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Particulate Air Pollution, Oxidative Stress Genes, and Heart Rate Variability in an Elderly Cohort

Teresa Chahine,1 Andrea Baccarelli,1,2 Augusto Litonjua,3 Robert O. Wright,1,3 Helen Suh,1 Diane R. Gold,1,3 David Sparrow,4 Pantel Vokonas,4 and Joel Schwartz1

1Department of Environmental Health, Harvard School of Public Health, Boston, Massachusetts, USA; 2Center of Molecular Epidemiology and Genetics; and EPOCA Epidemiology Research Center, University of Milan and IRCCS Maggiore Hospital, Mangiagalli and Regina Elena Foundation, Milan, Italy; 3Channing Laboratory, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts, USA; 4VA Normative Aging Study, Veterans Affairs Boston Healthcare System and the Department of Medicine, Boston University School of Medicine, Boston, Massachusetts, USA

BACKGROUND AND OBJECTIVES: We have previously shown that reduced defenses against oxidative stress due to glutathione S-transferase M1 (GSTM1) deletion modify the effects of PM2.5 (fine-particulate air pollution of <2.5 µm in aerodynamic diameter) on heart rate variability (HRV) in a cross-sectional analysis of the Normative Aging Study, an elderly cohort. We have extended this to include a longitudinal analysis with more subjects and examination of the GST short tandem repeat polymorphism in the heme oxygenase-1 (HMOX-1) promoter.

METHODS: HRV measurements were taken on 539 subjects. Linear mixed effects models were fit for the logarithm of HRV metrics—including standard deviation of normal-to-normal intervals (SDNN), high frequency (HF), and low frequency (LF)—and PM2.5 concentrations in the 48 hr preceding HRV measurement, controlling for confounders and a random subject effect.

RESULTS: PM2.5 was significantly associated with SDNN (p = 0.04) and HF (p = 0.03) in all subjects. There was no association in subjects with GSTM1, whereas there was a significant association with SDNN, HF, and LF in subjects with the deletion. Similarly, there was no association with any HRV measure in subjects with the short repeat variant of HMOX-1, and significant associations in subjects with any long repeat. We found a significant three-way interaction of PM2.5 with GSTM1 and HMOX-1 determining SDNN (p = 0.008), HF (p = 0.01) and LF (p = 0.04). In subjects with the GSTM1 deletion and the HMOX-1 long repeat, SDNN decreased by 13% [95% confidence interval (CI), −21% to −4%], HF decreased by 28% (95% CI, −43% to −9%), and LF decreased by 20% (95% CI, −35% to −3%) per 10 µg/m³ increase in PM.

CONCLUSIONS: Oxidative stress is an important pathway for the autonomic effects of particles.


Particulate air pollution (PM) is associated with increased risk of hospitalization and death from cardiovascular disease (Brook et al. 2004; Forastiere et al. 2005; Samet et al. 2000; Schwartz 1999; Zanobetti and Schwartz 2005), but the mechanisms underlying such effects are not fully understood. Reductions in heart rate variability (HRV), a noninvasive measure that independently predicts cardiovascular mortality (Tsuij et al. 1996), have been related to PM exposure, particularly to fine-particulate air pollution of <2.5 µm in aerodynamic diameter (PM2.5) (Creason et al. 2001; Devlin et al. 2003; Gold et al. 2000; Holguin et al. 2003; Liao et al. 2005a; Magari et al. 2002; Park et al. 2005; Pope et al. 2004; Schwartz et al. 2005a).

Animal experiments indicate that reactive oxygen species (ROS), which have established relevance in the pathogenesis of cardiovascular disease (Dhalla et al. 2000), are potent mediators for particle effects on HRV and other cardiovascular end points (Brook et al. 2004; Gurgueira et al. 2002; Nel 2005; Rhoden et al. 2004). While animal models can identify potential mechanisms of particle effects, the relative importance of these pathways in humans at lower doses is not clear and may be determined by examining subjects with genetically determined differences in oxidative-stress defenses. In elderly subjects living in the Boston, Massachusetts, metropolitan area, we recently showed that PM2.5 levels during the 48 hr before the study were associated with decreased HRV in individuals with the glutathione S-transferase M1 (GSTM1) deletion, but had no effect in subjects with GSTM1 present (Schwartz et al. 2005b).

Studies showing the effect of just one polymorphism are unlikely to correctly represent the complex etiology of common diseases, and failure to account for gene–gene interactions in the search for susceptibility genes has been widely suggested to explain the persisting difficulties in replicating significant findings (Mills et al. 2006). Particle exposure induces both heme oxygenase-1 (HMOX-1) and GSTM1 expression through activation of the genetic antioxidant response element (ARE) (Li et al. 2004). A high number of microsatellite (GT)n dinucleotide repeats in 5′-flanking region may reduce HMOX-1 inducibility by ROS and has been associated with increased risk of coronary artery disease in high-risk groups with hyperlipidemia, diabetes, or current smoking (Chen et al. 2002; Kaneda et al. 2002). Consequently, individuals with a high number of (GT)n repeats may be more susceptible to the effects of airborne particles.

We hypothesized that the gene encoding HMOX-1, which is involved in various aspects of responses against oxidative stress, may a) directly modify the effect of ambient PM on HRV and b) interact with GSTM1 to determine which subjects are susceptible to airborne particle effects. To establish the role of the antioxidant response pathway in determining the cardiovascular effects of airborne particles, we examined in the present study the association of PM2.5 with HRV in a repeated measure study of elderly subjects from the Boston metropolitan area, and evaluated how that association was affected by genetic variation in the HMOX-1 and GSTM1 loci.

Materials and Methods

Study population. Our study population consisted of 539 white males from the Normative Aging Study (NAS), a longitudinal study of aging established in 1963 by the U.S. Veterans Administration (Bell et al. 1972). Between January 2000 and June 2005, all participants still presenting for examination (n = 676) were evaluated for HRV. Of these, 137 subjects were excluded because of heart arrhythmias, measurement time <3.5 min, or
missing potential confounding variables or HMOX-1 data. Among the remaining 539 subjects, GSTM1 data were available for 476 subjects, who had one (n = 314) or two (n = 162) HRV measurements. In subjects with multiple HRV measurements, the time interval between measurements was approximately 3 years. This study was conducted in compliance with all applicable requirements of the U.S. and international regulations (including institutional review board approval). All subjects gave written informed consent prior to the study.

**HRV measurement.** HRV was measured at rest during normal breathing for 7 min using a two-channel (five-lead) ECG monitor (Trillium 3000; Forest Medical, East Syracuse, NY) while the subject was seated. Standard deviation of normal-to-normal intervals (SDNN), high frequency (HF) (0.15–0.4 Hz), and low frequency (LF) (0.04–0.15 Hz) were computed with a fast Fourier transform using software (Trillium 3000; Forest Medical) complying with established guidelines (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996). In the analysis, we used the 4 consecutive minutes of ECG reading that included the lowest number of artifacts.

**Air pollution and weather data.** Continuous PM2.5 was measured at a stationary monitoring site on the roof of Countway Library of Harvard University in downtown Boston using a Tapered Element Oscillating Microbalance (TEOM; Model 1400A, Rupprecht & Patashnick Co., East Greenbush, NY). Meteorological data was obtained from the Boston airport weather station. The 48-hr moving average of PM2.5 before each HRV measurement was used as the exposure index, as this exposure period has shown the strongest association in previous studies (Park et al. 2005).

**HMOX-1 and GSTM1 genotyping.** The HMOX-1 locus (UniGene Hs.301961; UniGene 2007a) was amplified at exons 4 and 5 by polymerase chain reaction (PCR) as previously described to differentiate between the null polymorphism and the presence of one or more copies of the gene (Schwartz et al. 2005b). The HMOX-1 (UniGene Hs.517581; UniGene 2007b) microsatellite (GT)n length assay was designed per Yamada and coworkers (Yamada et al. 2000). Briefly, the HMOX-1 locus was amplified by PCR at the 5’ promoter flanking region containing (GT)n repeats with primers as described by Yamada, and the sizes of the PCR products were analyzed with a laser-based automated DNA sequencer (AB 3100; Applied Biosystems, Foster City, CA). Although the exact cutoff for HMOX-1 modulation is still unknown, constructs with lengths of >25 repeats showed reduced HMOX-1 basal promoter activity and decreased transcriptional upregulation in response to various stimuli like H2O2 compared with lengths <25 repeats (Chen et al. 2002; Yamada et al. 2000). In the analysis of the data, we used the 25-repeat cutoff to categorize the study subjects in two categories [≤25 (GT)n repeats in both alleles or ≥25 (GT)n repeats in at least one allele] based on the HMOX-1 microsatellite length.

**Statistical analysis.** HRV measurements were log10-transformed to improve normality. The following potential confounders were chosen a priori and included in the analysis: age, body mass index (BMI), mean arterial pressure, fasting blood glucose, cigarette smoking (never/former/current), alcohol consumption (≥2 drinks a day, yes/no), use of beta-blockers, calcium channel blockers, acetylochinesterase (ACE) inhibitors, room temperature, season,
and 48-hr moving average of outdoor apparent temperature. Potential nonlinearity between apparent temperature and HRV was accounted for using a linear and quadratic term.

Because our data included repeated measures of HRV for many participants, our data may lack independence. To deal with this, we fit a mixed effects model (PROC MIXED in SAS version 9.0; SAS Institute Inc., Cary, NC). We assumed:

\[ Y_{it} = b_0 + u_i + b_1 X_{it} + \cdots + b_k X_{kt} + \beta_{Pollution} + \epsilon_{it}, \]

where \( Y_{it} \) is the logarithm of HRV in subject \( i \) at time \( t \), \( b_0 \) is the overall intercept, and \( u_i \) is the separate random intercept for subject \( i \). In the above, \( X_1 \text{--} X_k \) are the covariates measured at each of the visits in which the HRV measurements were taken. This captures the correlation among measurements within the same subject.

**Results**

Table 1 shows the levels and distribution of the variables used in this study, overall, and for the different combinations of GSTM1 genotype and HMOX-1 microsatellite repeat length. The study participants were all male, with average age of 72.8 years (SD = 6.6 years) at the first HRV measurement. No differences among the subpopulations defined by the combinations of GSTM1 genotype [wild-type or null] and HMOX-1 microsatellite repeat length (< 25 (GT)n repeats in both alleles or ≥ 25 (GT)n repeats in at least one allele) were found in age, BMI, systolic blood pressure, diastolic blood pressure, mean arterial pressure, heart rate, fasting blood glucose, total cholesterol, high-density lipoproteins (HDL), triglyceride, smoking status, alcohol intake (≥ 2 drinks/day), history of coronary heart disease (CHD), diabetes, hypertension, or stroke, and use of beta-blockers, calcium channel blockers, or ACE inhibitors (Table 1). The two genes were not associated with each other (\( p > 0.67 \)).

Table 2 shows the results of the analyses for the association of PM2.5 with changes in HRV for the entire population (model 1), by GSTM1 genotype (model 2), and by HMOX-1 microsatellite (GT)n repeat length (model 3).

For the entire population, we found that a 10 µg/m³ increase in ambient PM2.5 in the 48 hr before the HRV measurement was associated with a 6.8% decrease in SDNN [95% confidence interval (CI), –12.9 to –0.2; \( p = 0.043 \)] and with a 17.3% decrease in HF [95% CI, –30.0 to –2.3; \( p = 0.026 \)]. Ambient PM2.5 concentrations were also negatively associated with LF \( (\text{estimated change} = \text{–12.7% decrease in SDNN (95% CI, –22.8 to 2.2), but the result was not statistically significant (} p = 0.10 \).}

The PM2.5–HRV association was modified by GSTM1 genotype, with PM2.5 concentrations negatively associated with SDNN, HF, and LF in GSTM1-null subjects, whereas no association between PM2.5 and HRV was found in GSTM1–wild-type carriers. In subjects with the GSTM1-null deletion, a 10-µg/m³ increase in PM2.5 was associated with a 10.5% decrease in SDNN (95% CI, –18.2 to –2.2; \( p = 0.015 \)), a 24.2% decrease in HF (95% CI, –39.2 to –5.5; \( p = 0.014 \)), and a 17.0% decrease in LF (95% CI, –31.0 to –0.2; \( p = 0.048 \)). In GSTM1–wild-type subjects, the estimated decreases in HRV for a 10-µg/m³ increase in PM2.5 were 2.0% (95% CI, –11.3 to 8.3; \( p = 0.69 \)) for SDNN, 4.0% (95% CI, –24.8 to 22.6; \( p = 0.74 \)) for HF, and 0.6% (95% CI, –19.0 to 22.0; \( p = 0.95 \)) for LF. However, the \( p \)-values for statistical interactions between PM2.5 and GSTM1 genotype were not significant.

Similarly, we found that that PM2.5–HRV association was modified by HMOX-1 genotypes. Ambient PM2.5 concentrations were negatively associated with all three HRV outcomes of carriers of at least one allele with ≥ 25 microsatellite (GT)n repeats in the HMOX-1 promoter region, whereas no association between PM2.5 and HRV was present in carriers of < 25 repeats in both alleles. In subjects with at least one allele with ≥ 25 microsatellite (GT)n repeats, a 10-µg/m³ increase in PM2.5 was associated with a 8.5% decrease in SDNN (95% CI, –14.8 to –1.8; \( p = 0.014 \)), a 20.1% decrease in HF (95% CI, –32.9 to –5.0; \( p = 0.012 \)), and a 14.0% decrease in LF (95% CI, –25.7 to –0.5; \( p = 0.043 \)). The \( p \)-value for statistical interactions between PM2.5 and GSTM1 genotype was marginally significant (\( p = 0.059 \)) when the SDNN component of HRV was considered but was not significant for HF (\( p = 0.14 \)) and LF (\( p = 0.11 \)).

We further evaluated the relationship between PM2.5, GSTM1, and HMOX-1 by estimating the effect of PM2.5 on HRV within each combination of the GSTM1 genotypes and HMOX-1 microsatellite repeat length categories (Table 3). These results indicate a clear trend of increasingly negative coefficients as we move across gene categories. In carriers of both the GSTM1-null deletion and at least one allele with ≥ 25 HMOX-1 microsatellite (GT)n repeats, PM2.5 was negatively associated with all three HRV outcomes, whereas no significant association was found in subjects with any other combinations. In subjects carrying the GSTM1-null deletion and at least one allele with ≥ 25 HMOX-1 microsatellite (GT)n repeats, a 10-µg/m³ increase in PM2.5 in the 48 hr before the HRV measurement was associated with a –12.7% decrease in SDNN (95% CI, –20.6 to –3.9; \( p = 0.0059 \)), a 27.8% decrease in HF (95% CI, –43.0 to –8.5; \( p = 0.0073 \)), and a 20.1% decrease in LF (95% CI, –34.5 to –2.7; \( p = 0.0261 \)). GSTM1 genotypes and HMOX-1 microsatellite repeat lengths had a

**Table 2.** Adjusted percent change (95% CI) of heart rate variability (HRV) for each 10 µg/m³ of PM2.5 in the 48 hr before the measurement, by HMOX-1 microsatellite repeat length or GSTM1 polymorphism.

<table>
<thead>
<tr>
<th>HRV measurement</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PM2.5</td>
<td>GSTM1</td>
<td>HMOX-1</td>
</tr>
<tr>
<td>( \log_{10} ) SDNN</td>
<td>–8.8%</td>
<td>–2.0%</td>
<td>4.4%</td>
</tr>
<tr>
<td>(–12.9 to –0.2)</td>
<td>( p = 0.043 )</td>
<td>( p = 0.015 )</td>
<td>( p = 0.039 )</td>
</tr>
<tr>
<td>( \log_{10} ) HF</td>
<td>–17.3%</td>
<td>–4.0%</td>
<td>8.9%</td>
</tr>
<tr>
<td>(–30.0 to –2.3)</td>
<td>( p = 0.026 )</td>
<td>( p = 0.0139 )</td>
<td>( p = 0.0115 )</td>
</tr>
<tr>
<td>( \log_{10} ) LF</td>
<td>–11.2%</td>
<td>–0.8%</td>
<td>10.0%</td>
</tr>
<tr>
<td>(–22.8 to –2.2)</td>
<td>( p = 0.0986 )</td>
<td>( p = 0.0478 )</td>
<td>( p = 0.0430 )</td>
</tr>
</tbody>
</table>

All models adjusted for age, BMI, mean arterial pressure, fasting blood glucose, cigarette smoking (never/former/current), alcohol consumption (two or more drinks a day, yes/no), use of beta-blockers, use of calcium channel blockers, use of ACE inhibitor, room temperature, season, and 48-hr moving average of outdoor apparent temperature.

*Standard deviation of SDNN, power in HF (0.15–0.4 Hz) and LF (0.04–0.15 Hz) computed using a fast Fourier transform algorithm. *Carriers of ≥ 25 microsatellite (GT)n repeats in at least one allele.
combined effect on the association between PM<sub>2.5</sub> and HRV, as shown in Table 3 by the significant three-way interaction term between GSTM1, HMOX-1, and PM<sub>2.5</sub> (coded as PM<sub>2.5</sub> x a trend variable that is 1 for GSTM1 present and both short repeats, 2 for GSTM1 null and both short repeats, 2 for one long repeat and GSTM1 present, and 4 for GSTM1 null and at least one long repeat).

To test whether the effect modification by genotype was driven by a few individuals or represented a more general shifting of the distribution of PM<sub>2.5</sub>-HRV slopes, we refit our model with random slopes for the association of PM<sub>2.5</sub> level with HRV measurement. All models adjusted for age, BMI, mean arterial pressure, fasting blood glucose, cigarette smoking (never/former/current), alcohol consumption (two or more drinks a day, yes/no), use of beta-blockers, use of calcium channel blockers, use of ACE inhibitor, room temperature, season, and 48-hr moving average of outdoor apparent temperature.

### Table 3. Effect modification of HMOX-1 microsatellite repeat length and GSTM1 combinations on the adjusted percent change (95% CI) of HRV for each 10 µg/m<sup>3</sup> of PM<sub>2.5</sub> in the 48 hr before the measurement.

<table>
<thead>
<tr>
<th>HRV measurement&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HMOX-1 &lt; 25 repeats&lt;sup&gt;b&lt;/sup&gt; GSTM1 wild-type (no. of visits = 26&lt;sup&gt;c&lt;/sup&gt;)</th>
<th>HMOX-1 ≥ 25 repeats&lt;sup&gt;d&lt;/sup&gt; and GSTM1 null or GSTM1 wild-type (no. of visits = 30&lt;sup&gt;e&lt;/sup&gt;)</th>
<th>HMOX-1 ≥ 25 repeats&lt;sup&gt;c&lt;/sup&gt; GSTM1 null (no. of visits = 303&lt;sup&gt;f&lt;/sup&gt;)</th>
<th>Test for interaction&lt;sup&gt;g&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; SDNN</td>
<td>28.7% (–12.0 to 88.2) p = 0.1937</td>
<td>–2.7% (–11.5 to 6.8) p = 0.5620</td>
<td>–12.7% (–20.6 to –3.9) p = 0.0084</td>
<td>p = 0.0084</td>
</tr>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; HF</td>
<td>45.6% (–43.5 to 275.3) p = 0.4370</td>
<td>–5.8% (–25.7 to 18.8) p = 0.8153</td>
<td>–27.6% (–43.0 to –8.5) p = 0.0073</td>
<td>p = 0.0108</td>
</tr>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; LF</td>
<td>68.5% (–23.3 to 270.4) p = 0.1944</td>
<td>–2.8% (–19.9 to 18.0) p = 0.7722</td>
<td>–20.1% (–34.5 to –2.7) p = 0.0261</td>
<td>p = 0.0385</td>
</tr>
</tbody>
</table>

All models adjusted for age, BMI, mean arterial pressure, fasting blood glucose, cigarette smoking (never/former/current), alcohol consumption (two or more drinks a day, yes/no), use of beta-blockers, use of calcium channel blockers, use of ACE inhibitor, room temperature, season, and 48-hr moving average of outdoor apparent temperature.

<sup>a</sup>Standard deviation of SDNN, power in HF (0.15–0.4 Hz) and LF (0.04–0.15 Hz) computed using a fast Fourier transform algorithm. <sup>b</sup>Cars of < 25 microsatellite (GT) repeats in both alleles. <sup>c</sup>Cars of < 25 microsatellite (GT) repeats in at least one allele. <sup>d</sup>Number of HRV measurements.
endothelial cells (Liu et al. 2005). Our results showing interactions of particles with GSTM1 deletion and microsatellite (GT)n repeat length in the gene coding for HMOX-1 are consistent with these laboratory findings that suggest a prominent role of ROS in particle toxicity.

HMOX-1, the inducible heme oxygenase isoform, is expressed in multiple tissues, including vascular smooth muscle and endothelial cells (Exner et al. 2004). HMOX-1 expression has been shown to be upregulated in rat heart microvascular endothelial cells exposed to organic extracts of diesel exhaust particles (Furuyama et al. 2006), an effect that is likely to represent a response directed against ROS production (Morita 2005). Large individual differences in the ability to modulate the quantitative level of HMOX-1 activity in response to a given stimulus have been described, which correlate with differences in the length of a microsatellite (GT)n repeat in the 5’ flanking region of the HMOX-1 gene (Exner et al. 2004; Hirai et al. 2003; Yamada et al. 2000). The purine-pyrimidine alternating sequence in the (GT)n repeat has the potential to assume Z-DNA conformation, a left-handed double-helical structure that is thermodynamically unfavorable compared with B-DNA conformation (Rich et al. 1984) and has been described as negatively affecting transcriptional activity (Delic et al. 1991; Naylor and Clark 1990). Yamada and co-workers demonstrated by transient-transfection assay in cultured cell lines that the larger the number of (GT)n repeats in the HMOX-1 gene promoter, the lower is the HMOX-1 inducibility by ROS (Yamada et al. 2000).

In our study, HMOX-1 microsatellite (GT)n repeat length appeared to modulate the effects of PM2.5 on autonomic function, as measured by HRV variability. PM2.5 exhibited a negative correlation with SDNN, HF, and LF in individuals with ≥25 repeats, whereas no effect was seen in subjects with <25 repeats. Furthermore, the strongest effects of PM2.5 were found in subjects that were lacking efficiency in antioxidant responses due to the combination of the GSTM1 deletion and ≥25 microsatellite (GT)n repeats in the HMOX-1 promoter. While the p-values for statistical interaction between PM2.5 and GSTM1 and between PM2.5 and HMOX-1 were not significant, the tests for the three-way interaction among PM2.5, GSTM1, and HMOX-1 were highly significant, confirming that the stronger effect modification of PM2.5 effects on HRV is seen when both genes are considered. The statistical modeling we used to fit the three-way interaction tested whether the size of PM2.5 effects in carriers of GSTM1 null and at least one long HMOX-1 repeat was 2 times larger than that observed in subjects carrying either GSTM1 null or one long HMOX-1 repeat. Thus, our data strongly suggest that particle exposure interacts with individual variations in the antioxidant response pathway to determine its effects on HRV.

A potential limitation of this study is that we used ambient PM2.5 concentrations from a single monitoring site as a surrogate for recent exposure to PM2.5. A recent study comparing ambient concentrations at this site with personal exposures in Boston has shown a high longitudinal correlation (Sarnat et al. 2005) between the two measurements; the study also reported that PM2.5 concentrations were spatially homogenous over the Boston area. This suggests that our use of ambient concentrations is reasonable and the resulting exposure error is likely to be nondifferential. In our analyses, we considered several potential confounding factors that may have influenced HRV measures, as we adjusted our models for age, BMI, mean arterial pressure, fasting blood glucose, cigarette smoking, alcohol consumption, room temperature, outdoor apparent temperature, season, and use of beta-blockers, calcium channel blockers, and ACE inhibitors. Therefore, chances that the observed associations reflected bias due to confounders are minimized.

Our results can be generalized only to an aged population that consists of older males who are almost all white. The effect on women and children as well as different ethnic groups should be addressed in future studies, particularly in relation to the exposure of different population groups to PM2.5 with varying geographic location, occupation, socioeconomic status, and behavioral characteristics. Other health outcomes including respiratory responses may also be affected by responses to ROS in an interaction with PM2.5 exposure. Our findings provide new information to guide research on the breadth of the effect of PM2.5 exposure.

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