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Reconstructed Human Epidermis Response to Urban Particulate Matter Activates Multiple Stress-Related Pathways and Impacts the Skin Barrier Function

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Abstract

There is an increasing demand for identification and characterization of the effects of skin care products against air pollutants. Meeting this demand requires reliable, standard in vitro assays in appropriate skin models to investigate the protective effects of skin care ingredients and formulas. We used reconstructed human skin exposed to standardized urban dust and the usual approaches for gene and protein expression analyses, as well as trans-epidermal electrical resistance. We validated multiple biomarkers directly associated with the skin response to pollution, such as activation of detoxification and antioxidant pathways, inflammation, and impaired barrier function. This work stresses the fact that the skin response to pollution entails cross-talk between several signaling pathways that should be studied together to assess the effects of potential protective compounds. Our data demonstrate the usefulness of in vitro assays based on a reconstructed human epidermis model to assess the antipollution and detoxification potency of investigational compounds.

INTRODUCTION

Skin aging and inflammatory conditions are a primary concern for millions of people around the world. Such skin alterations not only occur because of time and genetics but are also the consequence of exposure to different environmental factors.

The increase in air pollution over the years has been recognized to have major effects on human skin, especially in large industrial cities such as Mexico City, Shanghai, or Sao Paulo, where exposure to air pollutants has been linked through population studies to skin aging, inflammation and allergy [1-7]. These studies, strengthened by preliminary *in vitro* assays, have shown that the main effects of ambient air particulate matter are linked to free radical generation, inducing molecular damage and inflammatory cascades. The skin's response to pollution will subsequently lead to impairment of the skin barrier and extracellular matrix as well as to pigmentation disorders [1-3, 8-9]. The molecular targets and cellular pathways that could be modulated to dampen the effects of pollution are currently under investigation.

The limited availability of mechanistic data and the need for specific and cost-effective bioassays led us to develop robust *in vitro* assays using a 3D reconstructed human epidermis (RHE) and to validate relevant biomarkers allowing the evaluation of protective compound candidates. We used the SRM1649b urban particulate matter (UbD) as a standard reference material and investigated the response of RHE to this mixture of pollutants by combining gene expression analyses and bioassays. Our final goal was to create a screening tool based on a panel of gene signatures that reflects potential gene expression variations in skin epidermis exposed to urban pollution particles.

EXPERIMENTAL

Cell and tissue culture

Reconstituted human epidermis (RHE) was produced by StratiCELL, Isnes, Belgium. Normal human epidermal keratinocytes (NHEK) and normal human dermal fibroblasts (NHDF) were purchased from Lonza, Verviers, Belgium. All cultures were maintained in culture media in a humid atmosphere at 37 °C with 5 % CO_2 according to the manufacturer's instructions.

Urban dust particulate and other reference materials

We used the standard reference material SRM 1649b from the National Institute of

Standards and Technology (Gaithersburg, MD, USA) as an established surrogate for authentic urban dust. SRM 1649b is prepared from atmospheric particulate material collected in the Washington, DC area in 1976 and 1977. It should generally typify atmospheric particulate matter obtained from an urban area. The certificate of analysis can be obtained online at the following address: https://www-s.nist.gov/srmors/certificates/1649b.pdf [10].

The urban dust particulates were suspended in DMSO before use either in the culture medium or for topical application to RHE tissues. Although the actual concentrations of particles in the environment vary greatly from place to place and time to time, the subtoxic concentrations that were chosen to perform this study were in line with the fact that real-life situations rarely induce acute skin toxicity but still induce biological effects like those observed in this study. The dose selection was also in accordance with observations from previous studies [11-12]. Dexamethasone and carnosic acid were from Sigma, Overijse, Belgium. Recombinant TGFβ was from Bio-Techne, Oxon, UK, and ascorbic acid from VWR BDH Prolabo, Oud-Heverlee, Belgium. DMSO 0.1 % was used as the control condition in every experiment.

Cell and tissue toxicity study

To determine the working concentrations for UbD and the references, preliminary experiments were conducted on culture triplicates. NHEK, seeded in 24-well plates, and RHE, after 14 days at the air-liquid interface, were exposed to UbD in their culture medium or topically for either 24 or 72 hours. At the end of treatment, the cell viability was then assessed by an MTS assay (3-(4,5-dimethythiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium).

RHE samples were also fixed in formaldehyde and embedded in paraffin. Then the histology was verified after hematoxylin and eosin staining. UbD concentrations used in the different assays were subtoxic (15 or 120 µg per cm² for systemic or topical application, respectively).

Reactive oxygen species

Analysis of the effects of UbD on the production of reactive oxygen species (ROS) by NHEK and RHE was carried out using the prefluorescent probe H2D-CFDA (2′, 7′-dichlorodihydrofluorescein diacetate). At the end of culture, the medium was replaced by PBS, and RHE was incubated for 30 minutes with the probe, which diffused into the tissue cells. The probe could then be oxidized by free radicals present in the cells, producing the fluorescent compound DCF (2′, 7′-dichlorofluorescein). This allowed quantification of the production of free radicals according to the level of fluorescence emitted by the oxidized probe. After the incubation with the probe, the PBS was changed and the cells or RHE were irradiated or not with UVA (5 J or 10 J/cm², respectively) using a Bio-Sun illuminator (Vilber, Eberhardzell, Germany). The subsequent oxidation of the probe was quantified at excitation and emission wavelengths of 485 and 520 nm, respectively.

Quantification of inflammatory mediators
To determine the influence of UbD on the release of inflammatory mediators in reconstructed human epidermis, RHE were exposed to different concentrations of UbD for 24 hours in the presence or absence of dexamethasone. At the end of the experiment, culture supernatants were collected and used for quantification of interleukins IL-1α, IL-6, IL-8, and TNFα. The quantification of inflammatory markers was performed using specific ELISA kits for IL-1α, IL-6, IL-8, and TNFα, all from R&D Systems / Bio-Techne, Oxon, UK). The methods followed the supplier's specifications based on standard curves.

Immunohistochemistry

RHE samples fixed in formaldehyde were dehydrated with ethanol and isopropanol before paraffin inclusion and storage. Paraffin-embedded sections were de-paraffined and re-hydrated for histological assessment (eosin and hematoxylin staining) or immune labeling (fluorescence detection). When needed, antigen unmasking was performed using a citrate buffer (pH = 6). Sections were incubated with primary antibody after blocking in the presence of bovine serum albumin. Slides were mounted with Mowiol medium (Sigma, Overijse, Belgium) and examined with a fluorescence microscope (Leica DM 2000, lens 40x) equipped with a digital camera (Leica DFC420C, Diegem, Belgium). Antibodies were bought from Acris Antibodies/Sanbio (mouse anti-filaggrin, Uden, Netherlands) and Molecular Probes/Thermo Fisher (anti-mouse Alexa-488, Merelbeke, Belgium). Nuclei were stained with DAPI (Invitrogen / Thermo Fisher).

Transepidermal electrical resistance

The skin barrier function of RHE samples was determined by measuring the transepidermal electrical resistance (TEER) using the Millicell ERS Ohmmeter (Millipore, Overijse, Belgium). An empty insert was used as the blank. All measurements were performed on 12 wells plates filled with PBS.

Transcriptomics analyses

RNA samples were amplified using the Ribo-SPIA technology, purified with the Agencourt RNA Clean up XP Beads kit (Agencourt – Beckman Coulter Genomics, Suarlée, Belgium), and then fragmented and labeled with biotin using the NuGEN Encore Biotin Module (NuGEN, Leek, Netherlands). Hybridization was carried out on Human Gene 2.0 ST DNA chips (Affymetrix/Thermo Fisher, Merelbeke, Belgium) according to the protocol defined by Affymetrix. Intensity measurements were scanned with the GeneChip Scanner 3000 (Affymetrix). Raw data processing was carried out using R (v3.1.1) [13] and the oligo package (v1.30.0) of the BioConductor project (v3.0) [14]. The latest version of the libraries provided by Affymetrix built on version 19 of the human genome (UCSC Human genome 19) and the RMA method described by *Irizarri et al.* [15] were used to guide and perform pre-processing and sequence annotation. The quality of the samples was checked before and after amplification using the Agilent Bioanalyzer (electrophoretic profiles and RIN values). The cDNA profiles were also verified after fragmentation and biotin labeling. At the end of pre-processing of the hybridization data, several representations were generated

Table I List of References Used in the TaqMan Gene Expression Assays

Gene symbol	Gene name	TaqMan Assay Id
ALDH3A1	aldehyde dehydrogenase 3 family, member A1	Hs00964880_m1
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	Hs01054797_g1
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	Hs02382916_s1
CYP4F11	cytochrome P450, family 4, subfamily F, polypeptide 11	Hs01680107_m1
FLG	filaggrin	Hs00856927_g1
FMO4	flavin containing monooxygenase 4	Hs00157614_m1
FTL	ferritin, light polypeptide	Hs00830226_gH
GADD45A	growth arrest and DNA-damage-inducible, alpha	Hs00169255_m1
GCLM	glutamate-cysteine ligase, modifier subunit	Hs00157694_m1
GPX2	glutathione peroxidase 2 (gastrointestinal)	Hs01591589_m1
GSTM1	glutathione S-transferase mu 1	Hs01683722_gH
IL1B	interleukin 1, beta	Hs00174097_m1
IL8	interleukin 8	Hs00174103_m1
LCE3E	late cornified envelope 3E	Hs01631234_sH
MMP1	matrix metallopeptidase 1 (interstitial collagenase)	Hs00899658_m1
MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	Hs00968305_m1
MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	Hs00234579_m1
MT1G	metallothionein 1G	Hs02578922_gH
MT4	metallothionein 4	Hs00262914_m1
NQO1	NAD(P)H dehydrogenase, quinone 1	Hs02512143_s1
SPRR1A	small proline-rich protein 1A	Hs00954595_s1
SQSTM1	sequestosome 1	Hs01061917_g1
TGM1	transglutaminase 1	Hs00165929_m1

to assess the quality of the pretreatment (boxplots, ma-plots, etc.). Principal component analysis and hierarchical clustering were carried out in order to verify that the samples associate according to their labels and to identify the samples for which the acquired data show deviant behavior. The main component analysis was carried out on all the probesets represented on the GeneChip Human Gene 2.0 ST chips. Hierarchical clustering (Euclidian distance, average linkage) was performed on a subset of the probesets: only the annotated probesets in the public databases were retained, with one probe per gene in case of multiplicity (the most informative with maximum variability). Individual statistical analysis of the genes/transcripts was carried out using the Moderated t method implemented in the R Limma 3.22.1 package [16]. Gene annotation and definition of gene clusters for over-representation analysis using the hypergeometric test method were carried out with our proprietary »StratiCELL Skin Knowledge Database«, which models StratiCELL's expertise in skin biology in a dermocosmetic context and integrates data from public databases.

Gene expression analysis using qRT-PCR

Gene expression changes were analyzed using TaqMan qPCR assays. RNA extraction was performed with the RNeasy Mini Kit (Qiagen, Antwerp, Belgium) according to the supplier's recommendations. The RNA samples were stored frozen at -80°C. The RNA concentrations were determined by spectrophotometric measurements and the RNA quality was analyzed by capillary electrophoresis (Agilent Bioanalyzer 2100, Diegem, Belgium). After reverse transcription the TaqMan assays were processed as described in the manufacturer's instructions (TaqMan™ Assays, Applied Biosystems/Thermo Fisher, Merelbeke, Belgium). In brief, cDNAs were mixed in 96-well plates with a specific buffer and probes specific to the target genes. Plates were sealed and qPCRs were run by a 7900HT Fast Real-Time System (Applied Biosystems). Relative quantification of gene expression was performed using the comparative Ct (ΔΔCt) method. The references for the TaqMan genes expression assays (Applied Biosystems) are listed in ***Table I***.

Statistical analyses

Experimental data were analyzed for statistical significance using Student t tests. $p \leq 0.05$ was considered significant (*), $p \leq 0.01$ very significant (**) and $p \leq 0.001$ highly significant (***). Normal distribution was assumed from prior empirical data using similar models and assays.

RESULTS

Main effects of pollution *in vitro* in RHE exposed to UbD

We first evaluated the relevance of RHE exposed to UbD as an *in vitro* model to study the effects of pollution and protective compounds on skin inflammatory and oxidative stress responses (see experimental section for methods). Subtoxic testing concentrations of UbD were selected during preliminary dose finding experiments in order to guarantee the absence of detrimental effects on viability and histology ***(Figure 1***).

There were significant statistical differences between UVA-treated RHE with and without exposure to UbD (*) as well as between UVA and UbD treated cells with and without

vitamin C 300 µg/ml (*), as evident in ***Figure 2***. Both systemic and topical exposure of RHE to UbD were tested with similar results (15 µg/cm² or 60 µg/ml, and 120 µg/cm², respectively). In the presence of the chosen concentrations, exposure to urban dust by itself was not sufficient to significantly increase the level of reactive oxygen species. However, the oxidative stress induced by UVA was significantly amplified when RHE was previously exposed to UbD for 48 h ***(Figure 2)***.

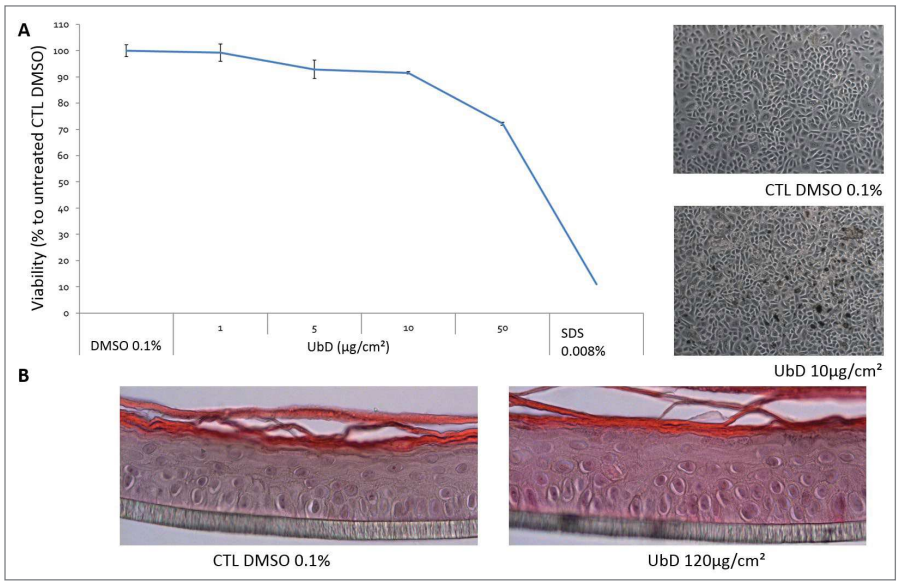

Figure 1 Cell and tissue toxicity of UbD. A) Means and standard deviations of the percentage of NHEK cell viability normalized to untreated DMSO control after 48 h of culture as a function of UbD concentration (MTS assay, SDS = cytotoxic reference, representative images on right side) and B) H&E staining of RHE tissues exposed or not to UbD at the working concentration of 120 µg/cm².

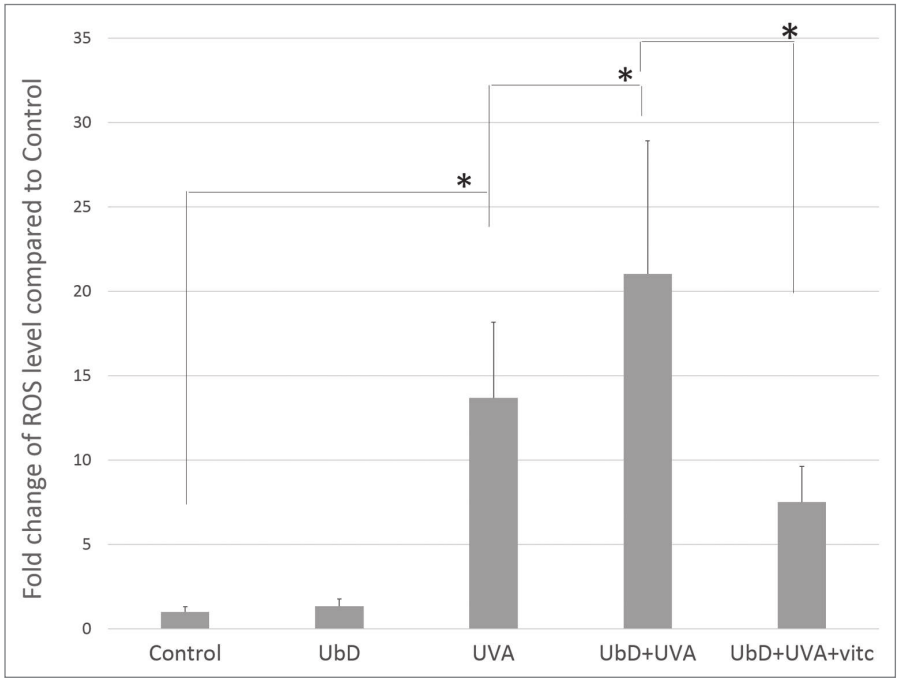

Figure 2 Relative measurement of the intensity of oxidative stress induced in RHE after 48 hours of culture in the presence of UbD in the culture medium and exposure to UVA 10 J/cm² (positive reference = Vitamin C, means of triplicates + standard deviations of fold change values normalized to control). Student's t-tests were performed to compare the UBD-induced condition with other conditions (* $p<0.05$).

In contrast, exposure of RHE to urban dust alone for 24 h at subtoxic concentrations induced a strong inflammatory response by the tissues, which released TNFα, IL-6, IL-8, and IL-1α into the culture medium ***(Figure 3)***. Both systemic and topical exposure of RHE to UbD were tested with similar results (15 µg/cm² or 60 µg/ml, and 120 µg/cm², respectively). The inflammatory cytokine release was partially (TNFα, IL-8, IL-1α) or totally (Il-6) inhibited in the presence of dexamethasone. These observations were also made in keratinocyte monolayers (data not shown).

We then assessed the impact of air pollutants on the integrity of the RHE skin barrier function by measuring transepidermal electrical resistance (TEER) after 7 days of exposure to UbD ***(Figure 4)***. We observed a loss of TEER that can be directly interpreted as a decrease in the barrier function. Both systemic and topical exposure of RHE to UbD were tested with similar results (15 µg/cm² or 60 µg/ml, and 120 µg/cm² respectively).

To further study the effects of UbD in this context, we quantified the variation in the abundance of filaggrin between exposed and non-exposed RHE samples ***(Figure 5)***. The significant decrease in filaggrin in the presence of UbD corroborates the fact that urban pollution weakens the skin barrier function. Since exposure to pollution has also been correlated with premature aging and metalloproteinases (MMP) [6], we sought to determine if UbD at subtoxic concentrations would induce MMP release from dermal fibroblasts. In addition to the studies on RHE, we observed a significant overexpression of pro-MMP1 as well as a release of the pro-MMP1 precursor in the culture medium of fibroblasts exposed to UbD ***(Figure 6)***.

Multiple stress-related pathways activated in RHE exposed to UbD

With the aim of further characterizing the biomolecular response of the RHE model to UbD, we performed preliminary transcriptomics analyses on NHEK. The cells were exposed to UbD in the culture me-

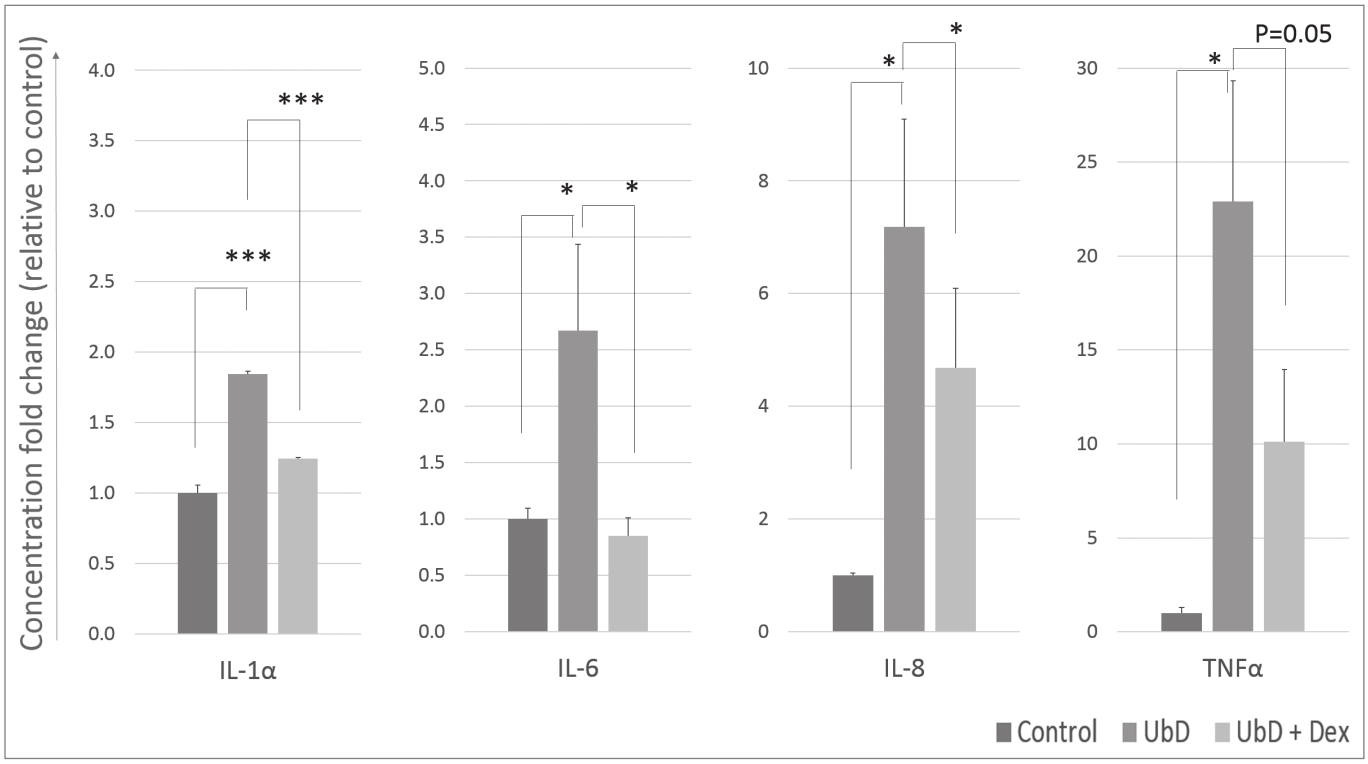

Figure 3 Effect of urban dust (UbD) in the culture medium on release of inflammatory markers in reconstructed human epidermis (UbD conc. 150 µg/cm²) in the presence or absence of dexamethasone (Dex) at 10 µM. Results are normalized as fold changes to control values and shown as mean + standard deviation of triplicates. Student's t tests were performed to compare the UBD-induced condition with other conditions (* $p<0.05$, *** $p<0.001$).

dium for 24 hours and then the RNA was processed for Affymetrix analyses at the whole transcriptome level as explained in the Experimental section. Filtering the data through our proprietary Skin Knowledge Database, we observed that UbD had mul-

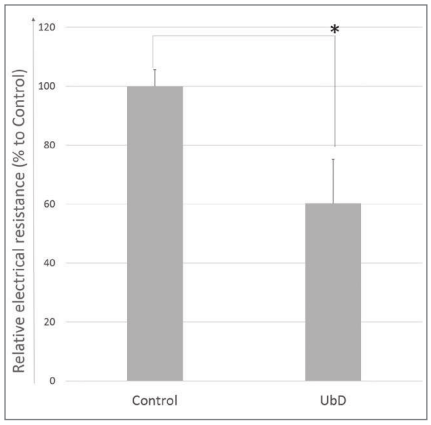

Figure 4 Change in the transepidermal electric resistance (TEER) of reconstructed human epidermis exposed to UbD for 7 days (results normalized as percentages of control values and shown in the graph as mean + standard deviation of triplicates). Student's t-tests were performed to compare the UBD-induced condition with control (* $p<0.05$).

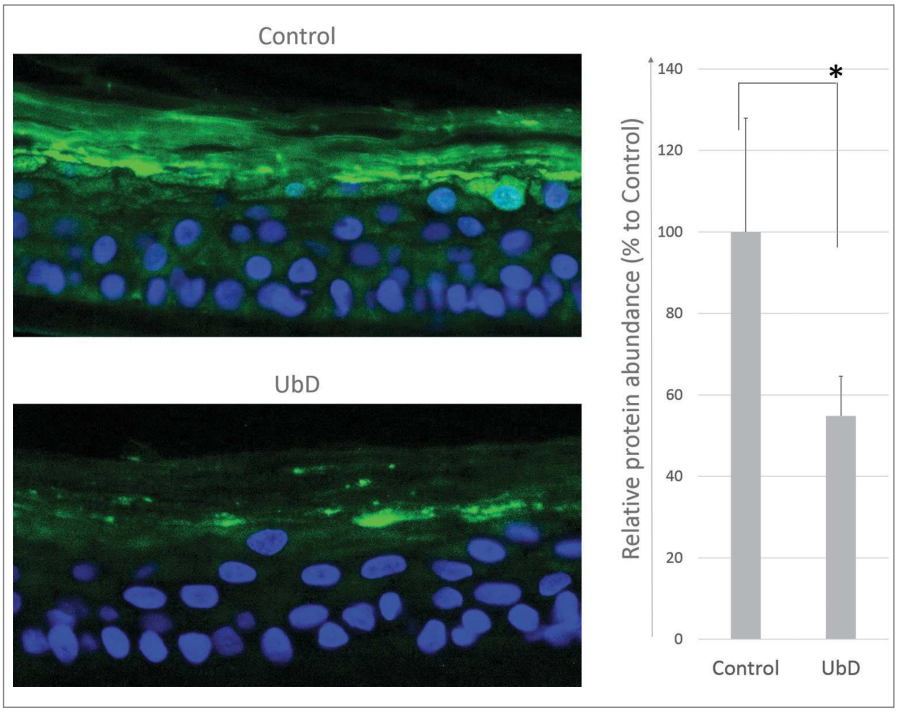

Figure 5 Decrease in filaggrin expression in reconstructed human epidermis exposed to urban dust. RHE were topically exposed to urban dust (120 µg/cm²) for 48 h and then processed for immunohistochemistry and stained with anti-filaggrin antibody. The abundance of filaggrin (representative pictures on the figure) was quantified and normalized to the surface area and number of nucleated cells. The graph on the right side shows the mean + standard deviation of values as percentages relative to control (* $p<0.05$).

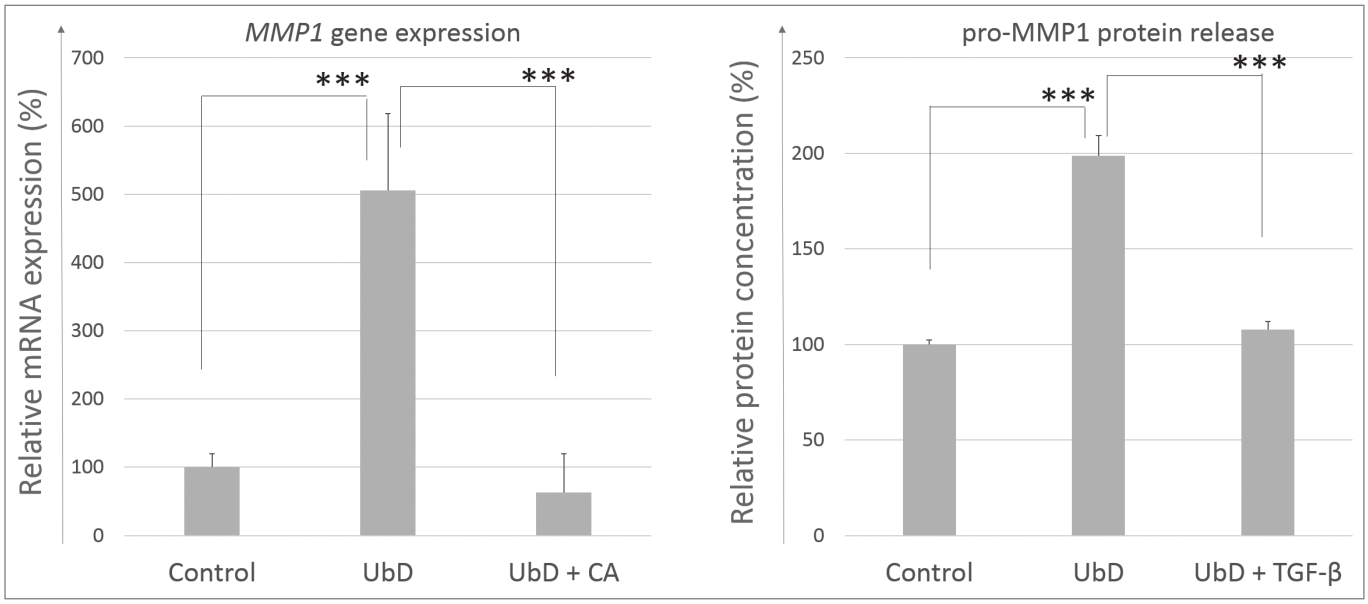

Figure 6 Relative expression levels of gene (left) and protein (right) of pro-matrix metalloproteinase 1 (MMP1) in normal human dermal fibroblasts (NHDF) and their culture supernatants, respectively, after 72 hours of culture. Positive references = carnosic acid (CA, 2 µM) and TGFβ (20 ng/ml). The graphs present the means and standard deviations (from 3 independent cultures) of pro-MMP-1 levels as percentages relative to control (*** $p < 0.001$).

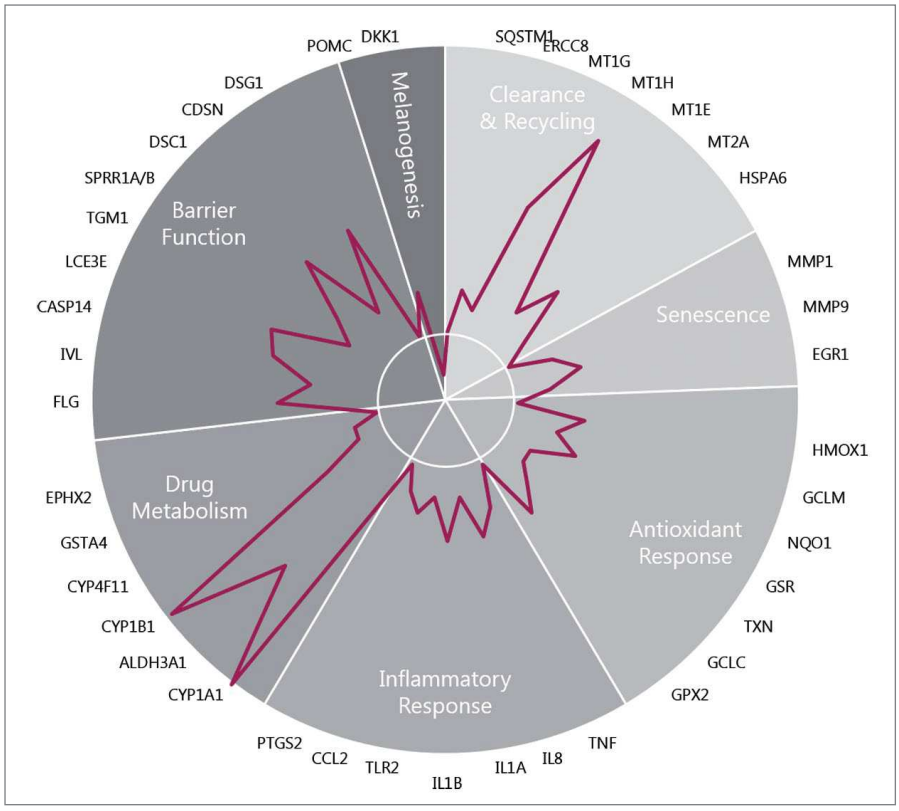

Figure 7 Radar plot of a gene expression analysis using the Skin Knowledge Database. Using statistical and fold change thresholds of $p < 0.05$ and $FC > 1.3$, respectively, for individual gene expressions and filtering the data through our proprietary Skin Knowledge Database, we identified 807 genes of which expression was modulated by UbD. The radar chart shows in a base-10 log scale the proportional magnitude of expression changes compared with control for several genes involved in key cellular functions as depicted. The white circle at the center is the baseline control level 0 (i.e. FC = 1), while the edge of the figure equals 2 (i.e. FC = 100). The farther from the center, the more expressed the gene is compared with the control.

tiple effects on stress-related pathways of the skin. An illustration of the data for a UbD concentration of 60 µg/ml in the culture medium can be seen in ***Figure 7*** in the form of a Radar plot.

We used these data to design a specific TaqMan Low Density Array (TLDA) to study simultaneously the expression of 96 genes involved in these different pathways/functions of the skin associated with environmental stress. This TLDA was then used to analyze the gene expression changes from the 3D RHE model exposed to UbD. The results presented in ***Table II*** clearly show that urban dust influences multiple cell signaling pathways, including inflammation, antioxidant defense, cellular stress response and xenobiotic metabolism, but also epidermal biology and differentiation, as well as genes coding for extracellular matrix metalloproteinases. Many of the effects of urban dust on RHE gene expressions corroborated those observed in NHEKs, except for genes involved in terminal differentiation and barrier function ***(Table II)***. Indeed, the primary keratinocytes exposed to urban dust present a strong overexpression of these genes, while the differentiated RHE showed a decreased expression of the same genes under the same conditions.

Table II Expression Fold Changes of Genes Involved in Response to Urban Dust Exposure in RHE and NHEK Exposed to UbD in the Culture (60 µg/ml) Medium for 24 Hours (Gene expression was analyzed using TaqMan qPCR assays. The table shows the means of expression fold changes relative to control. Genes are grouped by cell function or signaling pathways.)

Cell signaling or function	Gene name	RHE		NHEK	
		Fold change	P-value	Fold change	P-value
Antioxidant response (Nrf2/EpRE)	FTL	1.39	6.80E-02	1.33	9.70E-02
	MT4	-2.30	4.00E-02	1.41	7.47E-01
	MT1G	1.61	2.06E-01	9.82	3.82E-02
	GPX2	2.15	6.00E-03	3.18	1.70E-03
	NQO1	2.34	0.00E+00	3.82	3.09E-02
	GSTM1	1.78	3.70E-02	1.19	4.42E-02
	GCLM	1.38	7.00E-02	1.12	3.79E-01
Autophagy	SQSTM1	1.20	3.40E-02	1.99	1.52E-02
DNA damage & repair	GADD45A	1.48	2.10E-02	1.03	2.94E-01
Drug metabolismphase I (AhR)	ALDH3A1	2.36	1.10E-02	13.28	3.82E-02
	CYP1A1	23.19	1.00E-03	101.43	3.88E-02
	CYP1B1	7.77	0.00E+00	89.25	8.40E-03
	CYP4F11	1.84	6.10E-02	3.93	1.09E-02
	FMO4	-1.23	1.20E-02	-1.43	2.56E-01
Inflammatory response	IL1B	2.05	4.60E-02	3.18	4.28E-02
	IL8	1.66	1.05E-01	9.48	1.55E-02
Matrix metalloproteinases	MMP1	3.41	1.40E-02	2.19	8.40E-03
	MMP3	3.76	1.93E-01	1.53	3.70E-01
	MMP9	1.66	4.20E-02	1.73	1.09E-02
Terminal differentiation & barrierfunction	LCE3E	1.27	8.70E-02	1.52	4.12E-01
	SPRR1A	1.17	3.50E-02	2.10	3.09E-02
	TGM1	-1.47	2.32E-01	1.75	6.03E-04
	FLG	1.04	8.24E-01	5.19	6.03E-04

Concerning the other cell functions significantly modulated by UbD, we noted that many target genes of the aryl hydrocarbon receptor (AhR) were overexpressed. Several of these genes are required for biotransformation of xenobiotics through the successive phases of detoxification and elimination [17]. Most of the related genes that are overexpressed code for enzymes involved in the first phase, such as CYP1A1 (23.2x, $p=0.001$) CYP1B1 (7.8x, $p<0.001$) and CYP4F11 (1.8x, $p=0.061$), which are three members of the CYP450 family of oxidases, as well as the aldehyde dehydrogenase ALDH3A1 (2.4x, $p=0.011$). Genes coding for phase II enzymes included glutathione-S-transferases like GSTM1 (1.8x, $p=0.037$), glutathione peroxidases like GPX2 (2.1x, $p=0.006$), and other antioxidant enzymes like NAD(P)H Quinone Dehydrogenase 1 (NQO1, 2.3x, $p<0.001$). The effects of UbD on metalloproteinases were also observed in the RHE model, with an overexpression of the genes MMP1 (3.4x, $p=0.014$) and MMP9 (1.7x, $p=0.042$).

DISCUSSION

The effects of UbD on the RHE inflammatory response ***(Figure 3)*** and barrier function integrity ***(Figures 4, 5)*** corroborate the observations from populations and animal studies [8, 18-19] and are therefore directly relevant for assessing the possible benefits of protective compounds.

When looking at the other changes in gene expression induced by urban dust, our first observation is that, except for the genes involved in terminal differentiation and barrier function, most gene expression modulated by UbD in RHE tissues are similarly modified in NHEK cells ***(Table II)***. Primary keratinocytes exposed to urban dust present a strong overexpression of these genes. This can be understood as a result of cell differentiation stimulated by the pollutants. In contrast, the expression of these genes, which is already high in a healthy differentiated RHE, is decreased after exposure of such tissue models to UbD. These observations lead us to hypothesize that atmospheric pollution could have a double negative effect on differentiation of the epidermis. On one hand, it would decrease the regeneration capacity of the basal layer, while on the other hand it would disturb the normal differentiation of the upper layers of the epidermis, with detrimental consequences on the barrier function as observed in ***Figures 4*** and ***5***.

In this study we used two routes of exposure to urban dust. NHEK cells were exposed through the culture medium, while we tested both systemic and topical application on RHE. When using a systemic treatment, we consequently focused the

study on the effects of soluble compounds present in UbD. Different hypotheses have been proposed regarding the initiation of skin damages by particulate matter [12]. Smaller particles may enter skin either through hair follicles or transepidermally. Ambient pollutants also carry metal ions and organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), which can penetrate the skin surface. Particulate matter induces detrimental effects probably through both penetration into the skin and indirect effects via an outside-inside signaling cascade [12].

Another important response of the epidermis to UbD is activation of the AhR pathway required for biotransformation of pollutants, such as PAHs, through the different phases of detoxification. Phase I introduces chemically reactive groups into xenobiotics in order for these compounds to be conjugated to polar compounds in phase II reactions. Finally, in a third phase the conjugated xenobiotics are further processed to facilitate their excretion out of the cell. We observed that UbD induces expression of many genes of the first phase but not so many of the second phase. It should be noted that the first phase enzymes of the detoxification process can produce intermediate metabolites even more reactive than their exogenous precursors [17] and it is therefore of primary importance for the cell to achieve complete transformation and detoxification of such metabolites through the expression of genes coding for phase II enzymes. It is also important to note the fact that phase II gene expression is dependent on the oxidative stress sensor Nrf2, a transcription factor interacting closely with the AhR pathway to protect the cell against oxidative stress [17] triggered by pollutants.

The equilibrium between these different pathways is very important in order to eliminate toxic compounds while keeping the oxidative status of the cell at a safe level. In our study we observed that urban dust at subtoxic concentrations was not sufficient to significantly increase the level of reactive oxygen species in RHE, at least in a steady manner. This could mean that the endogenous detoxification and antioxidant response of the epidermis is sufficient to protect the skin under moderate exposure to pollutants. However, the oxidative stress induced by UVA was significantly amplified when RHE was exposed to UbD ***(Figure 2)***, revealing that endogenous cell protection systems are readily overwhelmed by other oxidative stresses when they have to cope with pollutants. Disruption of these endogenous mechanisms leads to excessive levels of reactive oxygen species and molecular damage, activating other stress sensors such as the NFkB transcription factor, which together with AhR is responsible for triggering the inflammatory response and expression of metalloproteinases [20]. Such inflammation is indeed observed in RHE exposed to UbD, both at the protein level with the release of interleukins into the culture medium ***(Figure 3)*** and also at the gene level, for example, with induced expression of IL1B (2.1x, p=0.046, ***Table II***).

Table III UbD-Induced Gene Expression of Genes from the Epidermal Differentiation Complex*

Epidermal Differentiation Complex (29 out of 63 genes, p<0.001)		
$log_{10}p$	FC	Gene name
3.29	19.85	LCE2C
2.73	16.92	LCE2B
3.33	12.98	C1orf68
3.5	11.25	LCE2D
3.25	9.84	LCE1F
3.07	9.04	SPRR2G
3.39	9.01	FLG2
2.77	8.54	LOR
4.21	8.23	LCE1C
3.44	7.99	LCE3D
3.65	7.22	LCE6A
3.77	7.13	LCE3E
3.85	7.09	SPRR2B
3.73	6.55	LCE1A
4.2	5.96	KPRP
3.22	5.19	FLG
2.71	5.07	SPRR2E
3.66	4.62	SPRR4
3.14	4.03	LCE2A
4.71	3.55	S100A8
3.5	3.41	SPRR1B
3.73	3.05	S100A9
2.63	2.99	S100A7
4.23	2.89	SPRR1A
4.05	2.84	CRCT1
4.96	2.8	IVL
2.43	2.28	LCE1B
2.39	2.14	SPRR2A
2.25	1.53	S100A12
2.57	1.45	SPRR3
2.06	1.43	FLG-AS1
1.46	1.41	PGLYRP4

* According to over-representation analysis using the hypergeometric test method the most significant group of genes with 29 out of 63 genes significantly modulated (p<0.001) by UbD).

In view of the data obtained, it is important to remember the fact that AhR has pleiotropic functions and that specific interactions with other clearance and protective responses can greatly impact the effects of pollutants on skin conditions. While this transcription factor has been well studied for its role in drug metabolism and detoxification, it is also known to modulate immune cell maturation, inflammation, matrix remodeling, and keratinocyte differentiation [20-21]. This last function was clearly identified in our gene expression study on NHEK, with a drastic overexpression of many genes involved in epidermis maturation and barrier function after exposure to urban dust (***Table III*** and ***Figure 7***). Since termination of our study, recent observations by Kim and colleagues [22] have strengthened our conclusions regarding skin barrier defects, as they also performed a transcriptome investigation of human normal epidermal keratinocytes exposed to fine particles, and gene ontology enrichment analysis showed that the most significantly enriched gene ontology terms were associated with epidermis-related biological processes, such as »epidermis development (GO: 0008544)« and »keratinocyte differentiation (GO: 0030216)«.

Interestingly, endogenous protective responses from skin cells appear to be readily activated in the presence on pollutants but are then easily overwhelmed under worse conditions. Candidate protective compounds could therefore act at different levels, leading to different observations of the biological responses in the presence of pollutants. On one hand, a hypothetical compound could act as a shield or as a scavenger to decrease the endogenous stress. As a result, the expression of biomarkers related to the endogenous protective responses would be expected to decrease. On the other hand, it could activate these endogenous mechanisms without harmful side effects, leading to higher expression of the associated biomarkers. In line with this second strategy, an active that could increase the level of phase II enzymes would help to keep the oxidative stress under control, as explained above [19].

CONCLUSION

Recent studies showing detrimental effects of air pollution on skin health have raised the interest of scientists in a better characterization of the mechanisms involved in order to enable them to develop and market better protective strategies. Using a reconstructed human epidermis model and a standardized urban particulate matter, we could demonstrate that such urban pollutants activate multiple stress-related pathways and impact the skin barrier function *in vitro*. We validated multiple biomarkers directly associated with skin response to pollution, including detoxification and oxidative stress, inflammation, and impaired barrier function, emphasizing the fact that skin response to pollution entails a cross-talk between several signaling pathways that should be studied together to assess the effects of potential protective compounds. In support of functional studies, or as a screening tool, our work also led us to design a TaqMan Low Density qPCR Array to study simultaneously 96 target genes of these multiple pathways. Overall, our data support the efficiency of *in vitro* assays based on a reconstructed human epidermis model to assess the antipollution and detoxification potency of investigational compounds.

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