Ambient Pollution-related Reprogramming of the Human Small Airway Epithelial Transcriptome

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Abstract

Rationale: Epidemiologic studies have demonstrated that exposure to particulate matter ambient pollution has adverse effects on lung health, exacerbated by cigarette smoking. Particulate matter less than or equal to 2.5 μm in aerodynamic diameter ($\text{PM}_{2.5}$) is among the most harmful urban pollutants and is closely linked to respiratory disease.

Objectives: Based on the knowledge that the small airway epithelium (SAE) plays a central role in the pathogenesis of smoking-related lung disease, we hypothesized that elevated $\text{PM}_{2.5}$ levels are associated with dysregulation of SAE gene expression, which may contribute to the development of respiratory disease.

Methods: From 2009 to 2012, healthy nonsmoker ($n = 29$) and smoker ($n = 129$) residents of New York City underwent bronchoscopy with SAE brushing (2.6 ± 1.3 samples/subject; total of 405 samples). SAE gene expression was assessed by Affymetrix HG-U133 Plus 2.0 microarray. New York City $\text{PM}_{2.5}$ levels

(Environmental Protection Agency data) were averaged for the 30 days before bronchoscopy. A linear mixed model was used to assess $\text{PM}_{2.5}$ -related gene dysregulation accounting for multiple clinical and methodologic variables.

Measurements and Main Results: Thirty-day mean $\text{PM}_{2.5}$ levels varied from 6.2 to 18 $\mu\text{g}/\text{m}^3$. In nonsmokers, there was no dysregulation of SAE gene expression associated with ambient $\text{PM}_{2.5}$ levels. In marked contrast, $n = 219$ genes were significantly dysregulated in association with $\text{PM}_{2.5}$ levels in the SAE of smokers. Many of these genes relate to cell growth and transcription regulation. Interestingly, 11% of genes were mitochondria associated.

Conclusions: $\text{PM}_{2.5}$ exposure contributes to significant dysregulation of the SAE transcriptome of smokers, linking pollution and airway epithelial biology in the risk of development of respiratory disease in susceptible individuals.

Keywords: particulate matter; smoker; pollution; chronic obstructive pulmonary disease; oxidative stress

The London Fog of 1952, a major air pollution disaster associated with more than 4,000 excess deaths, brought attention to the adverse health consequences of ambient pollution (1). Since then, multiple studies have linked exposure to ambient pollution to increased morbidity and mortality

(2–10). Particulate matter (PM), particularly PM less than or equal to 2.5 μm in aerodynamic diameter ($\text{PM}_{2.5}$), small enough to be deposited in the small airways (11, 12), is one of the most harmful urban pollutants (13) and is closely linked to respiratory disease (14). Pollution and

$\text{PM}_{2.5}$ exposure have been associated with increased hospital admissions, all-cause and respiratory-related mortality (2–10, 15), lung function impairment (16, 17), incidence and exacerbations of asthma (18–21), chronic obstructive pulmonary disease (COPD) (22–25), and lung cancer

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At a Glance Commentary

Scientific Knowledge on the

Subject: Exposure to air pollution is associated with many adverse effects, including increased mortality and the development and/or worsening of lung diseases, such as chronic obstructive pulmonary disease. However the mechanisms by which this occurs are not well understood.

What This Study Adds to the

Field: We demonstrate through analysis of the small airway transcriptome of nonsmoker and smoker New York City residents that fine particulate matter exposure results in significant dysregulation of small airway gene expression in smokers but not in nonsmokers. This suggests that smokers are more susceptible to the detrimental effects of fine particulate matter than nonsmokers even when fine particulate matter concentrations remain in a “good” to “moderate” range as defined by the Environmental Protection Agency. The study provides insight into the biologic mechanisms underlying pollution-associated respiratory disease in susceptible individuals.

(14, 26). Smokers and individuals with lung disease are particularly susceptible to the effects of PM_{2.5} (7).

Although the link between pollution exposure and pulmonary disease is well recognized, the biologic mechanism by which pollution is harmful remains poorly understood. Animal models and *in vitro* experimental work have linked PM_{2.5} exposure to oxidative stress in the airways, inducing inflammatory responses and cytokine production (27, 28). In humans, studies of acute controlled exposure to pollutants, including diesel exhaust fumes, suggest induction of pulmonary and systemic inflammation (29–31), although these findings have been inconsistent, particularly at low exposure levels (32). Studies of exhaled breath and systemic biomarkers have linked the presence of airway inflammation and oxidative stress to exposure to PM pollution (33, 34), and transcriptomic profiling of circulating leukocytes have demonstrated gene

expression profiles consistent with inflammation associated with pollution exposure (35, 36). Additionally, epigenetic modifications, including changes in global DNA methylation patterns in blood leukocytes of individuals with both acute and chronic pollution exposure, have been described (37, 38), as have alterations in microRNA expression both in *in vitro* models and human studies (39, 40).

Based on the knowledge that the small airway epithelium (SAE) plays a central role in the pathogenesis of COPD and lung cancer (41, 42), we hypothesized that elevated ambient mean PM_{2.5} levels are associated with alterations in SAE gene expression that may contribute to the development of respiratory disease. To evaluate this hypothesis, we capitalized on our assessment of 405 samples of the SAE transcriptome obtained by bronchoscopy in New York City (NYC) resident nonsmokers and smokers over a 4-year period (2009–2012). Genes expressed in the SAE were analyzed using a linear mixed model with Environmental Protection Agency (EPA) 30-day mean NYC PM_{2.5} levels over the same period. The data demonstrate that whereas 30-day mean PM_{2.5} levels of 6.2–18 µg/m³ observed in NYC has no effect on the SAE transcriptome of nonsmokers, this level of pollution has a significant effect on the SAE transcriptome of smokers, dysregulating SAE biology relevant to the risk of development of pollution-related respiratory disease.

Methods

Study Population

Healthy nonsmokers ($n = 29$) and smokers ($n = 129$ total; healthy smokers $n = 71$ and COPD smokers $n = 58$) were recruited under institutional review board–approved protocols and provided informed consent. Eligibility was determined after detailed screening including medical history, physical examination, laboratory investigations, chest radiograph, electrocardiogram, and full pulmonary function tests (see METHODS section in the online supplement for details regarding full inclusion/exclusion criteria). Details of the demographic features of the study population are in Table 1, where the healthy and COPD smokers are combined, and Table E1 in the online supplement, which details the healthy and COPD

smokers separately. All of the healthy smokers had normal lung function, whereas the COPD smokers had lung function in categories of 64% Global Initiative for Chronic Obstructive Lung Disease (GOLD) I, 34% GOLD II, and 2% GOLD III (43).

SAE Sampling and Gene Expression Analysis

Eligible subjects underwent serial research bronchoscopy with brushing of the SAE (10th- to 12th-order bronchi) as previously described (44). Each individual underwent a maximum of four bronchoscopies over the course of the study with procedures scheduled at 0-, 3-, 6-, and 12-month time points. A total of 405 bronchoscopies to sample the SAE were performed over the 4-year period. Table E2 provides full details of the number of bronchoscopies per subject and phenotype.

After brushing of the small airway, SAE cells were dislodged from the cytology brush by flicking into 5 ml of ice-cold Bronchial Epithelium Basal Medium (Lonza) and kept on ice until processed. An aliquot was removed for cell viability and differential analysis. The total number of cells recovered was determined by counting on a hemocytometer. To quantify the percentage of epithelial and inflammatory cells and the proportions of basal, ciliated, secretory, and columnar cells recovered, cells were prepared by centrifugation (Cytospin 11, Shandon Instruments) and stained with Diff-Quik (Baxter Healthcare). The remaining sample was immediately processed and stored in TRIzol reagent (Invitrogen) at -80°C until subsequent RNA purification.

Total RNA was extracted using the TRIzol method with final sample clean-up using RNeasy columns (Qiagen). RNA quantity and quality were assessed by Nanodrop ND-1000 (Thermo Scientific) and Bioanalyzer (Agilent Technologies), respectively. Total RNA was prepared for microarray transcriptome analysis using the 3'IVT Express Kit.

For processing on HG-U133 Plus 2.0 microarrays (Affymetrix), total RNA (1–2 µg) was used to synthesize double-stranded cDNA, and Affymetrix kits were used to quantify the biotin-labeled cDNA yield. RNA was hybridized on the arrays with probes for more than 54,000 genome-wide transcripts, using Affymetrix protocols, hardware, and software. SAE samples were

Table 1. Demographics of Nonsmokers and Smokers*†

Parameter	Nonsmokers	Smokers†	Smokers vs. Nonsmokers P Value
<i>n</i>	29	129	
Sex, M/F	15/14	107/22	<0.0003
Age, yr	38 ± 12	44 ± 9	<0.0002
Race, B/W/H/O	10/8/11/0	72/22/31/4	>0.1
Body mass index, kg/m ²	26 ± 5	27 ± 4	>0.5
Smoking history			
Age of initiation, yr	NA	16 ± 3	NA
Duration of smoking, pack-years	NA	27 ± 14	NA
Urine nicotine, ng/ml‡	0	1,084 ± 1,235	<10 ⁻¹⁰
Urine cotinine, ng/ml‡	0	1,506 ± 950	<10 ⁻¹⁰
Carboxyhemoglobin, %	0	3.1 ± 1.3	<10 ⁻¹⁰
Pulmonary function parameters§			
FVC, %	107 ± 11	109 ± 15	>0.3
FEV ₁ , % predicted	103 ± 12	95 ± 20	<0.008
% Change FEV ₁ post-bronchodilator	2.3 ± 3.2	4.3 ± 5.5	<0.009
FEV ₁ /FVC	80 ± 6	70 ± 11	<10 ⁻⁹
TLC, % predicted	98 ± 16	97 ± 12	>0.8
D _{CO} , % predicted	91 ± 11	80 ± 14	<10 ⁻⁴
Cough score	0.5 ± 0.6	1.7 ± 1.4	<10 ⁻¹⁰
Sputum score	0.5 ± 0.5	1.4 ± 1.2	<10 ⁻⁸
30-d mean PM _{2.5} level, µg/m ³	11.3 ± 2.5	11.3 ± 2.7	>0.8
SAE cell differential, %			
Epithelial cells	98.9 ± 1.0	99.2 ± 1.0	<0.04
Inflammatory cells	1.1 ± 1.0	1.2 ± 5.7	>0.7
Ciliated cells	68.9 ± 6.2	62.3 ± 7.5	<10 ⁻¹⁰
Secretory cells	10.4 ± 4.6	14.6 ± 6.9	<10 ⁻¹⁰
Undifferentiated cells	12.3 ± 5.3	15.5 ± 7.3	<10 ⁻⁵
Basal cells	7.3 ± 5.0	6.6 ± 5.0	>0.2

Definition of abbreviations: B = black; GOLD = Global Initiative for Chronic Obstructive Lung Disease; H = Hispanic; NA = not applicable; O = other; PM_{2.5} = particulate matter less than or equal to 2.5 µm in aerodynamic diameter; SAE = small airway epithelium; W = white.

*Data are presented as mean ± SD. *P* values of numeric parameters were calculated using a two-tailed Student's *t* test with unequal variance. *P* value of categorical parameters were calculated using a chi-square test for screening date.

†Includes *n* = 71 healthy smokers and *n* = 58 chronic obstructive pulmonary disease smokers (*n* = 37 GOLD I, *n* = 20 GOLD II, *n* = 1 GOLD III) (see Table E1 for full demographic details).

‡Undetectable urine nicotine <2 ng/ml; cotinine <5 ng/ml.

§Pulmonary function testing parameters are given as % of predicted value with the exception of FEV₁/FVC, which is reported as % observed.

||Cough and sputum score were each evaluated on a scale of 0–4: 0 = not at all; 1 = only with chest infections; 2 = a few days a month; 3 = several days per week; 4 = most days per week (72).

divided over five microarray chips with individuals from each phenotype included on all microarrays chips. Microarray quality was verified by signal intensity ratio of GAPDH 3' to 5' probe sets less than or equal to 3.0 and multichip normalization scaling factor less than or equal to 10.0. Gene expression was normalized using the Single-Channel Array Normalization/Universal exPression Codes method and University of Michigan HG-U133 Plus 2.0 microarray probeset definitions, version 21 (45). Genes with a Universal exPression Code value of less than 0.2 in all samples were excluded (46, 47). The raw data are publicly available at the Gene Expression Omnibus website

(www.ncbi.nlm.nih.gov/geo/) under accession number GSE108134.

Ambient PM_{2.5} Measurements

To characterize NYC air quality, daily mean PM_{2.5} levels were obtained from the EPA Air Quality System's Database (https://www.epa.gov/outdoor-air-quality-data) for sites around the city and averaged. In all cases, parameter code 88,502 (acceptable PM_{2.5} AQI and speciation mass) data were used. The mean PM_{2.5} concentration for the 30 days before each bronchoscopy was calculated. The 30-day mean PM_{2.5} level was chosen to reflect the effects of subacute PM_{2.5} exposure on the SAE transcriptome rather than daily PM_{2.5}

levels where analysis could be influenced by brief fluctuations in PM_{2.5} concentrations and daily variations in individuals' exposure to outdoor air.

Statistical Analysis

A linear mixed model was used to account for the presence of longitudinal data (48). Expression of each SAE gene was modeled as a function of 30-day mean PM_{2.5} level before the date of the bronchoscopy with covariates selected to account for known factors that can impact gene expression. Covariates included demographic, lung function, bronchoscopy sampling, and RNA technical variables for the nonsmoker cohort with additional smoking variables included as covariates for the smoker cohort (see Table 2 and Table E3 for full covariates details, and Figure E1 for covariate effects). To account for seasonality over the course of the study, a 1-year sine term was included in the model. *P* values for the regression coefficient for the pollution variable (PM_{2.5}) for each gene were calculated using a two-sided Wald test. A total of 15,384 genes were tested and a Benjamini-Hochberg correction for multiple tests was applied with a cutoff of 0.05 to assess significance. Corrected SAE gene expression residuals were calculated by subtracting the linear mixed model-predicted random effects and all fixed effects except for 30-day mean PM_{2.5} concentration (see Figure E2 for examples of expression of the genes most significantly upregulated and downregulated in response to PM_{2.5} levels in all subjects).

Assessment of biologic processes, molecular function, cellular components, and Reactome Pathways was performed on PM_{2.5}-associated genes to reveal overrepresented or underrepresented categories using PANTHER (protein annotation through evolutionary relationship), a classification system for protein-coding genes that uses Gene Ontology (GO) or in the case of "PANTHER GO-slim" a curated subset of GO terms and the Reactome biologic pathway resource (49).

Results

Over the course of the study from December 2009 through June 2012, subjects were exposed to varying ambient PM_{2.5} levels in the month before SAE sampling

Table 2. Linear Mixed Model Covariates*

Demographic Covariates	Smoking Covariates	PFT Covariates	Sample Covariates	RNA Covariates
Subject ID	Smoking status	FVC pre	Region	Time to chip
Month	Smoking years	FVC post	Bronchoscopist	RNA technician
1 yr sine term	Pack-years	FEV ₁ pre	SAE cell differential	Chip technician
Age	Packs per day	FEV ₁ post	% inflammatory cells	RNA concentration
Sex	Urine nicotine	FEV ₁ /FVC pre	% ciliated cells	RIN
Ethnicity	Urine cotinine	FEV ₁ /FVC post	% secretory cells	Chiplot
BMI	Urine 3-OH cotinine level	TLC	% undifferentiated cells	Background
Height	Urine anabasine	D _{LCO}	% basal cells	Scaling factor
30-d mean PM _{2.5} level	CO Hb level	Cough score [†]		GAPDH
		Sputum score [†]		β-Actin

Definition of abbreviations: BMI = body mass index; CO Hb = carboxyhemoglobin; PFT = pulmonary function test; PM_{2.5} = particulate matter less than or equal to 2.5 μm in aerodynamic diameter; post = post-bronchodilator; Pre = prebronchodilator; RIN = RNA integrity number; SAE = small airway epithelium.

*Complete list of covariates used in the linear mixed model (see Table E3 for additional covariate details).

[†]Cough and sputum score were each evaluated on a scale of 0–4: 0 = not at all; 1 = only with chest infections; 2 = a few days a month; 3 = several days per week; 4 = most days per week (72).

(Figure 1A). Highest PM_{2.5} levels in NYC were observed during the summer and winter months, with the lowest PM_{2.5} values in the spring and fall. There was a 2.6-fold difference between the lowest and highest 30-day mean PM_{2.5} levels. The PM_{2.5} level of 12 $\mu\text{g}/\text{m}^3$ separates the EPA designations of “moderate” and “good” PM_{2.5} levels (50). Within these ranges, the 30-day moderate levels ranged from 12.1 to 18 $\mu\text{g}/\text{m}^3$ and the good levels from 6 to 12 $\mu\text{g}/\text{m}^3$. The timing of the bronchoscopies to sample the SAE was spread over the 2009–2012 period thus representing the variations in PM_{2.5} levels (Figure 1B). The number of SAE samples collected each month ranged from 23 to 44, with a monthly mean of 34.9 ± 7.2 bronchoscopies. Each subject underwent a mean of 2.6 ± 1.3 bronchoscopies with brushing of the SAE as detailed in Table E2. There was no significant difference in the 30-day mean PM_{2.5} exposure level among phenotype groups (Table 1). Additionally, there were no significant changes in annual meteorologic conditions in NYC over the study period (51).

SAE gene expression was assessed in a total of 405 SAE samples ($n = 98$ nonsmoker samples, $n = 176$ healthy smoker samples, $n = 131$ COPD smoker samples). After Benjamini-Hochberg correction, no genes were associated with ambient pollution levels in nonsmokers (Figure 2A). In contrast, $n = 219$ genes were significantly associated with PM_{2.5} levels in smokers ($n = 126$ upregulated, $n = 93$ downregulated) (Figure 2B; see Table E4), indicating differences in susceptibility of smokers to

effects of PM_{2.5} exposure. GO analysis of the biologic process categories of the SAE PM_{2.5}-responsive genes in all smokers combined showed a broad distribution dominated by genes related to cell growth and proliferation, apoptosis, immune responses,

metabolism, transcription, signal transduction, regulation, and transport (Figure 2C).

The most significant PM_{2.5} dysregulated genes in the SAE of all smokers included RBBP7 (RB-binding

Figure 1. Daily 30-day mean particulate matter less than or equal to 2.5 μm in aerodynamic diameter (PM_{2.5}) levels in New York City in 2009–2012 and the individual bronchoscopies performed in nonsmokers and smokers during this study period. (A) PM_{2.5} 30-day mean values. Daily data were obtained from the Environmental Protection Agency, and the 30-day city mean was calculated. Over the course of the study, PM_{2.5} levels varied with highest PM_{2.5} levels observed during the summer and winter months. The dashed line indicates the cutoff between Environmental Protection Agency–designated “moderate” and “good” PM_{2.5} levels (50). (B) Dates at which a bronchoscopy was performed to sample the small airway epithelium of nonsmokers and smokers. Individual dots represent each of the 309 days over which the 405 small airway epithelium samples were collected. Letters indicate months in order, starting with December.

Figure 2. Significantly dysregulated particulate matter less than or equal to $2.5\ \mu\text{m}$ in aerodynamic diameter ($\text{PM}_{2.5}$)-associated genes in the small airway epithelium of nonsmokers and smokers. (A) Nonsmokers ($n = 0$ significant genes). (B) Smokers ($n = 219$ significant genes, $n = 126$ upregulated, $n = 93$ downregulated). y-axis, $-\log_{10}$ of pBH value; x-axis, regression coefficients. The Benjamini-Hochberg procedure was used to determine the significance threshold separately for each phenotype. Gray dots represent the Benjamini-Hochberg nonsignificant genes, red dots are Benjamini-Hochberg significant genes. (C) Biologic process categories of $\text{PM}_{2.5}$ -associated dysregulated genes in the small airway epithelium of smokers. y-axis, log of P value incorporating the direction of expression change; x-axis, biologic process. See Table 3 for the 25 most significant $\text{PM}_{2.5}$ -responsive genes and Table E4 for the complete list. pBH = Benjamini-Hochberg corrected P value.

protein 7, chromatin remodeling factor), which encodes a histone-binding protein that acts as a transcriptional repressor (52); AIMP1 (aminoacyl tRNA synthetase complex interacting multifunctional protein 1), a proinflammatory cytokine linked to the development of emphysema in mice (53); and UBXN11 (UBX domain protein 11), which is linked to Rho signaling regulation of cell functions including cytoskeleton dynamics and cell movement (54) (Table 3; see Table E4 for the complete list).

To evaluate whether exposure to increasing $\text{PM}_{2.5}$ levels leads to greater dysregulation of SAE gene expression, SAE samples obtained from smokers were divided into two groups based on 30-day mean $\text{PM}_{2.5}$ exposure level: “good” ($\text{PM}_{2.5} \leq 12\ \mu\text{g}/\text{m}^3$) and “moderate” ($\text{PM}_{2.5}$ 12–35.4 $\mu\text{g}/\text{m}^3$) using EPA definitions (50) and corrected expression of the most significant genes identified in the analysis assessed (Figure 3). This analysis demonstrated that, at least for these dysregulated genes in smokers, exposure to increasing $\text{PM}_{2.5}$

levels was associated with significantly greater changes in gene expression in the SAE.

PANTHER statistical overrepresentation testing using a curated group of GO molecular function categories (GO-Slim) suggested that higher levels of $\text{PM}_{2.5}$ -associated genes were linked to activation of catalytic processes in the SAE (false discovery rate [FDR], $P < 0.01$) (see Table E5). A PANTHER overrepresentation test of GO annotated cellular components revealed enrichment of the aminoacyl-tRNA

Table 3. Top 25 PM_{2.5}-associated Genes in the Small Airway Epithelium of Healthy and COPD Smokers*

Gene Symbol	Gene Name	P Value	pBH	Regression Coefficient [†]	95% Confidence Interval
RBBP7	Retinoblastoma-binding protein 7	3.0×10^{-7}	3.3×10^{-3}	0.009	0.006 to 0.013
UBXN11	UBX domain protein 11	4.3×10^{-7}	3.3×10^{-3}	-0.029	-0.039 to -0.018
MFSD14A	Major facilitator superfamily domain-containing 14A	6.8×10^{-7}	3.5×10^{-3}	0.010	0.006 to 0.013
ATN1	Atrophin 1	2.0×10^{-6}	7.8×10^{-3}	-0.009	-0.013 to -0.006
HAP1	Huntingtin-associated protein 1	4.0×10^{-6}	1.0×10^{-2}	-0.005	-0.007 to -0.003
PRKACA	Protein kinase, cAMP-dependent, catalytic, α	4.7×10^{-6}	1.0×10^{-2}	-0.006	-0.009 to -0.004
CSNK1G3	Casein kinase 1, gamma 3	5.8×10^{-6}	1.0×10^{-2}	0.008	0.005 to 0.011
HSD17B10	Hydroxysteroid (17- β) dehydrogenase 10	5.8×10^{-6}	1.0×10^{-2}	0.009	0.005 to 0.013
PIBF1	Progesterone immunomodulatory binding factor 1	5.9×10^{-6}	1.0×10^{-2}	0.012	0.007 to 0.016
OCIAD2	OCIA domain-containing 2	7.3×10^{-6}	1.1×10^{-2}	0.012	0.007 to 0.017
ZNF440	Zinc finger protein 440	9.9×10^{-6}	1.2×10^{-2}	-0.015	-0.021 to -0.008
MFSD1	Major facilitator superfamily domain-containing 1	1.0×10^{-5}	1.2×10^{-2}	0.011	0.006 to 0.016
LOC105371450	Uncharacterized LOC105371450	1.0×10^{-5}	1.2×10^{-2}	-0.009	-0.013 to -0.005
AIMP1	Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1	1.1×10^{-5}	1.2×10^{-2}	0.006	0.003 to 0.008
TUNAR	TCL1 upstream neural differentiation-associated RNA	1.1×10^{-5}	1.2×10^{-2}	-0.007	-0.010 to -0.004
ESPN	Espin	1.4×10^{-5}	1.3×10^{-2}	-0.009	-0.013 to -0.005
TYMP	Thymidine phosphorylase	1.5×10^{-5}	1.3×10^{-2}	-0.029	-0.041 to -0.016
SLC37A3	Solute carrier family 37 member 3	1.5×10^{-5}	1.3×10^{-2}	0.009	0.005 to 0.013
HTR7	5-Hydroxytryptamine (serotonin) receptor 7 (adenylate cyclase-coupled)	1.7×10^{-5}	1.4×10^{-2}	-0.004	-0.006 to -0.002
RTCB	RNA 2',3'-cyclic phosphate and 5'-OH ligase	1.8×10^{-5}	1.4×10^{-2}	0.008	0.004 to 0.011
BTBD2	BTB domain-containing 2	1.9×10^{-5}	1.4×10^{-2}	-0.009	-0.013 to -0.005
SLC30A6	Solute carrier family 30 (zinc transporter), member 6	1.9×10^{-5}	1.8×10^{-2}	0.006	0.003 to 0.009
LINGO1	Leucine rich repeat and Ig domain-containing 1	2.1×10^{-5}	1.4×10^{-2}	0.005	
MARS	Methionyl-tRNA synthetase	2.2×10^{-5}	1.4×10^{-2}	0.008	0.004 to 0.011
MYO5B	Myosin VB	2.4×10^{-5}	1.4×10^{-2}	0.008	0.004 to 0.011

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; pBH = Benjamini-Hochberg corrected *P* value; PM_{2.5} = particulate matter less than or equal to 2.5 μ m in aerodynamic diameter.

*Twenty-five most significant genes upregulated and downregulated in the small airway epithelium of healthy and COPD smokers in response to PM_{2.5} exposure (see Table E4 for the complete list).

[†]Regression coefficients from the linear mixed model indicate the direction and magnitude of gene expression of the fitted pollution effect.

synthetase multienzyme complex, a component of which is AIMP1, which is involved in translation and protein biosynthesis (55), and overrepresentation of cytoskeleton, cytosol, organelles and extracellular exosome components (all FDR, $P < 0.05$) (see Table E6). Interestingly, 11% of the genes significantly associated with PM_{2.5} levels in smokers encode proteins with mitochondrial localization, including HSD17B10 (hydroxysteroid 17- β dehydrogenase 10), ETFB (electron transfer flavoprotein β subunit), ATP5A1 (ATP synthase F1 subunit α), MGME1 (mitochondrial genome maintenance exonuclease 1), HSPA9 (heat shock protein family A [Hsp70] member 9), and BCL2L1 (BCL2 like 1) and its chaperone FKBP8 (FK506-binding protein 8) (see Table 4 for the complete list) (56). PANTHER Reactome Pathway statistical overrepresentation testing revealed overrepresentation of pathways related to

incorporation of selenomethionine into proteins (FDR, $P < 0.05$) (see Table E7).

The detailed analyses described above focused on all smokers combined to increase the analysis power and the robustness of the study findings. Separate analysis of healthy smokers (i.e., normal lung function) and COPD smokers with similar cohort sizes demonstrated that PM_{2.5} exposure, either in healthy smokers or COPD smokers, was associated with dysregulation of SAE gene expression with $n = 134$ and $n = 92$ genes significantly dysregulated by PM_{2.5} exposure, respectively (see Figure E3 and Tables E8 and E9).

Discussion

Elevated levels of PM pollution are recognized as a risk for respiratory morbidity and mortality (2–4, 6–8, 14, 16, 22–26). This study demonstrates that

although exposure to PM_{2.5} levels within a “moderate” to “good” range as observed in NYC between 2009 and 2012 was not associated with any significant alterations in the SAE transcriptome of nonsmokers, this level of pollution had a significant effect on the SAE transcriptome of smokers, dysregulating SAE biology relevant to the risk of development of pollution-related respiratory disease. This finding suggests that exposure to ambient PM_{2.5} even below the current EPA designation of “unhealthy for sensitive groups” (50) may be harmful to susceptible individuals, at least at the biologic level. In addition to illustrating that cigarette smoking population groups are more susceptible to the effects of PM_{2.5} exposure, the study also demonstrates evidence of a dose–response in SAE of smokers with increasing dysregulation of gene expression with increasing PM_{2.5} exposure.

Figure 3. Examples of the most significant particulate matter less than or equal to 2.5 μm in aerodynamic diameter ($\text{PM}_{2.5}$)-dysregulated small airway epithelial (SAE) genes in SAE samples obtained from smokers with “good” and “moderate” levels of ambient $\text{PM}_{2.5}$ exposure. All smokers were divided into two groups based on 30-day mean $\text{PM}_{2.5}$ exposure level (“good,” $\text{PM}_{2.5} \leq 12 \mu\text{g}/\text{m}^3$; “moderate,” $\text{PM}_{2.5} > 12 \mu\text{g}/\text{m}^3$ based on Environmental Protection Agency criteria) and corrected expression of the genes most significantly related to $\text{PM}_{2.5}$ level in the SAE of smokers identified using a linear mixed model plotted. Corrected SAE gene expression residuals were calculated by subtracting the linear mixed model predicted random effects and all fixed effects except for 30-day mean $\text{PM}_{2.5}$ concentration. (A) $\text{PM}_{2.5}$ -related upregulated genes. (B) $\text{PM}_{2.5}$ -related downregulated genes. y-axis, corrected SAE gene expression; x-axis, $\text{PM}_{2.5}$ exposure group. *P* values were determined by Student’s *t* test. ATN1 = atrophin 1; CSNK1G3 = casein kinase 1 gamma 3; HAP1 = huntingtin-associated protein 1; HSD17B10 = hydroxysteroid 17- β dehydrogenase 10; MFSD14A = major facilitator superfamily domain-containing 14A; PIBF1 = progesterone immunomodulatory binding factor 1; PRKACA = protein kinase CAMP-activated catalytic subunit α ; RBBP7 = RB-binding protein 7, chromatin remodeling factor; UBXN11 = UBX domain protein 11; ZNF440 = zinc finger protein 440.

The epidemiologic evidence clearly demonstrates that PM exposure is associated with airflow obstruction and the development of COPD (16, 17, 22–25), but the biologic mechanisms underlying these clinical observations are not well understood. Studies have shown that concentrations of inhaled PM in distal airways greatly exceed those in the large

airways (11, 12). An autopsy study of nonsmoking females exposed to chronically elevated PM levels in Mexico City demonstrated evidence of small airway remodeling with increased fibrous tissue and muscle in airway walls and deposition of pigmented particles in the respiratory bronchioles compared with the small airway of control individuals living in

Vancouver (5-yr mean $\text{PM}_{2.5}$, $15 \mu\text{g}/\text{m}^3$) (57). Similarly, pathologic examination of the lungs of both nonsmokers and smokers from the central valley of California (mean $\text{PM}_{2.5}$, $22 \mu\text{g}/\text{m}^3$ during the study period) found particle accumulation and airway wall thickening and remodeling, most marked in the small airways, with more severe changes present in smokers (12). Consistent with these observations, our study demonstrates that, with chronic exposure to $\text{PM}_{2.5}$ below the levels associated with airway remodeling in nonsmokers (57), smokers show evidence of $\text{PM}_{2.5}$ -associated dysregulation of the transcriptome, which may be the precursor to the development of overt airway remodeling.

It has been postulated that smoking and PM exposure may act synergistically to cause airway disease. The consequences of smoking include impairment of pulmonary defense mechanisms, such as mucociliary clearance, in part because of a reduction in the number and function of ciliated cells and an increase in secretory cells in the airway of smokers, as observed in this cohort, which may compound the effects of PM on the airway epithelium. It has been shown that smokers have a reduced ability to clear PM (58), and pollution itself impairs mucociliary function (59). Additionally, increased deposition of small particulates in the small airways has been reported in smokers with evidence of peripheral airways disease, potentially exacerbating their effect (60). A study examining the additive effect of smoking and pollution assessed the pulmonary function of nonsmokers and smokers in areas with higher and lower pollution levels in Beijing found that smokers living in higher pollution areas had a significantly lower FEV₁ and FVC than smokers in lower pollution areas (61).

$\text{PM}_{2.5}$ pollution is a heterogeneous mixture of solid and liquid components that varies depending on the source and meteorologic conditions. Oxidative stress plays an important role in the detrimental effects of $\text{PM}_{2.5}$ pollution, and particle oxidative properties and components, including sulfate, nitrate, metals, organic compounds, biologic materials, and surface-absorbed gases, such as ozone, may be relevant to its toxicity (13). In NYC, most $\text{PM}_{2.5}$ pollutants are generated through the combustion of fuel by vehicles, power plants, and heating systems (62).

Table 4. Mitochondria-related PM_{2.5}-associated Genes in the Small Airway Epithelium of Healthy and COPD Smokers*

Gene Symbol	Gene Name	P Value	pBH	Regression Coefficient [†]	95% Confidence Interval
HSD17B10	Hydroxysteroid (17-β) dehydrogenase 10	5.8×10^{-6}	1.0×10^{-2}	0.009	0.005 to 0.013
OCIAD2	OCIA domain-containing 2	7.3×10^{-6}	1.1×10^{-2}	0.012	0.007 to 0.017
SLC30A6	Solute carrier family 30 (zinc transporter), member 6	1.9×10^{-5}	1.4×10^{-2}	0.006	0.003 to 0.009
ETFB	Electron-transfer-flavoprotein, β polypeptide	2.9×10^{-5}	1.5×10^{-2}	0.010	0.006 to 0.015
ATIC	5-Aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	3.1×10^{-5}	1.5×10^{-2}	0.008	0.004 to 0.012
VWA8	von Willebrand factor A domain-containing 8	6.9×10^{-5}	2.2×10^{-2}	0.006	0.003 to 0.009
RMDN1	Regulator of microtubule dynamics 1	6.2×10^{-5}	2.2×10^{-2}	0.006	0.003 to 0.009
ECHDC1	Ethylmalonyl-CoA decarboxylase 1	1.1×10^{-4}	2.6×10^{-2}	0.008	0.004 to 0.012
MTIF2	Mitochondrial translational initiation factor 2	1.5×10^{-4}	2.8×10^{-2}	0.007	0.003 to 0.010
NUDT19	Nudix hydrolase 19	1.2×10^{-4}	2.8×10^{-2}	0.009	0.004 to 0.013
MGME1	Mitochondrial genome maintenance exonuclease 1	1.8×10^{-4}	2.9×10^{-2}	0.007	0.003 to 0.010
THNSL1	Threonine synthase-like 1	2.2×10^{-4}	3.3×10^{-2}	0.007	0.003 to 0.011
BCL2L1	BCL2-like 1	2.7×10^{-4}	3.7×10^{-2}	-0.010	-0.015 to -0.005
KARS	Lysyl-tRNA synthetase	3.2×10^{-4}	4.0×10^{-2}	0.007	0.003 to 0.011
ME2	Malic enzyme 2, NAD(+) dependent, mitochondrial	3.8×10^{-4}	4.2×10^{-2}	0.007	0.003 to 0.011
ECI1	Enoyl-CoA delta isomerase 1	4.3×10^{-4}	4.4×10^{-2}	0.008	0.004 to 0.013
MTPAP	Mitochondrial poly(A) polymerase	4.7×10^{-4}	4.4×10^{-2}	0.005	0.002 to 0.008
ATP5A1	ATP synthase F1 subunit α	6.1×10^{-4}	4.6×10^{-2}	0.009	0.004 to 0.014
FKBP8	FK506-binding protein 8, 38kDa	6.2×10^{-4}	4.6×10^{-2}	-0.010	-0.016 to -0.004
HSPA9	Heat shock 70kDa protein 9 (mortalin)	6.1×10^{-4}	4.6×10^{-2}	0.007	0.003 to 0.011
GPAM	Glycerol-3-phosphate acyltransferase, mitochondrial	5.4×10^{-4}	4.6×10^{-2}	0.006	0.003 to 0.010
MRPL38	Mitochondrial ribosomal protein L38	5.6×10^{-4}	4.6×10^{-2}	-0.009	-0.014 to -0.004
PDE12	Phosphodiesterase 12	5.7×10^{-4}	4.6×10^{-2}	0.004	0.002 to 0.006

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; pBH = Benjamini-Hochberg corrected *P* value; PM_{2.5} = particulate matter less than or equal to 2.5 μm in aerodynamic diameter.

*Genes encoding proteins with mitochondrial localization dysregulated in the small airway epithelium of smokers in response to PM_{2.5} exposure (56).

[†]Regression coefficients from the linear mixed model indicate the direction and magnitude of gene expression of the fitted pollution effect.

Mitochondria are complex organelles that control many critical cellular functions, including redox homeostasis, apoptosis, and autophagy. Mitochondrial dysfunction has been linked to lung diseases, including COPD (63), and PM_{2.5} pollution has been reported to induce oxidative stress and mitochondrial toxicity (64). Abnormalities in mitochondrial gene expression have previously been associated with PM₁₀ pollution exposure in human circulating leukocytes (65). The observation in this study that many mitochondria-related genes are dysregulated in the SAE of smokers in response to PM_{2.5} pollution exposure potentially links PM_{2.5}-induced oxidative stress and mitochondrial dysfunction to the development of pollution-related respiratory disease.

Relevant to the pathophysiologic consequences of small airway biologic function, many of the genes observed to be most significantly dysregulated by PM_{2.5} exposure in the SAE of smokers relate to cell growth, proliferation, and transcription regulation. Pollution is recognized to

induce epigenetic changes in the human genome (37, 38). Interestingly, RBBP7, the gene most significantly associated with PM_{2.5} levels, encodes a histone-binding protein that serves as a transcriptional repressor, DNA damage repair, and chromatin assembly (52). Other transcription- and translation-related genes dysregulated in association with PM_{2.5} include RTCB (an RNA ligase) (66) and ZNF440 (zinc finger protein 440), which binds nucleic acids, while CDC27 (cell division cycle 27) and HCFC1 (host cell factor C1) are linked to cell cycle control (67, 68). AIMP1, a proinflammatory cytokine upregulated by cellular stresses, including oxidative stress (53), was also significantly associated with PM_{2.5} levels. AIMP1 is an antiangiogenic cytokine that induces apoptosis in endothelial cells after cleavage to its active form by proteinases including caspase-3 (53), also significantly associated with PM_{2.5} levels. Both AIMP1 and caspase-3 have been linked to the pathogenesis of emphysema and COPD (69) and may represent one pathway through which PM_{2.5} induces lung disease.

Another related gene significantly associated with PM_{2.5} levels in smokers was AIMP2 (aminoacyl tRNA synthetase complex interacting multifunctional protein 2), which is linked to oxidative stress and apoptosis (70), again suggesting that perturbations of oxidative stress response pathways play a role in pollution-related respiratory disease.

Limitations of the study include demographic differences among the phenotype groups, which were corrected for by inclusion of appropriate covariates in the linear mixed model. Additionally, there were fewer nonsmokers included compared with the healthy and COPD smokers. However, when each phenotype group was analyzed individually it was possible to detect significant SAE transcriptome changes in COPD smokers, a phenotype with a similar subject number to nonsmokers. Additionally, the findings in nonsmokers are consistent with prior studies suggesting that smokers and individuals with COPD are more susceptible than nonsmokers to the adverse health effects of ambient pollution (2, 12, 61).

Although smoking and COPD *per se* modify SAE biology, the data in the present study demonstrate that PM_{2.5} exposure also contributes to significant changes in the SAE transcriptome of these populations, suggesting prior dysregulation of the SAE is exacerbated by pollution exposure. These findings provide novel insight into the biologic mechanisms linking pollution to the risk

of development of respiratory disease. Additionally, these data suggest that PM_{2.5} exposure, even at levels below EPA and World Health Organization guidelines for “safe” PM_{2.5} levels (50, 71), is associated with alterations in the SAE transcriptome in smokers, suggesting that there may be no safe threshold for pollution exposure, particularly in susceptible individuals. ■

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