Key Points:
- Exposure to desert dust generates high levels of ROS, leading to mitochondrial damage
- Cellular adaptation to stress exerted by desert dust is through activation of Nrf2 transcription factor

Supporting Information:
- Supporting Information S1

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Mitochondria-mediated oxidative stress induced by desert dust in rat alveolar macrophages

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Abstract Exposure to ambient particulate matter (PM), including PM from resuspension of soils and dusts, increases the risk for respiratory diseases. However, the exact mechanism of PM-mediated damage to the lungs remains unclear. Due to recent increases in the frequency of dust storms in many areas, we examined the cytotoxic effects of soil-dust samples collected in an arid zone in Israel on rat lung macrophages. The desert soil contains soil crusts and low levels of toxic metal content. Exposure of cells to water extracts from the dust samples caused significant reduction in the concentration of live cells and overall cell viability. The dust samples induced cell death through apoptosis, mitochondrial dysfunction, and increased mitochondrial lipid peroxidation. The dust samples generated more reactive oxygen species (ROS) compared to control-treated samples and National Institute of Standards and Technology San Joaquin Valley standard reference material. To assess whether the oxidative imbalance induced by dust extract also interferes with the antioxidant defense, we evaluated phase II detoxifying genes, which are Nrf2 classical targets. The Nrf2 transcription factor is a master regulator of cellular adaptation to stress. The dust extracts produced a significant increase in phase II detoxifying genes. This work suggests that the health-related injury observed in rat lung cells exposed to dust extracts is associated with ROS generation, mitochondrial dysfunction, mitochondrial lipid peroxidation, and cellular antioxidant imbalance. Damage to lung mitochondria may be an important mechanism by which dust-containing bacterial material induces lung injury upon inhalation.

1. Introduction

Particulate matter (PM), an important component of global ambient pollution, is estimated to be the third leading contributor to disability-adjusted life years lost associated with respiratory diseases [Greco et al., 2016; Hou et al., 2015; Lim et al., 2012]. Much of the research on health effects due to PM exposure focused on anthropogenic urban and industrial pollution. Less studies have concentrated on exposure to airborne mineral dust and soils from nonurban or nonindustrial sources. Insufficient data are available on the exposure levels and the associated health effects due to inhalation of natural resuspended soil or dust from wind erosion. By mass, mineral dust is the dominant component of coarse and fine PM in many regions of the world, including North Africa, the Middle East, East Asia, and western USA [Chin et al., 2007; Ginoux et al., 2012]. Using high-resolution EMAC (European Centre/Hamburg/Modular Earth Submodel System MESSy Atmospheric Chemistry) atmospheric chemistry model and health impact functions from epidemiological studies, Giannadaki et al. [2014] estimated that the global premature mortality due to mineral dust exposure was about 400,000 in 2005, with about 3.47 million associated years of life lost per year. While the global fraction of the cardiopulmonary deaths caused by desert dust is estimated to be about 1.8% on a global scale, in dust-laden countries [Chin et al., 2007; de Longueville et al., 2013; Ginoux et al., 2012] as in North Africa, across the Middle East, and in South Asia and East Asia, the contribution increases up to 50% [Giannadaki et al., 2014]. Due to regular dust events, ambient PM10 and PM2.5 levels in these regions often exceed the World Health Organization exposure recommendations and therefore increases the potential risk for people’s health [de Longueville et al., 2013]. Lelieveld et al. [2015] note that the health impacts due to exposure to dust are less certain than adverse health effects due to anthropogenic air pollution due to lack of cohort studies.

Sources of airborne dusts are diverse. They include dried bed lakes, dry agricultural and arid soils, regional dust storms, and volcano ash deposits [Belnap, 2003; Chin et al., 2007; Ginoux et al., 2012; Pointing and Belnap, 2014]. Changes in land use and desertification are also common sources of airborne dust. In addition to the mineral components, transported dust particles can contain and distribute a diverse array of chemicals...
and biological components that adhere to the dust surface [Barberan et al., 2015; Mazar et al., 2016; Yamaguchi et al., 2012], including anthropogenic pollutants and organic compounds [Falkovich and Rudich, 2001], toxins, heavy metals, pesticides, bacteria, and mold, originating either at the source or through secondary atmospheric processes [Katra et al., 2014; Madaniyazi et al., 2015]. The adsorbed agents can add to the intrinsic effects caused by bare mineral dust particles. Natural desert soils often contain biological material [Garcia-Pichel et al., 2013; Metcalf et al., 2012] originating from biological crusts which are cohesive soil surface structures comprising of diverse biological species that can vary across climatic and precipitation gradients [Belnap, 2003] and land uses [Katra et al., 2016b]. The soil crusts and their dusts usually contain high content of cyanobacteria (particularly Microcoleus sp.) [Garcia-Pichel et al., 2013; Metcalf et al., 2012], heterotrophic bacteria, algae, fungi, mosses and lichens [Belnap, 2003], and associated toxins [Richer et al., 2015]. A possible human exposure route to cyanotoxins is through inhalation of dusts or marine aerosols containing cyanobacteria or their fragments [Lang-Yona et al., 2014; Stommel et al., 2013; Zhang et al., 2016]. Cultured cyanobacteria from Qatar desert crusts produced β-N-methylamino-L-alanine and 2,4 diaminobutyric acid neurotoxins that are responsible for amyotrophic lateral sclerosis/Parkinsonism-dementia complex [Cox et al., 2009]. It was suggested that ambient dust particles in this region contain desert crust and hence may transport toxin-producing cyanobacteria and thereby present a risk to human health [Cox et al., 2009].

Various adverse health effects, specifically respiratory diseases (such as asthma and pneumonia), cardiovascular diseases, cardiopulmonary diseases, and even mortality are associated with exposure to PM in general and to fine mineral dust particles in particular [Baccarelli et al., 2008; Badding et al., 2014; Belnap, 2003; Brook et al., 2004; Chen et al., 2006; Giannadaki et al., 2014; Lim et al., 2012; Metcalf et al., 2012; Perez Garcia-Pando et al., 2014; Soukup et al., 2000; West et al., 2016]. Exposure to PM often leads to oxidative stress in living organisms, through the formation of excess reactive oxygen species (ROS) that can induce inflammation, cell damage, and cell death [Adam-Vizi and Chinopoulos, 2006; Antognelli et al., 2014; Hamad et al., 2016; Kermanizadeh et al., 2015; Lodovici and Bigagli, 2011; Øvrevik et al., 2015; Shuster-Meiseles et al., 2016]. A recent study showed that exposure of macrophages to Asian dust particles induced tumor necrosis factor-α (TNF-α) production and nuclear factor-κB activation accompanied by ROS generation [Higashisaka et al., 2014]. While mitochondria are considered the major intracellular source and primary target of ROS, PM exposure has also been associated with increased mitochondrial DNA damage [Hou et al., 2010].

The response of cells to stress may lead to alterations in prooxidant and antioxidant activities [Kurutas, 2015; Lodovici and Bigagli, 2011; Øvrevik et al., 2015]. A redox sensitive regulator of cellular adaptation to stress is the Nrf2 transcription factor. Using both in vitro and in vivo models, we have recently shown that exposure to soluble metals present in resuspended urban dust can influence health-related mechanisms by alternating oxidative stress status and the Nrf2 transcription factor [Pardo et al., 2015; Pardo et al., 2014; Pardo et al., 2016]. Since the mechanisms that explain the respiratory effects of dust are mainly based on air pollution studies, it is important to study the specific effects caused by exposure to authentic desert dusts and soils. In this study we show that exposure of rat alveolar macrophages to water soluble dust extracts from unperturbed, crust-containing desert soil causes significant loss of cell viability. This was accompanied by mitochondrial dysfunction, oxidative damage, and redox imbalance that were evaluated by measuring increased amount of ROS, oxidation of lipids, and induction of Nrf2 phase II detoxifying genes.

2. Materials and Methods

2.1. Sample Collection and Preparations

Soil dust was collected in an arid Loess soil in Israel. This soil is associated with dust emissions following strong regional wind events [Katra et al., 2016b]. The soil material was collected from the upper layer (0–2 cm) of a bare surface in a natural preserve that is not subjected to human activities. The typical bare surfaces are characterized by an extensive cover of biological soil crust. Six replicates (50 g soil material each) were extracted and mixed, from which 100 g soil material was used for the experiment. The soil samples were sieved to <63 μm using a #230 mesh stainless steel sieve in order to investigate potential effects of the size fraction that can potentially become airborne [Kok et al., 2012]. Most of this size fraction is composed of cohesive fine particles (<10 μm) that are aggregated in the samples but represent at the bulk potential of exposure risk of suspended dust from soil sources.
To ensure reproducible exposure conditions, extracts were prepared from the collected soil fraction and for comparison also from National Institute of Standards and Technology standard reference material (SRM) particles (SRM2907) (the National Institute of Standards and Technology (Gaithersburg, MD)), which had been reported to cause a significant adverse health effects by generation of ROS [Mirowsky et al., 2015]. Ten mg of soil and SRM were extracted with 12.0 mL of ultrapure water. The samples were shaken for 6 h in the dark in precleaned polypropylene tubes. The water extracts were then taken for chemical analysis. Method blanks were prepared replicating the process procedure and named ("Control"). The PM samples were named “Dust extract” and “SRM”.

2.2. Chemical and Biological Analyses of the Soil Sample

Elemental composition was obtained by the X-ray fluorescence (XRF) method using an XRF spectrometer, PANalytical Co., model Axios (wavelength dispersive-WDXRF; 1 kW). The measurement was conducted on 1 g of air-dried powdered soil. Omnia software was used for quantitative analysis.

The bacterial community in the soil sample was analyzed by polymerase chain reaction (PCR) amplification and sequencing; Total DNA from each soil sample was independently extracted twice from 0.5 g soil sample using the PowerSoil DNA isolation kit according to manufacturer’s instructions (MoBio Laboratories, Carlsbad, CA, USA). The DNA content was measured with a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total genomic DNA was analyzed (DNA Services Facility, University of Illinois at Chicago), with PCR amplification using the 27 F and 534R primers (HMPC, 2012), for V1-V3 hypervariable regions of the bacterial 16S rRNA gene. Amplicons were sequenced in a paired end format using the Illumina MiSeq platform. Total of 423,762 bacterial sequence reads were recovered and subjected to the software package QIIME, leaving a total of 388,217 bacterial reads after chimeric and primer sequences removal and 14,411–25,613 bacterial reads for each sample. Sequence data were clustered into operational taxonomic units (OTU) at 97% similarity and classified with the nonredundant Greengenes reference OTU build (version 13.8; 97% similarity database; 99,322 sequences [Katra et al., 2016a].

2.3. Cell Culture

Rat alveolar macrophages, NR8383 (CRL 2192), were grown in Hams F12K medium (Biological Industries, Beit-Haemek, Israel) supplemented with 15% (w/v) fetal calf serum, 1% (w/v) glutamine and penicillin-streptomycin 1% (w/v) at 37°C in a humidified atmosphere consisting of 95% air and 5% CO2. Exposure to the Israel dust extracts was performed for 6 and 24 h. All samples were buffered with salts-glucose media; the buffer comprised 50 mM Hepes, 100 mM NaCl, 5 mM KCl, 2 mM CaCl2, and 5 mM Glucose; pH7.2 prior to use in the in vitro assays in order to maintain osmolarity.

2.4. WST-1 Cell Viability Assay

Cells were incubated for 24 h with dust extracts for assessing cell survival. Cell viability evaluated by WST-1 (Abcam, UK) assay according to the manufacturer’s instructions. The WST-1 is based on the cleavage of tetrazolium salt to formazan by a complex cellular mechanism that occurs primarily at the cell surface. The absorbance of the samples was measured at 440 nm and 650 nm by a microplate reader.

2.5. Determination of Cell Death and Viability

Flow cytometry (FACSCalibur, BD, Foster City, CA) analysis was used to evaluate the type of cell death as well as cell viability; Annexin V-fluorescein isothiocyanate (V-FITC) and the DNA intercalating dye propidium iodide (PI) (the MEBCYTO, MBL) were used to distinguish between apoptosis or necrosis cell death mechanisms, whereas, PI is excluded by viable cells. The following fluorescence settings were used: excitation at 488 nm and emission at 575 nm. Data are collected from 20 000 cells.

2.6. Measurement of Intracellular ROS

Reactive oxygen species (ROS) measurements were performed using the dichlorodihydrofluorescein diacetate (H2DCF-DA) fluorescent probe as previously described [Pardo et al., 2006]. Hydrogen peroxide (H2O2) was used as positive control.

2.7. Mitochondrial Physiology

For probing mitochondrial involvement and/or dysfunction, the XF96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, USA) was used. This technology is able to estimate cellular bioenergetics
parameters such as glycolysis, adenosine triphosphate (ATP) production, oxygen consumption rates, and respiratory capacity by measuring extracellular variations of oxygen and protons in the medium surrounding the cells [Badding et al., 2014]. In order to identify changes in mitochondria respiration, the NR8383 rat alveolar macrophages were seeded in a special XF96-well microplates at a density of 6 × 10⁴ cells/well. Following 24 h of incubation with the dust extracts at 37°C in 5% CO₂, each well was added: oligomycin, 0.5 μM; carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), 1 μM; and Rotenone, 0.5 μM. The XF96 Analyzer measured basal cellular respiration, oxygen consumption rate (OCR), and extracellular acidification rate (ECAR). OCR and ECAR were normalized to the number of cells per well using Hoechst staining.

2.8. Isolation of Mitochondria

Mitochondria isolation was performed as was previously reported by [Pardo et al., 2006]. NR8383 cells were exposed to dust extract for 24 h, then the cells were collected and washed in a buffer containing 134 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, and 2.5 mM Tris (pH 7.5). To rupture cells membrane, cells were suspended in a hypotonic-swelling buffer containing 10 mM NaCl, 1.5 mM CaCl₂, and 10 mM Tris (pH 7.5). The cells were also homogenized with a Teflon pestle homogenizer on ice. A concentrated sucrose solution (5×) with 1.65 M sucrose, 30 mM ethylenediaminetetraacetic acid (EDTA), and 43 mM Tris (pH 7.5) was added in order to reach 1X solution with the total volume of the cells. Nuclei, debris, and unbroken cells were removed by centrifugation from the suspension at 600 g for 10 min. The upper supernatant that contained the desired mitochondria was centrifuged at 10,000 g for 10 min. Mitochondria were resuspended in 1X sucrose solution.

2.9. Lipid Oxidation

Lipid oxidation was evaluated on isolated mitochondria from NR8383 cells, using the thiobarbituric acid (TBA) method [Ohkawa et al., 1979]. Isolated mitochondria were mixed with 50% trichloroacetic acid (1:1) to precipitate proteins in the sample. Following centrifugation, the supernatant reacted with TBA (280 mg in 100 mL) in a boiling water bath for 10 min. The samples were cooled, and the absorbance was measured at 532 nm with a spectrophotometer. Standard curve was performed with MDA tetrabutylammonium salt (Sigma-Aldrich, St. Louis, MO, USA).

2.10. Gene Expression; RNA Isolation, Reverse Transcription (RT), and Real-Time PCR

Gene expression was performed as was previously described [Pardo et al., 2014]. Briefly, total RNA was extracted from NR8383 cells, using RNeasy RNA kit (QiAGEN, Hilden, Germany). Total RNA (1 μg) was reverse-transcribed to cDNA using random hexamers (ABI, California, USA). For quantification of mRNA expression, real-time PCR were performed using the Fast SYBR Green PCR mix (Applied Biosystems, Foster City, CA, USA) in StepOnePlus Reverse Transcription (RT) PCR instrument. The following cycling conditions were used: 40 cycles at 95°C for 30 s and 60°C for 30 s and extension at 72°C for 30 s. β-actin and 18S genes were used as endogenous controls. Melt curve analysis for each primers set showed a single peak, and primers slopes were –3.4 for both target and endogenous control genes. Threshold cycle (CT) was set between 22 and 29 according to the cDNA dilution, whereas ΔCT between the target and the endogenous control genes was set to be no more than five cycles. Primers were purchased from Sigma-Aldrich (Rehovot, Israel) and are described in the following Table 1.

2.11. Statistical Analysis

Results are expressed as means ± standard deviation. One-way analysis of variance was used in multivariable analyses. Statistical evaluation was performed with OriginLab (Data Analysis and Graphing Software, Northampton, MA, USA). Differences were considered significant at probability level p < 0.05 using the Fisher protected least-significant difference method.

Table 1. List of Rattus norvegicus; Rat Primers Sequences

| Name   | Forward            | Reverse            |
|--------|--------------------|--------------------|
| Catalase| 5’-AGCCAGAAGAGAAACCACA-3’ | 5’-CTCCAGAAGTCCAGCACCAT-3’ |
| Gpx1   | 5’-TCCAGAAAAATCCTCGAGA-3’ | 5’-AGTGTGAACACCTGTTT-3’ |
| HO-1   | 5’-AACACCCACAAGGCAA-3’ | 5’-CTCTTCCTGACCCCTGTGTT-3’ |
| MT2    | 5’-CACCTCGAGACAGAAAG-3’ | 5’-TTTGTAATTTACACCATGTGAG-3’ |
| β-Actin| 5’-TTCATAATGAGTGCTTGTTG-3’ | 5’-GGGGGTGTGAAGGCTCAAA-3’ |
| 18S    | 5’-ACGGAAAGGGCACCACAGGA-3’ | 5’-AATGTGCCTGCTGCTTTA-3’ |
3. Results

3.1. Dust Extracts Analyses

Dust samples used in this study were collected from arid Loess soil in Israel. Tables 2 and 3 list the dust sample’s elemental content and compound by XRF. The dust consists mostly of Si (21%) and Ca (21%) and common metals in soils (Al, Fe, Mg, and K). The elemental composition indicates typical minerals present in desert dust samples, including quartz (SiO₂; 44%), carbonates (CaO; 30%), and clays (Al₂O₃; 11%). The biological analysis of dust samples (Table 4) by genomic sequencing indicates that cyanobacteria and actinobacteria are the dominant biological species in the dust extracts with relative abundance of bacterial 16S rRNA genes of 36.3% and 29.7%, respectively.

3.2. Dust Samples Reduce Cell Viability

To determine the degree of dust-induced cytotoxicity, viability assays were carried out in NR8383 cells following 24 h treatments with dust extracts prepared at a concentration of 0.3 μg/μL. The dust extracts caused significant reduction in cell viability as evidenced by a WST-1 assay (Figure 1a). A flow cytometry cytotoxicity assay was used to determine the mechanism of cell death. Cells were stained with PI and annexin V to assess the prevailing type of cell death, apoptosis or necrosis (respectively). Total cell death was the highest in the dust-treated samples compared to the Control and SRM (Figures 1b and 1c). It was found that cell death involves both apoptosis and necrosis types, with a greater contribution to apoptosis, suggesting that the dust extracts from Israel are cytotoxic.

3.3. Intracellular ROS Production by Dust Samples

An intracellular ROS assay (2,7-dichlorodihydrofluorescein diacetate) was used to investigate the impact of dust extracts on ROS production in the cells. Changes in fluorescence were monitored starting at 6 h and up to 24 h following exposure (Figure 2). Hydrogen peroxide (H₂O₂) was used as positive control. Both SRM and the dust samples increased ROS compared to the control, with no statistically significant differences between the SRM and the dust sample. In addition, only a minimal change in ROS production between the two time points (6 and 24 h) was observed for both SRM and dust extracts (Figures 2a and 2b). These findings indicate that exposure of cells to the dust extracts for 6 or 24 h induced ROS production that could lead to oxidative stress and damage.

3.4. Changes in Bioenergetics and Mitochondrial Function Following Exposure to Dust Extracts

Mitochondria are an important source of ROS inside most mammalian cells [Adam-Vizi and Chinopoulos, 2006; Murphy, 2009]. Mitochondrial respiration was measured as an additional indication for dust’s cytotoxicity and its effects on cells viability. The Extracellular Flux (XF) Analyzer (Seahorse

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| Table 2. Israel Desert Soil Elemental Content |
|---------------------------------------------|
| Element          | Concentration (%) |
|------------------|-------------------|
| Ca               | 21.216            |
| Si               | 20.521            |
| Al               | 5.842             |
| Fe               | 5.826             |
| Mg               | 1.516             |
| K                | 1.497             |
| Ti               | 1.098             |
| Na               | 0.209             |
| Zr               | 0.121             |
| Mn               | 0.105             |
| Sr               | 0.062             |
| Ce               | 0.05              |
| Cr               | 0.032             |
| V                | 0.02              |
| Ni               | 0.015             |
| Zn               | 0.014             |
| Co               | 0.013             |
| Pb               | 0.013             |
| Cu               | 0.011             |
| Rb               | 0.007             |
| Ga               | 0.004             |
| Y                | 0.004             |

| Table 3. Israel Desert Soil Mineral Content |
|--------------------------------------------|
| Compound         | Concentration (%) |
|------------------|-------------------|
| SiO₂             | 43.901            |
| CaO              | 29.685            |
| Al₂O₃            | 11.039            |
| Fe₂O₃            | 8.329             |
| MgO              | 2.514             |
| TiO₂             | 1.832             |
| K₂O              | 1.803             |
| Na₂O             | 0.282             |
| ZrO₂             | 0.164             |
| MnO              | 0.136             |
| SrO              | 0.073             |
| CeO₂             | 0.062             |
| Cr₂O₃            | 0.047             |
| V₂O₅             | 0.035             |
| NiO              | 0.019             |
| Co₃O₄            | 0.017             |
| ZnO              | 0.017             |
| PbO              | 0.014             |
| CuO              | 0.013             |
| Rb₂O             | 0.008             |
| Ga₂O₃            | 0.006             |
| Y₂O₃             | 0.005             |


the mitochondrial stress test was used to measure live changes in mitochondrial respiration. Oxygen consumption rates (OCR) following 24 h exposure to the dust extracts are shown in Figure 3a from a selected experiment. A period of basal readings was followed by the addition of compounds that are used to estimate mitochondrial bioenergetic functions within the cells. Oligomycin inhibits the ATP synthase (Complex V) to define the level by which oxygen consumption is linked to ATP production. FCCP, an uncoupler that allows the transport of hydrogen ions, increases respiration without the production of ATP. Rotenone inhibits the transfer of electrons from complex I to complex III with the formation of ROS, and it was used to suppress oxygen consumption. The changes in these parameters indicate reduction in mitochondrial respiration following the cells’ exposure to the dust and to the SRM extracts (Figure 3a). Pretreatment of the cells with the dust extracts not only prompted changes in the way mitochondria responded to the inhibitors but also showed lowered levels of the basal OCRs. While spiking with mitochondria inhibitors leads to maximal respiration in normal cells, OCR was mitigated in cells that were exposed to the dust extract. Although the effects for the dust extract were larger than for the SRM extract, they were not statistically different from each other. However, the reserve/spare capacity (maximum oxygen consumption in the basal state) was reduced compared to control-treated cells in a statistically significant manner. This measurement represents the mitochondria respiratory ranges and limits accessible for cells in response to various stressors that increase

### Table 4. Relative Abundance of Bacterial 16S rRNA Genes Reads (in %) at the Phylum Level

| Phylum             | % of Reads |
|--------------------|------------|
| Cyanobacteria      | 36.3       |
| Actinobacteria     | 29.7       |
| Proteobacteria     | 15.0       |
| Chloroflex         | 7.9        |
| Acidobacteria      | 1.8        |
| Gemmatimonadetes   | 1.5        |
| Planctomycetes     | 0.9        |
| Firmicutes         | 0.8        |
| Verrucomicrobia    | 0.6        |
| FBP                | 0.4        |
| Ammatimonadetes    | 0.3        |
| TM7                | 0.1        |
| Thermi             | 0.1        |
| Nitrospira         | 0.1        |
| Unclassified phylum| 0.1        |

Figure 1. Dust extracts reduce cell viability in alveolar macrophages. Alveolar macrophages were exposed to dust extracts for 24 h. Following 24 h of exposure, cell cytotoxicity was determined using (a) WST-1 assay, (b) flow cytometry analysis for apoptosis or necrosis cell death, and (c) flow cytometry histograms. The data represent mean ± standard error of the mean (SEM), and the asterisk sign means significantly higher at \( p < 0.05 \) than their controls. These experiments were performed in triplicate and were repeated twice.
Figure 2. Cellular ROS increased following 6 and 24 h exposure to dust. ROS levels measured by H$_2$DCF-DA detection kit as described in material and method section. (a) Histogram panel, the Y axis represents the number of events (cell count), and the X axis represents fluorescence signal intensity. (b) DCF fluorescence quantitation after 6 h. (c) DCF fluorescence quantitation after 24 h. (d) Histogram panel of cellular ROS following 6 h exposure to filtered and sterile dust extract. (e) DCF Fluorescence quantitation after 6 h. The data represent mean ± SEM, and the asterisk sign means significantly higher at $p < 0.05$ than their controls. These experiments were performed in triplicate and were repeated three times.
bioenergetic demands such as oxidative stress [Badding et al., 2014; Modis et al., 2012], which is induced by the dust extract.

Reduced respiration rate accompanied by increased ROS generation may lead to lipid oxidation by reactions with the polyunsaturated fatty acids of the cells’ membrane and constituents, initiating a self-propagating chain reaction [Mylonas and Kouretas, 1999]. To examine oxidative damage, we isolated mitochondria from the cells and tested for lipid oxidation after exposure to the dust extract. As shown in Figure 3b, the level of lipid oxidation was higher in mitochondria from dust-treated cells, supporting mitochondrial damage.

Figure 3. Mitochondrial dysfunction following exposure to dust extracts. Cells were treated with dust extracts for 24 h. Determination of the mitochondrial oxygen consumption rate (OCR) at different time points was performed using the mitochondrial stress test kit (Seahorse Biosciences). (a) Selected experiment showing basal and mean OCR following injections of the inhibitors and substances. (b) Isolated mitochondria-lipid peroxidation measured by MDA. These experiments were performed in quadruplets and represent three independent experiments. The data represent mean ± SEM, and the asterisk sign means significantly higher at p<0.05 than their controls.

Figure 4. Increased Nrf2-related genes following exposure to dust extracts for 6 or 24 h. Quantitative analysis of Nrf2-target genes was performed by RT and real-time PCR for mRNA levels of (a) HO-1, (b) NQO1, (c) catalase, (d) Gpx-1, and (e) Nrf2. Values are expressed as fold change of gene expression compared to a calibrator (endogenous control; β-Actin). The data represent mean ± SEM, the asterisk sign means significantly different at p < 0.05 than their controls. These experiments were performed in triplicate and were repeated two times.
3.5. Nrf2 Protective Signaling Pathway

We have recently demonstrated that Nrf2 target genes were involved in lung-PM response following intratracheal instillation [Pardo et al., 2015; Pardo et al., 2016]. To assess whether the dust extracts affects antioxidant defense, we evaluated phase II detoxifying and antioxidant enzymes, which are typical targets of Nrf2. Nrf2 target genes such as heme oxygenase-1 (HO-1), catalase, and glutathione peroxidase (Gpx-1) were evaluated following exposure to dust extract by real-time PCR. Indeed, exposure to the dust extract for 6 or 24 h produced a significant (twofold and fourfold, respectively) rise in the stress-induced protein heme oxygenase-1 (HO-1) (Figure 4a) compared to control. Antioxidant genes such as nqo1 also increased by 1.5-fold (Figure 4b) with exposure to dust extracts at 24 h, and catalase and glutathione peroxidase (gpx-1) showed smaller increase following dust exposure (Figures 4c and 4d) for 24 h. Significant twofold increase in Nrf2 mRNA levels was observed following dust exposure (Figure 4e) for 24 h and by 1.5-fold after 6 h of exposure. These results demonstrate enhancement of the Nrf2 antioxidant defense response following exposure to dust extracts starting at 6 h of exposure up to approximately 24 h.

4. Discussion and Conclusions

Extensive evidence indicates that oxidative stress and inflammation lead to adverse health effects associated with exposure to ambient and occupational PM [Li et al., 2002; Li et al., 2003; Øvrevik et al., 2015]. In this study we investigated the possible health effects due to exposure to desert dust from unperturbed soils which are rich in biological crusts. In the eastern Mediterranean, the frequency of dust storms and exposure of population to local and transported dusts is increasing, possibly due to global drying and warming trends [Georgoulas et al., 2016; Krasnov et al., 2016; Zittis et al., 2016]. In the southwestern U.S., springtime regional mean PM2.5 dust concentrations have increased in the last 20 years, due to early onset of the spring dust season by 1 to 2 weeks [Hand et al., 2016]. Dust storms are also common in Asia and Africa where millions of people are exposed to high levels of atmospheric soil-dust PM. These global trends lead to overall increased exposure to dusts. We investigated the mechanism by which such dust may have toxic influences and whether this presents a health hazard to exposed humans. A novel mechanism for dust toxicity was identified in which exposure induces ROS production, reduces mitochondrial respiration while causing oxidative damage that finally leads to cell death mainly by apoptosis. This is consistent with previous studies which identified mitochondrial dysfunction as a potential mechanism affected by exposure to PM [Ghio et al., 2014; Hou et al., 2010; Huang et al., 2003; Li et al., 2015; Meyer et al., 2013]. Dust toxicity was also accompanied with induction of protective mechanisms governed by Nrf2.

Alveolar macrophages (AM) are constantly exposed to the ambient environment. Exposure to environmental particulates such as residual oil fly ash (ROFA) [Huang et al., 2003; Magnani et al., 2013] or PM from various sources [Hamad et al., 2016; Li et al., 2015; Soukup et al., 2000], produces large quantities of ROS. The mitochondrial electron transport chain is the main source for ROS production in eukaryotic cells stimulated by PM. In isolated human AM, three mitochondrial inhibitors reduce ROFA-stimulated ROS production [Huang et al., 2003]. Reduced mitochondrial respiration was also observed upon exposure of mice macrophages to welding fumes [Badding et al., 2014], and mitochondrial DNA damage was illustrated when rats were exposed to PM2.5 [Li et al., 2015; Meyer et al., 2013]. In this study, increased ROS production was observed following exposure to dust extracts 6 and 24 h following exposure, and the mitochondrial function assay provided specific bioenergetics parameters that were altered following exposure. In support of reduced mitochondrial respiration, we also measured lipid peroxidation in mitochondria isolated from the exposed rat AM. This observation confirms that mild oxidative damage occurred in the mitochondria, suggesting that although the cells are mostly viable following this exposure level, the mitochondrial function was affected due to excess ROS production.

Oxidative stress occurs on the intersection of redox-sensitive pathways which can lead to different biological outcomes such as inflammation and cell death, e.g., the regulation of mitochondria-mediated apoptosis, death receptors, and the endoplasmic reticulum [Lodovici and Bigagli, 2011; Redza-Dutordoir and Averill-Bates, 2016]. The soil/dust sample studied here also induced apoptosis, as shown by annexin V staining. Together with the data on reduced mitochondrial respiration, it suggests that the exposure to dust activates the mitochondrial pathway of apoptosis. These findings are consistent with a previous study showing that Arizona dust induced oxidant production and apoptosis in BEAS-2B cells [Ghio et al., 2014].
Cells’ response to increased oxidative stress involves the induction of protective mechanisms (Limón-Pacheco and Gonsebatt, 2009). When oxidative stress is rather low, cellular defense mechanisms such as the induction of Nrf2 transcription factor and its related phase II protective genes counteract ROS production with antioxidative and detoxifying enzymatic processes, thus protecting the cells from adverse biological damage [Kang et al., 2005]. For example, Nrf2 protects against PM, polycyclic aromatic hydrocarbons, and gaseous matter in vitro cellular animal models [Pardo et al., 2014; Rubio et al., 2010] and in vivo models [Pardo et al., 2015; Pardo et al., 2016]. Additionally, activation of Nrf2-related genes was also identified in BEAS-2B cells exposed to isoprene-derived secondary organic aerosol (SOA) [Lin et al., 2016]. Data presented here support the induction of Nrf2 as exposure of rat alveolar macrophages to dust PM increased the transcription of heme oxygenase-1, catalase and glutathione peroxidase, and even Nrf2 itself.

The positive association between exposure to extracts from dust and adverse effects on mitochondrial respiration could result from increased ROS/oxidative stress initiated by the exposure. ROS-induced oxidative stress is believed to play dual roles in cell response; on the one hand, protective mechanisms are induced for cell survival and repair of damage. On the other hand, dysfunction in the mitochondria respiration may lead to additional ROS production, resulting in oxidative damage and cell death [Wang et al., 2013]. Association between mitochondrial DNA damage and PM exposure were previously reported, consistent with our observation of mitochondrial dysfunction caused by exposure [Hou et al., 2010].

Dust can have a wide range of characteristics that can drive oxidative stress, varying, for example, in chemical composition, grain size, and particle morphology. Since extracts were used in this study, physical characteristics of the particles have no significance. Using both in vitro and in vivo models, we recently showed that exposure to engineered inorganic nanoparticles and to PM extracts from resuspended urban dust alters oxidative stress status and induces the Nrf2 transcription factor. These studies suggested that soluble metals are the main drivers for these effects [Pardo et al., 2015; Pardo et al., 2014; Pardo et al., 2016; Shuster-Meiseles et al., 2016]. Hence, presence of metals in dust extracts can potentially induce oxidative damage. The concentrations of soluble metals such as chromium (Cr), iron (Fe), copper (Cu), manganese (Mn), and nickel (Ni) [Badding et al., 2014; Shuster-Meiseles et al., 2016] in the dust extracts were low. In addition, qPCR analysis of MT-2, a gene that regulates metallothionine, a protein associated with protection against heavy metals by reacting with sulfhydryl groups [Ling et al., 2016], showed that their levels did not change compared to control (Figure S1 in the supporting information). Together, these observations suggest that the effects observed in this study were not induced by the presence of metals in the extracts.

The origin of the dust used in this study was from desert soil that contains biological crust. These crusts contain a high number of cyanobacteria species [Garcia-Pichel et al., 2013; Metcalf et al., 2012], as was verified by a genomic analysis of the local desert soils [Katra et al., 2016b]. While exposure to desert extracts increased ROS in the cells, exposure after sterilization and filtration showed ROS levels similar to the control (Figure 2). Sterilization and filtration are expected to remove large biological components such as toxins and bacterial residues but not to affect the concentrations of soluble metals in the sample. This observation suggests that the active factor in the dust cannot be attributed to metals, but may be toxins or bacteria components, as was previously shown in Metcalf et al. [Metcalf et al., 2012]. Cyanobacteria or toxins can attach to mineral dust particles and can be transported over long distances [Griffin, 2007; Mazar et al., 2016]. Microcystin-LR, a toxin produced by cyanobacteria, has been shown to be toxic when applied to mice as a nasal spray and inhaled [Benson et al., 2005]. The presence of bacteria, bacterial fragments, or toxins in desert crusts raises the possibility that desert dust storms are a major route for adverse health effects following exposure.

Further studies are required in order to comprehend the health influences and mechanisms due to human exposure to desert dust and airborne soil particles. An understanding of the risks of inhaling particles containing cyanotoxins is also warranted as human activities, especially agriculture, are closely associated with soil dust generation and increased exposure to PM for the general population as well as for agricultural workers and population in rural areas.

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