Fine Ambient Particles Induce Oxidative Stress and Metal Binding Genes in Human Alveolar Macrophages

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Exposure to pollutant particles increased respiratory morbidity and mortality. The alveolar macrophages (AMs) are one cell type in the lung directly exposed to particles. Upon contact with particles, AMs are activated and produce reactive oxygen species, but the scope of this oxidative stress response remains poorly defined. In this study, we determined the gene expression profile in human AMs exposed to particles, and sought to characterize the global response of pro- and antioxidant genes. We exposed AMs obtained by bronchoscopy from normal individuals to Chapel Hill particulate matter of 2.5- μ m diameter or smaller (PM_{2.5}; 1 μ g/ml) or vehicle for 4 hours ($n = 6$ independent samples). mRNAs were extracted, amplified, and hybridized to Agilent human 1A microarray. Significant genes were identified by significance analysis of microarrays (false discovery rate, 10%; $P \leq 0.05$) and mapped with Gene Ontology in the Database for Annotation, Visualization, and Integrated Discovery. We found 34 and 41 up- and down-regulated genes, respectively; 22 genes (~30%) were involved in metal binding, and 11 were linked to oxidative stress, including up-regulation of five metallothionein (MT)-1 isoforms. Exogenous MT1 attenuated PM_{2.5}-induced H₂O₂ release. PM_{2.5} pre-mixed with MT1 stimulated less H₂O₂ release. Knockdown of MT1F gene increased PM_{2.5}-induced H₂O₂ release. PM_{2.5} at 1 μ g/ml did not increase H₂O₂ release. Mount St. Helens PM_{2.5} and acid-extracted Chapel Hill PM_{2.5}, both poor in metals, did not induce MT1F or H₂O₂ release. Our results show that PM_{2.5} induced a gene expression profile prevalent with genes related to metal binding and oxidative stress in human AMs, independent of oxidative stress. Metals associated with PM may play an important role in particle-induced gene changes.

Keywords: particulate matter; air pollution; microarray; metallothionein

Exposure to particulate matter (PM) is consistently associated with increased morbidity and mortality, attributable, in part, to respiratory illnesses (1, 2). These adverse effects include increased hospital admissions, asthma attacks, pulmonary infection, and mortality (3, 4). The mechanisms by which PM increases morbidity and mortality are not entirely clear, but pulmonary oxidative stress and inflammation induced by PM appear to play an important role (5–11).

The alveolar macrophage (AM) is one major cell type in the lung constantly exposed to ambient pollutants. Upon contact with environmental particulate pollutants, AMs are activated, and produce a large quantity of reactive oxygen species (ROS)

from various enzymatic sources (5, 12–16). PM exposure may also increase or decrease antioxidant defense mechanisms in the lung, which further modulates oxidative stress and enhances pulmonary and systemic inflammation (13, 14, 17–25). The scope of this pro- and antioxidant response, however, remains poorly defined, in part because of the multiple enzymatic sources of ROS, and the numerous antioxidant enzymes that can be affected by PM.

To better understand how this complex network of pro- and antioxidant enzymes may be affected by PM, we examined the gene expression profile of human AM exposed to Chapel Hill fine particles of 2.5- μ m diameter or smaller (PM_{2.5}). The gene expression analysis highlighted the role of genes involved in metal binding and oxidative stress. The functional role of *metallothionein (MT)-1* in response to PM was verified by RNA interference.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma, Inc. (St. Louis, MO) unless otherwise stated.

Particle Collection and Extraction

Particles used in this study were collected in April of 2002 in Chapel Hill, North Carolina, outside the U.S. Environmental Protection Agency Human Studies Facility using a ChemVol Model 2,400 high-volume cascade impactor (Rupprecht and Patashnick Co., Albany, NY) (5). PM_{2.5} on polyurethane foam (McMaster-Carr, Atlanta, GA), which were previously cleaned with methanol and water, and dried under sterile conditions, were retrieved weekly. The foam or filter was pre-etched with a small amount of 70% ethanol, and the endotoxin-free water was added to a total volume of 40 ml. The particles were removed from the foam or filter by sonication for 1 hour in a water bath (FS220, Fisher Scientific, Atlanta, GA). The foam was removed, and particles were then lyophilized. The metal constituents of PM_{2.5} of April 2002 were: Al, 930.0 ng/mg; As, 44.7 ng/mg; Cr, 10.2 ng/mg; Cu, 77.7 ng/mg; Fe, 1,713.0 ng/mg; Pb, 133.2 ng/mg; Ni, 26.7 ng/mg; Se, 70.8 ng/mg; Si, 729.0 ng/mg; Ti, 21.0 ng/mg; V, 66.0 ng/mg; and Zn, 522 (5).

Isolation of Human AMs and Bronchial Epithelial Cells

Human AMs and bronchial epithelial cells (BECs) were obtained by bronchoalveolar lavage and bronchial brushings from normal individuals according to procedures described previously (26, 27). Subjects were informed of the procedures and potential risks, and each signed an informed consent. The protocol was approved by the University of North Carolina School of Medicine Committee on Protection of the Rights of Human Subjects.

Procedures for human AM isolation and culture have been described previously (5, 13). Bronchoalveolar lavage samples were put on ice immediately and centrifuged at $300 \times g$ for 10 minutes at 4°C. The lavaged cells were washed once with ice-cold RPMI 1640 medium with 20 mg/ml gentamicin (Life Technologies, Rockville, MD). Cell counts were performed with a hemacytometer. Cytocentrifuge slides were prepared and stained with Diff Quick (Leukostat Solution; Fisher Scientific) to check for AM purity. The cell preparation consisted of 85–95% AMs. The viability of AMs was determined by trypan blue

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exclusion. Viability exceeded 85% in all samples. AMs were suspended in 1 ml of RPMI 1,640 supplemented with 2.5% FBS (Life Technologies, Gaithersburg, MD) in 5-ml polypropylene tubes. The suspended cells were then incubated with vehicle or PM_{2.5}.

Procedures for human BEC isolation and culture have been described previously (5). BECs adherent to the brushes were treated for 15 minutes at room temperature with 1 mM DTT in BEC growth medium (BEGM; Clonetics, San Diego CA) supplemented with bovine pituitary extract, 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 50 µg/ml gentamicin, 0.1 ng/ml retinoic acid, 10 µg/ml transferrin, 6.5 ng/ml triiodothyronine, 0.5 µg/ml epinephrine, and 0.5 ng/ml human epidermal growth factor, and dislodged from the brushes by pipeting. The cells were then plated at a density of 1×10^5 viable cells/cm² in six-well cluster plates (surface area, 9.6 cm²) that were precoated with 50 mg/ml human placental collagen type IV and used after passage 2 or 3.

Exposure to Chapel Hill PM_{2.5} and RNA Isolation

A suspension of AMs (1×10^6 cells) were incubated with control vehicle or Chapel Hill PM_{2.5} (1 µg/ml) for 4 hours in a static condition. At the end of the 4-hour incubation, total cellular RNA was extracted from AMs with Trizol reagent (GIBCO BRL/Life Technologies, Gaithersburg, MD), and further purified with phenol/chloroform. The RNA was amplified with a fluorescent linear amplification kit (no. 5184-3523; Agilent Technologies, Inc., Palo Alto, CA) according to the manufacturer's recommended procedures. The RNA integrity was assessed with an Agilent 2,100 bioanalyzer (Agilent Technologies, Inc.). The 260 nm:280 nm ratios for all RNAs were greater than 2.0.

Hybridization of RNA

Total RNAs (250 ng) were labeled using a Low Input Linear Amplification kit (Agilent Technologies) according to the manufacturer's protocol. Briefly, first- and second-strand cDNA synthesis was immediately followed by an *in vitro* transcription reaction, during which Cy3- or Cy5-UTP

were incorporated. Labeled cRNA was purified on RNeasy columns (Qiagen, Valencia, CA) and quantified using NanoDrop ND-1,000 UV-Vis spectrophotometer; 1 µg each of Cy3- and Cy5-cRNA were combined, defragmented, and hybridized on Agilent's *In Situ* Oligo Human 1A version 1 arrays per Agilent's protocol. Hybridizations were performed overnight (16–18 h) at 60°C in a rotisserie oven. Slides were washed using Agilent's sodium chloride–sodium citrate wash buffers and procedures, and scanned on an Axon 4000B confocal scanner (Molecular Devices, Sunnyvale, CA).

Microarray Analysis

The data discussed in this article have been deposited in the Gene Expression Omnibus (accession no. GSE10394; <http://www.ncbi.nlm.nih.gov/geo/>). All analyses were performed using TM4 software suite (28). Intensities were extracted from raw tiff images using TIGR Spotfinder. Cy3 and Cy5 intensities were lowest normalized for each individual hybridization using TIGR MIDAS. Dye flip replicas were filtered, merged to produce a single expression ratio measure for each gene, and log–base 2 transformed. Differentially expressed genes were identified using significance analysis of microarrays with 100 permutations (29). FDR was set at 10%.

Functional Classification

We also mapped the significant genes with Gene Ontology in the Database for Annotation, Visualization, and Integrated Discovery (DAVID Bioinformatic Resources 2007, National Institute of Allergy and Infectious Disease, <http://apps1.niaid.nih.gov/david/>) (30).

Quantitative PCR

Quantitative PCR (Q-PCR) was performed for selected genes. cDNAs were synthesized from 0.4 µg of total RNA in 100 µl of buffer containing 5 µM random hexaoligonucleotide primers (Pharmacia,

TABLE 1. GENES UP-REGULATED BY PARTICULATE MATTER OF 2.5-µM DIAMETER OR SMALLER

UniGene	Symbol	Fold	Gene Description	Functions
HS.534330	MT1F	3.164	Metallothionein 1F (functional)	Metal binding, antioxidant
HS.36102	MT1E	3.055	Metallothionein 1E (functional)	Metal binding, antioxidant
HS.181768	NCF1	2.908	Neutrophil cytosolic factor 1, (chronic granulomatous disease, autosomal 1)	Pro-oxidant
HS.188518	MT1H	2.765	Metallothionein 1H (functional)	Metal binding, antioxidant
HS.513626	MT1G	2.760	Metallothionein 1G (functional)	Metal binding, antioxidant
HS.645456	MT1B	2.743	Metallothionein 1B (functional)	Metal binding, antioxidant
HS.559477	NINJ1	2.714	Ninjurin 1	
HS.441047	ADM	2.655	Adrenomedullin	
HS.325978	IL18BP	2.573	Interleukin 18 binding protein	
HS.127022	RELB	2.319	V-rel reticuloendotheliosis viral oncogene homolog b, nuclear factor of kappa light polypeptide gene enhancer in b-cells 3 (avian)	
HS.154654	CYP1B1	2.258	Cytochrome p450, family 1, subfamily b, polypeptide 1	Metal binding, pro-oxidant
HS.517145	ENO1	2.133	Enolase 1, (alpha)	Metal binding
HS.31210	BCL3	2.119	B-cell cll/lymphoma 3	
HS.284491	PFKP	2.105	Phosphofructokinase, platelet	Metal binding
HS.494457	NQO1	2.007	NAD(P)H dehydrogenase, quinone 1	Antioxidant
NA	ENO1B	1.989	Enolase-α, lung specific	Metal binding
HS.297413	MMP9	1.985	Matrix metalloproteinase 9	Metal binding
HS.76556	PTPRE	1.983	Protein tyrosine phosphatase, receptor type, e	
HS.479728	GAPDH	1.953	Glyceraldehyde 3-phosphate dehydrogenase	Antioxidant
HS.470126	KYNU	1.949	Kynureninase (l-kynurenine hydrolase)	
HS.520708	DFNA5	1.862	Deafness, autosomal dominant 5	
HS.117062	AIFM2	1.817	Apoptosis-inducing factor, mitochondrion-associated, 2 (AMID)	Pro-oxidant
HS.636480	TUBB	1.687	Tubulin-β	
HS.493919	MPZL1	1.683	Myelin protein zero-like 1	
HS.307905	TFPI	1.625	Tissue factor pathway inhibitor	
HS.589369	PDXK	1.620	Pyridoxal (pyridoxine, vitamin B6) kinase	Metal binding
HS.26010	PPP1R15A	1.602	Protein phosphatase 1, regulatory (inhibitor) subunit 15a	
HS.125213	ATOX1	1.578	Atx1 antioxidant protein 1 homolog (yeast)	Metal binding, antioxidant
HS.406515	NR1H3	1.548	Nuclear receptor subfamily 1, group h, member 3	Metal binding
HS.1422	FGR	1.512	Gardner-rasheed feline sarcoma viral (v-fgr) oncogene homolog	
HS.438462	MYOD1	1.496	Myogenic differentiation 1	
HS.556274	CHST11	1.396	Carbohydrate (chondroitin 4) sulfotransferase 11	
HS.66542	GPR3	1.392	G protein-coupled receptor 3	
HS.438863	NRBP	1.368	Nuclear receptor binding protein 1	

Genes that are involved in metal binding and oxidative stress are indicated under "Functions."

Piscataway, NJ), 10 U/μl Moloney murine leukemia virus reverse transcriptase (GIBCO BRL/Life Technologies), 1 U/μl RNase inhibitor (RNasin; Promega, Madison, WI), 0.5 mM deoxynucleotide triphosphate solution (Pharmacia), 50 mM KCl, 3 mM MgCl₂, and 10 mM Tris-HCl (pH 9.3) for 1 hour at 39°C. Reverse transcriptase was heat inactivated at 94°C for 4 minutes.

Q-PCR of specimen and standard cDNA was completed using TaqMan predeveloped assay reagents. Quantitative fluorogenic amplification of cDNA was performed using the ABI Prism 7,500 Sequence Detection System, primers and probes of interest, and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The relative abundance of mRNA levels was determined from standard curves generated from a serially diluted standard pool of cDNA prepared from control human pulmonary artery endothelial cell cultures. The relative abundance of glyceraldehyde-3-phosphate dehydrogenase (Unigene accession no. 544,577) mRNA was used to normalize levels of the mRNAs of interest. For Q-PCR verification, RNA from six additional experiments was collected. RNA samples for microarray and Q-PCR were collected from different experiments, and do not represent the same sample.

Measurement of H₂O₂

Release of H₂O₂ into the medium was measured by the Amplex red reagent (10-acetyl-3,7-dihydroxyphenoxazine) (Molecular Probes/Invitrogen Corp., Carlsbad, CA). Amplex red reacts with H₂O₂ to produce highly fluorescent resorufin in the presence of horseradish

peroxidase. The fluorescent signals were measured over 120 minutes with a Bioassay HTS7,000 plate reader (Perkin Elmer, Inc., Wellesley, MA) with HTSoft v1.0 software (PE Applied Biosystems, Weiterstadt, Germany). The excitation and emission wavelengths were 530 and 590 nm, respectively. Generation of H₂O₂ was calculated by subtracting the fluorescence signal at 0 minutes (baseline) from that at 120 minutes, and expressed as optical density of resorufin. Positive control samples using H₂O₂ (0.5–2.0 μM) were included for all Amplex red assays. Human AMs were also incubated with PM_{2.5} with or without rabbit liver zinc-containing MT1 (Alexis Biochemicals, San Diego, CA). MT was added 20 minutes before Amplex red.

Transfection of Human BECs with *MT1F* siRNA

Human BECs were grown to 60–70% confluency and then transfected with Gene Silencer transfecting agent plus (Gene Therapy System, Inc., San Diego, CA) with *MT1F* short interfering RNA (siRNA; 100 nM) (Ambion, Austin, TX) (cat. no. 16,708/siRNA, ID no. 44,734/lot no. ASO04QXP/Locus ID 4,494/RefSeq no. NM_005949) in serum-free basic growth medium-2 for 3 hours, according to the manufacturer's recommendations. All control cells were incubated with Gene Silencer select negative control no. 1 siRNA (part no. 4390844) (Ambion). Fresh endothelial growth medium-2 with 2% FBS was then added, and cells were cultured for an additional 48 hours. Cell lysates from some wells were collected for *MT1F* gene expression by RT-PCR. Other wells were used for measuring H₂O₂ release.

TABLE 2. GENES DOWN-REGULATED BY PARTICULATE MATTER OF 2.5-μM DIAMETER OR SMALLER

UniGene	Symbol	Fold	Gene Description	Function
HS.567398	CCL4	−5.464	Chemokine (C-C motif) ligand 4	Metal binding
HS.416073	S100A8	−4.016	S100 calcium binding protein a8 (calgranulin a)	
HS.51120	CAMP	−3.610	Cathelicidin antimicrobial peptide	
HS.647049	LAT2	−3.534	Linker for activation of T cells family, member 2	
HS.7884	SLCO2B1	−2.941	Solute carrier organic anion transporter family, member 2b1	Metal binding
HS.233389	CPVL	−2.849	Carboxypeptidase, vitellogenic-like	
HS.153837	MNDA	−2.725	Myeloid cell nuclear differentiation antigen	
HS.75182	MRC1	−2.674	Mannose receptor, c type 1	
HS.515061	EDG6	−2.577	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 6	Metal binding
HS.64016	PROS1	−2.481	Protein S (α)	
HS.20315	IFIT1	−2.463	Interferon-induced protein with tetratricopeptide repeats 1	
HS.377894	GCA	−2.445	Grancalcin, EF-hand calcium binding protein	
HS.2551	ADRB2	−2.404	Adrenergic, β-2-, receptor, surface	Metal binding
HS.119192	H2AFZ	−2.392	H2a histone family, member z	
HS.75360	CPE	−2.320	Carboxypeptidase e	
HS.504657	CLEC4A	−2.315	C-type lectin domain family 4, member a	
HS.479208	FBXL5	−2.309	F-box and leucine-rich repeat protein 5	Metal binding
HS.75765	CXCL2	−2.198	Chemokine (C-X-C motif) ligand 2	
HS.12056	ASGR1	−2.183	Asialoglycoprotein receptor 1	
HS.528055	HIST1H4A	−2.183	H4 histone, family 2	
HS.202	BZRP	−2.179	Benzodiazapine receptor (peripheral)	Metal binding
HS.12333	RNF13	−2.174	Ring finger protein 13	
HS.25155	NET1	−2.160	Neuroepithelial cell transforming gene 1	
HS.518198	CSTA	−2.146	Cystatin a (stefin a)	
HS.499094	PYCARD	−2.141	PYD and CARD domain containing	Metal binding
HS.520989	FGL2	−2.132	Fibrinogen-like 2	
HS.446123	CAPZA2	−2.105	Capping protein (actin filament) muscle z-line, α 2	
HS.516834	C20ORF46	−2.092	Chromosome 20 open reading frame 46	
HS.486410	ECHDC1	−2.088	Enoyl coenzyme a hydratase domain containing 1	Metal binding
HS.26136	PIGY	−2.079	Phosphatidylinositol glycan anchor biosynthesis, class y	
HS.75367	SLA	−2.079	Src-like-adaptor	
HS.209983	STMN1	−2.075	Stathmin 1/oncoprotein 18	
HS.514412	PECAM1	−2.062	Platelet/endothelial cell adhesion molecule (CD31 antigen)	Metal binding
HS.70327	CRIP1	−2.058	Cysteine-rich protein 1 (intestinal)	
HS.523702	MS4A6A	−2.058	CD20-like precursor	
HS.484990	HIST1H3H	−2.049	Histone 1, h3a	
HS.325960	MS4A4A	−2.041	Membrane-spanning 4-domains, subfamily a, member 4	Metal binding
HS.8904	VSIG4	−2.020	V-set and immunoglobulin domain containing 4	
HS.558359	BIRC1	−2.016	Baculoviral IAP repeat-containing 1	
HS.282410	CALM2	−2.016	Calmodulin 1 (phosphorylase kinase, δ)	
HS.180878	LPL	−2.016	Lipoprotein lipase	

Genes that are involved in metal binding are indicated under "Function."

Statistical Analysis

Data are presented as mean values ($\pm$ SE). Data from nonmicroarray experiments were analyzed by one-way ANOVA, followed by Scheffé's test for *post hoc* comparisons. The statistical analysis was performed using commercially available software (Statview version 5.0.1; SAS Institute Inc., Cary, NC). A *P* value of less than 0.05 was taken as statistically significant.

RESULTS

Genes Induced by PM_{2.5} in Cultured AMs

Using the *above* statistical algorithm, we identified 38 and 43 probes that were up- and down-regulated, respectively, at 10% FDR in AMs exposed to PM_{2.5}. After eliminating probes with no corresponding genes, we reduced the list to 34 up-regulated genes (Table 1) and 41 down-regulated genes (Table 2). Expression of 13 up- and down-regulated genes was confirmed by Q-PCR (Figures 1 and 2). We further categorized these differentially expressed genes by their molecular functions using Gene Ontology. We found that 22 genes were involved in metal binding (Tables 1 and 2) (GOTERM_MF_3; *P* = 0.049).

Many of these genes, such as *MT1F*, *MT1E*, *MT1H*, *MT1G*, *MT1B*, *ATOX1*, *CYP1B1*, and *MMP9*, are also involved in oxidative stress (Tables 1 and 2). Other differentially expressed genes involved in oxidative stress included up-regulation of *NCF1*, *NQO1*, and *GAPD* (Tables 1 and 2). Hierarchical clustering analyses of significant genes are shown in Figure 3, which shows two main clusters in up-regulated genes. One cluster contains all MT1 isoform genes, *NCF1*, *NQO1*, and *MMP9*, and the other contains *CYP1B1*, *GAPD*, *AMID*, and *ATOX1*. Because of up-regulation of multiple *MT1* isoform genes, we further investigated the role of *MT1F*, one of the main isoforms in the lung (31–33), in oxidative stress induced by PM_{2.5}.

Effects of MT1 on H₂O₂ Production

Incubation of human AM with PM_{2.5} increased the extracellular H₂O₂ release detected by Amplex red in a dose-dependent manner, which was inhibited completely by catalase (200 U/ml) (Figure 4A). The enhanced release of H₂O₂ was also inhibited by the addition of MT1 protein in the medium (Figure 4B). These concentrations of PM_{2.5} were not cytotoxic, as there was no increase in lactic dehydrogenase release (data not shown). In addition, PM_{2.5} preincubated with 100 μ M MT1 for 30 minutes

before being added to the medium induced lower levels of H₂O₂ release compared with PM_{2.5} preincubated with BSA (10 μ g/ml) in human AM (Figure 4C) or cell-free system (Figure 4D), indicating that metal chelation is one mechanism by which MT1 decreases PM_{2.5}-induced H₂O₂ release.

Effects of MT1F Knockdown on H₂O₂ Release

To determine how MT1F regulates H₂O₂ release, we knocked down *MT1F* gene expression using siRNA in human BECs. We used human BECs instead of human AMs for these siRNA experiments, because siRNA was ineffective in human AMs. We verified that human BECs, like human AMs, increased *MT1F* and *CYP1B1* gene expression (measured by Q-PCR) by five- and sixfold, respectively, after PM_{2.5} exposure (100 μ g/ml or 10 μ g/cm²). Incubation with siRNA reduced *MT1F* gene expression by approximately 50% (Figure 5A) and increased PM_{2.5}-induced H₂O₂ release by approximately 30% (Figure 5B).

Effects of PM Metal Components on H₂O₂ Release

To determine whether or not the metal components of PM are important in inducing *MT1F*, human AMs were incubated with untreated PM_{2.5} (1 μ g/ml), metal-poor Chapel Hill PM_{2.5} and Mount St. Helens (MSH) PM_{2.5} (1 μ g/ml), which contains little metal. To prepare metal-poor PM_{2.5}, 1 μ g/ml of Chapel Hill PM_{2.5} was washed with 1 N HCl three times, and the pellet was resuspended in sterile water (34, 35). Human AMs incubated with acid-extracted Chapel Hill PM_{2.5} and MSH PM_{2.5} showed no increase in *MT1F* gene expression (Figure 6A). Neither the acid-extracted Chapel Hill PM_{2.5} nor MSH PM_{2.5} at 1 μ g/ml increased H₂O₂ release (Figure 6B). We also tested higher doses of these metal-poor particle preparations, and there was no increase in H₂O₂ release at doses up to 100 μ g/ml (data not shown). These results indicate that metals associated with Chapel Hill PM_{2.5} play an important role in gene expression changes induced by the whole particles, independent of oxidative stress.

DISCUSSION

Our findings suggest that metal components associated with PM may be important in inducing gene changes, independent of oxidative stress. Using microarray analysis and gene ontology, we found that about one-third of the differentially expressed genes in AMs challenged with PM_{2.5} had molecular functions

Figure 1. PCR confirmation of selected up-regulated genes (*n* = 6 independent experiments for quantitative PCR [Q-PCR]). Solid bars, microarray; shaded bars, PCR.

Figure 2. PCR confirmation of selected down-regulated genes ($n = 6$ independent experiments for Q-PCR). Solid bars, microarray; shaded bars, PCR.

related to metal binding. We also noted that five MT-1 isoform genes (*MTIF*, *MTIE*, *MTIH*, *MTIG*, and *MTIB*) were among the top 10 up-regulated genes. The MT1 genes are known to be involved in heavy metal binding. The MT1 proteins are also important antioxidant enzymes. Three other metal-binding genes, *ATOX1*, *CYP1B1*, and *MMP9*, are also linked to oxidative stress. Furthermore, four other genes not in the metal-binding category, including *NCF1*, *NQO1*, *AIFM2*, and

GAPD, are also known to be involved in oxidative stress. Thus, a total of 11 genes could be included in the pro- and antioxidant network. The 11 up-regulated oxidative stress genes fall into two main groups that include MT1 isoform genes and *CYP1B1*, respectively. Seven genes are involved in calcium binding (*MMP9*, *GCA*, *PROS1*, *ASGR1*, *CALM2*, *MRC1*, and *S100A8*), and may further modulate oxidant signaling by altering intracellular calcium (36–39).

Figure 3. Hierarchical clustering images for (A) up-regulated and (B) down-regulated genes. Hierarchical clustering on genes and samples was performed using Euclidean distance metric.

Figure 4. Effects of metallothionein (MT)-1 on H₂O₂ release in human alveolar macrophages (AMs). (A) Particulate matter (PM) of 2.5-μm diameter or smaller (PM_{2.5}) dose-dependently increases H₂O₂ release. Catalase inhibited H₂O₂ release stimulated by PM_{2.5}. The dose of PM_{2.5} used in the catalase experiments was 30 μg/ml. (B) Addition of MT1 into the medium decreases PM_{2.5}-induced H₂O₂ release detected by increases in resorufin signals. PM_{2.5} preincubated with MT1 (100 μM) for 30 minutes induced lower levels of H₂O₂ release compared with PM_{2.5} preincubated with BSA (10 μg/ml) in human AMs (C) or cell-free medium (D). The concentration of PM_{2.5} was 30 μg/ml. **P* < 0.05; *n* = 4–6 each.

PM is known to produce oxidative stress in lung cells. Our microarray study identified three pro-oxidant genes that were up-regulated: *NCF1*, *CYP1B1*, and *AIFM2*. *NCF1* encodes a 47-kD cytosolic subunit of the membrane reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme responsible for the respiratory burst. The membrane NADPH oxidase on AMs may be activated by PM exposure and produce ROS (5, 12, 16). *CYP1B1* encodes a mono-oxygenase in the cytochrome p450 system localized in the endoplasmic reticulum. *CYP1B1* is part of the phase-I xenobiotic enzyme system. It may be up-regulated by PM exposure, and is a major source of ROS in cells stimulated with PM (40–43). *AIFM2* (*AMID*) encodes a mitochondrial protein with significant homology with reduced nicotinamide adenine dinucleotide oxidoreductase and apoptosis-inducing factor. Up-regulation of this gene by PM has not been reported. *AIFM2* may be one of the important sources of mitochondrial ROS produced by AMs during PM exposure (13, 15, 44), and is probably linked to a caspase-independent mitochondrial effector of apoptotic cell death.

The up-regulation of multiple MT1 isoform genes is intriguing. MTs are low-molecular weight, metal and sulfur-rich proteins that are widely distributed in all organs, including the lung (32, 45). These intracellular proteins are thought to be involved in both heavy metal detoxification and the homeostasis of essential trace metals, such as zinc and copper. The metal-binding property and the abundance of cysteines (18–23 residues) in MTs have also been implicated as the mechanisms by which MTs protect cells from oxidative injury (46–49). We have shown that addition of exogenous MT1 protein attenuated the extracellular resorufin signals. In human BECs that also showed up-regulation of *MT1F* and *CYP1B1* gene expression after PM_{2.5} exposure similar to human AMs, knocking down *MT1F* gene expression by more than 50% was associated with an approximately 30% increase in PM_{2.5}-induced H₂O₂ release. In addition, particles preincubated with MT1 before being added to the cells attenuated the release of H₂O₂. These results confirm the capability of MT1 to scavenge ROS and bind metals in human lung cells during PM exposure. MT1 may bind to redox-active metals associated with PM via its cysteine residues, and decrease hydroxyl radicals generated from the Fenton reaction. Binding of MT1 to PM may also interfere with the engulfment of MT1–PM complex by the AMs and inhibit downstream oxidant pathways. The abundant thiol groups present in MT1 also may directly scavenge oxygen radicals, independent of its metal-binding capability (49).

We further noted that approximately 30% of the 75 differentially expressed genes have molecular functions related to metal ion binding. Of these, the five MT1 isoforms are known to be essential in detoxifying environmental heavy metals, such as arsenic, cadmium, and mercury (50–53). MT1 levels in various tissues may also increase in animals exposed to environmental zinc (54, 55). The MT1 genes also are involved in binding of intracellular metals, such as zinc and copper (32, 56–58), and,

Figure 5. Effects of *MT1F* knockdown on H_2O_2 release in primary human bronchial epithelial cells. (A) Incubation of siRNA against *MT1F* inhibited gene expression of *MT1F* induced by $PM_{2.5}$ by more than 50%. (B) Knockdown of *MT1F* enhanced $PM_{2.5}$ -induced H_2O_2 release. The concentration of $PM_{2.5}$ was 100 $\mu g/ml$ or 10 $\mu g/cm^2$. * $P < 0.05$; $n = 4-6$ each.

thus, may regulate signaling mediated by these trace metals. *ATOX1* encodes a copper chaperone that binds and transports cytosolic copper to ATPase proteins in the trans-Golgi network for later incorporation to the ceruloplasmin (59–61). Thus, our gene profiling study indicates that PM exposure may induce host defense mechanisms that are normally invoked to combat heavy metal toxicity, and that molecular signaling pathways mediated by zinc and copper may be important in PM-induced health effects.

Metals associated with PM likely were the primary inducer for gene changes, as metal-poor PM preparations did not induce MT1. Oxidative stress was unlikely the inducer for changes in gene expression, because $PM_{2.5}$ at 1 $\mu g/ml$, which altered gene expression, did not increase H_2O_2 release. The induction of MT1 genes further indicates that certain metal components in PM may be the biologically active constituents for pulmonary health effects. MT1 genes may be responsive specifically to some heavy metals that are normally present in PM, such as zinc and copper (32, 50, 62). The $PM_{2.5}$ sample used in this study contains approximately 500 ng/mg of zinc and 70 ng/mg of copper (5). Zinc is a major metal element detected in traffic-derived PM (63–66), presumably from tire wear. Incubation of human BECs with zinc up-regulated MT1 isoform genes (33). Copper in PM has also been linked to traffic sources, as copper

Figure 6. Effects of $PM_{2.5}$ -associated metals on *MT1F* gene expression (A) and H_2O_2 release (B). Human AMs were treated with vehicle (control), untreated Chapel Hill $PM_{2.5}$ (1 $\mu g/ml$), or Chapel Hill $PM_{2.5}$ (1 $\mu g/ml$) extracted with 1 N HCl or Mount St. Helens $PM_{2.5}$ (1 $\mu g/ml$). * $P < 0.05$; $n = 4-6$ each.

is an element in car brakes (65, 67, 68). Zinc and copper may also come from certain industrial sources (63, 65). In a previous study exposing healthy volunteers to Chapel Hill $PM_{2.5}$, the principal component analysis identified a factor containing zinc and copper correlating with increased blood fibrinogen (26). Also, in an *in vitro* study of cultured human BECs, the principal component analysis identified a copper-containing factor that was associated with Chapel Hill PM-induced IL-6 release (5).

AMs have an important role in clearing particles from the lungs (69). Binding and subsequent engulfment of particles may activate signaling pathways, resulting in production of ROS by various pro-oxidant enzymes, such as NADPH (16). Peroxynitrite may also be formed from the interaction between ROS and nitric oxide (25). PM effects may be recognized via binding receptors on AM, which activates downstream inflammatory pathways (70). Thus, interactions between PM and cell membrane proteins of AMs may be a critical event that triggers subsequent oxidative stress and inflammation. Our results demonstrate changes in the expression of genes encoding membrane proteins, such as *PTPRE*, *MRC1*, *ADRB2*, *CLEC4A*, *ASGRI*, *MS4A4A*, and *MS4A6A*. All of these genes, except *PTPRE*, were down-regulated. How PM-induced changes in these novel membrane proteins affect oxidative stress and inflammation, and thus health effects, will need to be investigated in future studies.

In summary, our gene profiling experiments identified an array of gene expression response in human AMs exposed to $PM_{2.5}$, independent of oxidative stress. This profile is most notable for genes unique to heavy metal binding and oxidative stress. Metals associated with PM may be important in inducing host defense mechanisms normally invoked to combat heavy metal toxicity, and molecular signaling pathways mediated by metals may be important in PM-induced health effects.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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