The Cytotoxicity of PM2.5 and its Effect on the Secretome of Normal Human Bronchial Epithelial Cells

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Abstract

Exposure to airborne fine particulate matter ($PM_{2.5}$) induced various adverse health effects, such as metabolic syndrome, systemic inflammation and respiratory infection. Many works have studied the influence of $PM_{2.5}$ exposure to intracellular proteome and the underlying mechanism. But the extracellular proteome changes under $PM_{2.5}$ exposure, and the correlation between secretome changes and $PM_{2.5}$-induced cytotoxicity remains confusing. Herein, the cytotoxicity of $PM_{2.5}$ on normal human bronchial epithelia cells (BEAS-2B) was evaluated and the secretome profile of BEAS-2B cells before and after $PM_{2.5}$ exposure was investigated. The secretion of 83 proteins (58 up-regulated and 25 down-regulated) was differentially expressed upon $PM_{2.5}$ treatment. In addition to apoptosis, extracellular matrix (ECM) organization, complement activation and RNA splicing were also found to be involved in $PM_{2.5}$ mediated cytotoxicity. These results provide an insight into the underlying mechanism of respiratory injury caused by $PM_{2.5}$.

Introduction

Air pollution, especially pollution of airborne fine particulate matter ($PM_{2.5}$), can cause adverse health effect and result in many diseases, such as lung disease, heart disease, stroke, hypertension, and cardiovascular diseases (Englert 2004; Li et al. 2003; Lippmann 2014; Pope et al. 2002). In the past decade, many time-series air pollution epidemiological studies have assumed that long-term exposure to $PM_{2.5}$ greatly increases the incidence of lung-related diseases (Adam et al. 2015; Kloog et al. 2013). It is also reported that the correlation between $PM_{2.5}$ with the mortality risk of respiratory diseases is much higher than that between $PM_{2.5}$ and the mortality risk of cardiovascular disease (U.S.EPA 2020). Although many studies have reported that $PM_{2.5}$ can trigger the increase of intracellular oxidative stress, which play critical role in the development of lung diseases (Oh et al. 2011; Valavanidis et al. 2013; Xu et al. 2021), the biological mechanisms for respiratory injury caused by $PM_{2.5}$ exposure are not fully elucidated.

Recently, proteomics has been widely used to explore the toxic effects of $PM_{2.5}$. Xue et al. found that MAPK and PI3K/AKT pathways may be involved in $PM_{2.5}$-induced lung injury (Xue et al. 2019). Huang et al. found abnormal protein synthesis and degradation were major factors in human lung cytotoxicity of water soluble $PM_{2.5}$ extracts by MALDI-TOF-TOFMS (Huang et al. 2015). In addition to intracellular proteins motioned above, extracellular proteins, such as secretory proteins and exsomal proteins, have also been reported to be involved in the $PM_{2.5}$ induced diseases. The latest research found that $PM_{2.5}$ exposure promotes the secretion of pro-inflammatory cytokines in human lung carcinoma cell (A549) (Zhao et al. 2019). Xu et al. found that the exosomes secreted by A549 lung cancer cells promoted the progression of A549 tumor in vivo after $PM_{2.5}$ treatment (Xu et al. 2019a). However, few studies have explored the extracellular proteome changes of respiratory tract cells exposed to $PM_{2.5}$.

In this study, a strategy combing metabolic labeling, protein “equalization,” protein fractionation, and filter-aided sample preparation (FASP), called MLEFF, was applied to explore the correlation between secretome changes of BEAS-2B cells and cytotoxicity caused by $PM_{2.5}$ exposure. The secretome analysis demonstrated that some biological processes, such as apoptosis, extracellular matrix (ECM) organization, complement activation and RNA splicing were significantly interfered in BEAS-2B cells exposed to $PM_{2.5}$. The results will improve our understanding of the mechanism of respiratory injury caused by $PM_{2.5}$ exposure.

Materials And Methods

Sample collection and component analysis of $PM_{2.5}$

$PM_{2.5}$ samples were collected on quartz fiber membrane using air monitoring systems (AMS®, Pesaro, Italy) from November 2016 to March 2017 in Dalian, a city in northeast China. For the collection of inorganic components, each membrane were weighed and cut into 2cm×2cm squares. The scraps of paper were submerged in ultrapure water (Millipore, Darmstadt, Germany) followed by sonication (20min per time, 6 times). For the collection of heavy metal elements, each membrane were weighed and cut into 1cm×1cm squares and submerged in aqua regia followed by microwave digestion. Then, the inorganic components and heavy metal elements were detected by ion chromatography (Thermo Fisher, Waltham, MA, US) and ICP-MS (PerkinElmer NexION 300D, Santa Clara, CA, US). The 5cm×5cm cuts of each membrane were picked up for collection of organic components. The organic components such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) were measured using a gas chromatograph equipped with a mass spectroscopy detector (Thermo Fisher) and gas chromatograph coupled with a DFS mass spectrometer (Thermo Fisher). The 9 cm^2 pieces of each membrane were collected and the sonication suspensions were evaporated under vacuum. The vacuum dried powder were blended in ultrapure water to make stock solution (500 mg mL^{-1}), which was stored at -80 °C for further exposure. Before usage, stock solution were UV irradiated for 2h to prevent contamination.

Cell culture and $PM_{2.5}$ exposure

Human lung-bronchial epithelium cells BEAS-2B (ATCC®,CRL-9609™) were cultured in BEBM medium (LONZA CC-3170, Basel, Switzerland) at 37 °C, supplied with 5% CO_2. For proteomic analysis, cells were labeled using stable isotope labeling with amino acids in cell culture (SILAC) medium (Thermo Fisher). For the “medium” labeling media, SILAC DMEM medium were supplemented with [4,4,5,5-D_4] L-lysine (100 μg/mL) and [$^{13}C_6$] L-arginine (100 μg/mL), 10% dialyzed fetal bovine serum (FBS), and 1% penicillin/streptomycin mixture. For the “heavy” labeling media, only [4,4,5,5-D_4] L-lysine and [$^{13}C_6$] L-arginine were replaced with [$^{13}C_6$, $^{15}N_2$] L-lysine and [$^{13}C_6$, $^{15}N_4$] L-arginine. The control group and exposure groups were grown in the “heavy” and “medium” media, respectively. After 24 h, the cells and conditioned media were both harvested and were mixed based on equal number of each cell type. For

exposure treatment, the vacuum dried $PM_{2.5}$ particles were dilute to 1000 μg mL^{-1} with medium. By using CCK-8 assay (Dojindo, Mashikimachi, Japan), the 24h-IC_{50} of $PM_{2.5}$ was calculated with the dosage range of 5–500 μg mL^{-1} (9 dose groups) to ascertain the appropriate exposure dosage. Finally, BEAS-2B cells for in vitro exposure were treated with 10, 50, 100 and 250 μg mL^{-1} of $PM_{2.5}$ for 24 h base on the IC_{50} value.

Cell viability and apoptosis assay

BEAS-2B cells were seeded in 6-well plates and exposed to different doses of $PM_{2.5}$ for 24 hours. After exposure, Calcein-AM/PI Double Staining Kit and Hoechst 33258 (Dojindo) were used to identify living cells, living cell nucleus and dead cells. Concisely, the cells were collected from each well (1×10^6 cells) and resuspended in 1 mL PBS, followed by adding 500μl staining solution. After incubation for 15 min at 37 °C, live cells with green fluorescence and dead cells with red fluorescence were observed (E_x = 490 nm; E_m = 545 nm) by fluorescence microscope. Cell and nuclei were stained with Hoechst 33258 for 15 minutes, and stained nuclei were observed (E_x = 350 nm; E_m = 460 nm) by fluorescence microscope. For apoptosis assay, after 24 hours exposure, the apoptotic rate of cells from different groups was determined with Annexin V-FITC/PI apoptosis kit (Liankebio, Hangzhou, China). Briefly, the cells were resuspended in the 500 μL 1× binding buffer. The 5 μL Annexin V/FITC and 10 μL PI were added, respectively. After incubation for 5 minutes at room temperature in dark, the cells were examined by the Automatic Personal Cell Sorter SH800 (SONY, Tokyo, Japan). Annexin V-FITC was detected by FITC channel (Ex = 488 nm; Em = 530 nm) and PI was detected by PI channel (E_x = 535 nm; E_m = 615 nm). Data were analyzed using the Cell Sorter Software (SONY).

ROS assay

The ability of $PM_{2.5}$ to generate intracellular ROS in BEAS-2B cells were measured by using the fluorescent indicator 2',7'-dichlorodihydrofluorescein diacetate (DCFH/DA), in which fluorescence appears if reactive oxygen species (ROS) are present, according to the instructions of the ROS assay kits (njjcbio, Nanjing, China).

Secretome analysis

The flow chat of secretome analysis was illustrated in Fig. 1. BEAS-2B cells in control groups (heavy labeled) and exposure groups (medium labeled) were treated with 0 μg and 10 μg mL^{-1} of $PM_{2.5}$ for 24 h to study the secretome changes caused by $PM_{2.5}$ exposure. conditioned media were processed according to our previously reported MLEFF strategy and one sample (Weng et al. 2016),by which eight fractions were collected from a single sample. All experiment were performed in three biological replicates and digested peptides were stored at −80 °C for further analysis.

The peptides were analyzed with a nano-RPLC-MS/MS on a Q-Exactive MS coupled with an Easynano LC system (Thermo Fisher). Buffers A is 2% acetonitrile, 98% water, and 0.1% formic acid whereas Buffer B is

98% acetonitrile, 2% water, and 0.1% formic acid. The gradient was comprised of 90 min of 6%–22% buffer B, followed by a 20 min of 22%–35% buffer B. The spray voltage was set to 2.5 kV, and temperature of the ion transfer capillary was set to 275 °C. The 10 most intense ions were subjected to HCD fragmentation with normalized collision energy at 28%. The MS scans were performed at a resolution of 70 000 from m/z 300 to 1800, and the data were acquired in profile mode. The MS/MS scans were performed at a resolution of 17 500, and the data were acquired in centroid mode using a 20 s exclusion window. The unassigned ions or those with a charge of +1 and > +7 were rejected. One microscan was acquired for each MS and MS/MS scan. Raw data were processed by Proteome Discoverer (PD, version 1.4.1.14) with Mascot (2.3.2) and searched against the UniProtKB Human complete proteome sequence database (release 2017_06, 24,148 entries). The reverse sequences were appended for an FDR evaluation. The mass tolerances were set at 0.5 Da for the parent ions and at 10 ppm for the fragments. The peptides were searched using tryptic cleavage constraints, and a maximum of two missed cleavages were allowed. The minimal peptide length was six amino acids. Carbamidomethylation (C, + 57.0215 Da) was used as the fixed modification. Oxidation (M, + 15.9949 Da) and acetylation (protein N-termini, + 42.0106 Da) were searched as variable modifications. Two SILAC-based labels, (Lys 4, + 4.0251 Da) and (Lys 8, + 8.0142 Da), were used as variable modification. The peptide and protein identifications were filtered by PD to keep the FDR less than 1%. At least one unique peptide was required for each protein identification. The clustering strategy of extracellular proteins as follows: a protein was defined as a classical secreted protein when it was annotated to contain a “signal peptide” or had the keyword “secreted” in UniProtKB (http://www.uniprot.org), or it was predicted by the Signal P 4.1 server to had a “signal peptide”; those proteins that did not contain a “signal peptide” and were predicted by Secretome P 2.0 with an NN score > 0.5, were defined as non-classical secreted proteins; the exosomal proteins were matched by the ExoCarta database (http://exocarta.org). The Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analysis were performed using DAVID 6.8 (https://david.ncifcrf.gov). ECM-related proteins were matched by the matrisome database (http://matrixdb.univ-lyon1.fr). Interaction network analysis was generated using STRING and visualized using the Cytoscape.

Western blotting analysis

Sample of BEAS-2B cells (5×10^6) were collected and extracted with 4% SDS containing 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich, MA, US). After centrifugation at 15000 rpm at 4 °C for 20 min, the supernatant was collected and the protein concentration was determined by bicinchoninic acid (BCA) assay (Beyotime, Shanhai, China). 20 μg of proteins were separated on 12% SDS-PAGE, and then transferred onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad, CA, US). The PVDF membrane was blocked in Tris-buffered saline containing 5% milk for 1 h at room temperature, followed by incubation with the antibodies including SFEP4 and CD9 (Genetex, CA, US) overnight at 4 °C. After incubation with anti-HRP conjugate (Thermo, MA), bands were visualized with a chemiluminescence reagent (Thermo Fisher) and detected using the ChemiDoc system (Bio-Rad).

Results

Components analysis of $PM_{2.5}$

We analyzed water-soluble ions, heavy metals and organic components in $PM_{2.5}$ particles (data not shown). The elements analysis showed SO_4^{2-}, NH_4^+, NO_3^- and Cl^- were higher than other water soluble ions. Toxic heavy metals (including Mn, Cd, Cr, Ni, Mn, Ti, As and Pb) were detected in $PM_{2.5}$. The content of PAHs with 5- and 6-ring included BbF, BkF, IcbP and BghiP, which had the most contribution of total $\sum_{16}$PAHs. The 1234678-HpCDD, OCDD, OCDF and 234678-HpCDF in $PM_{2.5}$ particles were the most abundant of $\sum_{17}$PCDD/Fs. The PCB-157 was the most abundant of $\sum_{14}$PCB in $PM_{2.5}$ particles.

Cytotoxicity and oxidative stress induced by $PM_{2.5}$ exposure

To determine the exposure dose of $PM_{2.5}$, we detected the cell viability via CCK-8 assay. Significant differences compared to control were observed starting at the concentration of 1μg mL^{-1} and up to 500 μg mL^{-1}. The cell viability showed a decline along with the increase of the exposure doses (Fig. 2(a)), with the half inhibitory concentration dose (IC_{50}) at 227.1 μg mL^{-1}. All of these indicated that $PM_{2.5}$ induced more serious cytotoxicity of lung-bronchial epithelial cells. To study the mechanisms of the cytotoxicity induced by $PM_{2.5}$ exposure, the concentrations of $PM_{2.5}$ were set up at 10 μg mL^{-1}, 50 μg mL^{-1}, 100 μg mL^{-1} and 250 μg mL^{-1}, fluctuated based on IC_{50} value, as well as the time of exposure was 24 hours.

To further explore $PM_{2.5}$-induced toxic effect in BEAS-2B cells, we measured the level of intracellular ROS. As shown in Fig. 2(b), the intracellular ROS level in exposure groups increased significantly compared to the control group. The fluorescence intensity of $PM_{2.5}$ exposure groups was 1.08-fold, 1.16-fold, 1.29-fold and 1.61-fold higher than that of control group, respectively. Intracellular ROS level were elevated along with the increase of $PM_{2.5}$ concentration in a dose dependent manner. The variation in the intracellular ROS level revealed that $PM_{2.5}$ exacerbated the ROS generation and induced oxidative stress in BEAS-2B cell. In addition, research have shown that $PM_{2.5}$ can cause apoptosis. To investigate the apoptosis might be caused by $PM_{2.5}$ exposure in BEAS-2B cells, Annexin V-FITC/PI fluorescence double staining was used to measure the apoptotic rate of cell via the flow cytometry. In comparison with the control group, the apoptotic rates were significantly increased in $PM_{2.5}$ exposure groups, showing 7.7-fold, 15.6-fold, 23.6-fold and 28.4-fold respectively, higher than that of the control group (Fig. 2(c)). The increase in apoptotic rate revealed that $PM_{2.5}$ exposure accelerated apoptosis in the BEAS-2B cells in a dose dependent mode.

Secretome Analysis of conditioned media from BEAS-2B cells exposed to $PM_{2.5}$

To investigate the secretome changes in BEAS-2B cells after $PM_{2.5}$ exposure, a LC-MS/MS based quantitative proteomics analysis were performed. The SILAC labeled control cells (medium labelled) and treated cells (heavy labelled) were used to ensure the quantitative accuracy. The conditioned media were

subjected to our recently developed MLEFF strategy (Weng et al. 2016), overcoming the challenge to proteomics analysis of serum-containing conditioned media, where the low-abundant secreted proteins were seriously masked by high-abundant proteins derived from fetal bovine serum (FBS).

In total, 481 proteins were quantified in all three replicates (Table S-1), including 8 growth factors, 37 proteases, 15 protease inhibitors and 18 ECM structural proteins (Table S-2). Meanwhile, we analyzed the subcellular location of 184 proteins (Table S-3), which were identified in all three replicates and with t-test *p*-value less than 0.05 (n=3). Among them, **89** proteins (**48.6**%) had signal peptide and were identified as classical secreted proteins, **16** proteins were predicted as non-classical secreted proteins by Secretome P 2.0, and **47** proteins were identified as extracellular exosome based on uniprot database (Fig. 3). Collectively, **83.1**% of these quantified proteins were extracellular-region located proteins, showing this extracellular proteome profiling a real profile of cell secretion in the presence of even 10% (v/v) FBS in cell culture media.

For further analysis, proteins with ratios more than 1.5 or less than 0.67 (Log_2 ratio (exposure / control) > 0.585 or < -0.585) and *p*-value of ≤0.05 were considered as differentially expressed proteins. Base on this criterion, 58 proteins were identified up-regulated in the conditioned media of BEAS-2B cell in exposure to $PM_{2.5}$, whereas 25 proteins were down-regulated (Fig. 4).

By using the software DAVID, the Gene Ontology (GO) analysis of these differentially expressed proteins was performed (*p*-value < 0.01), as shown in Fig. 5. As expected, the top 4 ranked GO terms of cellular component categories were extracellular-related (Fig. 5(a)). The highest ranked term was extracellular exosome, indicating a huge variation in secretion of exosomal proteins. Among the enriched 9 GO terms of the molecular function category (Fig. 5(b)), the mostly enriched terms were heparin binding and receptor binding, which play important roles in the ECM organization. Biological process clustering revealed 12 GO terms, showing the most abundant terms related to cell adhesion and ECM organization (Fig. 5(c)). Other terms were also enriched, such as negative regulation of endopeptidase activity, ECM disassembly and proteolysis. Among Kyoto Encyclopedia of Genes and Genomes (KEGG) enriched from the differentially expressed proteins using DAVID software (Fig. 4(d)), the most significant pathways included ECM-receptor interaction, spliceosome, focal adhesion, and complement and coagulation cascades.

Differentially expressed proteins were mapped onto STRING to build a protein–protein interaction networks (Fig. 6) and the pathways including ECM organization, RNA splicing, regulation of complement activation, response to stimulus and secretion by cell were changed under the $PM_{2.5}$ exposure. The proteins that were clustered into pathways such as ECM organization, response to stimulus and secretion included more up/down-regulated proteins, indicating the sever dysregulations of these pathways.

Western blotting analysis

To validate the expression changes of DSG2, ECM1 and PAI1 before and after $PM_{2.5}$ exposure, their expression levels were analyzed via Western blot analysis. Significant up-regulated expression of DSG2

and down-regulated expression of ECM1 and PAI1 were detected in cells exposed to $PM_{2.5}$ (Fig. 7), which was consistent with the MS quantification.

Discussion

Chemical Characterization and Cytotoxicity of $PM_{2.5}$

As shown in our study, the viability of BEAS-2B cells decreased with increasing exposure dose, which was in a dose-response mode. This result was coincident with other studies using different cell lines such as endothelial cells and placenta cells, that the cell proliferation was inhibited by $PM_{2.5}$, indicated the $PM_{2.5}$ could have adverse effects on living cells (Rui et al. 2016; Wang et al. 2017; Kim et al. 2018).

A growing number of evidence showed that the generation of the ROS and oxidative stress played a critical character in the process of cytotoxicity of $PM_{2.5}$ (Hong et al. 2016; Deng et al. 2013). Therefore, we detected the ROS generation by $PM_{2.5}$ and our results showed that the $PM_{2.5}$ aggravated the level of intracellular ROS. Similar to our results, it was reported that exposure of $PM_{2.5}$ led to an up-regulated-ROS production and oxidative stress in fatty cells, red blood cell and airway cells (Yan et al. 2015; Xu et al. 2019b; Torres-Ramos et al. 2011). It could be seen that $PM_{2.5}$ could directly stimulate the increase of intracellular ROS level and induce oxidative damage in cells. Therefore, it is necessary to study the relationship between the composition of $PM_{2.5}$ particles versus the increased intracellular ROS level and oxidative damage. In our another study (data not shown), we found the positive correlation between heavy metals (Mn, Cd, Cr et al.) and PAHs in $PM_{2.5}$ and the intracellular ROS level, but not water-soluble ions. Some studies also demonstrated that organic ingredients and transition metals in $PM_{2.5}$ contributed most to the generation of ROS (Liu et al. 2015; Hippelein et al. 1996). The heavy metals would combined the $PM_{2.5}$ to deposit in the lung, which led to the ROS increase (Huang et al. 2018). Heavy metals such as Pb, Sb, Cd could induce adverse health effects via cytotoxicity or genotoxicity (Huang et al. 2009). Previous research found that PAHs toxicity mainly depends on high ring number (4~6 rings) PAHs (Liu et al. 2015), which was coincident with our results. Moreover, it was found that polycyclic aromatic hydrocarbons and PCDD of $PM_{2.5}$ could induce oxidative stress (Bae et al. 2010; Zhang et al. 2019). Therefore, the chemical composition might be part of the cause of oxidative stress induced by $PM_{2.5}$. Our results indicated that $PM_{2.5}$ was complex and harmful mixture which could induce adverse effects on lung-bronchial epithelial cells.

Corresponding to the cell survival rate analysis, we also found that $PM_{2.5}$ could trigger apoptosis in BEAS-2B cells and the apoptosis rate increased significantly with increasing exposure dose of $PM_{2.5}$. Similarly, a recent study showed $PM_{2.5}$ induced higher apoptosis rate than PM_{10} in macrophages (Reyes-Zárate et al. 2016). It's reported that the increased intracellular ROS could induce apoptosis by participating in signal pathways such as P53 and Caspase (Circu and Aw 2010; Johnson et al. 1996). Another research showed that $PM_{2.5}$ induce skin cells apoptosis by elevating intracellular ROS level and mitochondrial damage (Piao et al. 2018). Previous study also demonstrated that $PM_{2.5}$ could increase ROS production

in keratinocytes and inhibit the intracellular antioxidant system, resulting in cell viability decreased (Hu et al. 2017). Herein, in the current study, our results have revealed that $PM_{2.5}$ could increase intracellular ROS levels that played an important role in induction of cell apoptosis.

The secretome analysis of BEAS-2B cells exposed to $PM_{2.5}$

Toxicological experiments showed that $PM_{2.5}$ could cause oxidative damage and apoptosis in BEAS-2B cells. Consistent with this, secretome profile also showed that $PM_{2.5}$ exposure led to abnormal expression of apoptosis-related proteins. It was reported that short-term exposure to $PM_{2.5}$ induced apoptosis in human epithelia lung cells (L132) through activation of tumor necrosis factor and mitochondrial pathway (Dagher et al. 2006). In the secretome, two proteins involved in positive regulation of apoptosis, growth arrest-specific protein 1 (GAS1) and Desmoglein-2 (DSG2), were up-regulated. It is reported that GAS1 can induce cell death through an intrinsic apoptotic pathway and the down-regulation of Dsg2 protects epithelial cells from apoptosis (Zarco et al. 2012; Nava et al. 2007). Up-regulation of these proteins was consistent with the results of cytotoxicity assay, which showed that $PM_{2.5}$ significantly increased cell apoptosis rate in a dose-dependent manner. However, some inhibitor of apoptosis, such as cell cycle and apoptosis regulator 2 (CCAR2) and macrophage migration inhibitory factor (MIF), were also up-regulated in the media. CCAR2 can promote cell survival during mitochondrial stress-induced apoptosis and MIF can reduces pro-oxidative stress-induced apoptosis (Kim et al. 2017; Nguyen et al. 2003). The up-regulation of them indicated that some antiapoptotic signal cascades were also activated in BEAS-2B cells exposed to $PM_{2.5}$

In addition, the largest pathway in the protein-protein interaction networks concentrated on ECM organization and the expression of involved proteins was severely disordered. Among the 83 significantly up/down-regulated proteins, we found 36 ECM-related proteins (Table S-4), which could be divided into three subtypes, constituent proteins (22 proteins), ECM regulators (12proteins) and secreted factors (2proteins). The abnormal secretion of these proteins indicated the dysregulated ECM arrangement caused by $PM_{2.5}$ exposure. Firstly, some cell adhesion promoting molecules were increased in secretome of $PM_{2.5}$ treated cells, such as Fibulin-7 (FBLN7) and Fibulin-2 (FBLN2). They are members of the fibulin protein family, a group of cell-secreted glycoproteins that interact with other matrix proteins (Sarangi et al. 2018). Over-expression of FBLN7 and FBLN2 increased cell adhesion to ECM proteins (Olijnyk et al. 2014). Besides, Desmoglein-2 (DSG2), a transmembrane glycoprotein component of desmosomes, was also up-regulated. Desmosomes are one of the strongest cell-to-cell adhesion types and DSG2 is localized to desmosome structures at regions of cell-cell contact and functions to structurally adhere adjacent cells together (Schlipp et al. 2014). In contrast, changes in secretion of ECM regulators showed that $PM_{2.5}$ exposure led to ECM degradation. Cysteine-types peptidase cathepsin H (CTSH) was up-regulated and it involved in the degradation and remodeling of the ECM and were associated with cellular motility and adhesion. Notably, two inhibitors of matrix metalloproteinases (MMPs), extracellular matrix protein-1 (ECM1) and plasminogen activator inhibitor-1 (PAI1), were down-regulated. ECM1 can reduce the proteolytic activity of matrix metalloproteinase 9 (MMP9). A dose-dependent down-regulation of ECM1

was detected in primary human lung cells (type II pneumocytes) subjected to stimulation with cigarette smoke extract (Heinbockel et al. 2018). PAI1 can extensively inhibit the activities of several MMPs, which is a key regulator of the ECM and involves in the degradation of various ECM proteins. (Freeberg et al. 2018). Many studies have shown that MMPs played important role in ECM degradation. The reduced secretion of MMPs inhibitor indicated $PM_{2.5}$ exposure might aggravate the degradation of ECM and disturb the ECM organization of BEAS-2B cells.

The interaction networks also included the pathway of response to stimulus because of the expression of some proteins involved in stimulus response were detected. For example, the regulator of Wnt signaling Dickkopf1 (DKK1, 0.15 fold) showed a dramatic change in $PM_{2.5}$-exposed BEAS-2B cells. DKK1 is a potent antagonist of Wnt signaling (Niida et al. 2004). Inhibition of DKK1 could activate Wnt/beta-catenin signaling and attenuated $PM_{2.5}$-induced pulmonary fibrosis in mice (Yang et al. 2020). The significant decrease in secretion of DKK1 indicated abnormal activation of Wnt signaling of BEAS-2B cells in response to $PM_{2.5}$ exposure.

Interestingly, all proteins involved in regulation of complement activation and RNA splicing pathways were up-regulated, indicating their potential role in the damage mechanism caused by $PM_{2.5}$ exposure. Epidemiological studies have shown a strong correlation between $PM_{2.5}$ and the risk of lung inflammation. Various steps of inflammatory response were actively regulated by complement system, in which component C3 (C3) plays a crucial role (Pei et al. 2019; Kang et al. 2010). In this work, KEGG analysis showed that complement system was most significantly altered in the BEAS-2B cells exposed to $PM_{2.5}$. Complement C3 showed the very high rate of change (5.0 fold) in all differentially expressed proteins. In an investigation based on a panel of 175 older adults, short-term exposure to $PM_{2.5}$ could result in inflammatory reaction with a significant increase in serum level of C3 (Pei et al. 2019). Meanwhile, other proteins involved in complement activation, such as complement C1s (C1S) and complement C1r (C1R) were all increased, indicating a strong association between $PM_{2.5}$ exposure and complement activation. Meanwhile, All RNA splicing pathway-related proteins were also up-regulated. Among them, small nuclear ribonucleoprotein 70 kDa (SNRNP70) was reported significantly up-regulated in epithelial cell A549 upon $PM_{2.5}$ exposure and splicing factor heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) was found highly expressed in a variety of cancers, indicating the dysfunction of RNA splicing may has a potential driving role in $PM_{2.5}$ induced cytotoxicity (Zhao et al. 2019; David et al. 2010; Golan-Gerstl et al. 2011).

Conclusion

Herein, we evaluated the cytotoxicity of $PM_{2.5}$ exposure on BEAS-2B cells and investigated the corresponding variation in secretome. $PM_{2.5}$ exposure was showed to increase oxidative stress and enhance apoptosis in dose dependent manner. For secretome analysis, many differentially expressed proteins were closely related to apotosis, ECM organization, complement activation and RNA splicing.

These novel findings from an extracellular perspective provided insights into the underlying mechanisms on adverse health effects of $PM_{2.5}$ exposure.

Declarations

Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

Conceptualisation: SZ, CJ and ZL; methodology: SZ, SX and WY; writing—original draft preparation: SZ, SX and WY; writing—review and editing: LJ, ZB and LZ; supervision: CJ, ZL and ZY; funding acquisition: ZL, SZ and SX. All authors read and approved the final manuscript.

Data availability

All data generated or analyzed during this study are included in this published article.

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Figures

Figure 1

The flow chat of secretome analysis

Figure 2

(a) The cell viability in BEAS-2B cells exposed to PM2.5; (b) The ROS level in BEAS-2B cells exposed to PM2.5; (c) The apoptosis rate of BEAS-2B cells exposed to PM2.5

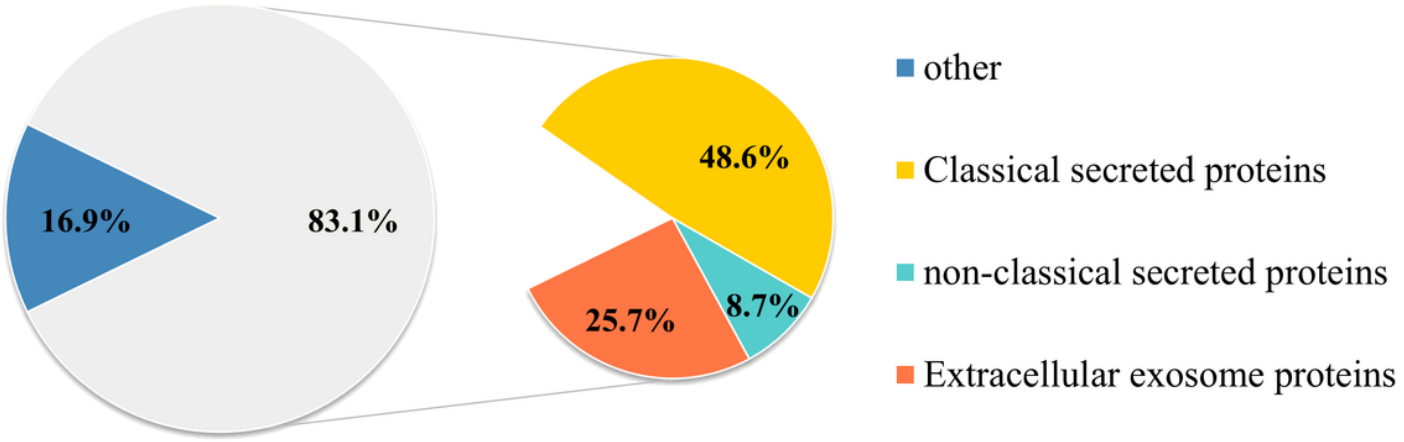

Figure 3

Classification of the differentially expressed proteins

Figure 4

Volcano plot of the quantified proteins. The components of each type of protein were shown in the pie chart. Experiment contains 3 biological replicates

Figure 5

Gene Ontology (GO) analysis of the extracellular proteome (Only the terms with p-value less than 0.01 were shown. (a) cell component; (b) molecular function; (c) biological process); D: KEGG pathway analysis

Figure 6

Interaction networks of differentially expressed proteins in major process

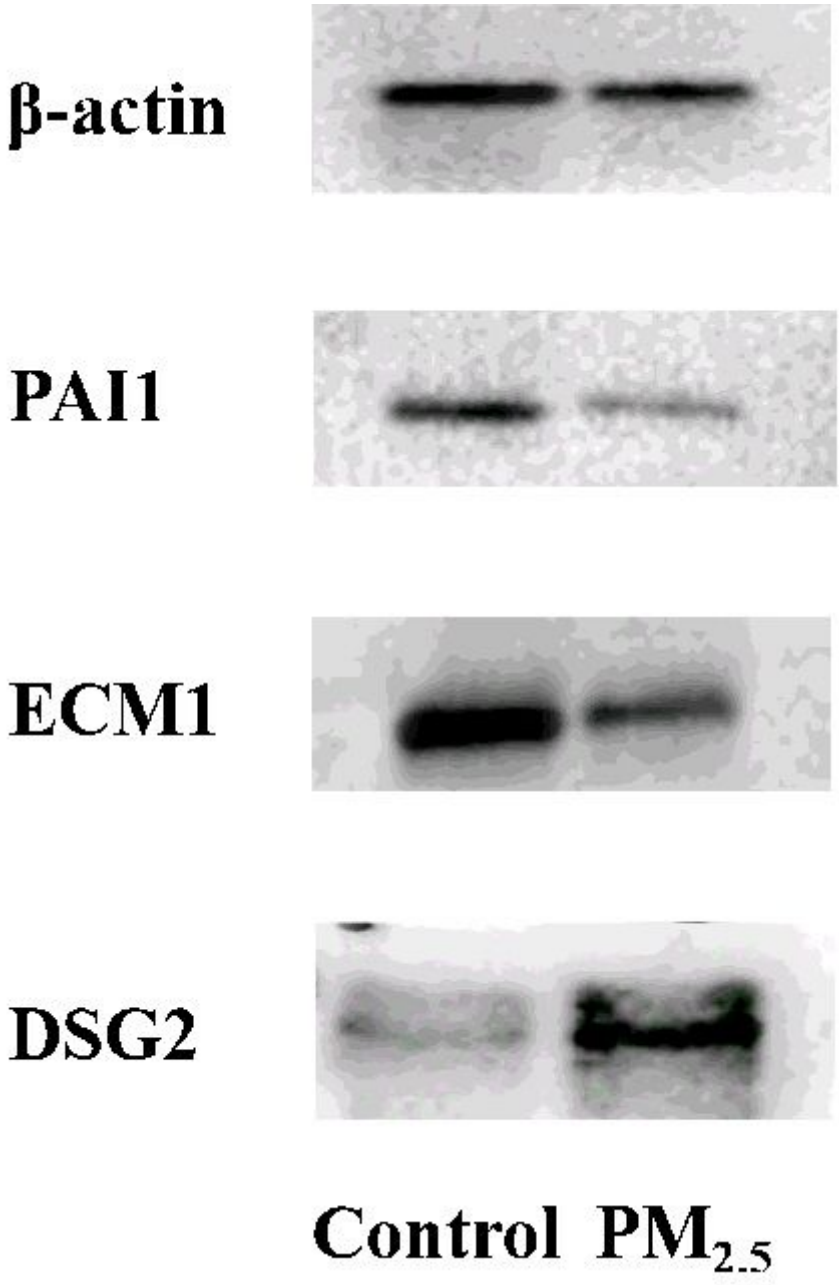

Figure 7

Western blot analysis

Supplementary Files

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- tableS1S4.xlsx