Particulate Matter Exposure-Induced Cholesterol Metabolism Disorder In Lung Epithelial Cells

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Abstract

Background: Particulate matter (PM) has been the worldwide problem to threaten human health. Although many work have well investigated physiological effect of PM on different tissues, few work can systemically evaluate intrinsic molecular mechanism of PM effect at molecular pathway levels and how to lead cellular toxicity.

Methods: Here, RNA sequencing was employed to explore molecular mechanism of micro-meter PMs obtained from air on ferret lung epithelial cells. PM particles were firstly characterized using SEM, XPS and IR to examine morphology and elemental components. Then, dosage-dependent cellular toxicity of PM was examined using MTT method, and it displayed dosage-dependent cellular apoptosis.

Results: By performing bioinformatics analysis, we found that 190 down-regulated and 56 up-regulated genes were identified after exposure to PM particles with higher concentration (500 $\mu\text{g/mL}$). By employing gene ontology (GO) and gene score enrichment analysis (GSEA), results demonstrated that “cholesterol metabolism” pathway was substantially down-regulated, which was confirmed using qRT-PCR and free cholesterol measurement. The intrinsic mechanism to lead dysfunction of cholesterol metabolism is oxidative stress with imbalance of GSSH/GSH and NADP/NADPH ratios, leading by higher ROS and disorder of mitochondrial membrane potential.

Conclusion: These results give a new insight in physiological effect of PMs on lung epithelial cells and provide the potential intervention approaches to rescue respiratory-related diseases caused by exposure to particulate matters.

1 Introduction

More evidence reveal that air pollution has become the key reason to lead various respiratory-related diseases[1–3]. Air pollution mainly is comprised by aerosol, which contains particulate matters (PM) and gas components[4]. Based on particle size, PM can be divided into three subtypes: ultrafine (0.1 μm), fine (0-2.5 μm), and coarse (2.5–10 μm)[5]. However components of coarse PMs are not constant, which are always highly associated with seasons, environment temperature, and geographical spatial distribution. For example, water solubility of $\text{NO}_3^-/\text{SO}_4^{2-}$ increasing from Spring to Fall,[6] early PM_{2.5} pollution including 23 elements from 2001 to 2003[7], sulfate as major water-soluble ions in Mediterranean coastal site[8]. Therefore, cellular toxicity to further explore the precise molecular mechanism can provide more clear insight into PM-induced disease development.

In the past decade, too many clinical investigation indicated that exposure to PMs can lead pulmonary inflammation and further damage respiratory system[9, 10]. To focus on lung functions (including lung volume and flows), higher concentration exposure to PM₁₀ can dramatically promote the decline of lung function, especially for small airway function[11, 12]. By investigation PM_{2.5} and hospital visits, exposure to PM pollution displays the linear relationship with adult asthma in Beijing City.[13] To determine intrinsic molecular mechanism, inflammation response plays an essential role in inducing cell

dysfunction, for example increase of oxidative stress by PM[14, 15], DNA damages by radicals[16], inducing cell apoptosis[17–19]. However, these investigation only focused on one aspect to determine effect of PMs, and neglected the systematical interaction in cellular signaling network. Therefore, systematical investigation is helpful to comprehensively understand PM-induced toxicity.

To assess health risk of PMs, various types of techniques, for example cell viability screening[20, 21], histological characterization[22–24], examination of blood indexes[25–27] and omics characterization[28–30], have been employed. These examinations provided different aspects into molecular mechanism of PM toxicity in biointerface interaction between PMs and tissue. To examine the impact of PMs on epithelial cells, lots of literature have reported using different methods, for example A549 to explore the genotoxicity of fine and coarse PMs[31, 32], normal bronchial epithelial human cells BEAS-2B to determine the roles of PM_{2.5} in pro-inflammatory and oxidative stress[33]. Most of investigation focused on the inflammatory response or apoptosis process, and most of these work did not systematically determine how PMs induced inflammatory response or apoptosis at global molecular networks. As one of critical techniques to explore the effects of whole gene expression, RNA sequencing can provide the expression profile of total genes after external intervention, for example cellular components, molecular functions or molecular processes. Compared to other techniques, RNA sequencing can provide the global insight in how PMs regulate molecular networks and which plays the critical role for external response after intervention. Consequently, RNA sequencing approach is an excellent technique for exploring the potential dysfunction of solid particles on cellular processes.

In this study, we collected PMs using the sedimentation method and further examined the components of these solid samples using XPS and IR methods. Then, ferret lung epithelial cells were employed to evaluate the biological impact of PMs depending on different intervention dosages. The total RNA of these biological samples was collected and further characterized by the RNA sequencing technique. RNA sequencing data were further analyzed using the bioinformatics method, including DESeq, gene ontology analysis, protein-protein interaction (PPI) network analysis, gene set enrichment analysis (GSEA). By performing bioinformatics analysis, the hub genes and relative disrupted pathways can be enriched, and these results were confirmed by quantitative reverse transcription PCR (qRT-PCR). Using confocal laser scanning microscopy, ROS level and mitochondrial membrane potentials after PM treatment. Finally, ATP, GSSG/GSH, and NDAP/NADPH were examined to assess oxidative stress in PM-treated cells. Our investigation aims to explore the chronic impact of suction-type PMs on lung epithelial cells, and these results may give a glance at molecular mechanism of PM-induced toxicity, which provides the potential intervention strategies for air pollution-induced pulmonary diseases.

2 Materials And Methods

2.1 Materials

Mv 1 Lu (NBL-7) cell line was purchased from ATCC cell line bank. Phosphate buffered saline (PBS), Fetal bovine serum (FBS), dulbecco's modified eagle medium (DMEM), penicillin-streptomycin and 0.25% (w/v)

trypsin solution were purchased from Gibco Life Technologies (USA). Cell counting kit-8 (CCK8) was obtained from Dojindo. Milli-Q water was purified via the Milli-Q Plus System (Millipore, USA). Silicon dioxide (diameter 2.0 μm , purity > 99.99% metal basis) was purchased from Aladdin company (Shanghai, China).

2.2 Air solid particles sampling and preparation

All the samples of air solid particles were collected nearby Yellow river, Kaifeng City, China. The sampling method was performed using literature report[34]. Samples were collected during November 2020 to January 2021. Samples were suspended in de-ionized water (> 18.4 $\text{M}\Omega\cdot\text{cm}$) and utilized for further analysis.

PM adsorbate (upper solution) was prepared using following method: PM particles were suspended into PBS solution with specific concentration, and treated with ultrasonic (20 W for 5 min at 37 $^{\circ}\text{C}$). Then, suspended mixture was centrifuged with 15000 rpm for 10 min, and upper layer was PM adsorbate solution. The concentration of PM adsorbate was labeled based on total PM concentration.

2.3 Cell culture and treatment

1×10^4 NBL-7 cells were seeded to 96-well dishes at 37 $^{\circ}\text{C}$ with 5% CO_2 , and the component of cell culture medium includes DMEM, FBS (5%), 100 U/mL penicillin-streptomycin. Solid particles was suspended with DMEM medium before treatment at different intervention dosage, and then incubated for 48 h. The cell viability was examined using CCK8 method as describing in literature reports[35].

2.4 Cholesterol, GSH/GSSG and NADP/NADPH quantification

Total cholesterol quantification was performed using Micro Total Cholestenone (TC) Content Assay Kit (Cat.BC1890, Solarbio Life Science Company). Therefore, 10^6 cells were treated by PM upper solution for 12 h, 24 h and 48 h. Then, 10^6 cells were collected and lysed using extraction solution. This mixture was sonicated using Ultrasonic Homogenize (Scientz-IIID, Ningbo Xinzhi Company) with power 300W (2 s sonication with 3 s gap, total time 3 min). Mixture was centrifugated (8000 g, 4 $^{\circ}\text{C}$, 10 min), and then upper solution was collected for examination using manufacturer's manuals. Before centrifugation, protein concentration was examined using Detergent Compatible Bradford Protein Assay Kit (Cat.P0006C, Beyotime, China).

ATP levels were quantified with ATP Bioluminescence Assay Kit (Cat.S0026, Beyotime, China). GSSG/GSH ratios were quantified with GSH and GSSG Assay Kit (Cat. S0053, Beyotime, China). NADP/NADPH ratio were characterized using NADP $^{+}$ /NADPH Assay Kit with WST-8 (Cat.S0179, Beyotime, China). All the processes were in accordance with the manufacturer's recommendations. A total of 1 to 2×10^6 cells/well were used for examining relative concentration.

2.5 Measurement of ROS levels and mitochondrial membrane potential

Intracellular ROS levels were measured using DCFH-DA (Cat.ID3130, Solarbio Life Science Company). Cells (10^4 cells/well) were seeded into coverslips, which were coated by poly-L-lysine (Cat. P2100, Solarbio Life Science Company), and then cultured for 48 h (5% CO₂, 37°C). After cells were treated by the PM upper solution for 48 h, old medium was removed, and FBS-free medium containing DCFH-DA (10 µM) was added for further incubating for 1 h. Cells were washed by with 0.9% NaCl solution, and then fixed by 4% paraformaldehyde for 20 min. Cellular nucleus was stained by DAPI dye for 10 min, and washed by 0.9% NaCl solution for 3 times. Samples were characterized using confocal laser scanning microscopy (TCS SP8 STED 3X).

To measure the mitochondrial membrane potentials $\Delta\Psi_m$, 1×10^4 cells were seeded onto coverslips as described above. After cells were treated by PM upper solution for 48 h, cells were stained using Mitochondrial Membrane Potential Assay Kit with JC-1 (Cat.M8650, Solarbio Life Science Company). The characterization protocols were performed using manufacturer's manuals. Here, the positive control for mitochondrial dysfunction was treated using CCCP (Carbonyl cyanide 3-chlorophenylhydrazone). Cellular nucleus was stained using DAPI and fixed using 4% paraformaldehyde. Samples were characterized using confocal laser scanning microscopy (TCS SP8 STED 3X).

2.6 Total RNA extraction, RNA sequencing and real time-PCT assay

1×10^6 Cells were seeded into 6-well microplate, and then incubated with solid particles for 48 h at two different intervention dosage: 250 µg/mL and 500 µg/mL. The total RNA of samples were collected using Total RNA Extraction Kit (Solarbio life sciences, R1200) under manufacture's instruction. Quantification of total RNA samples was employed using Nanodrop700 (ThermoFisher, MA, USA) to evaluate the quality. RNA-seq was performed by Illumina Hiseq 2500 (Novo Gene company, Tianjin, China). The screening throughout is 150 bp and 6.0 G. The RNA raw data was processed by Novo gene and we obtained the gene expression count matrix for further analysis. qRT-PCR assays were conducted using Roche LightCycler 480 system, and the designation of primers was conducted in NCBI website. All the RNA seq data were deposited to GEO website and released now (GEO series NO. GSE186322, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE186322>). The primer sequences were listed as follows:

Table 1
Primer sequences utilized in this study.

Genes	Forward sequence (5' to 3')	Reverse sequence (3' to 5')
<i>IL1A</i>	GCCTGAGTCAGCAAAGAAGTC	TGCAGTCCTCATGAAGTGGG
<i>TM7SF2</i>	TGCCTCATGAATGCTGTTGG	GCTGTGGGGATGGTCTCAAG
<i>PMVK</i>	CCACTCAAGGAACAGTACGCT	CGTGTCACCTCACCAGCCAGAT
<i>MVK</i>	GGTTCATGGCAAGGTAGCACT	CCTGCTCCAGAAAGCTCGTA
<i>FDPS</i>	GTTCTACCTCCCTGTGGCTG	GGTCGAGGTAATCGTCCTGG
<i>LSS</i>	CCTGAGGATCTGTACACGCC	GGTTTTTCGAGATCGGGCCAA
<i>MVD</i>	CCTTCCCGCCCATCTCTTAC	ACGTGTATGCCACCTTGGTC
<i>HMGCR</i>	AGGTGTATCCATTCGCCGAC	AACAGGCTCCCATCACCAAG
<i>SOD1</i>	GCATGGATTCCACGTCCATC	GGTCTCCAACATGCCTCTCTT
<i>NDUFA3</i>	ACTCGGAGAGGGAAGACTCTG	CCGGCAGAATTATAGCGAGGC
<i>CAT</i>	GTGGAGACGTGAGAACCGAG	TCAGGACATCGGGTTTCTGC
<i>ACTB</i>	CCGACTCCCTTCGTAGTTCG	CCACCATCACGCCCTGG

2.7 Bioinformatics analysis

Gene ontology (GO) analysis was conducted using the database g: Profiler (<https://biit.cs.ut.ee/gprofiler/gost>). Protein-protein interaction network analysis was employed to analyze the enriched terms using STRING website (<https://www.string-db.org/>). The minimum required interaction score was set as 0.700 and method of network clustering is kmean clustering method. The PPI network images were obtained using CytoScape 3.8.2 software. Gene set enrichment analysis (GSEA) was performed using GSEA v4.1.0 software with molecular signatures database (<http://www.gsea-msigdb.org/gsea/index.jsp>). All the parameters were set as default. The threshold values considered as significance in this study were set as: false discovery rate q-value (FDR) < 0.25, p-value < 0.05, normalized enrichment score (NES) > 1.0. Venn diagram of DEGs and genes was performed using Venn website (<https://www.vandeppeerlab.org/tools/venn-diagrams>). The volcano plot was conducted using Rstudio software. All the statistical analysis were performed using Graphpad prism 9.0.

3 Results

3.1 Properties of sandstorm silica particles

In the north China region, the air pollution mainly contains particulate matter (PM) and organic compounds, which are majorly originated from dust and industrial activities[36]. To obtain PM samples, we collected the particles samples nearby the Yellow river (Fig. 1A) using the natural air descending

method, which is the major resource of particle pollution in north China cities. We firstly examined the morphology of these particles using scanning electronic microscopy (SEM), and we can observe that size distribution of these samples was ranged from ten nanometers to micrometer levels (Fig. 1B). This data suggests that these particles will not only induce mechanical damage, but also generate biointerface interaction between cells and PM particles.

Firstly, infra-red (IR) spectra were employed to examine these samples compared to commercial silica powder. As shown in Fig. 1C, the presence of these peaks, i.e. 530 cm^{-1} , 687 cm^{-1} , 1045 cm^{-1} , and 2074 cm^{-1} , in IR spectrum of PM particles indicated that organic chemicals were observed, implying that these particles may absorb organic chemicals or contains the organic components. Then, X-ray photoelectron spectroscopy (XPS) was employed to examine the elemental components. As shown in Fig. 1D, elements, for example carbon, sodium, aluminium, calcium and nitrogen, were observed in XPS spectra, except silicon and oxygen. By analyzing the N1s narrow scanning spectra (Fig. 1E), the further fitting results of nitrogen spectrum indicated that these organic chemicals mainly contain the amide (399.8 eV) and aromatic (400.95 eV) chemical bonds. Moreover, The C1s narrow scanning spectra (Fig. 1F) clearly showed the presence of C = O (289.25 eV), C-O (286.45 eV) and C-C (284.75 eV) chemical bonds. These results confirmed the presence of organic chemicals adsorbed onto PM particles.

3.2 RNA sequencing and gene ontology analysis

To explore the cellular toxicity of PM particles, endothelial cells were treated by PM and examined the dosage-dependent cell survival rate for 48 h. As shown in Fig. 2A, IC_{50} value of cellular toxicity is approximately $500\text{ }\mu\text{g/mL}$, indicating the low toxicity of these particles on endothelial cells. However, cell viability assays cannot precisely display the biological impact of PM particles on endothelial cells. Therefore, we performed the RNA sequencing technique to identify the dosage-dependent transcriptome levels of endothelial cells after treating with particles ($250\text{ }\mu\text{g/mL}$ and $500\text{ }\mu\text{g/mL}$, respectively). The quality of RNA sequencing data was showed in SI Fig.S1-2, which confirmed that quality of these RNA samples can be performed in the following analysis. Volcano plot displayed the expression profile of detected genes after treated with PM particles compared to control group. The threshold value to identify the significantly differentially expressed genes (DEGs) was set as $|\text{Log}_2\text{FoldChange}| > 1.0$ & $p\text{-adj} < 0.05$ (highlighted by green color). For $250\text{ }\mu\text{g/mL}$ treatment group (Fig. 2B), only two genes were identified as DEGs, i.e. *ABCG1* and *DAD1*. *ABCG1* is one critical member of ABC transporter to participate into lipid homeostasis process[37], and *DAD1* is the anti-apoptotic genes located at ER[38]. For $500\text{ }\mu\text{g/mL}$ treatment group (Fig. 2C), 190 up-regulated and 56 down-regulated genes are identified as DEGs, which are displayed in heatmap (Fig. 2C). These results suggested that these DEGs may play a critical role in response for PM treatment or inducing cellular toxicity.

To further determine the biological effect of PM particles on endothelial cells, gene ontology (GO) analysis was employed at molecular pathway levels. For $250\text{ }\mu\text{g/mL}$ treatment group, GO analysis revealed that lipid-regulated process and macromolecular biosynthesis may be disrupted to affect the molecular network (SI Fig.S3), including regulation of lipid metabolic process, regulation of sterol

transport, cellular macromolecules biosynthetic process, protein N-linked glycosylation via asparagine, and protein N-linked glycosylation. When interventional dosage was increased to 500 µg/mL, top 10 enriched terms strongly revealed that higher PM particles may disrupt lipid-related pathways, especially for cholesterol process (SI Fig.S4), i.e. regulation of cholesterol metabolic process, mevalonate pathway, cholesterol biosynthesis pathway, cholesterol biosynthesis, cholesterol metabolism, regulation of sterol biosynthetic process, regulation of cholesterol biosynthetic process. Thus, these results implied that PM particles may disrupt sterol-related pathways to induce cellular toxicity.

3.3 Exposure to high concentration PM particles disrupts cholesterol metabolism pathway

GO results demonstrated that exposure to PM particles may affect cholesterol-related pathways. To further examine the biological effect of PM particles, gene set enrichment analysis (GSEA) was employed to identify critical pathways in this process. As shown in Fig. 3A, GSEA clearly revealed the 10 top-ranked pathways, which included GPCR class C metabotropic glutamate pheromone, cytoplasmic ribosomal proteins, cytokines and inflammatory response, retinoblastoma gene in cancer, nephrogenesis, metabolic reprogramming in colon cancer, miRNA targets in ECM and membrane receptors DNA replication, pathogenesis of SARS-CoV2 mediated by NSP9/NSP10 complex, cholesterol metabolism includes both Bloch and Kandutsch-Russell pathways. Among these pathways, cholesterol metabolism pathway was presented in Fig. 3B, in which ABC-related transporters focused on cholesterol efflux.

GSEA analysis (Fig. 3C) showed that the “cholesterol metabolism pathway” was significantly down-regulated in PM-treated cells, owing that the low normalized enrichment score (NES) is less than -1.8. By overlapping the DEGs set and cholesterol metabolism pathway-related gene set (Fig. 3D), 6 DEGs were identified, i.e. *TM7SF2*, *PMVK*, *MVK*, *FDPS*, *LSS*, and *MVD*. Heatmap displayed that these DEGs were significantly down-regulated (Fig. 3E). Using qRT-PCR assays (Fig. 3F), we observed that these DEGs were corroborated as significant down-regulation. *HMGCR* plays a critical role in mevalonate biosynthesis, which is an essential molecule for cholesterol biosynthesis[39]. We examined time-dependent mRNA expression levels of *HMGCR* gene after PM treatment, and these results confirmed that PM can significantly decrease the expression level of *HMGCR* gene (Fig. 3G). To examine the concentration of free cholesterol in cells, quantitative measurement revealed that PM can significantly down-regulate free cholesterol concentration (Fig. 3H). These results confirmed that PM particles can inhibit cholesterol metabolism pathway.

3.4 Exposure to high concentration PM adsorbate up-regulates ROS to lead OXPHOS dysfunction

Components of PM particles are complex and contain amount of inorganic or organic entities, which are absorbed[40, 41]. To examine critical components of PM particles to lead cellular toxicity, purity SiO_2 particles, PM particles, washed PM particles and adsorbate solution (upper solution) obtained by washing PM. Released lactate dehydrogenase (LDH) can be as a critical biomarker to examine the integrity of cell membrane after exterior intervention[42]. As shown in SI Fig.S5, LDH assays revealed that PM or upper

solution did not damage integrity of cell membrane, implying that the cellular toxicity of PM particles is not originated from cell membrane damage.

Adsorbate solution was obtained using the centrifugation method (as shown in Fig. 4A). By treating cells with washed PM and upper solution, we can find that the cell viability was significantly increased with different concentrations (Fig. 4B). To confirm that adsorbate can lead to cellular apoptosis, we performed the flow cytometry to examine apoptotic percentage. Flow cytometry assay result clearly revealed that PM adsorbate significantly increased cellular apoptotic percentage from 0.83–9.32%, confirming the PM adsorbate led to cellular apoptosis. These results implied that the cell toxicity is mainly originated from PM adsorbate.

ROS always plays a critical role in the cellular apoptosis process[43, 44]. Here, we examined cellular ROS levels after upper solution treatment using H2DCFDA molecular probe. As shown in Fig. 4C, these results clearly showed that cellular ROS level was significantly up-regulated after treatment. Furthermore, JC-1 molecular probe revealed that PM adsorbate induced dysfunction of mitochondrial membrane potential (Fig. 4D). GSEA term (Fig. 4E and SI Fig.S7) revealed that oxidative phosphorylation (OXPHOS) pathway was substantially down-regulated in PM treated cells. Dosage-dependent ATP down-regulation (Fig. 4F) was consistent with GSEA, JC-1 and ROS assays. These results suggested that PM, mainly originating from PM adsorbate, can up-regulate cellular ROS level and mitochondrial membrane potential disorder to impair OXPHOS.

3.5 Impaired GSSG/GSH and NADP/NADPH ratios lead to oxidative stress

Owing to the disorder of oxidative phosphorylation in PM-treated cells, we sought to explore whether GSH and NADPH levels were linked to the disorder of oxidative phosphorylation. As shown in Fig. 5A, GSH and NADPH play a central role in maintaining oxidative homeostasis[45]. After PM treatment, ROS-related enzymes (Fig. 5B), for example, *SOD1* and *CAT*, were significantly up-regulated, consistent with the above mention. Then, we examined GSSG/GSH and NADP/NADPH ratios after washed PM and upper solution treatment (Fig. 5C-D), and assay results revealed that upper solution treatment can significantly increase GSSG/GSH and NADP/NADPH ratios, demonstrating the increases of oxidative stress. Our results suggested that disorder of GSSG/GSH and NADP/NADPH ratios leads to oxidative stress and further induces cellular apoptosis.

Moreover, GSEA ranking results (Fig. 4C) also implied the association with an inflammatory response. GSEA term (SI Fig.S8) indicated that the “cytokines and inflammatory response” pathway was up-regulated in PM-treated cells. By overlapping between gene set and DGEs (SI Fig.S8B), only *IL1A* was strongly affected among the gene set of inflammatory response (SI Fig.S8C). Using RT-qPCR, mRNA expression level of *IL1A* gene was significantly up-regulated with ~ 1.5 folds. This result suggests that bio-interaction between PM particles and cells may induce the *IL1A*-related inflammatory response.

4 Discussions

Particulate matter (PM) is the major component for environment pollution and further leads to respiratory system-related diseases[46–49]. In our study, we attempted to explore the toxicological impact of PMs on lung epithelial cells, which may be associated with lung-related diseases. PM samples were collected nearby Yellow river, which is the major PM resource for Kaifeng City, a Chinese city located at north China, in winter. These particles were characterized using SEM, XPS and IR techniques (Fig. 1C, 1D, 1E, 1F), and results indicated that these PMs contain amount of adsorbate, which include Na^+ , Ca^{2+} , N , Al^{3+} , etc. To evaluate the effect of different components of PM on epithelial cells, PM particles, washed PM particles and adsorbate separated from PMs were examined using the MTT method (Fig. 2A), which displayed the dosage-dependent toxicity. Moreover, wash PMs with the removal of adsorbate displayed lower toxicity compared to PMs. These results suggested that toxicity of PMs may be mainly originated from PM adsorbate.

To systematically explore the effect of PM particles on epithelial cells, we utilized RNA sequencing technique to examine the expression profile of total genes after treatment (Fig. 2B, 2C). The dosage-dependent RNA sequencing data present that exposure to higher PMs can significantly affect genetic profiles. To assess this effect on epithelial cells, GO (SI Fig.S4B) and GSEA analysis (Fig. 3A) results implied that exposure to higher PMs can affect cholesterol metabolism-related pathways. To corroborate this hypothesis, we examined the gene expression of critical genes using qRT-PCR (Fig. 3F), and results indicated that “cholesterol metabolism pathway” was substantially down-regulated. Other critical biomarkers, *HMGCR* (Fig. 3G) and free cholesterol (Fig. 3H) also displayed the time-dependent attenuation, confirming the down-regulation of cholesterol metabolism pathway.

To determine what drives PM toxicity, we firstly examined the ROS levels (Fig. 4C) and mitochondrial membrane potential (Fig. 4D), which always led the toxicity and further induced cell apoptosis[43, 50]. The confocal laser scanning microscopy indicated that adsorbate solution (upper solution) can significantly increase cellular ROS level and dysfunctional mitochondrial membrane potential. GSEA analysis (Fig. 4E) and ATP concentration (Fig. 4F) is consistent with CLSM results, confirming the disorder of oxidative phosphorylation. To our knowledge, disorder of oxidative phosphorylation is always associated with oxidative stress, and higher oxidative stress can induce expression of disease-related downstream genes, for example, inflammation response[51], DNA damage[52], chromosomal aberrations[53]. Our experimental results indicated that the adsorbate solution can significantly promote expression levels of anti-oxidation-related genes (Fig. 5B), i.e. *SOD1* and *CAT*, and oxidative stress biomarkers (Fig. 5C, 5D), GSH and NADPH, displayed a higher oxidation states. These results demonstrated that PM adsorbate solution can up-regulated cellular oxidative stress.

Cholesterol synthesis and homeostasis play an important role in the maintenance of physical processes, including bile acid and steroid hormone synthesis[54]. Dysfunction of cholesterol pathways is highly associated with various diseases, for example cardiac health[55], nervous system diseases[56–58], and tumorigenesis[54, 59, 60]. In the respiratory system, cholesterol homeostasis can modulate the angiogenic sprouting[61] and affects the nitric oxide synthase in the vascular system[62, 63]. The external intervention will lead the immunological response[64–66], and further leads the respiratory

system diseases. Moreover, cholesterol homeostasis also plays a critical role in the assistant of virus infection[67, 68]. Our results showed that PMs can induce the down-regulation of the cholesterol-related pathway. These results implied that long-term exposure to PM may promote respiratory diseases by the disorder of cholesterol metabolism pathways.

5 Conclusions

In conclusion, our results showed that exposure to the higher concentration of PM induced dosage-dependent cellular toxicity to respiratory endothelial cells. To further explore the molecular mechanism of PM-induced toxicity, dosage-dependent RNA sequencing clearly reveals that exposure to higher PM can lead to dysfunction of cholesterol metabolism pathway. Our investigation indicated that PM adsorbate is a major factor to lead to cellular toxicity by up-regulating cellular ROS levels and disorder of mitochondrial membrane potential, which further lead oxidative stress through impairing GSH and NADPH levels in vivo. Nevertheless, we systematically report that PM particles collected from atmosphere can alter cholesterol metabolism with exposure to the higher concentration of PM particles, and this exposure can lead ROS-lead oxidative stress through dysfunction of mitochondrial membrane potential, GSSG/GSH, and NADP/NADPH. Our investigation provides the potential intervention approach to rescue health risk of higher PM exposure.

Declarations

The Supporting Information

Experimental condition for LDH and cell apoptosis; Table for RNA sequencing data; RNA expression states of total samples; PCA cluster of RNA sequencing data; GO analysis of *ABCG1* and *DAD1* genes; Protein-protein interaction network of DEGs and GO analysis; LDH release assay after PM treatment; flow cytometry after PM treatment; overlapping between DEGs and gene set; GSEA reveals the up-regulation of inflammation response pathways and expression level of *IL 1A* gene.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All the RNA seq data were deposited to GEO website and released now (GEO series NO. GSE186322, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE186322>).

Competing interests

The authors declare no conflict of interest.

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Author contributions

Conceived and designed the experiments: T.Y., Y.L., X.C. and H.W.. Performed the experiments: Y.L. and R.D.. Analyzed the data: Z.D. and L.Q.. Contributed reagents/materials/analysis tools: H.W.. Wrote the paper: T.Y. and H.W.. Support financially and administratively, final approval of manuscript: T.Y..

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Figures

Figure 1

Properties of collected particulate matters. **A.** Geographical information of sampling site. **B.** SEM morphology of sandstorm particles. Scale bar is 1 μm . **C.** IR spectrum of sandstorm particles and silica. **D.** XPS spectra of sandstorm particles. The major elements were labeled. **E.** N 1s narrow scanning spectra from 390 eV to 410 eV. **F.** C 1s narrow scanning spectra from 282 eV and 292 eV. The major components of narrow scanning spectrum were analyzed by fitting.

Figure 2

Higher concentration exposure of PM particles induces cellular toxicity. **A.** Cell viability of endothelial cells depends on different intervention dosages. IC_{50} of PM on endothelial cells was approximately 505 $\mu\text{g/mL}$. **B.** Volcano plot presenting expression levels of differentially expressed genes between 250 $\mu\text{g/mL}$ treated group and control group. *ABCG1* and *DAD1* are enriched in up-regulated genes with threshold value ($|\log_2\text{FoldChange}| > 1.0$ & $\text{FDR p-adj} < 0.05$). DEGs were highlighted by green color. **C.** Volcano plot presenting expression levels of differentially expressed genes between 500 $\mu\text{g/mL}$ treated group and control group. 56 genes and 190 genes are identified as significantly up-regulated genes and down-regulated genes. Cholesterol metabolism-related genes identified as DEGs were labeled by green color (threshold value, $|\log_2\text{FoldChange}| > 1.0$ & $\text{FDR p-adj} < 0.05$). **D.** Heatmaps of up- and down-

signatures between 500 µg/mL PM treated and control group. Expression levels of relative genes are presented by Z-score, and scale bar was displayed from -2 to +2. N=3 per group.

Figure 3

Higher exposure of PM induces the dysfunction of cholesterol metabolism pathway. **A.** Canonical pathway analysis demonstrated the inhibition of cholesterol metabolism includes both bloch and kandutschrussell pathway. **B.** Schematic illustration of cholesterol synthesis pathway and export. **C.** GSEA enrichment plot shows down-regulation of cholesterol metabolism pathway. **D.** Venn diagram showing the overlaps between cholesterol metabolism pathway gene set and DEGs after treatment with 500 µg/mL for 48 h. **E.** Heatmap of enriched genes obtained from Venn diagram between 500 µg/mL treated group and control. N=3. Expression profile of DEGs was normalized by Z-score method. **F.** q-RT-PCR of DEGs cholesterol metabolism pathways between 500 µg/mL treated group and control group. N=3 biological independent samples. ***, p<0.001; ****, p<0.0001. **G.** Time-dependent qRT-PCR of *HMGCR*

gene, which focused on synthesis of mevalonate from HMG-CoA. N=3 biological independent samples. *, p<0.05; **, p<0.01; ***, p<0.001. H. Quantification of time-dependent free cholesterol concentration incubated with 500 µg/mL PM particles compared to control group. N=10 biological independent samples. *, p<0.05; ***, p<0.001.

Figure 4

PM adsorbate leads cellular oxidative stress and mitochondrial dysfunction. **A.** Schematic illustration of experimental protocols to obtain PM adsorbate solution. **B.** Dosage-dependent cellular toxicity of washed PM particles and PM adsorbate solution (250µg/mL and 500 µg/mL). N=3 biological independent samples, data are present as Mean ± SD. *, p<0.05; **, p<0.01. **C.** Representative micrographs displaying

cellular ROS levels after treated with PM upper solution using H2DCFDA. Scale bar is 50 μ m. **D.** Representative micrographs showing the mitochondrial dysfunction after treated with PM upper solution for 24 h. Cells treated with CCCP is as positive control. Scale bar is 20 μ m. Cellular nucleus is stained by DAPI. **E.** GSEA enrichment plot shows down-regulation of oxidative phosphorylation pathway. **F.** Quantification of time-dependent (24 h, 48 h) free ATP concentration incubated with 500 μ g/mL PM upper solution compared to control group. Cells treated with CCCP is as positive control. N=4 biological independent samples, data are present as Mean $\pm$ SD. **, $p<0.01$; ***, $p<0.001$.

Figure 5

PM treatment causes oxidative stress by increasing oxidation/reduction ratio. **A.** Schematic illustration of GSH/GSSG and NADP/NADPH conversion in *in vivo* regulated by ROS. **B.** mRNA expression levels of *SOD1* and *CAT* gene after upper solution treatment. **C.** Impact of PM on GSSG/GSH ratio after treatment for 48 h. **D.** Impact of upper solution on NADP/NADPH ratio after treatment for 48 h. Data was present by Mean $\pm$ SD (n=3); ns, no significance; **, $p<0.01$; ***, $p<0.001$

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