppressing the production of TNF-α, IL-6 [12]. These findings led us to postulate that S.C. lignans might protect skin cell functions against urban pollution and could be used as cosmetic agents.

Thus, the first aim of this study was to evaluate the effect of active extract from S.C. on the protection of cell damages caused by Urban dust to evaluate an anti-pollutant effect. The activation of Nrf2 transcription factor, AhR, and NF-kB in basal conditions and after a pollutant treatment on human keratinocytes was evaluated. Secondly, using reconstructed 3D human skin models with human primary cells (fibroblasts/keratinocytes) (Lab Skin Creations, Lyon, France), we evaluated the impact of this extract on S.C. extract on proinflammatory cytokines, lipoperoxidation, and on epidermal functions.

Materials and Methods

The Urban dust chosen was SRM1649b from Sigma including PAHs and nitro-PAHs, Polychlorobiphenyls, Chlorinated pesticides, Decabromodiphenyl Ether, Polychlorinated Dibenzo-p-Dioxins and Dibenzofurans, Inorganic constituents & heavy metals, and Particulate Matter (PM2.5 and PM10).

After the cytotoxicity evaluationfor determination of cell viability using WST1 assay, Normal Human Epidermal Keratinocytes (NHEK) cells were cultured overnight at a 5000 cells/well of density in a 96 well plate, at 37°C, 5 % CO_2. The cells were treated then 24 hours with the S.C. extract 0.0.1 %or control, then exposed during 6h to Urban dust (80 µg.ml-1) and S.C. extract. The cells were fixed with formalin and the expression of AhR, Nrf2, DJ1, and NF-kB were detected by immunofluorescence. After a step of permeabilization/saturation, staining of the treated cells with antibodies (anti-AhR polyclonal, anti-Nrf2 polyclonal, anti-DJ1 polyclonal and anti-NFkB polyclonal) was performed overnight at 4°C.The secondary antibody AlexaFluor 488 tagged goat antibody was applied 1h at RT. Fluorescent labeling was imaged and quantified by automated microscopy (Array Scan Cellomics TM). The fluorescence was quantified by the bioapplication Compartmental Analysis.

The following analyses on skin equivalent models were also performed:

- Quantitative immunoassays to evaluate IL-6 and Prostaglandin E2 (PGE2) (Elisa, R&D Systems, France)
- Analysis of malondialdehyde (MDA) and filaggrin were evaluated using immunohistochemistry
- Occludin was measured by immunofluorescence.

The 3D reconstructed full-thickness model procotol was as follow: 6 days pre-treatment with S.C. extract 0.01 % + 1 day co-treatment S.C. extract 0.01 % and Urban dust 80 µg.ml-1. Statistical analyses were carried out on Urban dust treatments with S.C. to study the relevance of the protective effect of S.C. against the pollutant treatment, using a non-parametric test (Mann-Whitney). Statistical significance was set at $p<0.05$, 95 % of confidence.

Results

Fig. 3 shows the effects of Urban dust and co-treatment of Urban pollution and S.C. extract on NF-kB expression in cy-

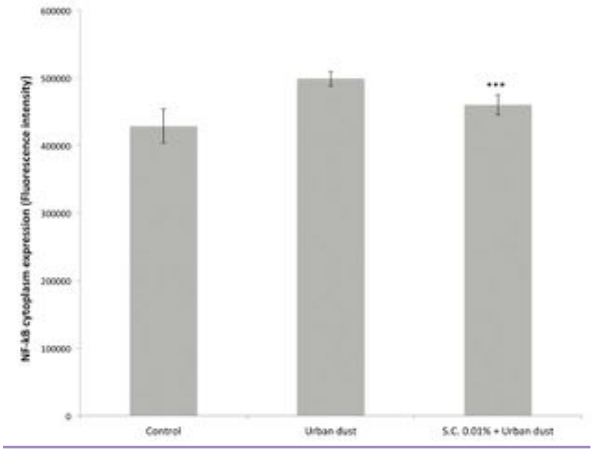

Fig. 3 NF-kB cytoplasm expression after Urban dust exposure or co-treatment with Urban dust and S.C. extract. *** p< 0.001 *versus* Urban dust.

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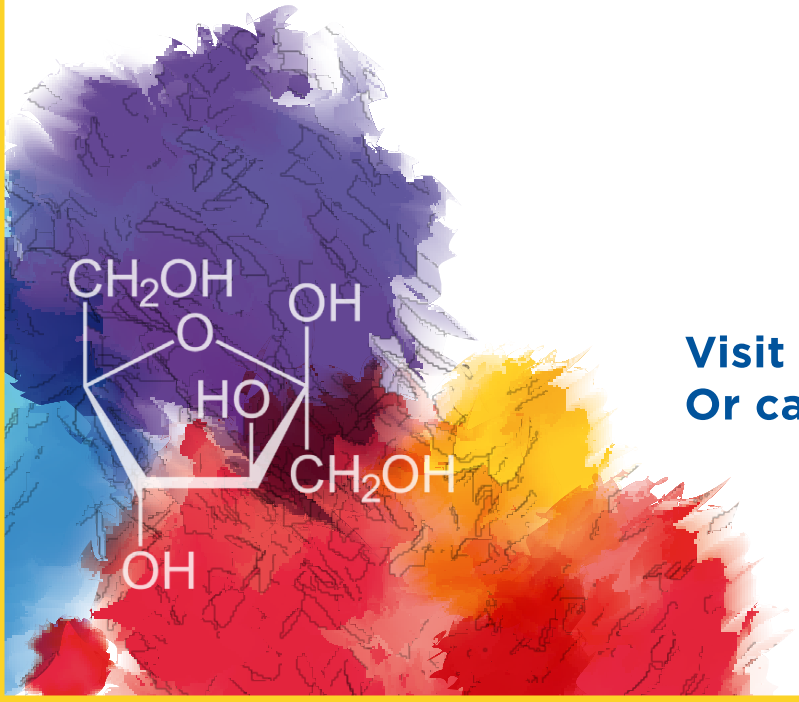

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toplasm (A). We noted that the co-treatment induced a significant inhibition of NF-kB expression (-6 %: p < 0.001), when compared to Urban dust alone. At the same time, the Urban dust is able to activate AhR and Nrf2 pathways (**Figs. 4** and **5**). It is also able to activate DJ-1 (**Fig. 6**). Co-treatment decreased significantly AhR expression (-33 %: p < 0.001) and demonstrated a significative activity on Nrf2 and DJ-1 (33 %: p < 0.001) (**Figs. 5** and **6**).

Inflammation observed after 24 hours of Urban dust treatment (elevation of PGE2 and IL-6) in reconstructed skin supernatant was abolished after the addition of S.C. extract (**Fig. 7**).

Expression of MDA in reconstructed skin was significantly reduced (**Fig. 8**) with co-treatment (-22 %, p < 0.05). This co-treatment also induced a significant increase in Occludin expression (32 %: p < 0.05), this protein being implicated in close junction stability and barrier function, and also a significant increase in filaggrin, which is implicated in cornification process and skin hydration (160 %: p < 0.05) (data don't show).

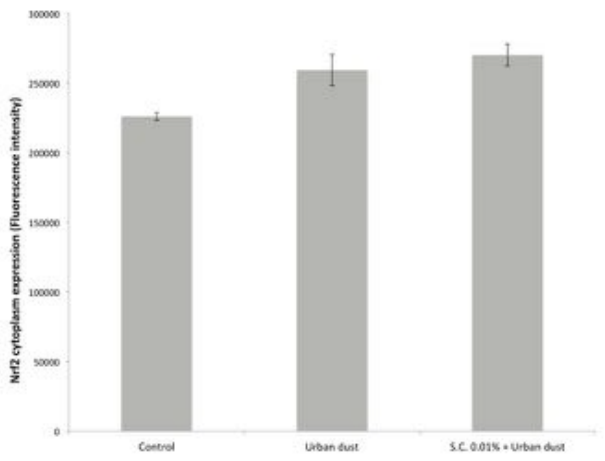

Fig. 5 Nrf2 cytoplasm expression after Urban dust exposure or co-treatment with Urban dust and S.C. extract.

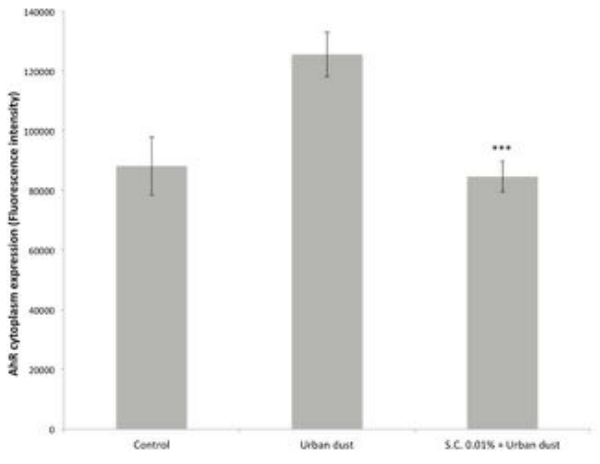

Fig. 4 AhR cytoplasm expression after Urban dust exposure or co-treatment with Urban dust and S.C. extract. *** p< 0.001 *versus* Urban dust.

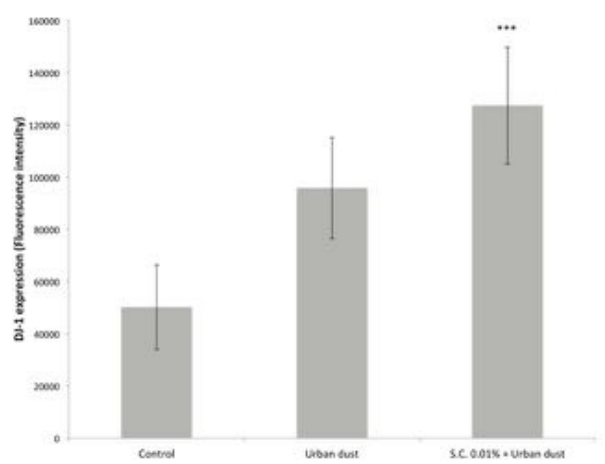

Fig. 6 DJ-1 expression after Urban dust exposure or co-treatment with Urban dust and S.C. extract. *** p< 0.001 *versus* Urban dust.

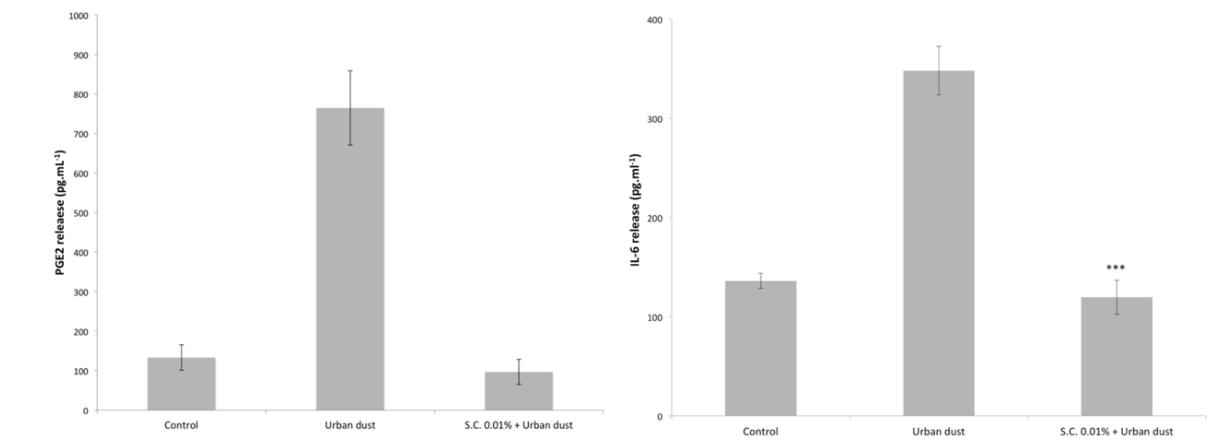

Fig. 7 Secretion of PGE2 and Il-6 in polluted or non-reconstructed skin supernatant treated or not with S.C. extract. Analysis has been done 24h after pollutant stress. *** p< 0.001 *versus* Urban dust.

Discussion

Skin is inevitably exposed to environmental pollutants. Oxidative stress by these factors accelerates skin aging and induces skin inflammation. In this study, we have demonstrated the protective effect of S.C. extract, which increased DJ-1 protein levels, Nrf2 expression and decreased AHR and NF-kB in cytoplasm even if the cells are under stress pollution. These results are in line with those of *Lin et al.*, [13], who recently reported that Schisandrin B, an active ingredient extracted from S.C., blocked the activation of NF-kB, and activated Nrf2, which resulted in the inhibition of inflammatory response. The decrease in inflammation after S.C. extract into the reconstructed skin (**Fig. 7**) is in line with this assumption.

As previously mentioned, Nrf2 is an important transcription factor that plays a critical role in protecting cells from oxidative stress. Activation of Nrf2 could regulate and induce the expression of numerous detoxifying and antioxidant genes. Moreover, the increase of DJ-1 noted in our study after co-treatment of Urban dust and S.C. extract is an important result, DJ-1 quenching the activity of ROS and protecting mitochondrial function [14]. It is also known that DJ-1 promotes Nrf2 binding to antioxidant response elements by which Nrf2 can regulate the expression of several endogenous antioxidative enzymes and reduce ROS production to protect mitochondria and can also respond to oxidative stress. This mechanism is mediated by the fact that DJ-1 sequesters Keap1, an Nrf2 binding protein in the cytosol promoting Nrf2 degradation. In reconstructed skin, we noted that the quantity of MDA in the epidermis decreased in polluted skin when preventive S.C. extract was performed, reflecting a protection of cell membrane integrity from oxidative stress induced by pollution.

AhRs are chemical sensors that are abundantly expressed in epidermal keratinocytes and mediate the production of ROS. Many Nrf2-mediated antioxidant phytochemicals are capa-

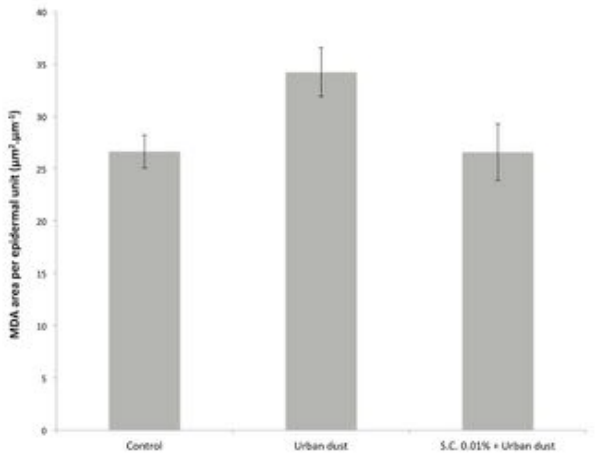

Fig. 8 MDA expression in epidermis of skin equivalent model. Analysis has been done 24h after pollutant stress.

ble of up- and down-modulating AhR signaling. The precise mechanisms by which these phytochemicals differentially affect the AhR and Nrf2 system remain largely unknown and warrant future investigation [6]. Co-treatment of Urban dust and S.C. extract induced a significant decreased of AhR expression (**Fig. 4**), as compared to Urban dust alone. Pollutant-induced up-regulation of inflammation parameters is dependent on activation of AhR signaling pathway. Recently, *Lee et al.*, [15] noted that pre-topical application of AhR antagonists or antioxidants in vivo (in mice) protected the skin against PM-induced inflammatory responses. Moreover, by inhibition of phase I enzymes they reduce the oxidative transformation of precursor compounds into toxic intermediates. It has also been shown that the activation of AhR via air pollutants induced expression of epidermal hyper-innervation and inflammation, AhR activation and ARTN expression being positively correlated in the epidermis of patients with atopic dermatitis [16]. Finally, the changes induced by S.C. extract are reflected at the epidermal level by an increase of Filaggrin and Occludin expression. This, one can put forward the hypothesis that S.C. extract promotes cornification and improves skin hydration and increases barrier function stability.

Conclusion

In summary, using an *in vitro* model, we showed that co-treatment with Urban dust and S.C. extract was able to stimulate the expression of Nrf2 and DJ-1, as well as the decrease of NF-kB and AhR, suggesting that this active extract may provide wide protection for skin against daily environmental insults. This extract protected cells from oxidative stress induced by environmental pollutants. In a skin equivalent model stressed by Urban dust, the extract provided a global protection of skin components as shown by key proteins involved in the epidermal barrier. Even if further investigations are needed, we speculate that S.C. extract can be used to buffer the harmful effects of free radicals and enhance the skin barrier, due to air pollutant interactions with the skin, allowing a global preservation of cutaneous structures.

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