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Epigenetic Alterations Induced by Ambient Particulate Matter in Mouse Macrophages

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Abstract

Respiratory mortality and morbidity has been associated with exposure to particulate matter (PM). Experimental evidence suggests involvement of cytotoxicity, oxidative stress, and inflammation in the development of PM-associated pathological states; however, the exact mechanisms remain unclear. In the current study, we analyzed short-term epigenetic response to PM₁₀ (particles with aerodynamic diameter less than 10 µm) exposure in mouse ascitic RAW264.7 macrophages (BALB/C Abelson murine leukemia virus-induced tumor). Ambient PM₁₀ was collected using a high volume sampler in Little Rock, AR. Analysis revealed that PM₁₀ was composed mainly of Al and Fe, and the water soluble organic fraction was dominated by aliphatic and carbohydrate fragments and minor quantities of aromatic components. Exposure to PM₁₀ compromised the cellular epigenome at concentrations 10–200 µg/ml. Specifically, epigenetic alterations were evident as changes in the methylation and expression of repetitive element-associated DNA and associated DNA methylation machinery. These results suggest that epigenetic alterations, in concert with cytotoxicity, oxidative stress, and inflammation, might contribute to the pathogenesis of PM-associated respiratory diseases.

Keywords

air pollution; DNA methylation; repetitive elements

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AUTHOR CONTRIBUTIONS

A.B., I.G.K., and I.K. designed the study, I.R.M., M.C.B., X.W. conducted the research, I.R.M., M.C.B., N.A.B., X.W., A.B., I.G.K., and I.K. analyzed the data, I.G.K. and I.K. wrote the manuscript. All authors reviewed the manuscript.

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Additional Supporting Information may be found in the online version of this article.

INTRODUCTION

Exposure to particulate matter (PM) has been estimated to cause over 3 million excess deaths annually, primarily from cardiopulmonary disease and lung cancer [Lim et al., 2012]. Most recently, PM was recognized as carcinogenic to humans (Group 1) as an increased risk of lung cancer with increasing levels of exposure to PM has been shown [IARC, 2013]. Experimental evidence suggests that cytotoxicity, oxidative stress, and inflammation are involved in the development of PM-associated pathological states; however, the exact mechanisms are not well understood.

A growing number of studies report epigenetic alterations caused by environmental factors *in vitro*, *in vivo*, and in occupationally exposed human subjects [Bollati and Baccarelli, 2010; Feil and Fraga, 2011; Koturbash et al., 2011]. These findings have led to the recognition of the epigenome as an important target of environment-induced modification that functions as an interface between the inherited genome and the environment [Umer and Herceg, 2013]. DNA methylation is the most studied mechanism of epigenetic regulation and is crucial for normal development and maintenance of cellular homeostasis. Alterations in DNA methylation may lead to reactivation of oncogenes and repetitive DNA sequences, to silencing of tumor-suppressor genes, and can result in genomic instability and cancer [Howard et al., 2008]. Of particular interest is a loss of proper control over repetitive elements. Formerly, considered “junk” DNA, repetitive elements have become increasingly recognized as an important class of regulators of genetic expression [Roman et al., 2011; Cowley and Oakey, 2013]. Repetitive elements comprise nearly half of the entire genome and are one of the major contributors to cancer-related hypomethylation, which is associated with their reactivation and genomic instability [Weisenberger et al., 2005; Ehrlich et al., 2006].

Initial studies on the epigenetic effects of exposure to PM reported induction of histone acetylation in human A549 and BEAS-2B cell lines in response to ambient particles and diesel exhaust, respectively [Gilmour et al., 2004; Cao et al., 2007]. Animal studies have demonstrated global DNA hypermethylation in sperm after exposure to particulate air pollution in mice [Yauk et al., 2008] and in rats lung following 6 months of exposure to gasoline engine exhaust [Reed et al., 2008], as well as hypermethylation of p16^{INK4A} in the mouse lung after exposure to concentrated ambient PM_{2.5} [Soberanes et al., 2012]. Recent epidemiological studies have also demonstrated an association between PM exposure and changes in peripheral blood nuclear DNA methylation, primarily in transposable repetitive elements LINE1 and Alu (in mice known as SINE B1) [Baccarelli et al., 2009; Madrigano et al., 2011]. Despite significant progress in the field, there is lack of data relating to the effects of PM exposure on DNA methylation in the respiratory system.

Macrophages, the pro-inflammatory cells, are important constituents of the first line of defense against inhaled particles. In this study, we used the macrophage RAW264.7 cell line, an accepted model for studies of molecular effects caused by PM, to elucidate whether or not short-term exposure to ambient PM can result in substantial alterations in DNA methylation.

MATERIALS AND METHODS

PM Collection, Chemical Analysis, and Preparation

Wintertime atmospheric particles (PM₁₀; particles with aerodynamic diameter less than 10 µm) were collected in an urban area (Little Rock, AR) for 14 days on a quartz filter. A portion of the filter was analyzed for elements by inductively coupled plasma mass spectrometry, for water-soluble organic carbon (WSOC) by thermal optical reflectance method and for organic speciation by proton nuclear magnetic resonance (¹H-NMR) spectroscopy [Chalbot et al., 2013b]. For the cell culture studies, we obtained the aqueous extract of the PM by sonication for 1-hr with 50 ml of ultrapure H₂O. The extract was filtered through a 0.45-µm filter, lyophilized, weighed, and kept frozen. The extract was redissolved in H₂O prior to cell exposure.

Cell Culture and Exposure

RAW264.7 ascitic macrophages, derived from the BALB/C Abelson murine leukemia virus-induced tumor, were obtained from ATCC (Manassas, VA). Cultured cells were grown in Dulbecco's modified Eagle medium (DMEM) media and fetal bovine serum (FBS) supplemented with 1:100 penicillin/streptomycin (Life Technologies, Carlsbad, CA). Cells were exposed to 0, 10, 20, 50, 100, 200, 300, 400, 500, and 600 µg/ml of PM₁₀ added to the culture media. After 24 hr of incubation, cells were harvested and kept frozen at -80°C until molecular analyses. Experimental exposures were performed twice and four samples per treatment group were analyzed at each time point.

Lactate Dehydrogenase Release Assay

Cells were grown in a 96-well plate overnight to 80% confluence and then exposed with varying concentrations of the PM for 24 hr. Cell death was quantified by using the lactate dehydrogenase (LDH) release assay kit (Promega, Madison, WI) as per manufacturer's instructions.

TUNEL Assay

Cells were grown in 8-well glass chambers to sub-confluence, exposed to PM₁₀ for 24 and 48 hr, and then fixed with 5% formalin. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol.

Quantitative Reverse Transcription Real-Time Polymerase Chain Reaction

Total RNA was extracted from RAW264.7 cells using the AllPrep DNA/RNA extraction kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. DNA and RNA concentration and integrity were ascertained by Nanodrop 2000 (Thermo Scientific, Waltham, MA). The levels of gene transcripts were determined by Quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) using TaqMan Gene Expression Assays (Life Technologies, Carlsbad, CA; and Integrated DNA Technologies, Inc, Coralville, IA) (Supporting Information Table 1), according to the manufacturer's

protocol. The mRNA abundance of DNA repetitive elements was determined by qRT-PCR, as previously described [Martens et al., 2005] (Supporting Information Table 2).

Quantification Analysis of 5-Methylcytosine

Whole genome CpG methylation was assessed using a fluorescence-based immunoassay MethylFlash Methylated DNA Quantification Kit (Epigentek, Farmingdale, NY) according to the manufacturer's protocol.

Analysis of Methylation Status of DNA Repetitive Elements

The methylation status of DNA repetitive elements was determined by methylation-sensitive McrBC-qPCR assay as previously described [Martens et al., 2005]. Primer sequences can be found in the Supporting Information Table 2.

Statistical Analyses

All data are presented as mean $\pm$ standard error of mean of at least four independent samples per each group of treatment. The significance of difference in mean values between groups was examined with ANOVA followed by Tukey's HSD test (IBM SPSS 21.0). The $P < 0.05$ for fold changes from control of at least 1.5 was considered significant.

RESULTS AND DISCUSSION

PM Chemical Composition

The ambient PM₁₀ concentration was 46.9 $\mu\text{g}/\text{m}^3$. The total water-soluble extract (TWSE) and WSOC concentrations were 9.0 ± 0.5 and 3.1 ± 0.1 $\mu\text{g}/\text{m}^3$, respectively. The organic mass accounted for 4.2 $\mu\text{g}/\text{m}^3$ of the TWSE, which is comparable to the organic mass contribution on fine particles in the same area for the 2001–2010 [Chalbot et al., 2013a]. Chain methylene ($-\text{CH}_2-$) groups (1.2–1.8 ppm) were more abundant than methyl (CH_3-) groups (0.7–1.2 ppm) (Fig. 1). Aliphatic protons adjacent to a double bond ($\text{H}-\text{C}=\text{C}-$; 1.8–3.2 ppm) were attributed to mono/dicarboxylic acids and ketone groups. $\text{H}-\text{C}$ and $\text{H}-\text{C}-\text{C}=\text{C}$ protons accounted for 27.5% and 16.1% of H-atoms, respectively. The resonances in the 2.5–4.4 ppm region indicated the presence of amines ($\text{N}-\text{H}$), sulfonates (RSO_3-), hydroxy- ($\text{H}-\text{C}-\text{O}$), and ether-Hs ($\text{H}-\text{C}-\text{O}-\text{C}$), which are typically associated with carbohydrates and sugars. In agreement with previous studies, methanesulfonate (2.8 ppm) and levoglucosan (5.48 ppm), products of the marine dimethylsulfide and biomass burning aerosol, respectively, were identified [Chalbot et al., 2013a,b]. The percentage of H-atoms in the carbohydrate range (3.2–4.4 ppm; $\text{O}-\text{C}-\text{H}$) was 53.5%. Minor quantities of aldehydic-Hs (0.1% of H-atoms) were observed at 9.0–10.0 ppm. Aromatic-Hs (6.5–8.3 ppm) accounted for 2.7% of H-atoms. The total concentration of H-atoms was 0.17 $\mu\text{mol}/\text{m}^3$, resulting in a molar H/C ratio of 0.68. This was comparable to that observed for polyacids and organic aerosol from biomass burning.

The most abundant elements were Al and Fe (720 and 690 ng/m^3 , respectively). The concentrations of Cu, Zn, and Mn were 183, 31, and 27 ng/m^3 , respectively, while minor quantities were measured for Pb, V, As, Ni, and Cr (4, 2, 1, 1, and 1 ng/m^3 , respectively). Their contribution was 1.7 $\mu\text{g}/\text{m}^3$. Based on the elemental concentration ratios of Al/Si,

K/Fe, and Al/Ca, a total of up to 4.4 $\mu\text{g}/\text{m}^3$ was associated with metals. We previously determined that these metals mostly originate from mineral dust, primary traffic, diesel combustion, and biomass burning.

Effects of PM Exposure on Cytotoxicity, Inflammation, and Oxidative Stress

Cytotoxicity of PM₁₀ exposure is well-documented [Monn and Becker, 1999]. By LDH release assay, the PM₁₀ was cytotoxic beginning from 300 $\mu\text{g}/\text{ml}$, reaching statistical significance at 500 $\mu\text{g}/\text{ml}$ ($P < 0.01$) (Fig. 2A). TUNEL assay confirmed this finding by demonstrating the increase of TUNEL-positive cells at concentrations above 300 $\mu\text{g}/\text{ml}$ at 24 and 48 hr time-points (Fig. 2B). Therefore, to limit the influence of cytotoxicity on evaluated molecular parameters, exposures to PM₁₀ concentrations below 300 $\mu\text{g}/\text{ml}$ were considered further.

Macrophages drive the innate immune response induced by particles via the production of pro-inflammatory cytokines. Hence, we examined the expression of the generally accepted marker of inflammatory response—the cytokine Tnf α . Exposure to PM₁₀ resulted in a significant dose-dependent increase in Tnf α expression ($R^2 = 0.81$), starting at a concentration of 20 $\mu\text{g}/\text{ml}$ (Fig. 2C).

Analysis of the expression of genes involved in response to oxidative stress provides a valuable and rapid tool for determination of PM-induced parameters associated with changes in redox homeostasis and DNA damage [Brauner et al., 2007]. Therefore, we measured the expression of genes associated with oxidative stress. Exposure to PM resulted in a dose-dependent increase in gene expression of *Cat*, *Hmox1*, and *Sod2* ($R^2 = 0.90, 0.59$, and 0.67 , respectively). No significant differences were observed in the expression of *Ogg1* and *Gpx1* (Fig. 2C).

Effects of PM Exposure on DNA Methylation

Exposure to PM has been shown to impact DNA methylation (reviewed in [Wang et al., 2012]). In order to evaluate the effect of PM₁₀ on global DNA methylation in RAW264.7 macrophages, we measured the genome-wide levels of 5-mC in the DNA of control and exposed cells. Exposure for 24 hr did not result in significant alterations in the global 5-mC levels independently of PM₁₀ concentration (Fig. 3A).

Effects of PM Exposure on Repetitive Elements

We evaluated the methylation status of the five most abundant repetitive elements: LINE1, SINE B1, SINE B2, and major and minor satellites. These transposable elements comprise at least half of mammalian genomes and are believed to be silenced by DNA methylation [Roman et al., 2011; Fedoroff, 2012; Cowley and Oakey, 2013]. Exposure to 10 $\mu\text{g}/\text{ml}$ of PM₁₀ resulted in slight hypermethylation in the LINE1 (1.45-fold, $P < 0.1$) (Fig. 3B). Also, notable increases in methylation of LINE1, SINE B1, SINE B2, and minor satellites were detected after exposure to 20 $\mu\text{g}/\text{ml}$ of PM₁₀ (1.6- to 2-fold change), but these were not statistically significant. Opposite effects were observed in major satellites, where loss of DNA methylation was detected after exposure to 20 $\mu\text{g}/\text{ml}$ of PM₁₀ (1.7-fold, $P < 0.1$) reaching 4-fold decrease ($P < 0.01$) at 200 $\mu\text{g}/\text{ml}$, suggesting the re-distribution of

methylation patterns between repetitive elements. Several epidemiological studies report loss of LINE1 and Alu (SINE B1) methylation in human peripheral blood after exposure to PM_{2.5-10} [Tarantini et al., 2009]. These differences can possibly be due to different cell types that were investigated, duration of exposures, as well as different size (PM_{2.5}–PM₁₀) and composition of the PM. Interestingly, an association between repetitive elements hypermethylation and allergen sensitization has been recently reported [Sordillo et al., 2013]. Finally, alterations in DNA methylation associated with metals present in PM (such as iron, found at high concentrations in the PM₁₀ investigated in this study) were also previously demonstrated [Brocato and Costa, 2013].

Because DNA methylation plays an important role in transcriptional regulation of repetitive elements, we measured the expression of the latter in control and PM₁₀-exposed cells. Interestingly, we detected significant reactivation of SINE B2 and major and minor satellites upon PM₁₀ exposure; a dose-dependent increase was observed (statistically significant for SINE B2 at $P < 0.001$; F -value of 8.1) (Fig. 3C). In contrast, exposure to PM₁₀ resulted in transcriptional silencing of LINE1 for all PM₁₀ concentrations and no dose-dependent trends were observed. Transposable elements are vital maintainers of the stability and proper function of the genome. Via “copy and paste” (retrotransposons) or “cut and paste” (transposons) mechanisms, repetitive elements may influence the genome by insertion into ORF, provision of novel promoters and splice sites, introduction of transcription factors binding sites, and insulator activity [Roman et al., 2011; Fedoroff, 2012; Cowley and Oakey, 2013]. Observed alterations in repetitive elements expression, primarily associated with their reactivation, may significantly impact expression of genetic information; however, the persistence of these alterations in response to PM exposure needs to be further investigated.

Discrepancies between the methylation and transcriptional statuses of transposable elements suggest that histone modifications may also be involved in their silencing, and histone-mediated regulation may even precede regulatory effects of DNA methylation [Martens et al., 2005; Maksakova et al., 2008]. The limited amount of collected PM₁₀ material did not allow for a comprehensive analysis of histone modifications. Therefore, we performed an analysis of gene expression for the histone deacetylases *Hdac1* and *Hdac2*, whose expression has been shown to be tightly associated with the silencing of repetitive elements [Montoya-Durango et al., 2009]. While no changes were detected in the expression of *Hdac1* (data not shown), significant dose-dependent loss of expression was detected for *Hdac2* ($R^2 = 0.54$) (Fig. 3D). Additionally, we measured the expression of *Ezh2*, which serves as a histone methyltransferase for histone H3K27 trimethylation; *Prdm2*, which serves as methyltransferase for histone H3K9 trimethylation—the histone marks associated with chromatin condensation and transcriptional silencing; and two other genes that were shown to interact with repetitive elements—*Rb1* and *E2f1* [Montoya-Durango et al., 2009]. We detected a significant ($P < 0.05$) and dose-dependent ($R^2 = 0.74$) loss of expression of *Ezh2* and weak decreases in expression of *Rb1* and *E2f1* (Fig. 3D) suggesting the involvement of his-tone regulation in repetitive elements expression.

PM and DNA Methylation Machinery

Proper maintenance of DNA methylation is facilitated by the family of DNA methyltransferases (*Dnmt1*, *Dnmt3a*, *Dnmt3b*) and accessory proteins, such as *Uhrf1*, that recruit *Dnmt1* to the replication fork and hemimethylated sites. Treatment of cells with PM₁₀ resulted in a significant decrease of mRNA levels of all three DNA methyltransferases as well as *Uhrf1* (at $P < 0.05$; F -values higher than 9.0) in a dose-dependent manner ($R^2 = 0.68, 0.65, 0.63$, and 0.84 for *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Uhrf1*, respectively) (Fig. 4). The most pronounced changes were observed in the expression of the *de novo* methyltransferase *Dnmt3b*, where nearly a 2-fold decrease in mRNA levels were observed after exposure to 10 µg/ml and a 4-fold decrease after exposure to 200 µg/ml of PM₁₀. The expression of methyl-binding protein *Mecp2* was also significantly diminished, although to a lesser extent (1.5- to 1.6-fold), and did not resemble dose dependence. Loss of function of DNA methyltransferases may lead to an imbalance in the maintenance of normal methylation patterns and result in alterations in DNA methylation. Additionally, recent studies also indicate that the deletion of one of the following genes: *Dnmt1*, *Dnmt3a*, *Dnmt3b*, or *Uhrf1* results in profound losses of DNA methylation in some repetitive elements, including major satellites [Arand et al., 2012].

CONCLUSIONS

We demonstrate that short-term exposure to PM₁₀, in addition to causing generally accepted cytotoxicity, inflammation, and oxidative stress, may also compromise the cellular epigenome in cells as a first line of defense against the inhaled particles. Observed alterations were dose-dependent decrease in the expression of DNA methylation machinery, and re-distribution of methylation patterns within the repetitive elements and their reactivation. Our results indicate that the mixture of low, environmentally relevant, concentrations of metal and organic components was capable of causing epigenetic effects, in agreement with the results of epidemiological studies [Cantone et al., 2011]. Further studies, nevertheless, are needed to investigate the persistence of epigenetic effects of PM₁₀ exposure and to delineate which PM₁₀ fractions have the most pronounced effects on the cellular epigenome.

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Fig. 2.

Analysis of cytotoxicity (**A, B**), inflammatory, and oxidative (**C**) potentials of the water-soluble fraction of PM on the RAW264.7 cells. **A**: LDH release. Asterisks (*) denote significant ($P < 0.05$) difference from control. **B**: TUNNEL assay. Cells were mounted under cover slips with a Prolong® Antifade kit and acquired using the Olympus IX-51 inverted microscope equipped with a Hamamatsu ORCA-ER monochrome camera. **C**: The differential gene expression was determined by quantitative RT-PCR. Asterisks (*) denote significant ($P < 0.05$) difference from control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 3.

Effects of PM exposure on global DNA methylation (**A**), methylation (**B**), and expression (**C**) of repetitive elements and histone modifications machinery (**D**). **A**: Global DNA methylation as measured by quantification of the 5-methylcytosine (5-mC). Asterisks (*) denote significant ($P < 0.05$) difference from control. **B**: DNA methylation in repetitive elements as measured by methylation-sensitive MspBC-qPCR assay. Asterisks (*) denote significant ($P < 0.05$), and “a” ($P < 0.1$) difference from control. **C**: The differential expression of repetitive elements was determined by quantitative RT-PCR. Asterisks (*) denote significant ($P < 0.05$) difference from control. **D**: The differential gene expression was determined by quantitative RT-PCR. Asterisks (*) denote significant ($P < 0.05$) difference from control.

Fig. 4.

Effects (mean $\pm$ σ) of PM exposure on expression of genes involved in regulation of DNA methylation. Asterisks (*) denotes significant ($P < 0.05$) difference from control.