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Original Article

Proteome-wide changes in primary skin keratinocytes exposed to diesel particulate extract—A role for antioxidants in skin health

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ABSTRACT

Background: Skin acts as a protective barrier against direct contact with pollutants but inhalation and systemic exposure have indirect effect on keratinocytes. Exposure to diesel exhaust has been linked to increased oxidative stress.
Objective: To investigate global proteomic alterations in diesel particulate extract (DPE)/its vapor exposed skin keratinocytes.
Methods: We employed Tandem Mass Tag (TMT)-based proteomics to study effect of DPE/DPE vapor on primary skin keratinocytes.
Results: We observed an increased expression of oxidative stress response protein NRF2, upon chronic exposure of primary keratinocytes to DPE/its vapor which includes volatile components such as polycyclic aromatic hydrocarbons (PAHs). Mass spectrometry-based quantitative proteomics led to identification 4490 proteins of which 201 and 374 proteins were significantly dysregulated ($\geq$1.5 fold, $p \leq 0.05$) in each condition, respectively. Proteins involved in cellular processes such as cornification (cornifin A), wound healing (antileukoproteinase) and differentiation (suprabasin) were significantly downregulated in primary keratinocytes exposed to DPE/DPE vapor. These results were corroborated in 3D skin models chronically exposed to DPE/DPE vapor. Bioinformatics analyses indicate that DPE and its vapor affect distinct molecular processes in skin keratinocytes. Components of mitochondrial oxidative phosphorylation machinery were seen to be exclusively overexpressed upon chronic DPE vapor exposure. In addition, treatment with an antioxidant like vitamin E partially restores expression of proteins altered upon exposure to DPE/DPE vapor.

Abbreviations: DPE, Diesel particulate extract; PAHs, Polycyclic aromatic hydrocarbons; MMPs, Matrix metalloproteinases; ROS, Reactive oxygen species; ARE, Antioxidant Response Element; NHEK-Ad, Adult normal human epidermal keratinocytes; ECM, extracellular matrix membrane; HPRD, Human Protein Reference Database; TEABC, Triethyl ammonium bicarbonate; BCA, Bicinchoninic acid; TMT, Tandem Mass Tag; NADPH, Nicotinamide adenine dinucleotide phosphate; HCD, High energy Collision induced Dissociation; AGC, Automatic Gain Control.

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Conclusions: Our study highlights distinct adverse effects of chronic exposure to DPE/DPE vapor on skin keratinocytes and the potential role of vitamin E in alleviating adverse effects of environmental pollution.

1. Introduction

Air pollution is one of the major environmental risk factors known to adversely affect human health. Outdoor air pollution has been classified as carcinogenic to humans in a 2013 assessment report by the International Agency for Research on Cancer (IARC) [1]. The most common sources of outdoor air pollution include industrial and automobile emissions. Emissions from fuel combustion that include carbon monoxide, nitrogen oxides, ozone, sulphur dioxide and particulate matter (PM), are known to cause various lung ailments and cardiomyopathies [2,3]. Outdoor air pollution has also been linked to increasing incidence of cancers such as lung and breast cancer [4,5]. In addition, a growing body of literature highlights the adverse effects of air pollution on skin [6,7].

Diesel emissions are comprised of a heterogeneous mixture of solid and vapor phase components, that includes carbonaceous particulate matter of varying sizes, aliphatic hydrocarbons, polyaromatic hydrocarbons (PAHs) and their derivatives and gases such as carbon monoxide, sulphur and nitrous oxides [8]. Particulate matter in ambient air is one of the major by-products of diesel exhaust emissions and contributes to a number of ill-effects on human health [9]. Diesel exhaust consists of fine particles that range from 2 to 10 μm in size and particulate matter with an average diameter <10 μm (PM_{10}) has a greater likelihood of entering circulation and cause adverse oxidative and inflammatory effects [10]. A major mechanism by which diesel exhaust particles exert their detrimental effects is through the generation of oxidative stress [11] which is an important contributor to extrinsic skin aging [12,13]. Diesel exhaust is known to induce overexpression of genes for phase I [cytochrome P-450 1A1 (CYP1A1)] and phase II [NADPH quinone oxidoreductase-1 (NQO-1)] xenobiotics metabolizing enzymes in human airway epithelial cells [14]. One study also implicates the role of environmental pollutants such as diesel particulate extract in inducing the expression of inflammatory cytokines such as IL-8 and IL-1ß in normal human keratinocytes [15].

Apart from particulate matter, diesel exhaust also comprises of a mixture of volatile components such as aldehydes (formaldehyde, acetaldehyde and acrolein), benzene, 1,3-butadiene, PAHs and nitro-PAHs which can act as irritants in humans [16]. More than 300 putative and 40 established PAHs are known to be adsorbed on PM surface and account for majority of the volatile components in diesel exhaust emissions [17,18]. A number of studies have linked cellular metabolism of PAHs and ROS production to cytotoxicity in *in vitro* and *in vivo* systems. ROS-mediated oxidative DNA damage, DNA adduct formation and their associated cellular toxicity has been attributed not only to exposure to PM but also to surface adsorbed PAHs and nitro-PAHs [19,20]. Studies have shown that PAHs from diesel exhaust and cigarette smoke can trigger inflammatory responses in the respiratory tract [21,22]. In addition, the role of PAHs in contact hypersensitivity or dermatitis in skin has also been well-studied [23,24]. PAHs such as benzopyrene (BP) are known to induce oxidative stress related damage in skin through aryl hydrocarbon (AHR)-related pathway [25]. A recent study also highlights the toxic effect of PAHs in synergy with UVA1 by impairment of cellular homeostasis of skin keratinocytes [26]. Although there are some studies investigating the effects of vapor phase of diesel exhaust, they focus mainly on PAH components, and an unbiased study of total vapor phase is lacking. It is intuitive to expect that PM and volatile PAHs may exert distinct effects on cellular systems. To the best of our knowledge, there are no high throughput studies investigating the adverse effects of chronic exposure to DPE and its vapor in skin.

We therefore aimed to elucidate the effect of chronic diesel exhaust exposure on primary skin keratinocytes. We employed a quantitative proteomics approach to understand the molecular alterations brought about in primary human skin keratinocytes upon exposure to diesel particulate extract (DPE) or its vapor. Mass spectrometry-based proteomic analysis of primary skin cells exposed to DPE or its vapor led to quantitation of 4490 proteins. Of these, 201 and 374 proteins were found to be dysregulated by $\geq$1.5 fold (p-value ≤ 0.05) in DPE and DPE vapor phase exposed skin keratinocytes, respectively. Our study indicates that DPE and its vapor results in significantly altered expression of several proteins reported to be involved in maintenance of skin epithelial integrity, regulation of skin hydration and oxidative stress. Vitamin E is a well-studied antioxidant with known beneficial roles in skin [27]. We observed that treatment with vitamin E alleviated the adverse effects of chronic exposure to environmental pollution.

2. Materials and methods

2.1. Keratinocyte culture

Adult normal human epidermal keratinocytes (NHEK-Ad) from a single Hispanic non-smoker donor were purchased from Lonza (Walkersville, MD, USA (Catalog #00192627)). NHEK-Ad cells were cultured in KGM-Gold™ BulletKit™ (Lonza, Basel, Switzerland) supplemented with bovine pituitary extract, human epidermal growth factor, bovine insulin, hydrocortisone, gentamicin, amphotericin-B, epinephrine and transferrin. The cells were cultured in a 37 °C humidified air incubator with 5% CO_2.

2.2. Adapting skin keratinocytes to diesel particulate extract

Diesel particulate extract (DPE) was purchased from National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). NHEK-Ad cells were chronically treated with 0.05% (v/v) DPE in medium in a DPE exposure dedicated incubator. DPE concentration of 0.05% was selected based on cell cytotoxicity assays with varying concentrations of DPE (data not shown). Cells maintained in the DPE dedicated incubator and not directly treated with DPE were considered as DPE vapor exposed cells. Cells were chronically exposed to DPE or its vapor for 20 days. NHEK-Ad cells cultured in a regular incubator without DPE exposure were considered as control. Hereafter, unexposed parental cells will be referred to as NHEK-Ad cells, cells exposed directly to 0.05% DPE will be referred to as NHEK-Ad-DPE and cells exposed to the vapor effect of DPE will be referred to as NHEK-Ad-DPE-V. Parental cells and DPE and DPE vapor exposed keratinocytes treated with vitamin E (Sigma Aldrich, St. Louis, MO) (α-tocopherol $\geq$95.5%, 9 IU/ml for 72 h) will be referred to as NHEK-Ad-Vit-E and NHEK-Ad-DPE-Vit-E and NHEK-Ad-DPE-V-Vit-E, respectively.

2.3. Western blot analysis

Parental cells and cells exposed to DPE or its vapor were washed thrice with ice-cold 1X phosphate buffered saline (PBS) and harvested using RIPA lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton-X-100, 0.1% SDS containing protease and phosphatase inhibitor cocktails). Protein concentration was determined using BCA assay [28]. Thirty micrograms equivalent protein from each exposure condition was resolved using SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in PBS-T and incubated overnight with anti-NRF2 antibody (Santa Cruz Biotechnology, Dallas, TX, USA). HRP conjugated anti-rabbit IgG were used as secondary antibody. β-actin was used as loading control.

Western blot analysis for 3D skin models is provided in Supplementary Methods.

2.4. Sample preparation for mass spectrometry analysis

NHEK-Ad, NHEK-Ad-DPE and NHEK-Ad-DPE-V cells with and without vitamin E treatment were grown to 80% confluence and serum starved for 8 h. Following serum starvation, cells were washed thrice with ice-cold phosphate buffered saline (pH 7.2) and harvested in lysis buffer (2% SDS, 5 mM sodium fluoride, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate in 50 mM Triethylammonium bicarbonate (TEABC)). The cell lysates were sonicated, centrifuged and protein concentration was determined using BCA assay [28]. In-solution trypsin digestion of samples was carried out as described previously (details in Supplementary Methods) [29].

2.5. Tandem Mass Tag (TMT) labeling

Following trypsin digestion, the peptides were vacuum-dried and labeled with TMT reagents as per manufacturers' instructions. Peptide samples were dissolved in 50 mM TEABC (pH 8.0) and added to TMT reagents dissolved in anhydrous acetonitrile. Peptides from NHEK-Ad parental, NHEK-Ad-Vit-E, NHEK-Ad-DPE-V, NHEK-Ad-DPE-V-Vit-E, NHEK-Ad-DPE, and NHEK-Ad-DPE-Vit-E were labeled with TMT tags 126, 127N, 129C, 130N, 130C, 131, respectively. After incubation at room temperature for 1 h, the reaction was quenched with 5% hydroxylamine. The labeled samples from all conditions were pooled and subjected to fractionation and analyzed on Orbitrap Fusion Tribrid mass spectrometer (Thermo Electron, Bremen, Germany) as detailed in **Supplementary Methods**. Data from LC/MS analysis was searched using Proteome Discoverer (Version 2.1) software suite (Thermo Fisher Scientific, Bremen, Germany) (details of data analysis is provided in **Supplementary Methods**).

2.6. Data availability

Mass spectrometric data generated in this study was submitted to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [30] with the dataset identifier PXD006743.

2.7. Bioinformatics analysis

Information on the subcellular localization of differentially expressed proteins and the biological processes they are involved in was obtained from Human Protein Reference Database (HPRD; http://www.hprd.org) [31]. Statistical analyses were performed and volcano plots were generated using R program (version 3.2.5). P-value for proteins was calculated based on raw reporter ion intensities of each peptide spectral match (PSM) using two tail paired *t*-test. ClustVis, a web-based tool, was employed for Principal Component Analysis (http://www.biit.cs.ut.ee/clustvis/) [32]. Heatmap was generated using Morpheus (version 3.0.206) software (https://software.broadinstitute.org/morpheus/). Circos plots were created on Circos (http://www.circos.ca/) [33]. Network analysis was carried out using STRING (*version 10)* [34] to identify modulation of biological networks upon DPE exposure. Analysis was performed with a list of proteins dysregulated in response to DPE by a fold change of $\geq$1.5 and p-value $\leq$ 0.05. Interaction networks were generated for *Homo sapiens*.

3. Results and discussion

We developed a cellular model to investigate the effects of chronic exposure to diesel particulate extract and its vapor upon primary human skin keratinocytes. Diesel exhaust and its constituents such as PAHs and their derivatives are known to affect cellular signaling and redox balance by increasing ROS levels. Diesel exhaust particles have been shown to produce reactive oxygen species that lead to oxidative injury [35] and increased production or accumulation of reactive oxygen species (ROS) results in tissue damage. In addition, increased levels of ROS have been implicated in various cellular processes including aging and skin damage [36].

3.1. Chronic exposure to DPE and its vapor phase induces nuclear factor, erythroid 2-like 2 (NRF2) expression

To counteract the toxic effects of ROS, a number of antioxidant proteins and certain detoxifying enzymes such as NADPH quinone oxidoreductase (NQO1), gamma-glutamylcysteine synthetase (gGCS) and heme oxygenase-1 (HO-1) are activated by transcription factors through antioxidant response elements (ARE) [37–39]. Nuclear factor, erythroid 2-like 2 (NFE2L2 or NRF2) is a transcription factor which is responsible for both constitutive and inducible expression of ARE-regulated genes [40]. In order to investigate the oxidative changes upon chronic exposure to DPE, NRF2 expression was studied in skin keratinocytes exposed to DPE and its vapor phase. Western blot analysis showed relative increase in NRF2 expression in NHEK-Ad-DPE-V and NHEK-Ad-DPE cells compared to NHEK-Ad cells (Fig. 1**a**).

Based on our observations and prior literature evidence, we hypothesize that exposure to DPE or its vapor could result in global proteomic alterations in skin keratinocytes. We therefore went on to investigate proteome-wide changes in NHEK-Ad cells chronically exposed to DPE or its vapor phase.

3.2. Exposure to DPE and its vapor induces widespread proteomic alterations in skin keratinocytes

A TMT-based quantitative proteomic approach was used to investigate proteomic changes in skin keratinocytes chronically exposed to DPE or its vapor phase. The experimental framework is depicted in **Supplementary** Fig. 1. A total of 4490 proteins were quantified across all replicates, of which 374 proteins and 201 proteins were found to be dysregulated by $\geq$1.5 fold ($p \leq 0.05$) in NHEK-Ad-DPE-V and NHEK-Ad-DPE cells, respectively (Fig. 1**b; Supplementary** Table 1). The complete list of peptides identified in the study is provided in **Supplementary** Table 2.

A partial list of proteins dysregulated in NHEK-Ad-DPE and NHEK-Ad-DPE-V compared to NHEK-Ad cells is provided in Table 1. Amongst the dysregulated proteins were cornifin A (SPRR1A), suprabsin isoform 1 (SBSN) and antileukoproteinase (SLPI) in cells exposed to DPE and its vapor. Cornifin A belongs to the SPRR family of proteins and is a structural protein involved in the process of cornification [41]. Suprabasin (SBSN) has been reported to have a potential role in the process of epidermal differentiation based on

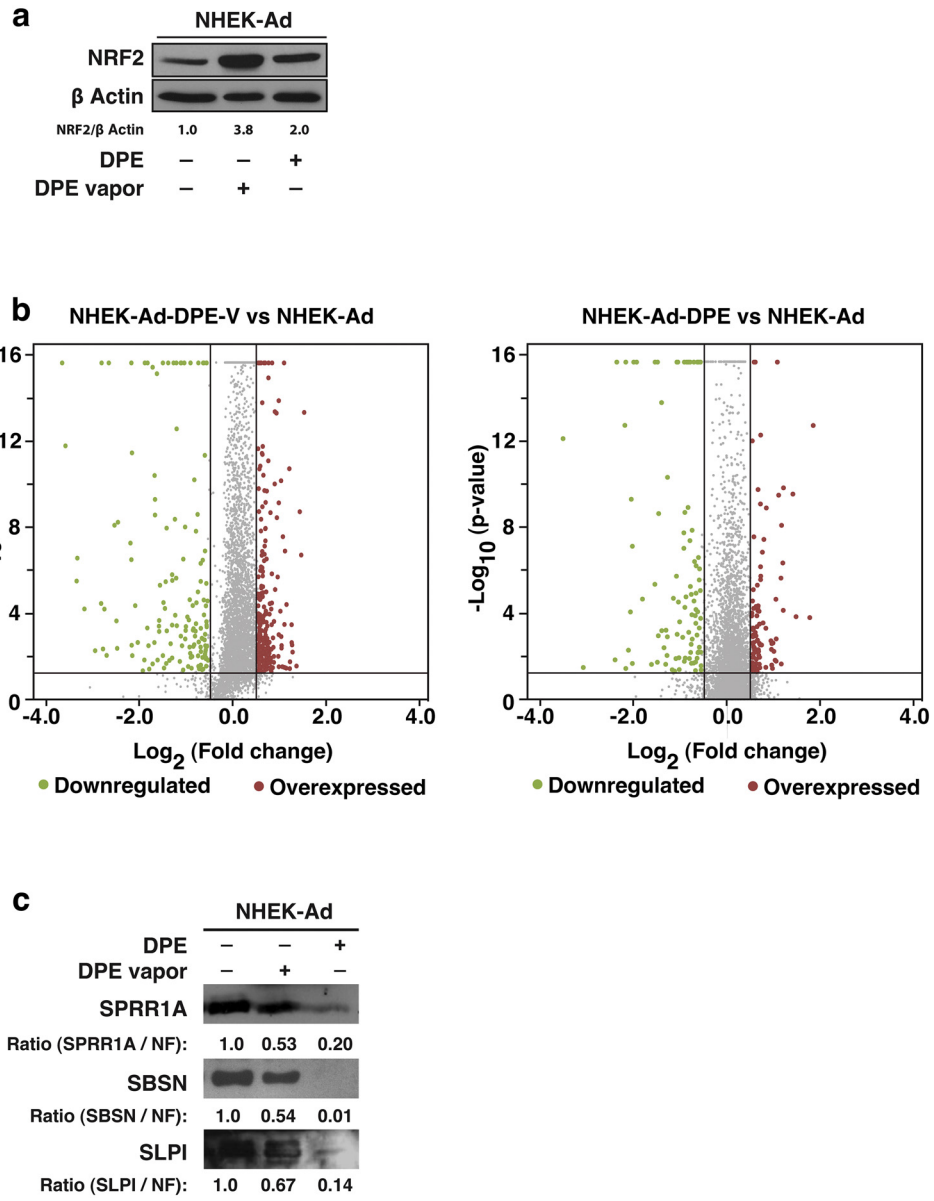

Fig. 1. DPE and DPE vapor induce molecular alterations in primary skin keratinocytes. (a) Expression of Nuclear factor, erythroid 2-like 2 (NFE2L2 or NRF2) in NHEK-Ad, NHEK-Ad-DPE-V and NHEK-Ad-DPE cells. β-actin was used as loading control. Volcano plots of log2 transformed fold change (x-axis) plotted against −log2 p-values (y-axis) for **(b)** primary skin keratinocytes exposed to diesel particulate extract vapor (NHEK-Ad-DPE-V) and primary skin keratinocytes exposed to diesel particulate extract (NHEK-Ad-DPE). Significantly ($p \leq 0.05$) overexpressed and downregulated proteins are depicted in red and green, respectively **(c)** Western blot analysis of Cornifin A (SPRR1A), Suprabasin (SBSN), Antileukoproteinase (SLPI) in RHE™ EpiSkin™ cells exposed to DPE or its vapor compared to unexposed cells. Normalization factor (NF) was determined using total protein amounts per lane.

Table 1
Partial list of proteins dysregulated in DPE and DPE vapor exposed skin cells.

Gene Symbol	Protein Name	[NHEK-Ad-DPE-V/NHEK-Ad]		[NHEK-Ad-DPE/NHEK-Ad]		Biological processes
		Median Fold change	p-value	Median Fold change	p-value	
MCM6	DNA replication licensing factor MCM6	2.2	1.2E-07	1.5	1.1E-03	DNA replication
CPT1A	carnitine O-palmitoyltransferase 1, liver isoform isoform 1	1.8	1.3E-03	2.1	1.6E-02	Palmitoyl CoA metabolism
UQCR10	cytochrome b-c1 complex subunit 9 isoform a	1.7	2.1E-03	2.0	2.3E-03	Electron transport chain
EVPL	envoplakin	0.5	2.2E-16	0.6	2.2E-16	Stratum corneum formation
SBSN	suprabasin isoform 1	0.1	2.2E-16	0.3	2.2E-16	Epidermal differentiation
FLG	filaggrin	0.1	1.6E-12	0.2	1.9E-13	Stratum corneum formation
SCEL	sciellin isoform 1	0.3	3.7E-11	0.5	2.1E-09	Stratum corneum formation
CASP14	caspase-14	0.4	5.6E-05	0.4	6.3E-04	Stratum corneum formation
SPRR1A	cornifin-A	0.1	5.2E-03	0.4	8.9E-03	Stratum corneum formation

expression studies performed on mouse epidermis [42,43]. Anti-leukoproteinase or secretory leukocyte peptidase inhibitor (SLPI) is a secreted inhibitor which protects epithelial tissues from serine proteases. Previous studies report a positive correlation between SLPI expression and increased cutaneous wound healing [44,45].

3D skin models such as reconstructed human epidermis EpiSkin (RHE™ EpiSkin™) consists of epidermal cells in different stages of development and are structurally and functionally similar to *in vivo* human epidermis. 3D skin models reflect the complexity of actual human skin more closely. In order to investigate whether DPE and its vapor have a comparable effect on 3D skin models, we cultured RHE™ EpiSkin™ cells in similar exposure conditions as NHEK-Ad cells. Western blot analysis of DPE and DPE vapor exposed 3D skin cell lysates revealed recapitulation of our observed 2D skin proteomics data (Fig. 1**c, Supplementary** Fig. 2**a**).

Normal keratinocytes were grown *in vitro* in culture medium that supports proliferation. However, exposure to DPE and DPE vapor resulted in the dysregulation of a number of proteins involved in cellular processes observed in differentiating keratinocytes. We employed bioinformatics analyses to elucidate the biological processes that may be affected due to chronic exposure to DPE or its vapor in skin keratinocytes.

3.3. Exposure to DPE and its vapor affects proteins related to skin barrier function and skin integrity

Multiple proteins known to play an essential role in skin biology were seen to be dysregulated in both NHEK-Ad-DPE-V and NHEK-Ad-DPE cells (Fig. 2**a, b**). We observed downregulation of members of the plakin family including periplakin (PPL), envoplakin (EVPL), involucrin (IVL) and epiplakin (EPPK1) in both DPE exposure models. The plakin family of proteins is required for the formation of cornified epithelium in skin keratinocytes and for maintenance of integrity of the epithelial barrier [46,47]. Enzymes such as transglutaminase 1 and 3 (TGM1 and TGM3) that play an essential role in cross-linking proteins including IVL and loricrin in the cornified epithelium [48] were also downregulated by ≥1.5 fold ($p \leq 0.05$). Proteins such as cystatin A (CSTA), cystatin B (CSTB), sciellin (SCEL), cornifin-A (SPRR1A), cornifin-B (SPRR1B) and small proline-rich protein 2D (SPRR2D) known to be involved in maintenance of epithelial barrier integrity [41] were significantly downregulated in both DPE exposure models. In addition, filaggrin (FLG), a protein with a well-established role in skin hydration [49] was seen to be down-regulated in skin keratinocytes exposed to both DPE and DPE vapor.

A marked downregulation in the expression of both type I and II keratin family of proteins was observed in response to both insults. Type II cytoskeletal keratin 80 (KRT80) which is involved in cell differentiation and required to maintain the structural integrity of epithelial cells was downregulated by 0.2 fold in NHEK-Ad-DPE-V and 0.5 fold in NHEK-Ad-DPE cells. Type II cytoskeletal keratin 1 (KRT1) and type I cytoskeletal keratin 10 (KRT10) pair are integral to the process of keratinization and imparts mechanical integrity to the cells and the whole epidermis [50,51]. The KRT1-KRT10 pair was downregulated in both DPE exposure models.

Processes involved in maintenance of skin integrity and cornification are known to be adversely affected upon exposure to air pollution [6,52]. Changes in expression of proteins known to be involved in maintaining skin architecture and transepidermal water loss may be attributed to adverse effects of DPE and DPE vapor exposure.

3.4. DPE vapor affects distinct cellular processes in skin keratinocytes grown in vitro

Vapor phase of DPE is composed of multiple volatile organic constituents including aldehydes, alkenes, nitro compounds,

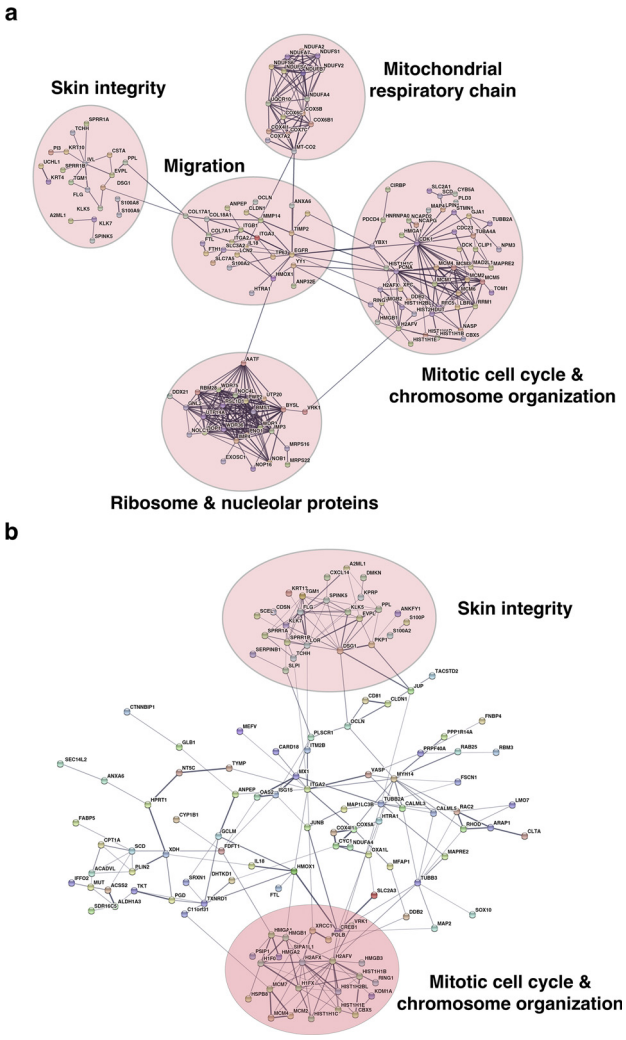

Fig. 2. Protein network analysis of dysregulated proteins. STRING v10 was employed to identify dysregulated networks in skin keratinocytes exposed to **(a)** Diesel particulate extract vapor (DPE-V) and **(b)** diesel particulate extract (DPE).

polyaromatic hydrocarbons (PAHs) and their derivatives [53,54]. A number of these constituents have been studied for their adverse effects on human skin. DPE vapor, especially PAHs and their derivatives, are known to induce extrinsic skin aging through mitochondrial ROS production and subsequent mitochondrial damage [55]. Protein network analysis revealed that NHEK-Ad-DPE-V cells displayed unique interactive clusters of proteins involved in mitochondrial oxidative phosphorylation (Fig. 2**a**).

3.4.1. Dysregulation of mitochondrial oxidative phosphorylation proteins upon exposure to DPE vapor

Protein-protein interaction analysis resulted in the identification of protein components of mitochondrial complex I such as NADH:ubiquinone oxidoreductase subunit A7 (NDUFA7), NDUFA4, NDUFV2, NDUFS4, NDUFA2, NDUFS6, NDUFB7, NDUFS1, that were overexpressed by ≥1.5 fold (p- ≤0.05). In addition, components of mitochondrial complex IV including cytochrome C oxidase subunit 7C (COX7C), COX5B, COX6C, COX2, COX7A2, COX4I1, COX6B1 and ubiquinol-cytochrome C reductase, complex III subunit X (UQCR10)- a component of complex III – were also overexpressed (fold change ≥1.5, $p \leq 0.05$) in DPE vapor exposed cells (Fig. 2**b**). Role of mitochondrial electron transport chain complex (ETC) in generation of reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl free radical ($^{\bullet}OH$)

has been widely documented [56,57]. Mitochondrial complex I forms the major entry-point for electrons into oxidative phosphorylation and increased activity of complex I and III leads to increased ROS production. In a variety of pathological conditions such as aging and Parkinson's disease, the primary source of ROS is reported to be Complex I [58,59]. It has also been shown that H_2S-mediated inhibition of complex IV leads to reduced ROS production in cardiomyocytes [60]. Overall, analysis of proteomic data reveals dysregulation of mitochondrial proteins involved in oxidative phosphorylation. This could cause increase in intracellular ROS which may be detrimental to skin.

3.4.2. DPE vapor exposure affects proteins that regulate cell migration

Several classes of proteins involved in maintenance of cell–cell and cell-matrix interactions are known to play an important role in cellular motility. Integrins are a class of heterodimeric, transmembrane receptors that are involved in keratinocyte migration and wound healing [61]. Integrin α2β1 pair has been shown to be necessary for migration of keratinocytes [62]. Overexpression of integrin α2β1 has also been documented at the migrating front during wound closure and in newly forming epidermis during wound healing [63,64]. A significant overexpression of integrin beta 1 (ITGB1), integrin alpha-2 (ITGA2) and integrin alpha-3 (ITGA3) was seen in NHEK-Ad-DPE-V cells. Apart from integrins, we also observed an overexpression of ECM proteins such as collagen alpha-1(VII) (COL7A1), collagen alpha-1(XVII) (COL17A1) and collagen alpha-1(XVIII) (COL18A1) in DPE vapor exposed cells. COL17A1 is known to participate in keratinocyte adhesion and p38MAPK-dependent migration. COL17A1 knockdown has also been implicated in reduced motility of keratinocytes [64].

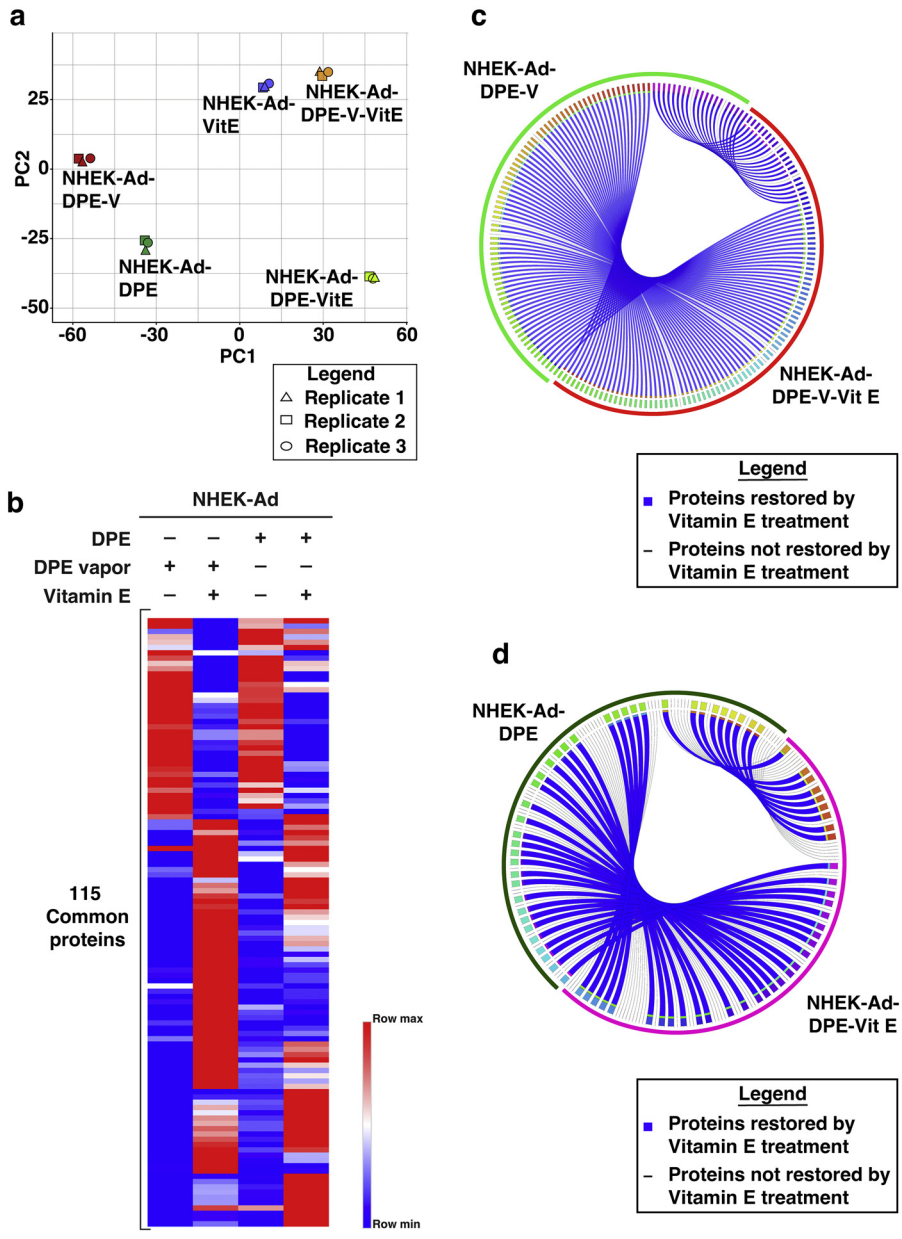

Fig. 3. Vitamin E partially restores molecular alterations induced by DPE and its vapor in primary skin keratinocytes (a) Principal component analysis (PCA) of NHEK-Ad-Vit E, NHEK-Ad-DPE-V, NHEK-Ad-DPE-V-Vit E, NHEK-Ad-DPE and NHEK-Ad-DPE-Vit E **(b)** Heat map depicting expression changes for proteins commonly dysregulated (1.5 fold; p-value ≤ 0.05) between NHEK-Ad-DPE-V and NHEK-Ad-DPE (n = 115). Circos plots depicting **(c)** 100 arbitrarily selected proteins out of 257 proteins uniquely dysregulated in NHEK-Ad-DPE-V cells and the pattern of restoration upon vitamin E treatment **(d)** 85 proteins uniquely dysregulated in NHEK-Ad-DPE cells and the pattern of restoration upon vitamin E treatment.

Matrix metalloproteases (MMPs) are endopeptidases involved in the turnover and degradation of ECM proteins [65]. In our proteomics data, we observed a significant overexpression of matrix metalloproteinase-14 (MMP14) and downregulation of tissue inhibitor of metalloproteinases (TIMP2) in NHEK-Ad-DPE-V cells. MMP14-mediated activation of pro- matrix metalloproteinase 2 (MMP2) has been demonstrated as important in cellular migration and epithelial wound healing [66]. TIMP2 is known to specifically inhibit MMP14-mediated activation of MMP2 [67,68]. It is interesting to note that proteins involved in cellular migration seem to be largely overexpressed in DPE vapor exposed cells (Fig. 2**a**) which may be a cellular defense response to DPE vapor. However, this response was not observed in DPE exposed cells and may highlight a stronger effect of direct DPE exposure in skin keratinocytes.

3.5. Vitamin E partially restores molecular alterations induced by DPE and its vapor in primary skin keratinocytes

As depicted in Fig. 3**a**, NHEK-Ad-DPE-V and NHEK-Ad-DPE cells cluster independently which iterates the differences observed upon network analysis. This may be a consequence of cellular responses elicited by two insults which differ inherently in their composition. Since oxidative stress is a well-known phenomenon induced by environmental factors such as DPE or its vapor, we also studied the effects of an antioxidant (vitamin E) on skin keratinocytes exposed to DPE and its vapor. NHEK-Ad-DPE and NHEK-Ad-DPE-V cells were treated with 9 IU/ml vitamin E to investigate its restorative effects in response to both insults. Treatment of DPE and DPE vapor exposed cells with Vitamin E treatment resulted in decreased expression of NRF2 in both DPE

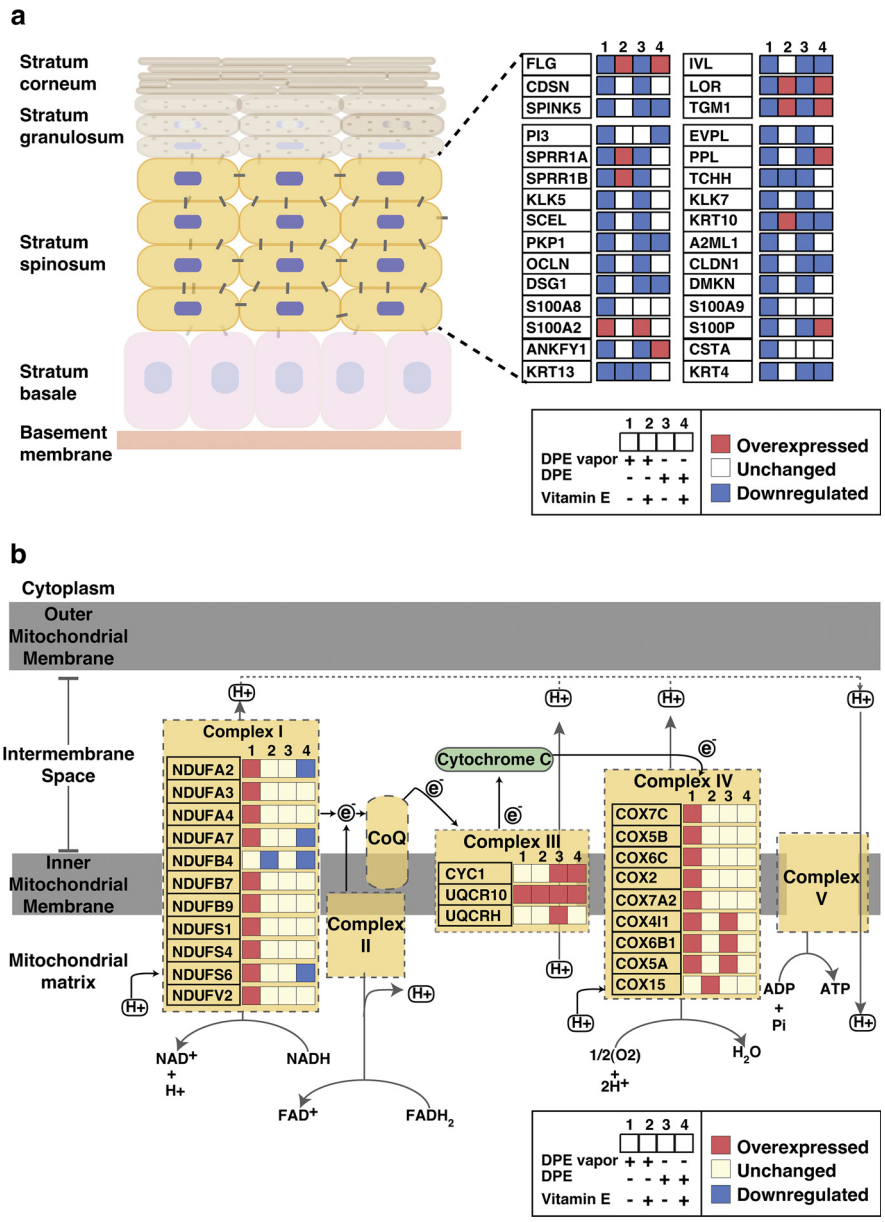

Fig. 4. Cellular processes affected in skin keratinocytes upon chronic exposure to DPE and its vapor. **(a)** Proteins involved in keratinocyte differentiation, stratum corneum formation and skin integrity were predominantly downregulated upon exposure to both DPE and its vapor. A large majority of these proteins were restored to baseline level upon treatment with vitamin E in both DPE and DPE vapor exposed cells **(b)** Proteins involved in mitochondrial oxidative phosphorylation were seen to be significantly overexpressed in DPE vapor exposed cells compared to DPE exposed cells. Treatment with Vitamin E restored these proteins back to basal levels in DPE vapor exposed cells.

vapor and DPE exposed cells (**Supplementary** Fig. 2**b**). DPE and DPE vapor exposed cells when treated with vitamin E also clustered distinctly highlighting its global effect on NHEK-Ad cells adversely affected by DPE and its vapor (Fig. 3**a**). Proteomic analysis revealed that vitamin E treatment restored ~97% of the 374 dysregulated proteins to basal levels in DPE vapor exposed cells as depicted in Fig. 3**b and c**. The restorative effect of vitamin E on proteins which were dysregulated both in DPE and its vapor exposed cells (n = 115) is depicted in Fig. 3**b.** In addition, proteins dysregulated uniquely in DPE vapor exposed cells and the effect of vitamin E on their expression is depicted in Fig. 3**c**. In contrast, only ~61% of the 201 dysregulated proteins in NHEK-Ad-DPE cells were restored to basal levels upon vitamin E treatment (Fig. 3**b, d**). Proteins dysregulated uniquely in DPE exposed cells and the effect of Vitamin E on their expression is depicted in Fig. 3**d**. Partial rescue by vitamin E in NHEK-Ad-DPE cells could indicate harsher effect of direct DPE exposure compared to vapor phase in skin keratinocytes.

The restorative effect of vitamin E on proteins involved in essential biological processes including maintenance of skin integrity, mitochondrial oxidative phosphorylation and cellular migration is discussed in ensuing sections.

As shown in Fig. 4**a**, vitamin E treatment in DPE and DPE vapor exposed cells is seen to restore the expression levels of proteins involved in formation of stratum corneum, desquamation, skin hydration and structural integrity in skin. This could possibly reflect its mitigating effects against DPE and DPE vapor exposure in primary skin keratinocytes.

In addition, Vitamin E treatment restored the expression levels of proteins belonging to both mitochondrial complex I and complex IV in DPE vapor exposed cells (Fig. 4**b**). This highlights the protective effect of antioxidants such as vitamin E in skin cells chronically affected by exposure to oxidants present in DPE vapor.

We also aimed to investigate the effect of vitamin E on the migratory ability of NHEK-Ad-DPE-V cells. As previously discussed, proteins with pro-migratory functions were seen to be overexpressed in DPE vapor exposed cells. Western blot analysis showed increased levels of activated MMP2 in DPE vapor exposed cells (**Supplementary** Fig. 3**a**). Dysregulation in expression of integrins, ECM proteins as well as the TIMP2/MMP14/MMP2 axis indicates an

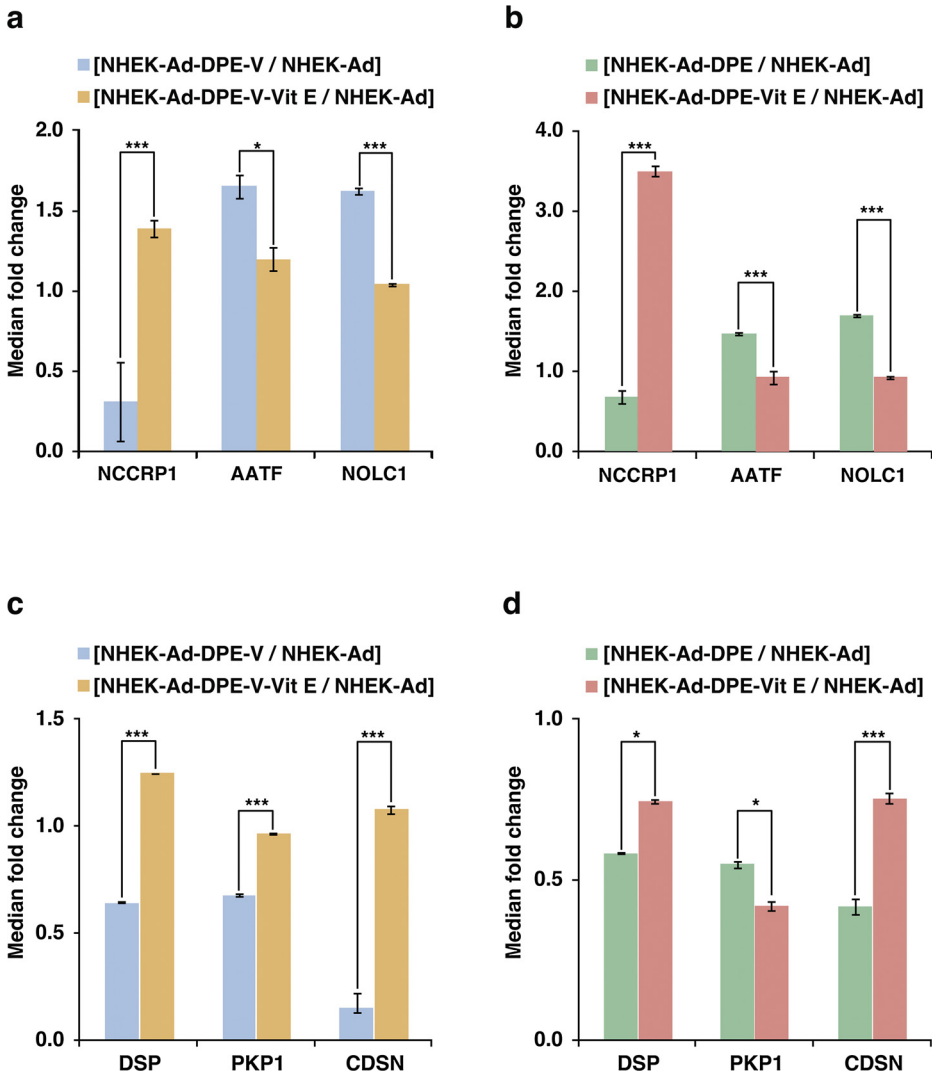

Fig. 5. Bar graphs depict expression levels of representative proteins novel to the study in **(a)** NHEK-Ad-DPE-V and NHEK-Ad-DPE-V-Vit E and **(b)** NHEK-Ad-DPE and NHEK-Ad-DPE-Vit E including non-specific cytotoxic cell receptor protein 1 homolog (NCCRP1), apoptosis antagonizing transcription factor (AATF) and nucleolar and coiled-body phosphoprotein 1 (NOLC1); proteins involved in maintenance of desmosomal integrity in **(c)** NHEK-Ad-DPE-V and NHEK-Ad-DPE-V-Vit E and **(d)** NHEK-Ad-DPE and NHEK-Ad-DPE-Vit E including desmoplakin (DSP), plakophilin 1 (PKP1) and corneodesmosin (CDSN). Vitamin E treatment restored all proteins back to basal levels in both DPE and DPE vapor exposed cells.

increase in migratory potential of NHEK-Ad-DPE-V cells. This is in conjunction with the wound healing assay results that indicate a 40% increase in migration of DPE vapor exposed cells compared to NHEK-Ad cells (**Supplementary** Fig. 3**b, c**). Vitamin E treatment restored these alterations in NHEK-Ad-DPE-V cells.

3.6. Identification of novel molecules dysregulated by DPE and its vapor in skin keratinocytes and restorative effect of vitamin E

Apart from proteins that are reported in literature to be altered due to external insults such as pollution, we identified a number of novel proteins dysregulated in DPE and DPE vapor exposed skin keratinocytes. Proteins such as nucleolar and coiled-body phosphoprotein 1 (NOLC1) and apoptosis antagonizing transcription factor (AATF) were overexpressed in NHEK-Ad-DPE-V and NHEK-Ad-DPE cells. In addition, we observed downregulation of proteins such as non-specific cytotoxic cell receptor protein 1 homolog (NCCRP1) in both NHEK-Ad-DPE-V as well as NHEK-Ad-DPE cells. Vitamin E treatment restored the levels of these proteins in both cells. Protein expression levels and restoration by vitamin E for these representative novel proteins is depicted in Fig. 5**a, b**. The biological consequence of these observations remains unclear and needs further in-depth investigation. A complete list of proteins dysregulated in response to DPE or its vapor and the restorative effect of Vitamin E on these proteins is provided in **Supplementary** Table 1.

3.7. Vitamin E restores expression of proteins involved in desmosomal integrity in DPE and DPE vapor exposed cells

Exposure to DPE or its vapor resulted in downregulation of several proteins related to desmosomal integrity such as desmoplakin (DSP), plakophilin-1 (PKP1) and corneodesmosin (CDSN). Mutation or loss-of-function in these desmosomal proteins have been implicated in several dermatological diseases [69–71]. Vitamin E treatment restores most of these proteins to levels similar to untreated cells in both DPE as well as DPE vapor exposed cells (Fig. 5**c, d**). It would be interesting to understand the biological significance of such proteins in skin biology in future studies.

Overall, our proteomic study highlights the differences in molecular alterations elicited by exposure to DPE and DPE vapor. Both direct and vapor forms of DPE were seen to alter expression levels of proteins involved in maintenance of skin integrity. These effects are recapitulated even in 3D skin models. Proteins related to mitotic cell cycle and chromosome organization were also significantly dysregulated in both DPE and DPE vapor exposed cells. In addition, DPE vapor distinctly affects biological processes such as mitochondrial oxidative phosphorylation and cellular migration. Treatment with a well-known antioxidant, vitamin E, resulted in partial restoration of altered proteins in DPE and DPE vapor exposed cells, underscoring its importance as a protective agent in response to pollution exposure.

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Author disclosure statement

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jdermsci.2018.05.003.

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