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Isoprene-Derived Secondary Organic Aerosol Induces the Expression of Oxidative Stress Response Genes in Human Lung Cells

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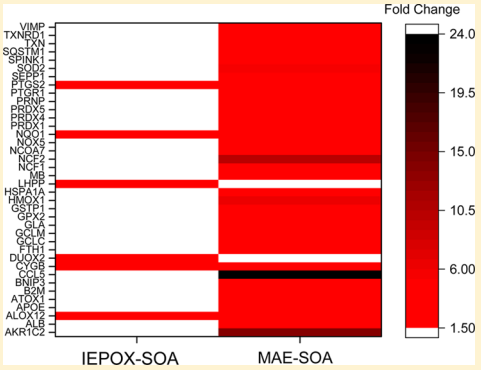
Supporting Information

ABSTRACT: Atmospheric oxidation of isoprene in the presence of acidic sulfate aerosol leads to secondary organic aerosol (SOA) that substantially contributes to the mass of outdoor fine particulate matter ($PM_{2.5}$). The potential adverse health effects resulting from exposure to this PM type are largely unknown. Isoprene-derived epoxides, isoprene epoxydiols (IEPOX) and methacrylic acid epoxide (MAE), have recently been identified as key gaseous intermediates leading to isoprene SOA formation through acid-catalyzed multiphase chemistry. Altered expression of oxidative stress-associated genes was assessed from exposure to laboratory-generated IEPOX- and MAE-derived SOA in an *in vitro* model of human airway epithelial cells (BEAS-2B). Exposure to SOA filter extracts is associated with an increased level of expression of oxidative stress response genes in human lung cells under noncytotoxic conditions, with MAE-derived SOA extracts showing greater potency than IEPOX-derived SOA extracts. Our findings highlight the importance of future work aimed at linking PM source, composition, exposure biomarkers, and health outcomes.



1. INTRODUCTION

Atmospheric fine particulate matter ($PM_{2.5}$, aerosols with of aerodynamic diameter of ≤2.5 μm) has been linked to a broad range of adverse health effects in epidemiological studies.[1,2] Although positive associations between $PM_{2.5}$ and exposure-induced health outcomes have been found, the specific $PM_{2.5}$ components associated with adverse health effects are diverse, depending on the physicochemical properties of $PM_{2.5}$.[3] $PM_{2.5}$ is a complex mixture of inorganic and organic components derived from both primary and secondary origins. Organic aerosol (OA), which is composed of a wide variety of individual organic compounds, constitutes a large fraction of $PM_{2.5}$ mass (20−90%) worldwide.[4] Currently, our knowledge is insufficient to quantitatively characterize the health risk from exposure to OA because its constituents are largely uncharacterized at the molecular level.[5]

Isoprene (2-methyl-1,3-butadiene) is the most abundant non-methane hydrocarbon emitted (~600 Tg $year^{-1}$)[6] into Earth's atmosphere and is derived primarily from broad-leaf trees. Secondary organic aerosol (SOA) produced from the hydroxyl radical (OH)-initiated oxidation (so-called photo-oxidation) of isoprene is a dominant source of $PM_{2.5}$ in many regions, particularly the southeastern United States, where high isoprene emissions interacting with surrounding anthropogenic pollutants enhance aerosol formation.[7,8] Isoprene photo-oxidation contributes ~30−50% of the global SOA burden.[9,10]

Laboratory studies have revealed that epoxide intermediates derived from photooxidation of isoprene, including isomeric isoprene epoxydiols (IEPOX)[11−13] and methacrylic acid epoxide (MAE),[14] are critical gas-phase precursors leading to isoprene SOA formation through acid-catalyzed multiphase chemistry.

Currently, little is known about the potential adverse health effects induced by exposure to isoprene-derived SOA. Prior work has indicated that exposure to a mixture of gaseous products from isoprene oxidation enhances the expression of proinflammatory cytokines in a human lung cell model (A549);[15] airway irritation has also been reported *in vivo*.[16−19] The role of isoprene SOA in ambient $PM_{2.5}$ exposures has not yet been examined. We have recently assessed the oxidative potential of isoprene-derived SOA using the acellular dithiothreitol (DTT) assay.[20] The objective of this study was to evaluate the potential contribution to toxicogenomic effects by isoprene SOA constituents based on the biological pathways hypothesized to link $PM_{2.5}$ exposure to cardiopulmonary mortality,[21−24] with a specific focus on pulmonary oxidative stress. We report results of *in vitro* gene

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expression in human bronchial epithelial cells (BEAS-2B) to evaluate the induced oxidative stress responses to the mixture of SOA components generated from the reactive uptake of *trans*-β-IEPOX and MAE onto acidic sulfate aerosols in controlled chamber experiments.

2. MATERIALS AND METHODS

2.1. Synthesis of SOA Precursors. *trans*-β-IEPOX and MAE were synthesized according to published synthetic procedures.[14,25] Identity and purity (>99%) were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance, gas chromatography/electron ionization mass spectrometry (GC/EI-MS) analysis with prior trimethylsilylation (TMS), or ultra performance liquid chromatography coupled to electrospray ionization high resolution quadrupole time-of flight mass spectrometry (UPLC/ESI-HR-QTOFMS).

2.2. Generation and Chemical Characterization of IEPOX- and MAE-SOA. Reactive uptake experiments were performed in a 10 m^3 flexible Teflon indoor chamber at The University of North Carolina. Detailed operating procedures for this chamber facility have been described previously[13] and are briefly summarized in the text of the Supporting Information. A summary of the experimental conditions is given in Table S1. Filter samples were chemically characterized by GC/EI-MS and UPLC/ESI-HR-QTOFMS. Detailed filter extraction procedures have been previously described by Lin et al.[13] The efficiency of removal of isoprene epoxide-derived SOA constituents from filters was estimated to be >90%. Detailed sample preparation, column conditions, and operating parameters for GC/EI-MS and UPLC/ESI-HR-QTOFMS have been published elsewhere.[26]

2.3. Cell Culture. BEAS-2B cells were cultured in keratinocyte growth medium (KGM BulletKit) (Lonza), which is serum-free keratinocyte basal medium (KBM) supplemented with bovine pituitary extract, human epidermal growth factor, insulin, hydrocortisone, and GA-1000 (gentamicin, amphotericin B). The cells were grown at 37 °C and 5% CO_2 in a humidified incubator.

2.4. Extraction of SOA Constituents for Cell Exposure. The Teflon filter membranes were extracted by sonication in high-purity methanol (LC/MS CHROMASOLV, Sigma-Aldrich) in the same manner that was described for chemical analysis. Multiple filter samples were combined to achieve the desired dose levels for both IEPOX- and MAE-derived SOA, and the combined filter extracts were dried under a gentle stream of nitrogen. Growth factor-deprived KBM medium was then added to the extraction vials to redissolve IEPOX- and MAE-derived SOA constituents for cell exposure. Control filters collected from acidified sulfate aerosol-only experiments were extracted and reconstituted in the same manner.

2.5. Cell Exposure. Cells were seeded in 24-well plates at a density of 2.5×10^4 cells/well in 250 μL of KGM 2 days prior to exposure. At the time of exposure when cells reached 60−70% confluence, cells were washed twice with the phosphate-buffered saline (PBS) buffer and then exposed to KBM medium containing 1, 0.1, or 0.01 mg/mL SOA extract of chamber-generated aerosol samples for 24 h. Experiments were conducted in triplicate per treatment group.

2.6. Assessment of Cytotoxicity. Cytotoxicity was assessed with the lactate dehydrogenase (LDH) cytotoxicity detection kit (Takara) according to the manufacturer's protocol to ensure toxicity of exposure levels would not interfere with gene expression analysis. After being exposed for 24 h, the supernatants were collected to assess LDH levels. Cells exposed to filter extracts from acidified sulfate aerosol-only experiments and cells maintained in KBM alone were treated as control groups.

2.7. Oxidative Stress-Associated Gene Expression Analysis. Cells were lysed with 350 μL of Trizol Reagent (Life Technologies) at the end of the exposure for total RNA isolation. Isolated RNA samples were further purified using the spin column-based Direct-zol RNA MiniPrep kit (Zymo Research). RNA quality and concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The 260 nm/280 nm absorbance ratios of all samples were determined to be >1.8. An aliquot of RNA (100 ng) was copied into cDNA using an RT^2 First Strand Kit (Qiagen). The pathway-focused Human Oxidative Stress Plus RT^2 Profiler PCR array (Qiagen, 96-well format, catalog no. PAHS-065Y) with 84 oxidative stress-associated genes (Table S2) was used to assess the exposure-induced differential gene expression with a Stratagene Mx3005P real time qPCR system (Agilent Technologies). Additionally, qRT-PCR assays (QuantiTect SYBR Green RT-PCR Kit, Qiagen) of selected individual genes, including prostaglandin-endoperoxide synthase 2 (*PTGS2*) and β-actin (*ACTB*, housekeeping gene), were also conducted for quality control.

2.8. Data Analysis. Relative levels of gene expression for exposure and control groups, given as fold changes, were calculated using the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method.[27] Transcriptional changes in cells exposed to SOA constituents were compared to changes in cells exposed to the extracts from acidic sulfate aerosol controls to assess the effects induced solely by the extracted SOA constituents. Differentially expressed genes were identified using Qiagen RT^2 Profiler Data Analysis version 3.5, with significance defined as $p < 0.05$. The p value adjusted for false discovery rate (FDR) was estimated to be 0.0005 (α/n; $\alpha = 0.05$, and $n = 84$ genes). Network-based analysis to identify canonical pathways and transcription factors was conducted using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Inc., Redwood City, CA). Gene networks representing enriched perturbed pathways were identified through enrichment analysis performed using the Fisher's Exact test as detailed previously.[28]

3. RESULTS AND DISCUSSION

3.1. Generation of SOA Constituents from Reactive Uptake of Epoxides. Time profiles of aerosol mass concentrations measured during the reactive uptake experiments are shown in Figure S1. SOA mass yields from the reactive uptake of *trans*-β-IEPOX onto acidified sulfate aerosol are substantially larger than those from reactive uptake of MAE under the same experimental conditions (<10% RH) and on the same time scale (2 h reaction time). These observations are consistent with recent flow tube studies of uptake kinetics that reported a higher reaction probability (γ) for *trans*-β-IEPOX than for MAE,[29,30] as well as with ambient measurements in the southeastern United States, which found the sum of IEPOX-derived SOA tracers (642−1225 ng m^{-3}) to be substantially larger than that of MAE-derived SOA tracers (~20 ng m^{-3}).[31]

3.2. Aerosol Chemical Composition. In Figure 1, the GC/MS total ion current (TIC) chromatograms of TMS-derivatized particle-phase reaction products from reactive uptake of *trans*-β-IEPOX (Figure 1A) and MAE (Figure 1B) in the chamber experiments are compared to that of an ambient $PM_{2.5}$ sample (Figure 1C) collected at a rural site in Yorkville,

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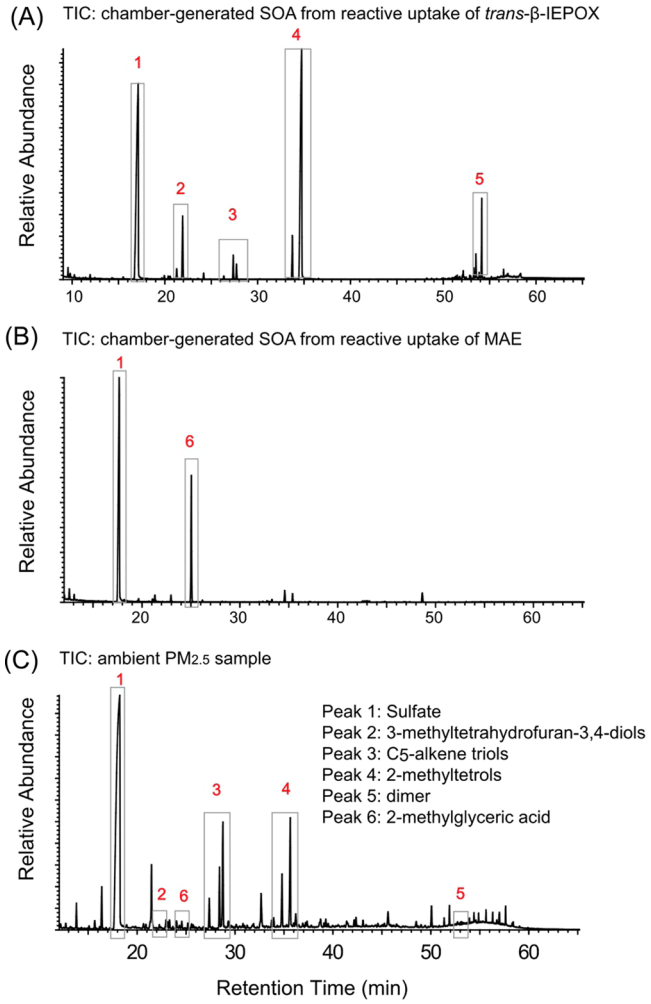

Figure 1. GC/MS total ion current chromatograms (TICs) of TMS-derivatized particle-phase reaction products from reactive uptake of (A) *trans*-β-IEPOX and (B) MAE onto acidified sulfate seed aerosol in chamber experiments and (C) a $PM_{2.5}$ field sample from Yorkville, GA. Mixtures of isomeric SOA products are grouped as one peak.

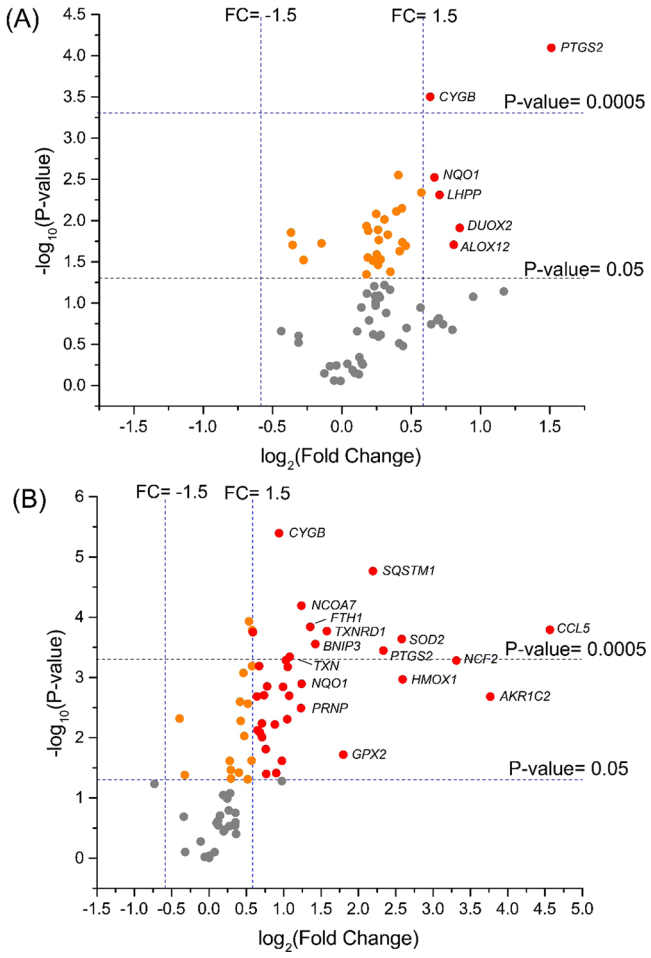

Figure 2. Volcano plots of differential gene expression in BEAS-2B cells upon exposure to (A) IEPOX-SOA and (B) MAE-SOA. A complete list of genes and p values is provided in the Supporting Information (Table S3).

GA, downwind of a coal-burning power plant and experiencing high isoprene emissions during the summer. The most abundant ion (peak 1) is the bis(trimethylsilyl) sulfate derivative of extractable inorganic particle sulfate.[31] The isoprene SOA tracers in chamber samples are identical to those in field samples. In Figure S2, TICs from UPLC/ESI-HR-QTOFMS analysis of the same samples are compared. The most abundant peaks in extracts of chamber samples represent the isomeric sulfate esters of 2-methyltetrol (m/z 215; $C_5H_{11}O_7S^-$) (Figure S2A) and the sulfate ester of 2-methylglyceric acid (m/z 199; $C_4H_7O_7S^-$) (Figure S2B).[12,14] Both ions are present in the extract of a typical ambient $PM_{2.5}$ sample (Figure 2C). Consistent with previous studies,[31] the epoxide-derived SOA products represent the major OA constituents of the ambient $PM_{2.5}$ samples collected from the southeastern United States during summertime and support the validity of the chamber experiments as being representative of ambient SOA composition.

3.3. Cytotoxicity Measurements. We observed significant cell death in acidified sulfate aerosol-only controls at a concentration of 1 mg/mL (cell death, ~27%; p = 0.02), while acidified sulfate aerosol-only concentrations of ≤0.1 mg/mL were not cytotoxic (cell death, ≤10%; $p > 0.05$). Therefore, cells exposed to a dose level of 0.1 mg/mL were selected for gene expression analysis.

3.4. Altered Expression of Genes Caused by Exposure to Isoprene-Derived SOA. Volcano plots of differential gene expression in BEAS-2B cells upon exposure to IEPOX-SOA and MAE-SOA are shown in Figure 2. A complete list of genes and p values is provided in Table S3. With a fold change cutoff value of 1.5, six oxidative stress-associated genes with significant fold changes were induced by exposure to IEPOX-SOA extract and 36 oxidative stress-associated genes by exposure to MAE-SOA extract. When FDR is considered, fold changes in two genes from exposure to IEPOX-SOA extract and in 13 genes from exposure to MAE-SOA extract remain significant.

3.5. Quality Check of Expression Changes through qRT-PCR. Expression of prostaglandin-endoperoxide synthase 2 (*PTGS2*) was selected as a quality control check for qRT-PCR analysis because exposure to both IEPOX- and MAE-derived SOA induced significant fold changes in expression. The comparison of fold change values between RT^2 Profiler PCR arrays and qRT-PCR is shown in Figure S3. Expression levels of *PTGS2* induced by exposure to IEPOX- or MAE-SOA extracts are normalized to the housekeeping gene (*ACTB*) and acidified sulfate aerosol exposure controls. At 0.1 mg/mL, MAE-derived SOA induces a level of *PTGS2* gene expression

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(7.09-fold; $p = 0.01$) significantly higher than that of IEPOX-derived SOA (3.29-fold; $p = 0.20$), consistent with RT^2 Profiler PCR array results (Table S3). At a low exposure concentration (0.01 mg/mL), induction of *PTGS2* gene expression was not significant for either IEPOX- or MAE-derived SOA ($p > 0.05$).

3.6. Pathway Enrichment Analysis Reveals the NRF2 Signaling Pathway Triggered upon Isoprene SOA Exposure. The 38 differentially expressed genes in the gene sets exposed to IEPOX- and MAE-derived SOA extracts were analyzed for enrichment within biological pathways. The canonical pathway for nuclear factor erythroid 2-like 2 (NRF2)-mediated oxidative stress response ($p = 10^{-16}$) was enriched in both sets, with one of six genes (16%) represented in the IEPOX-SOA set and 13 of 36 genes (36%) represented in the MAE set (Table S3), NADPH dehydrogenase, quinone 1 (*NQO1*) being represented in both sets at $p < 0.05$ (while it did not pass the stringent FDR, it does show differential expression as determined via RT-PCR).

3.7. Cellular Oxidative Stress Response and Oxidative Potential of PM. Our gene expression analysis indicates that the constituents of isoprene SOA generated from MAE (high-NO_x SOA precursor) are more potent as inducers of oxidative stress than those of SOA generated from IEPOX (low-NO_x SOA precursor). The difference in toxicity may be attributed to the distinct chemical composition of the SOA from the two epoxide precursors, which may determine bioavailability and chemical reactivity.[3] The oxidative potency of isoprene SOA extracts has been assessed by the dithiothreitol (DTT) assay.[20] MAE-derived SOA extracts were more strongly oxidizing [$(2.74 \pm 0.27) \times 10^{-3}$ nmol of DTT consumed min^{-1} (μg of sample)$^{-1}$] than IEPOX-derived SOA extracts [$(1.58 \pm 0.13) \times 10^{-3}$ nmol of DTT consumed min^{-1} (μg of sample)$^{-1}$]. This result is in accord with the oxidative stress responses of BEAS-2B cells observed in this study. Induction of *HMOX1* gene expression in BEAS-2B cells by MAE-SOA exposure (Table S3) is consistent with reports of strong correlation between DTT activity of PM samples and exposure-induced *HMOX1* gene expression.[32]

3.8. Environmental Health Implications. Oxidative stress is known to be associated with chronic pulmonary inflammation and contributes to adverse respiratory and cardiovascular health outcomes.[21,33,34] Our results suggest that exposure to isoprene-derived SOA extracts increases levels of oxidative stress responses in BEAS-2B cells. Differences in SOA chemical composition may result in different biological effects. Our observation of an enrichment of genes in the NRF2 network in cells exposed to isoprene SOA extracts further supports oxidative stress as a factor in cell damage. Additional studies are warranted to determine whether a specific gene or suite of genes might be useful biomarkers of exposure to isoprene SOA and to understand the role of the NRF2 pathway and identify populations that may be more susceptible to isoprene SOA exposure. As this is a first attempt to explore changes in gene expression induced specifically by IEPOX- and MAE-derived SOA constituents in whole cells, the use of BEAS-2B, which is an immortalized cell line, as well as methodological limitation of delivery of SOA PM to submerged cells should be noted. Previous studies showed airway irritation in mice caused by exposure to isoprene/O_3 mixtures.[16−19] Our results could provide motivation for *in vivo* investigation of the effects of IEPOX- and MAE-derived SOA. Further analysis at a transcriptome-wide level will help to identify other potential effects and eventually to provide mechanistic insights into adverse outcomes. In the southeastern United States, which is heavily impacted by isoprene emissions, ambient mass concentrations of known isoprene SOA tracers have been reported at levels of up to 1−2 μg m^{-3},[31,35,36] and that of the ensemble of isoprene SOA is even higher.[8,37] With regard to community exposure to high $PM_{2.5}$ concentrations and the associated adverse health effects, our study indicates the importance of the organic component of SOA and emphasizes the urgent need for additional work on mechanisms of airway pathology by $PM_{2.5}$ constituents.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.6b00151.

Summary of indoor chamber experiments (Table S1), gene symbols and full names of genes included in the RT^2 Profiler PCR Array Human Oxidative Stress Pathway Plus (Table S2), a complete list of genes and p values (Table S3), time profiles of aerosol mass concentrations during the chamber experiments (Figure S1), UPLC/ESI-HR-QTOFMS TICs of particle-phase constituents (Figure S2), quality check of *PTGS2* gene expression changes through qRT-PCR (Figure S3), and cell morphology comparing treatment, seed control, and unexposed groups (Figure S4) (PDF)

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Notes

The authors declare no competing financial interest.

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