

Nanotoxicology

ISSN: 1743-5390 (Print) 1743-5404 (Online) Journal homepage: http://www.tandfonline.com/loi/inan20

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To cite this article: Xiaobo Li, Chengcheng Zhang, Qian Bian, Na Gao, Xin Zhang, Qingtao Meng, Shenshen Wu, Shizhi Wang, Yankai Xia & Rui Chen (2016) Integrative functional transcriptomic analyses implicate specific molecular pathways in pulmonary toxicity from exposure to aluminum oxide nanoparticles, Nanotoxicology, 10:7, 957-969, DOI: 10.3109/17435390.2016.1149632

To link to this article: https://doi.org/10.3109/17435390.2016.1149632

View supplementary material

Accepted author version posted online: 01 Feb 2016.
Published online: 16 Mar 2016.

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http://informahealthcare.com/nan
ISSN: 1743-5390 (print), 1743-5404 (electronic)

Nanotoxicology

Nanotoxicology, 2016; 10(7): 957–969
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ORIGINAL ARTICLE

Integrative functional transcriptomic analyses implicate specific molecular pathways in pulmonary toxicity from exposure to aluminum oxide nanoparticles

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Abstract

Gene expression profiling has developed rapidly in recent years and it can predict and define mechanisms underlying chemical toxicity. Here, RNA microarray and computational technology were used to show that aluminum oxide nanoparticles (Al_2O_3 NPs) were capable of triggering up-regulation of genes related to the cell cycle and cell death in a human A549 lung adenocarcinoma cell line. Gene expression levels were validated in Al_2O_3 NPs exposed A549 cells and mice lung tissues, most of which showed consistent trends in regulation. Gene-transcription factor network analysis coupled with cell- and animal-based assays demonstrated that the genes encoding *PTPN6, RTN4, BAX* and *IER* play a role in the biological responses induced by the nanoparticle exposure, which caused cell death and cell cycle arrest in the G2/S phase. Further, down-regulated *PTPN6* expression demonstrated a core role in the network, thus expression level of *PTPN6* was rescued by plasmid transfection, which showed ameliorative effects of A549 cells against cell death and cell cycle arrest. These results demonstrate the feasibility of using gene expression profiling to predict cellular responses induced by nanomaterials, which could be used to develop a comprehensive knowledge of nanotoxicity.

Keywords

Aluminum oxide nanoparticle, cell cycle, cell death, microarray, nanotoxicology

History

Received 28 June 2015
Revised 24 January 2016
Accepted 27 January 2016
Published online 24 February 2016

Introduction

Because of the increased number of applications of nanoparticles (NPs) available today, protecting the human respiratory system from exposure to these manufactured ultrafine particulates has become a considerable health concern. Nanomaterials differ from their corresponding conventional materials not only in scale but also in their physical, chemical and biological characteristics. In general, nanoscale materials are more likely to be toxic than the same materials of conventional size and can be inhaled more deeply into the lungs. For this reason, the respiratory tract is considered the primary target organ for inhaled NPs. Moreover, the common mechanisms of clearance are often ineffective (Oberdorster et al., 2005), so inhaled nanosized materials can either remain within the respiratory system, which imply long-term contact between NPs and alveoli, or become translocated to other target organs. A549 cell line was first initiated from an explanted lung carcinoma. It is widely used as a model of type II alveolar epithelium, representing cells from the alveoli (Feliu et al., 2015; Ingvarsson et al., 2014; Mascelloni et al., 2015). In this study, A549 cells and mice were used to assess the potential respiratory toxicity of inhaled aluminum oxide (Al_2O_3) NPs.

Al_2O_3 NPs are widely studied and used in chemical, industrial and medical fields (Cho et al., 2006; Popat et al., 2004). Further, aluminum (AL) is one of the most common heavy metal components of airborne ultrafine particles (PM2.5) in an ambient environment (Reff et al., 2009; Veranth et al., 2007). They are associated with various adverse biological effects (Bell et al., 2010; Ebisu & Bell, 2012). Aluminum in the environment is relatively stable in the form of aluminum oxide and these ultrafine particles can enter the body through inhalation. In this way, both environmental and occupational exposures to Al_2O_3 NPs are likely increasing and may represent a serious health risk.

The toxicity of Al_2O_3 NPs has been evaluated in a wide range of studies, which have focused on lung tissue (Simon-Deckers et al., 2008; Wagner et al., 2007), the central nervous system (Li et al., 2009), liver (Hussain et al., 2005) and other vital organs (Di Virgilio et al., 2010). Because the respiratory tract is considered to be the primary target organ for inhaled NPs, the toxic effects of Al_2O_3 NPs on lung tissue and the mechanisms underlying these effects have been investigated thoroughly. For *in vivo* studies, aluminum oxide-based nanowhiskers were found to promote increases in the number of lung macrophages in mice (Adamcakova-Dodd et al., 2012). Wagner et al. (2007) showed

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a marginal increase in rat alveolar macrophage viability induced with 24 h continuous exposure to 100 μg/ml Al_2O_3 NPs. Also, 53 μg/cm^2 Al_2O_3 NP has been found to induce significant increases in the IL-6 response in human lung epithelial cells BEAS-2B and normal human bronchial epithelial cells *in vitro* (Veranth et al., 2007). Al_2O_3 NPs can enter A549 cells rapidly and travel into the cytoplasm and intracellular vesicles (Simon-Deckers et al., 2008). All these studies suggest a possible toxic mechanism involving inflammation, oxidative stress and cell death.

So far, the methods used to study the effects of Al_2O_3 NPs have been mostly based on traditional *in vitro* assays, which only permit analyses focused on specific biological processes and well-defined molecular pathways. Large-scale ''omic'' approaches can often show the effects of biological pathways and processes that had not previously been known (Cui & Paules, 2010). This makes them suitable for predicting toxicity and determining the mechanisms underlying changes induced by uncharacterized compounds (Blomme et al., 2009). Currently, the use of ''omic'' techniques in toxicology studies is paving the way to new fields such as predictive toxicology (Boverhof & Zacharewski, 2006). The use of gene expression profiling is reported to study the impact of nanomaterials, including metal or metal oxide NPs (Foldbjerg et al., 2012; Hanagata et al., 2011; Shim et al., 2012; Tuomela et al., 2013). However, few studies have focused on the effects of Al_2O_3 NPs on the respiratory cell lines. In this study, modulations in gene expression profiles in A549 cells exposed to Al_2O_3 NPs were investigated using an Agilent Array platform. Results demonstrated the potential systematic cellular responses to nanomaterials.

Methods

Nanomaterials

Al_2O_3 NPs were purchased from Plasmachem GmbH (Berlin, Germany) (purity >99.8%). The average size and zeta potential of Al_2O_3 NPs was 64.17 nm and 37.1 mV in culture medium, which was analyzed by a zetasizer (nano-zs90, Malvern Instruments, Malvern, UK) (Figure S1A and B). The morphology of Al_2O_3 NPs in PBS were determined under a transmission electron microscope (TEM) (Tecnai G2 F20, Heidelberg, the Netherlands), and the particle size of Al_2O_3 NPs in PBS suspension was <100 nm (Figure S1B). The aggregation of Al_2O_3 NPs in PBS suspension and culture medium increased in a time-dependent manner (Figure S1C).

Cell culture and RNA extraction

According to the references, 100 μg/ml Al_2O_3 NPs treatment could trigger toxic effects in pulmonary cells (Simon-Deckers et al., 2008; Wagner et al., 2007), therefore, A549 cells were treated with 100 μg/ml Al_2O_3 NPs, to explore the integrative transcriptomic alterations in this study. The human lung adenocarcinoma cell lineA549 (American Type Culture Collection) was maintained in Dulbecco's modified Eagle's medium (DMEM) at 37 °C in 5% CO_2. The culture medium was supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were seeded in 10 cm culture dishes and exposed to 100 μg/ml Al_2O_3 NPs with three biological replicates. Control groups were treated with culture medium. Then, 24 h after treatment, the complete medium was removed and the adherent cells were collected. Total RNA was extracted using the TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

RNA microarray and gene expression analysis

An Agilent Array platform (Agilent Technologies, Santa Clara, CA) was used for microarray analysis. mRNA was purified from total RNA after removal of rRNA using an mRNA-ONLY Eukaryotic MRNA Isolation Kit (Epicentre Biotechnologies, Madison, WI). Then each mRNA sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3′ bias using a random priming method. The labeled cRNAs were hybridized onto a Human LncRNA Array v3.0 (8×60 K; Arraystar) chip, which is designed for 26,109 coding genes. The arrays were scanned using an Agilent G2505C scanner and the density of fluorescence was analyzed using Agilent Feature Extraction software (version 11.0.1.1). Quantile normalization and subsequent data processing were performed using the GeneSpringGx v12.0 software package (Agilent Technologies). An absolute fold change of 1.5 or more and $p = 0.05$ were set as thresholds to evaluate the significance of gene expression differences of raw data. The raw data is available in a public database GEO with accession number GSE77355.

Functional group analysis

The DAVID 6.7 (Database for Annotation, Visualization and Integrated Discovery) functional annotation tool was used to analyze differentially expressed genes. The DAVID functional annotation cluster tool provides three structured networks of defined terms that describe the attributes of gene products. The p value was set to 0.1 to denote the significance of gene ontology (GO) term enrichment in the differentially expressed mRNA list. Then fold enrichment ((Count/Pop.Hits)/(List.Total/Pop.Total)) was set as 5 to denote the enrichment of a particular GO term in the input gene list compared with the population (the Homo sapiens genome).

Cell viability

Cellular viability was evaluated using a CCK-8 proliferation assay kit (Nanjing Jiancheng Bioengineering Institute, China). A549 cells were plated at a density of 1×10^4 per well in a 96-well plate and treated with 0, 12.5, 25, 50, 100, 250, 500, 1000 μg/ml Al_2O_3 NPs with eight biological replicates for each concentration. Then 10 μl CCK-8 was added to each well and the cells were incubated for 4 h at 37 °C and the absorbance was determined at 450 nm. Cell viability affected by Al_2O_3 NPs was monitored every 24 h up to 7 days.

Transmission electron microscopic observation

A549 cells were treated with 100 or 500 μg/ml Al_2O_3 NPs for 24 h, then collected and fixed with 2.5% glutaraldehyde in 0.1 M sodium dihydrogen phosphate (pH 7.4). The samples were then fixed in 1% OsO_4 for 1 h, dehydrated through a graded ethanol series and gradually infiltrated with epoxy resin. Ultra-thin sections were obtained on copper grids, stained with uranyl acetate and lead citrate and observed in a transmission electron microscope (JEOL-1010, Japan).

Cell cycle analysis

A549 cells were seeded in 6-well plates at a density of about 1×10^6 cells per well, and exposed to 100 or 500 μg/ml A Al_2O_3 NPs or control medium for 24 h. All experiments were performed in sextuplicate. The cells were washed with PBS and harvested with trypsin. The cells were fixed in 75% ethanol for 24 h and then stained with propidium iodide (PI) and analyzed with a FACS Calibur Flow Cytometry (BD Biosciences, East Rutherford, NJ) to quantify the cell cycle.

Cell apoptosis analysis

Al_2O_3 NPs-induced cell apoptosis were analyzed by flow cytometry with a KeyGENAnnexin V-FITC Apoptosis

Detection Kit (KeyGEN BioTECH, China) according to the manufacturer's instruction. In brief, after exposure to 100 or 500 μg/ml Al_2O_3 NPs for 24 h, A549 cells were harvested through trypsinization and washed twice with PBS. This was followed by centrifugation at 1000 rpm for 5 min, the pellet was resuspended in 500 μl binding buffer and incubated with 5 μl FITC-conjugated annexin V and 5 μl PI for 15 min at room temperature in the dark. The samples were analyzed by FACS Calibur Flow Cytometry (BD Biosciences).

Animal treatments

Eighteen male and 18 female ICR mice (20–22 g) were purchased from Shanghai SLRC Laboratory Animal Co., Ltd. (Shanghai, China). Animals were treated humanely and maintained and used in accordance with Guidelines of Committee on Animal Use and Care of Southeast University. The dynamic inhalation exposure chambers were outfitted with extensive air quality monitoring equipment and an aerosol generator (Beijing HuiRongHe Technology Co., Ltd., Beijing, China). Mice were divided into three groups (six female and six male mice each), here called the control, low, and high Al_2O_3 NPs treatment groups. Mice were housed six per polycarbonate cage on corncob bedding with *ad libitum* access to food and water. Exposure was carried out in three stainless-steel Hinners-type whole-body inhalation chambers; two groups received Al_2O_3 NPs, and the other received HEPA-filtered clean air at the same flow rate as the experimental group. Six male and six female mice were exposed to each chamber for 8 h per day for seven consecutive days. Light cycles were set on a 12:12 h light:dark cycle. The mean concentrations of Al_2O_3 NPs were 2 and 10 mg/m^3 for low and high treatments, respectively. The temperature in the chambers was set to 22.5 °C.

Aluminum burden

Mice were euthanized under ether anesthesia 1 h after the end of dynamic Al_2O_3 NPs exposure on the seventh day. All the mice were decapitated on an iced table. Lung, heart, liver, spleen and kidney tissue samples were collected and stored in liquid nitrogen.

Approximately 0.1 g of each tissue sample was digested with HNO_3 in a boiling water bath for 3 h. The aluminum burdens in different tissues were quantified using an inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700, USA).

RNA isolation and quantitative real-time PCR assay

A549 cells were seeded in 6-well plates at a density of $\sim 1 \times 10^6$ cells per well and exposed to 50, 100, 250 or 500 μg/ml Al_2O_3 NPs or control medium for 24 h, then cells were trypsinized and collected. For animal tissues, a piece of left lung tissues were prepared for qRT-PCR assay.

Total RNA of A549 cells and lung tissues was extracted using the GenEluteTM Mammalian Total RNA Miniprep Kit (Sigma, St Louis, MO) according to the manufacturer's protocol, and the concentration of total RNA was determined by measuring the absorbance at 260 nm using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA). cDNA synthesis for coding genes was performed with 1 μg of total RNA according to the manufacturer's instruction (Toyobo, Tokyo, Japan).

The mRNA levels for modulated genes were determined by reverse transcription of total RNA followed by quantitative real-time PCR analysis (qRT-PCR) on a Quant Studio 6 Flex system (Applied Biosystems, Life Technologies) using SYBR PCR Master Mix reagent kits (Toyobo, Singapore) in accordance with the manufacturer's protocol. Primers were designed for the modulated genes screened by RNA microarray. Primer sequences are shown in Tables S1 and S2. All experiments were performed in triplicate. The mRNA levels given here are relative to cyclophilin A for the indicated gene.

Gene-transcription factor network analysis

The gene-transcription factor network was analyzed based on the transcriptional factor target set in the Encyclopedia of DNA Elements (ENCODE) (Gerstein et al., 2012). The dataset was downloaded from file ''enets2.Proximal_filtered.xls'' in the Supplementary material of this article. The figure was drawn using a free online tool cytoscape.

siRNA knockdown, plasmids construction, transfections and immunodetection

For siRNA knockdown, we transfected A549 cells in a 6-well plate with siRNAs (Thermo Fisher Scientific, Waltham, MA) directed against control or *PTPN6* using DharmaFECT1 (Thermo Fisher Scientific). The final concentrations of *PTPN6* siRNA were 10, 20 and 40 nM, respectively. Wild-type (WT) human PTPN6 cDNA with 3′Myc epitope tagged CMV promoter-driven pCMV6-Entry expression vectors was purchased from Origene (OriGene Technologies, Beijing, China) as well as the pCMV6-Entry basic vector. Expression plasmids (100 ng/well) were used in transient transfection analyses. A549 cells were transfected using Lipofectamine 2000 (Invitrogen) in 6-well plates with a maximum of 500 ng total DNA per well. At 48 h post-transfection, cells were treated with 100 or 500 μg/ml Al_2O_3 NPs for another 24 h and then harvested.

Proteins were analyzed by immunoblotting with primary antibodies for the following antigens: human PTPN6 (1:500 dilution; Abcam, Cambridge, MA), α-tubulin (1:10,000 dilution; Sigma) or c-Myc (1:5000 dilution; Santa Cruz Biotechnology, Dallas, TX).

Data analysis

Values of cell viability and cell cycle assay are here expressed as means ± standard error of the mean (SEM). Statistically significant differences were determined using one-way ANOVA, followed by Dunnett's multiple comparison test. The $2^{-\Delta\Delta Ct}$ method was used to analyze the results of qRT-PCR in all experiments. Statistical analysis was performed by SPSS 12.0 and the significance was set at $p < 0.05$.

Results

Overview of mRNA microarray profiles

A total of 571 mRNAs were found to be differentially expressed between the control and Al_2O_3 NPs treated A549 cells, including 464 up-regulated and 107 down-regulated genes, which were under a screening criteria with a fold change of 1.5 and $p < 0.05$ (non-adjusted) (Figure S2A). The heatmap showed the induced and suppressed mRNA of samples exposed to Al_2O_3 relative to matched controls, which illustrates the agreement of results from volcano figure in terms of the fold-change values as 1.5, $p = 0.05$ (Figure S2B).

To identify relevant mRNA regulated in response to Al_2O_3 NPs exposure, a more restrictive set of criteria was used for further data analysis. As shown in Table S3, the expression of 11 gene symbols were found to be increased or decreased by at least 2-fold change as well as an adjusted p value <0.05 in the Al_2O_3 NPs-treated samples.

GO enrichment analysis reveal regulated categories of genes

GO enrichment analysis was performed in order to better understand the mRNA changes observed in cells exposed to