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Nrf2-related gene expression and exposure to traffic-related air pollution in elderly subjects with cardiovascular disease: An exploratory panel study

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Abstract

Gene expression changes are linked to air pollutant exposures in *in vitro* and animal experiments. However, limited data are available on how these outcomes relate to ambient air pollutant exposures in humans. We performed an exploratory analysis testing whether gene expression levels were associated with air pollution exposures in a Los Angeles area cohort of elderly subjects with coronary artery disease. Candidate genes (35) were selected from published studies of gene expression-pollutant associations. Expression levels were measured weekly in 43 subjects (12 weeks) using quantitative PCR. Exposures included gaseous pollutants O_3, nitrogen oxides (NO_x), and CO; particulate matter (PM) pollutants elemental and black carbon (EC, BC); and size-fractionated PM mass. We measured organic compounds from PM filter extracts, including polycyclic aromatic hydrocarbons (PAHs), and determined the *in vitro* oxidative potential of particle extracts. Associations between exposures and gene expression levels were analyzed using mixed-effects regression models. We found positive associations of traffic-related pollutants (EC, BC, primary organic carbon, $PM_{0.25-2.5}$ PAH and/or $PM_{0.25}$ PAH, and NO_x) with *NFE2L2*, Nrf2-

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mediated genes (*HMOX1*, *NQO1*, and *SOD2*), *CYP1B1*, *IL1B*, and *SELP*. Findings suggest that *NFE2L2* gene expression links associations of traffic-related air pollution with phase I and II enzyme genes at the promoter transcription level.

Keywords

longitudinal studies; oxidative stress; transcription; biochemical pathways; particulate matter; air pollution

INTRODUCTION

Air pollution has been linked with adverse cardiovascular outcomes in numerous epidemiological and toxicological studies.[1] Traffic-related air pollution is associated with increases in blood pressure, myocardial infarction, atherosclerotic progression, and other outcomes.[1] Previously, we showed increases in levels of circulating biomarkers of inflammation and platelet activation in association with exposure to primary (combustion-related) air pollutants in an elderly cohort panel with coronary artery disease (CAD).[2,3] That panel study contributed subject data to the present study. In the region of study (Los Angeles, CA), the markers of combustion-related air pollution used (e.g. elemental carbon, EC) are primarily from mobile sources (traffic). We hypothesize that gene expression levels in whole blood along biopathways relevant to these health outcomes will also be associated with traffic-related air pollutant exposures in our cohort.

Air pollutant exposure experiments *in vitro* show gene expression changes in cells involved in cardiovascular disease progression, including endothelial and epithelial cells, monocytes, and macrophages. Gong et al.[4] found diesel exhaust particle exposure increased expression levels in human endothelial cells of antioxidant genes (*SOD1*, *HMOX1*, and *NQO1*), genes linked with vascular inflammation (*IL8*, *CXCL1*, and *DUSP1*) and unfolded protein response (UPR) genes (*HSPA8*, *XBP1*, *ATF4*, and *ATF6*). *In vitro* genomic work by Gargalovic et al.[5] identified UPR genes of human endothelial cells as mediators of vascular inflammation and atherosclerosis, supporting a possible role for UPR genes in air pollution-related cardiovascular outcomes. Additionally, genes from the nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated oxidative stress response pathway were upregulated in airway epithelial cells after exposure to ambient outdoor particulate matter (PM).[6] There is evidence that oxidative stress, an important mechanism of cardiovascular disease progression,[7] is increased with elevated air pollution exposure.[8] Thus, the Nrf2-mediated oxidative stress response genes may be important in human responses to air pollution exposure.

Using inflammation-prone Sirt1 knock-out mice, Wu et al.[9] showed decreased expression of Kruppel-like factor 2 (Klf2) with PM exposure. Klf2, a key transcription factor for coagulation and thrombotic function, downregulates pro-inflammatory genes and has reduced expression levels in circulating monocytes from patients with CAD *versus* age-matched controls.[10] Thus, in a cohort with CAD, air pollution exposure-associated cardiovascular responses may be mediated by *KLF2* expression changes.

Although human studies have supported potential clinical impacts of traffic-related air pollution,[1] the ways that air pollutants bring about such changes on a cellular level are not well understood. Experimental data discussed above are beginning to shed light on this topic, including one study showing upregulation of inflammatory and oxidative stress pathway genes in peripheral blood mononuclear cells of five healthy human subjects exposed to diesel exhaust.[11] Studying gene expression in an epidemiologic study can help to support the potential public health relevance of experimental studies. However, to our knowledge, only three exploratory epidemiologic studies have been published on gene expression associations with air pollution from fossil fuel combustion or similar sources[12–15] (see McHale et al.[16] for review of human genome-wide studies of other inhaled exposures). On the basis of existing human studies, and *in vitro* and *in vivo* experiments, we selected 35 genes from 10 biological pathways relevant to air pollution exposure responses and examined changes in candidate gene expression in our cohort panel of elderly subjects with CAD.[2,3,17] Biopathways included: coagulation, Klf2-mediated immune response, NF-κB signaling, acute phase response, Nrf2-mediated oxidative stress response, endoplasmic reticulum stress (UPR), glutathione metabolism, phase I and phase II metabolism, endogenous reactive oxygen species (ROS) production, and cytokine signaling (Figure 1, Supplementary Table S1, which include additional references that were informative to the selection of genes). Exploring gene expression changes along these pathways, in an urban cohort panel, can support studies of potential mechanistic pathways through which air pollutants may cause adverse cardiovascular outcomes.

METHODS

Study Design and Population

This study was a cohort panel design consisting of repeated measures of outcomes and exposures for 43 elderly adults living in three retirement communities in the Los Angeles air basin. Each subject effectively serves as his/her own control. We followed subjects for 12 weeks, 6 weeks each during the warm and cool seasons to provide seasonal differences in air pollutant levels.[18] The cohort analyzed in this study represents a subset of three of four retirement communities in the Cardiovascular Health and Air Pollution Study cohort previously described[2,3,17] that were closest to the Los Angeles metro area.

Participants were over 65 years of age, with clinically confirmed CAD. This population is expected to have increased susceptibility to adverse associations with air pollution exposure.[19] Exclusion criteria included: smoking within previous 12 months; dialysis treatment or uncontrolled hypertension, which alter inflammation and other outcomes; and employment outside their retirement community (where daily exposures were measured). We collected time-invariant subject characteristics (e.g. age, sex, and comorbidities) with questionnaires. This study was approved by the Institutional Review Board of the University of California, Irvine. All subjects provided written informed consent before participation.

Gene Expression

We analyzed 35 candidate genes and 5 reference genes using RNA from whole blood. Blood is a suitable matrix to evaluate systemic impacts, reflecting alterations in both local and

distant environments.[20] Samples were collected each Friday, at the same time of day for each subject, in PaxGene vacuum tubes (BD Diagnostics, Franklin Lakes, NJ, USA) that inhibit RNA degradation and gene induction before testing.[21] Tubes were mixed, immediately frozen, transported to the laboratory and stored at − 80 °C until tested. Total RNA was isolated using a QIAcube™ and PAXgene Blood RNA kits (Qiagen, Valencia, CA, USA). Isolated RNA was reverse-transcribed into first-strand cDNA (ThermoScript RT-PCR kit, Invitrogen, CA, USA) for gene expression analysis.

Candidate genes (Figure 1, Supplementary Table S1) were selected based on either biological function or known pollutant exposure associations. Pathway information is derived from Ingenuity Pathway™ Software Analysis, the PANTHER classification system (http://www.pantherdb.org), and NCBI databases (http://www.ncbi.nlm.nih.gov). Gene expression levels were determined using end-point competitive PCR, with internal standards (the competitors) and sample cDNA co-amplified in the same reaction.[22] Products were resolved with linear Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MassARRAY™ Quantitative Gene Expression) and concentrations of the target transcripts were calculated from the ratio of the PCR products. All samples from a given subject were assayed on the same plate. Signal acquisition, allele assignment, peak area integration, data processing, and analysis were carried out using the MassARRAY™ platform and software (Sequenom, San Diego, CA, USA). Services were contracted with Immune Sciences Lab (David H. Murdock Research Institute, Kannapolis, NC, USA).

Transcript copy numbers were determined based on the EC_{50} calculated by non-linear regression analysis of the cDNA and competitor allele frequencies. Copy numbers were normalized using the geNorm algorithm described by Vandesompele et al.[23] We calculated the expression stability for a reference (housekeeping) gene as the average pairwise variation with other tested reference genes. We determined the most stable reference genes (*ACTB*, *B2M*, and *GAPDH*) by stepwise exclusion of genes with the highest instability. Gene expression normalization factors for each sample were generated from the geometric mean of reference genes. All calculations were carried out using the qBase^PLUS data-analysis and integrated geNORM software (Biogazelle™).

Exposure Measurement

Exposure variables and lags were selected based on *a priori* hypotheses of their relations to cardiovascular outcomes in a target population with high traffic-related air pollution exposure. The rationale for their measurement is detailed elsewhere.[17] The subset tested herein represents pollutants and averaging times with significant findings for cardiovascular and inflammation outcomes tested in our cohort.

We measured the concentrations of air pollutants in the immediate outdoor environment of participating retirement communities in the Los Angeles air basin, an area with some of the highest air pollutant levels in the United States. All exposure data were captured for each week preceding outcome measurements. We used standard federal reference methods to measure gases (nitrogen dioxide/nitrogen oxides, NO_2/NO_x; ozone, O_3; and carbon monoxide, CO). We also measured elemental and organic carbon (EC, OC) (OC_EC Analyzer, Model 3F, Sunset Laboratory, Tigard, OR, USA), and black carbon (BC)

(Aethalometer, Magee Scientific, Berkeley, CA, USA) in $PM_{2.5}$ (PM with aerodynamic diameter <2.5 μm). We estimated primary organic carbon (OC_{pri}) using the "EC tracer" method as we previously described,[24] and secondary organic carbon (SOC) as total OC minus OC_{pri}. The above exposures were averaged daily.

We determined daily size-fractionated particle mass for quasi-ultrafine particles (qUFP) <0.25 μm in aerodynamic diameter ($PM_{0.25}$), accumulation-mode particles 0.25–2.5 μm ($PM_{0.25-2.5}$) and coarse mode particles 2.5–10μm ($PM_{2.5-10}$), using collocated Sioutas Personal Cascade Impactors (SKC, Eighty-Four, PA, USA),[25] as discussed elsewhere.[26] We quantified organic components of $PM_{0.25-2.5}$ and qUFP from 5-day filter composites using GC/MS, as described elsewhere[3,27] (see Supplementary Table S2). Components included polycyclic aromatic hydrocarbons (PAHs), key toxic components of primary organic aerosols that, in Los Angeles, are primarily from fossil fuel combustion. We also measured hopanes, tracers of vehicle exhaust found in vehicle lubricant oils.[28] Exposure markers of secondary organic aerosols (SOA) included organic acids, primarily produced by photochemical aging of primary carbons,[29] and water-soluble organic carbon (WSOC), a tracer of both photochemically-produced SOA and biomass burning.[30,31] We estimated SOA as two times the difference between measured WSOC (from Sievers Total Organic Carbon, TOC; GE) and the fraction of secondary WSOC from wood smoke (estimated as 71% of OC from wood smoke, determined from a chemical mass balance model using levoglucosan as wood smoke tracer[32]), described in detail elsewhere.[27]

We measured particle oxidative potential from aqueous extracts of the 5-day filter composites of $PM_{0.25}$ and $PM_{0.25-2.5}$ using an *in vitro* assay of ROS described elsewhere[33] and in the Supplementary Material. Briefly, rat alveolar macrophages were exposed to PM extract and 2′7′-dicholorodihy-drofluorescein diacetate. Fluorescence intensity from cellular production of ROS was measured. Un-opsonized Zymosan (a β-1,3-polysachharide of D-glucose) was our positive control and results are reported in μg Zymosan equivalents/m^3 air.

Statistical Analysis

The distributions of the gene expression levels were evaluated after applying the natural logarithm of the Biogazelle normalized concentrations. The log-transformed gene expression data for an individual gene was retained if the distribution of all observations for that gene was sufficiently normal. If not, we applied an autoscale standardization method developed for use on the genes that were not log-normally distributed (*AHR*, *CCL2*, *F3*, *GCLC*, *IL6*, *IL6R*, *IL8*, *NQO1*, *SELP*, *TNF*, and *TNFRSF1B*).[34] This required centering the log-transformed gene expression data relative to the subject-specific mean and scaling the data by the ratio of the individual to the group standard deviations. This transformation normalized the distributions for the purposes of model fitting and parameter estimation. For those genes that were autoscaled, the presented fold-change estimates are interpreted relative to changes in the predictor of interest scaled by the ratio of the individual to the group standard deviation of the outcome.

We analyzed exposure–response relationships using linear mixed-effects models of gene expression predicted by air pollutant level. Each pollutant–gene expression relationship was examined in a separate model. We estimated random intercepts by subject, nested within

season and community, to account for correlated within-individual repeated measures. The best fitting covariance structure was autoregressive order 1, as determined by Akaike's information criterion. Pollutant concentrations were averaged for 1–7 days preceding blood draws and mean-centered by season and community, as in the previous analyses (described in the Supplementary Material).[3] We adjusted, *a priori*, for temperature over the same averaging time as the pollutant. We tested smoothed penalized spline terms adjusting for potential non-linear temperature associations; however, these did not improve the model fit and were not retained. In addition, we performed a *post hoc* analysis of distributed lag models for *NFE2L2* and *IL1B* to assess lag effects (Supplementary Figures S1 and S2). Much of the published research informing our selection of candidate genes was based on experimental designs, and would not be expected to have the same temporal relationships observed in free-living individuals in an epidemiological investigation like the present. Therefore, in this exploratory study, we evaluated moving averages for 1 through 7 days in linear mixed-effects models and lags 0 through 6 days the distributed lag models, rather than selecting specific lags for specific genes. Because these models concern within-subject associations with variation in air pollution levels, they were not adjusted for individual time-invariant characteristics. Differences in such characteristics between subjects are partly accounted for by the random subject intercepts. Person-weeks when subjects reported any infections were excluded from analysis because infections could markedly alter transcription levels along inflammatory and other pathways ($N = 11$ person-weeks, 3% of total). To standardize comparisons between pollutants, we expressed results for interquartile range (IQR) increases in pollutant levels. Due to power limitations, we were unable to assess effect modification in this analysis.

Gene expression profiling from whole blood is complicated by the heterogeneity of cell types and their possible differential responses to stressors; changes in the concentration of inflammatory cells may change measured expression levels. Also, expression changes may be diluted if a particular gene is only expressed in a small subset of cells.[35,36] However, the aim of this exploratory analysis was not to assess expression levels in particular cell types, but to characterize overall expression levels in peripheral whole blood. Due to IRB limitations in allowed total blood volumes, we were unable to collect repeated measurements of leukocyte differential counts. The use of repeated measures, with subjects effectively serving as their own controls, helps to reduce the effect of inherent biological variability of blood cell distribution between people on model results. Because within-person cell shifts may result in gene expression shifts, we *a priori* planned to utilize expression of cell surface markers as a means to adjust models for cell distribution as described in the online Supplementary Material. However, these results were not informative, and we present exposure–response models with the limitation that they were not adjusted for gene expression of cell surface markers.

Because we evaluated many models for this analysis, we attempted to quantify the extent of our multiple comparison problem. We used a permutation analysis to simulate the distribution of the Wald statistic for each association estimate under the strong null hypothesis that there is no relationship between gene expression level and pollutant level. We permuted the gene expression level outcomes 26,500 times for each exposure, within

each individual subject, and re-ran our mixed-effects model. We then compared our observed Wald statistic with the critical value corresponding to a family-wise level 0.05 test resulting from the simulated distribution after accounting for all comparisons. We used this method rather than the more conservative Bonferroni adjustment because the pollutant exposure levels are correlated, hence the resulting tests are also likely to be correlated. While this approach partially accounts for the observed correlation among tests, the permutation correction is still likely to produce somewhat conservative inference as it bounds the family-wise type I error relative to the maximum observed statistic across all comparisons. This computationally intensive, somewhat conservative adjustment method generated increased *P*-values that were no longer significant as might be expected. This may be attributable to the limited number of subjects and large number of models. We performed this simulation for a two gene subset (*IL1B* and *NFE2L2*) and present one, *NFE2L2*, as an example in the online Supplementary Material (Supplementary Figure S3, Supplementary Table S4). The observed values presented here are unadjusted with the caveat that these *P*-values are likely to be non-significant when adjusting the family-wise type I error rate at level 0.05. As such, we view the resulting regression parameter estimates as evidence of potential effects to generate hypotheses for future studies.

RESULTS

Population and Exposures

Table 1 shows cohort characteristics derived from subject questionnaires. Descriptive statistics of air pollutant exposures are listed in Table 2. The pollutant correlations are shown in Supplementary Tables S4 and S5. Briefly, primary combustion-related air pollutants (EC, BC, OC_{pri}, NO_X, and CO) were strongly correlated with each other and weakly correlated with secondary photochemically-related pollutants (O_3 and SOC). Individual components from a given particle size fraction were most highly correlated with the other components from that size fraction. Quasi-ultrafine $PM_{0.25}$ hopanes (a tracer of vehicular sources) and $PM_{0.25}$ PAH had a Spearman correlation coefficient of 0.77, supporting the view that PAH is largely attributable to traffic sources in the study area.

Gene Expression and Regression Models

We analyzed repeated quantitative PCR measures of 35 gene expression levels from 43 subjects (360 person-observations per gene on average). Expression levels of five of these candidate genes (*CYP1A1*, *PON1*, *SOD1*, *NOX1*, and *XDH*) were too low for quantification, leaving 30 genes for analysis. Using linear mixed-effects models, we found traffic-related air pollutants were associated with expression of genes in multiple pathways including inflammation, platelet activation, and Nrf2-mediated oxidative stress response (Figure 2). We present 95% confidence intervals (CIs) unadjusted for multiple testing because the computationally intense adjustment method, described in Materials and methods, confirmed that our study was underpowered for the number of models we explored. Many of the unadjusted 95% CIs for the fold-change estimates included 1. Figures 2 and 3 show model results for exposures with among the strongest associations. Nevertheless, there was a consistency of associations in terms of their magnitude and direction across each of the genes within the *a priori* defined pathways. Specifically, we found positive associations of

EC, BC, OC$_{pri}$, and NO$_X$ with the Nrf2 gene (*NFE2L2*), and Nrf2-mediated/linked genes, *HMOX1*, *NQO1*, and *SOD2*. The largest association with *NFE2L2* was for the 7-day average of OC$_{pri}$ (2.51 fold-change, unadjusted 95% CI: 1.18–5.53, per IQR increase). Traffic-related air pollutants were positively associated with increased expression of *IL1B*, *SELP*, and *CYP1B1* whose transcription is not directly Nrf2-mediated. The largest associations found for *SELP* and *CYP1B1* were with OC$_{pri}$ (1.53 fold-change in *SELP*, unadjusted 95% CI: 1–2.35, and 1.96 fold-change in *CYP1B1*, unadjusted 95% CI: 1.01–3.80, per IQR increase in 5-day OC$_{pri}$).

Figure 3 shows gene expression fold-change estimates for mass concentrations of PM size fractions, size-fractionated particle components, and oxidative potential of particle extracts. Findings for PAH from particle extracts in Figure 3 are consistent with those for other PM markers of primary combustion (EC, BC, and primary OC) (Figure 2). Overall, positive associations were seen for *CYP1B1*, *HMOX1*, *IL1B*, *NQO1*, *NFE2L2*, *SELP*, and *SOD2* in relation to PAHs, but did not differ substantially by PAH molecular weight class. Among the strongest of these associations was qUFP PAH and *IL1B*; we show a 1.33 fold-change in IL1B (unadjusted 95% CI: 1.08–1.64) per IQR increase in qUFP high molecular weight PAH. Accumulation mode PAH of high molecular weight had positive associations (unadjusted $P<0.05$) with *NQO1* (1.18 fold-change, unadjusted 95% CI: 1.06–1.31, per IQR increase) and *NFE2L2* (1.34 fold-change increase, unadjusted 95% CI: 1.01–1.77, per IQR increase), as well as positive but non-significant associations with *CYP1B1*, *HMOX1*, and *SELP*.

PM$_{0.25}$ *in vitro* ROS was associated with increases in *NFE2L2* gene expression (1.15 fold-change per IQR increase in PM$_{0.25}$ ROS, unadjusted 95% CI: 0.98–1.34). PM$_{0.25}$ *in vitro* ROS was also positively associated with *NQO1* and *CYP1B1*, while PM$_{0.25–2.5}$ ROS was only associated with *CYP1B1*. PM from biomass burning was positively associated (unadjusted $P<0.05$) with *HMOX1* (not shown), and was positively, but not significantly, associated with expression of several others (*ATF4*, *ATF6*, *GCLM*, *IL1B*, *KLF2*, *MPO*, and *XBP1*). Photochemically-related secondary pollutant exposures were not associated with gene expression, including size-fractionated particle components (SOA and organic acids), and PM$_{2.5}$ SOC and O$_3$ (see Supplementary Table S6). Other air pollutants measured daily (total OC and CO) were not associated with the expression of genes (not shown). We also found no trends of association of any pollutants with downregulation of genes.

Air pollutant exposures were not associated with expression of any of the following genes: *AHR*, *CCL2*, *CXCL1*, *DUSP1*, *F3*, *GCLC*, *GPX-1*, *GSTP1*, *HSPA8*, *IL6*, *IL6R*, *IL8*, *PTGS2*, *TNF*, and *TNFRS1B*.

The online Supplementary Material (Supplementary Figures S1 and S2) shows results of a sensitivity analysis to assess lag effects using a polynomial distributed lag model. Results for a few models showed that the strongest effects could be from exposure within 24 h, but most models were largely uninformative suggesting that cumulative average exposures were a better reflection of the underlying exposure–response relationships.

To assess the significance of our inference that genes mediated by Nrf2 were differentially expressed relative to those that are not, we performed a *post hoc* pathway analysis based on the methods described for Gene Set Analysis.[37] The details of this analysis can be found in the online Supplementary Material. Using this method, the *P*-value for the differential effect of the Nrf2 pathway was non-significant at 0.28.

DISCUSSION

We have shown that candidate gene expression from whole blood is associated with traffic-related air pollution exposures among elderly subjects with CAD living in the Los Angeles air basin. We found many positive associations within the Nrf2-mediated oxidative stress response pathway, providing preliminary support for our hypothesis that gene expression in pathways relevant to pro-oxidant exposures would be associated with traffic-related air pollutant exposures. This hypothesis-driven exploratory test of probable pathways for air pollutant responses generated results that could help bridge the gap between population-level epidemiological studies and both *in vivo* and *in vitro* toxicological experiments. To our knowledge, this is the first cohort panel study to show such gene expression changes in relation to urban air pollution exposures in the outdoor home environment.

Among the genes with positive associations, the Nrf2 gene is of particular interest. This transcription factor regulates Phase II and other antioxidant genes by binding antioxidant response elements (AREs) of their promoter regions. In a study of cardiac fibroblasts, Nrf2 expression was shown to be critical for total SOD and NQO1 enzymatic activity.[38] *In vitro* evidence showed that diesel exhaust particles increased Nrf2 protein expression,[39] while urban fine and ultrafine PM induced expression of both Nrf2-mediated oxidative stress response pathway genes and xenobiotic metabolism signaling pathway genes.[6] Mouse models showed that UFP exposure increased *NFE2L2*, *SOD2*, *CAT*, *NQO1*, and *ATF4* gene expression.[40] Our results in human subjects, together with these and other studies, support the probable role of Nrf2 in air pollution exposure–response relationships.

Further supporting the importance of Nrf2, we found positive associations of *NFE2L2* expression with PAHs. PAHs are markers of air pollution from fossil fuel combustion sources known to activate the aryl hydrocarbon receptor (AHR). Once bound, the AHR interacts with xenobiotic response elements (XREs) in the promoter regions of xenobiotic metabolism genes, initiating PAH metabolism to pro-oxidant compounds. Thus, PAH metabolites could then trigger Nrf2-ARE. Conversely, Nrf2 has been shown to bind to a functional ARE in the promoter of *AHR* in mouse embryonic fibroblasts.[41] Thus, *NFE2L2* expression can lead to AHR-XRE activation. Formerly considered separate response pathways, this recent evidence identifies crosstalk between the xenobiotic and antioxidant response pathways. We found increased *NFE2L2* expression along with increased expression in downstream genes in both AHR-XRE and Nrf2-ARE pathways. Much of the existing literature describes Nrf2 as a master regulator of antioxidant response,[38] and our novel findings here are in agreement with this. Our previous observations of this cohort panel showed that traffic-related air pollutants were associated with increased blood pressure,[42] ambulatory electrocardiographic evidence of cardiac ischemia (ST segment depression),[43] and ventricular arrhythmia.[44] Nrf2 pathway involvement in gene expression–

air pollution exposure associations suggests oxidative stress as a potential mode of action by which these exposures could cause the adverse cardiovascular outcomes observed in many air pollution studies.[1]

In our analysis of genes mediated by Nrf2, including *AHR* and *CYP1B1*, we found a non-significant maxmean statistic, though the trend among these genes suggests that they may be differentially expressed in association with the exposures we measured. This was partly driven by several Nrf2-linked genes that were not significantly associated with air pollutants (*AHR*, *ATF4*, *CAT*, *DUSP1*, *GCLC*, *GCLM*, *GSTP1*, and *TXNRD1*). We also considered the phase I and II metabolism and Nrf2-mediated oxidative stress response pathways linked, but expression levels of three candidate genes from the AHR-XRE pathway (*SOD1*, *CYP1A1*, and *PON1*) could not be included as they were below the limit of detection. Nevertheless, because of the established role of Nrf2 in regulating oxidative stress responses,[38] our findings highlight the possible importance of the Nrf2 pathway in humans with ambient traffic-related air pollution exposures.

It is unclear whether the associations of estimated biomass burning PM with *HMOX1* and *MPO* are meaningful and further work is needed. However, those results support the involvement of oxidative stress in the known associations of biomass burning with adverse respiratory outcomes.[45]

We also showed *IL1B* and *SELP* were positively associated with traffic-related air pollutant exposure. In our previous work, we found associations between levels of circulating sP-selectin (a protein marker of platelet activation encoded by *SELP*), and air pollution exposure, indicating that subjects at higher exposures had increased coagulation potential.[2] P-selectin has an established role in vascular disease progression.[46] IL-1β contributes to atherosclerosis progression by mediating vascular injury responses.[47] Together, the increases we found in *IL1B* and *SELP* may signal endothelial damage or dysfunction that could trigger heightened coagulation. Because these genes do not rely on the Nrf2-mediated oxidative stress response, and neither shows a strong association with PM oxidative potential, we speculate that induction of these genes may occur via non-oxidative stress mechanisms.

Though our study is limited in the number of subjects ($N = 43$), the use of many repeated measures for each individual (12) allowed us to detect trends in expression at low fold-change levels. While many unadjusted 95% CIs for the fold change estimates included 1, there was overall consistency in the associations. Using false discovery rate logic, we would expect approximately 140 significant associations (30 genes over 19 exposures with 5 lags yields 2850 models, at alpha = 0.05). However, this approach neglects correlations between our variables of interest and does not have an intrinsic known null distribution. Using a simulated null distribution for our variables, our results did not reach significance with adjustment for multiple comparisons, as our study is likely underpowered for the number of models we analyzed. Considering models for different averaging times, multiple positive associations of similar magnitude, even if some are non-significant, suggest a possible overall relationship between a given gene and air pollutant. In addition, if our findings resulted from chance, we would not expect to see trends within pollutant classes or grouping

of results by hypothesized biological pathways. Specifically, primary combustion-related air pollutants (EC, BC, OC_{pri}, NO_X, and CO) were strongly correlated with each other and likely were similar surrogates for the underlying causal pollutants components, which could have included PAH (a specific group of primary combustion-related air pollutant chemicals). Furthermore, we developed *a priori* hypotheses regarding specific candidate gene expression changes using published data; thus, our interpretations of these exploratory results are supported by biological plausibility and experimental evidence.

We are aware that some of our associations could have resulted from unmeasured confounding or high variability between individuals.[20] Many of the factors affecting variability identified by Dumeaux et al.[20] were either not relevant or controlled in our study. Of these, we could not control for blood cell subtype variation, but did exclude observations when subjects experienced infections to limit the effect infections would have had on cell type distribution and inflammation. There is work describing the use of methylation profiles to identify variations in cell type in whole blood.[48,49] However, we did not collect repeated DNA samples in this panel study; therefore, we were unable to utilize methods like these to further characterize the gene expression relations with specific cell subsets. While we modeled random intercepts in mixed models to account for time-invariant subject differences, unmeasured time-variant factors (e.g. diet), could have affected the study results. Furthermore, the dose–response relationships between pollutants and outcomes, and potential compensatory mechanisms and feedback pathways complicate these reported associations in ways we cannot capture.

We selected genes for their possible associations with cardiovascular outcomes, thus, in our population with pre-existing cardiovascular disease, changes in these genes may occur due to underlying cardiovascular disease changes, rather than air pollution exposure. Disease progression is an unmeasured time-variant factor, but we do not expect substantial, rapid progression for our entire cohort during or across these short 6-week study periods, which took place across a 5½ month period for each subject. In addition, these genes were also selected for their relevance to toxicant metabolism and detoxification pathways. While we cannot definitively state that our associations are robust, the results are of substantial interest in understanding our previously reported findings in the same cohort, namely, significant associations of protein biomarkers and acute cardiovascular outcomes with fossil fuel combustion-linked air pollutant exposures.[2,3,17,42,43,50] Additionally, we cannot generalize our results to younger, healthier, and more diverse populations, or other geographical areas because pollutant composition varies in urban areas other Los Angeles. We modeled associations using outdoor air pollutant levels. Our recent results show that, in our study locations, indoor levels of markers of traffic pollution, including EC and PAH, were strongly affected by outdoor levels.[51] In addition, we determined from daily diaries that the percentage of time spent outdoors while at home to be low (3.7%), and subjects spent an additional 7.4% of their time away from the community at both indoor and outdoor locations. Therefore, our exposure assessments based on measurements at outdoor community environments are likely to accurately reflect the exposures of our study participants. While similar combustion-related air pollutants could show comparable

associations in other regions, future studies are needed to examine these gene expression associations in other cohorts.

CONCLUSIONS

We report novel associations of gene expression changes with traffic-related air pollution exposures in a Los Angeles cohort of elderly subjects with CAD in this exploratory panel study analysis. We infer that the observed associations are coherent with experimental mechanistic data and with published associations between air pollution exposure from fossil fuel combustion sources and adverse cardiovascular outcomes, including hospital admissions and mortality.[1] Results suggest that further work is warranted to test whether *NFE2L2* gene expression may link air pollution from fossil fuel combustion with phase I and phase II enzyme genes via pathway crosstalk at the promoter transcription level. Additional research is needed to confirm these associations in other populations and to more thoroughly characterize the role of the Nrf2-mediated oxidative stress response pathway in cardiovascular responses to traffic-related and other air pollutant exposures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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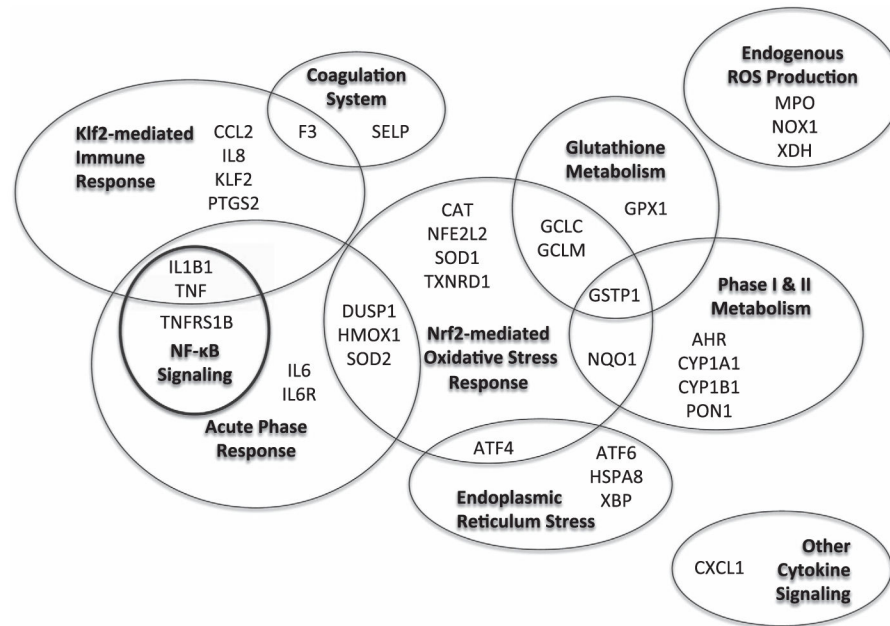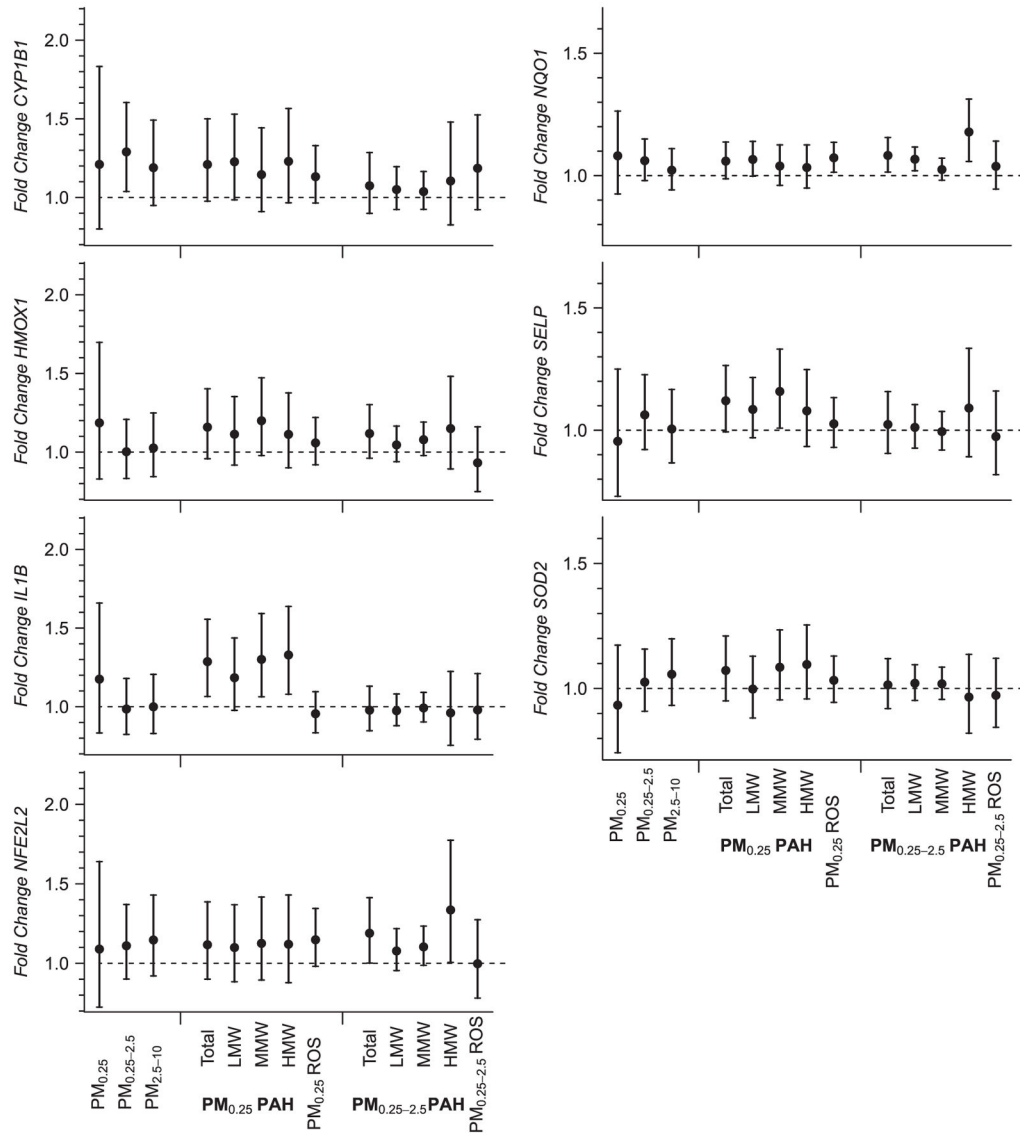
Figure 1.
Candidate genes grouped by biopathway. Gene abbreviations and NCBI identification numbers are listed in Supplementary Table S2.
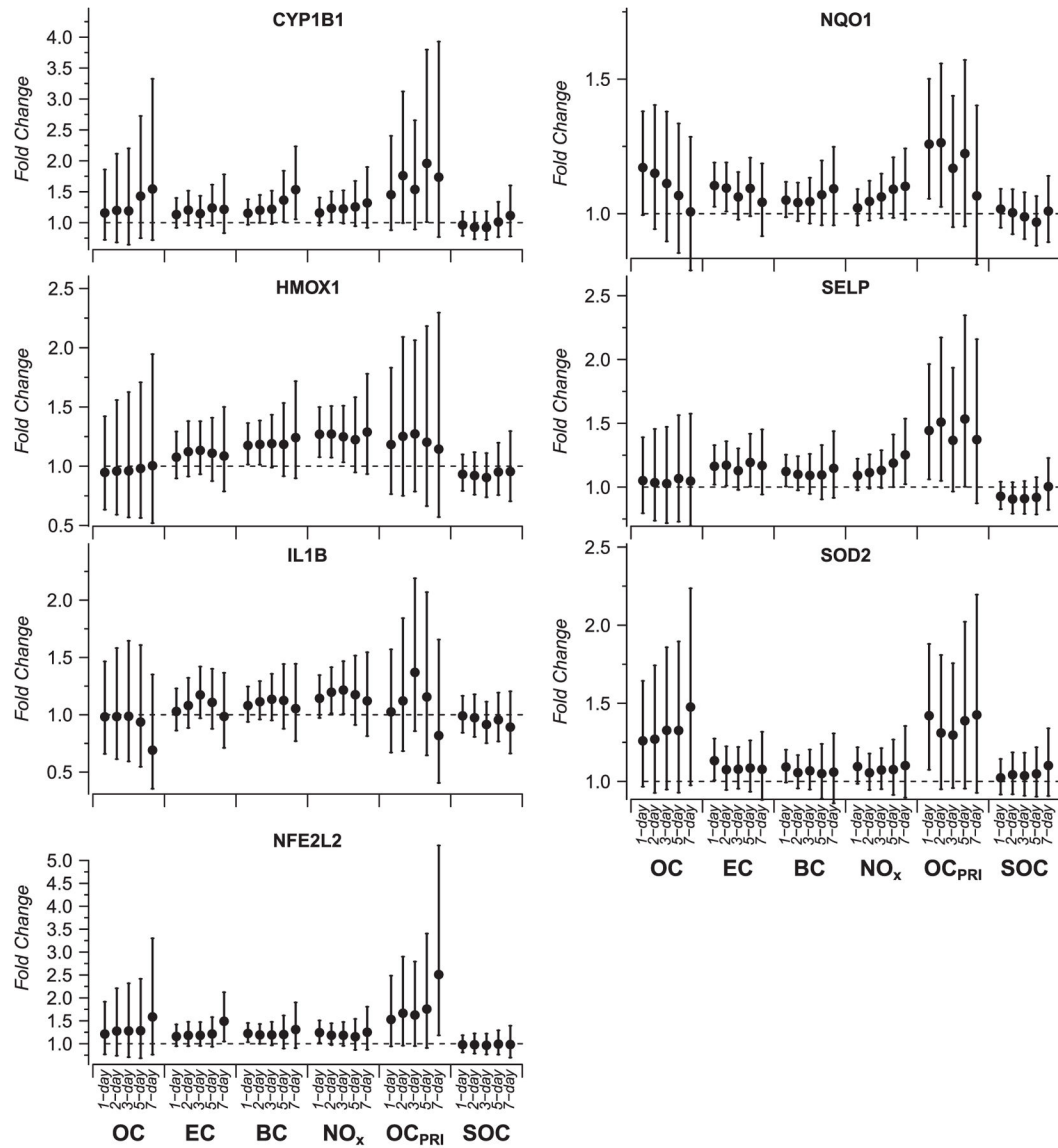
Figure 2.
Fold-change and 95% confidence interval for qPCR expression level of *CYP1B1*, *HMOX1*, *IL1B*, *NQO1*, *NFE2L2*, *SELP*, and *SOD2* in association with air pollutants measured hourly, including traffic-related air pollutants (elemental carbon, black carbon, primary organic carbon, and NO$_x$). Time period shown indicates the number of days preceding gene expression measurement over which pollutant concentration was averaged.

Figure 3.
Fold-change and 95% confidence interval for qPCR expression level of *CYP1B1*, *HMOX1*, *IL1B*, *NQO1*, *NFE2L2*, *SELP*, and *SOD2* in association with particulate matter size fractions, particulate matter polycyclic aromatic hydrocarbons, and particulate matter oxidative potential (measured as macrophage ROS production *in vitro*). Time period shown indicates the number of days preceding gene expression measurement over which pollutant concentration was averaged.

Table 1

Subject characteristics ($N = 43$).

Characteristic	Value
Age (years, Mean ±SD)	84.7 ±5.83
Female	53.5%
Race	
Hispanic	2.3%
White	97.7%
Cardiovascular history	
Confirmation of CAD	
Myocardial Infarction	44.2%
Coronary artery bypass graft or angioplasty	32.6%
Positive angiogram or stress test	16.3%
Clinical diagnosis	7.0%
Congestive heart failure	27.9%
Hypertension (by history)	76.7%
Hypercholesterolemia (by history)	67.4%
Medications	
ACE inhibitors and Angiotensin II receptor antagonists	41.9%
HMG-CoA reductase inhibitors (statins)	51.2%

Table 2

Descriptive statistics of outdoor air pollutant exposures in three retirement communities of the Los Angeles air basin.

Exposure[a]	Mean (SD)	Median	IQR	Min/Max
$PM_{0.25}$ mass ($\mu g/m^3$)	9.89 (3.97)	9.25	6.27	3.31/19.3
$PM_{0.25}$ macrophage ROS (μg Zymosan equivalents/m^3)	41.4 (38.5)	21.3	56.2	2.59/147
$PM_{0.25}$ organic components				
WSOC ($\mu g/m^3$)[b]	0.50 (0.22)	0.49	0.28	0.08/1.01
PAH total (ng/m^3)	1.13 (0.48)	0.97	0.46	0.55/2.70
PAH LMW (ng/m^3)	0.41 (0.15)	0.36	0.16	0.21/0.74
PAH MMW (ng/m^3)	0.37 (0.18)	0.34	0.18	0.11/0.96
PAH HMW (ng/m^3)	0.35 (0.20)	0.30	0.27	0.13/1.01
Hopanes (ng/m^3)	0.33 (0.31)	0.25	0.44	0.06/1.57
Organic Acids ($\mu g/m^3$)	0.26 (0.22)	0.19	0.35	0.06/0.96
$PM_{0.25-2.5}$ Mass ($\mu g/m^3$)	12.2 (5.31)	11	7.14	3.89/28.1
$PM_{0.25-2.5}$ Macrophage ROS (μg Zymosan equivalents/m^3)	84.9 (55.8)	84	97.8	9.03/203
$PM_{0.25-2.5}$ organic components				
WSOC ($\mu g/m^3$)[b]	0.50 (0.29)	0.48	0.36	0.16/1.37
PAH total (ng/m^3)	0.53 (0.17)	0.47	0.26	0.36/1.01
PAH LMW (ng/m^3)	0.17 (0.03)	0.15	0.03	0.14/0.30
PAH MMW (ng/m^3)	0.14 (0.09)	0.09	0.10	0.08/0.39
PAH HMW (ng/m^3)	0.22 (0.06)	0.21	0.07	0.13/0.41
Hopanes (ng/m^3)	0.49 (0.29)	0.39	0.25	0.16/1.45
Organic acids ($\mu g/m^3$)	48.8 (38.5)	40.7	41.6	9.74/150
$PM_{2.5}$ components				
Elemental carbon ($\mu g/m^3$)	1.63 (0.60)	1.58	0.82	0.36/3.34
Organic carbon ($\mu g/m^3$)	6.81 (2.80)	6.09	3.57	2.46/13.8
Black carbon ($\mu g/m^3$)	1.88 (0.76)	1.76	0.91	0.50/4.51
Primary organic carbon ($\mu g/m^3$)	4.37 (2.11)	3.62	2.39	1.41/10.6
Secondary organic carbon ($\mu g/m^3$)	2.76 (1.41)	2.61	1.82	0.27/7.65
Particle number (particle no./cm^3)	14,686 (5,910)	13,331	6,729	2,019/30,180
Gases				
NO_2 (ppb)	31.8 (9.58)	31.3	13.2	9.91/58.1
NO_x (ppb)	56.5 (30.3)	50.0	35.3	11.8/183
CO (ppm)	0.63 (0.27)	0.57	0.38	0.21/1.68
O_3 (ppb)	24.9 (11.4)	23.5	15.2	3.83/60.7
Temperature (°C)	19.6 (5.43)	19.6	8.92	8.42/31.4

Abbreviations: HMW, high molecular weight (>4 ring); IQR, interquartile range; LMW, low molecular weight (2–3 ring); MMW, medium molecular weight (4 ring); PAH, polycyclic aromatic hydrocarbon; ROS, reactive oxygen species; WSOC, water-soluble organic carbon.