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**Understanding the Early Biological Effects  
of Isoprene-Derived Particulate Matter  
Enhanced by Anthropogenic Pollutants**

Jason D. Surratt, Ying-Hsuan Lin, Maiko Arashiro,  
William G. Vizuete, Zhenfa Zhang, Avram Gold,  
Ilona Jaspers, and Rebecca C. Fry





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with a Critique by the HEI Review Committee

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Research Report 198

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# ABOUT HEI

The Health Effects Institute is a nonprofit corporation chartered in 1980 as an independent research organization to provide high-quality, impartial, and relevant science on the effects of air pollution on health. To accomplish its mission, the institute

- Identifies the highest-priority areas for health effects research;
- Competitively funds and oversees research projects;
- Provides intensive independent review of HEI-supported studies and related research;
- Integrates HEI's research results with those of other institutions into broader evaluations; and
- Communicates the results of HEI's research and analyses to public and private decision makers.

HEI typically receives balanced funding from the U.S. Environmental Protection Agency and the worldwide motor vehicle industry. Frequently, other public and private organizations in the United States and around the world also support major projects or research programs. HEI has funded more than 340 research projects in North America, Europe, Asia, and Latin America, the results of which have informed decisions regarding carbon monoxide, air toxics, nitrogen oxides, diesel exhaust, ozone, particulate matter, and other pollutants. These results have appeared in more than 260 comprehensive reports published by HEI, as well as in more than 1,000 articles in the peer-reviewed literature.

HEI's independent Board of Directors consists of leaders in science and policy who are committed to fostering the public–private partnership that is central to the organization. The Research Committee solicits input from HEI sponsors and other stakeholders and works with scientific staff to develop a Five-Year Strategic Plan, select research projects for funding, and oversee their conduct. The Review Committee, which has no role in selecting or overseeing studies, works with staff to evaluate and interpret the results of funded studies and related research.

All project results and accompanying comments by the Review Committee are widely disseminated through HEI's website ([www.healtheffects.org](http://www.healtheffects.org)), printed reports, newsletters and other publications, annual conferences, and presentations to legislative bodies and public agencies.



# ABOUT THIS REPORT

Research Report 198, *Understanding the Early Biological Effects of Isoprene-Derived Particulate Matter Enhanced by Anthropogenic Pollutants*, presents a research project funded by the Health Effects Institute and conducted by Dr. Jason D. Surratt of the University of North Carolina, Chapel Hill, and his colleagues. This research was funded under HEI's Walter A. Rosenblith New Investigator Award Program, which provides support to promising scientists in the early stages of their careers. The report contains three main sections.

**The HEI Statement**, prepared by staff at HEI, is a brief, nontechnical summary of the study and its findings; it also briefly describes the Review Committee's comments on the study.

**The Investigators' Report**, prepared by Surratt and colleagues, describes the scientific background, aims, methods, results, and conclusions of the study.

**The Critique**, prepared by members of the Review Committee with the assistance of HEI staff, places the study in a broader scientific context, points out its strengths and limitations, and discusses remaining uncertainties and implications of the study's findings for public health and future research.

This report has gone through HEI's rigorous review process. When an HEI-funded study is completed, the investigators submit a draft final report presenting the background and results of the study. This draft report is first examined by outside technical reviewers and a biostatistician. The report and the reviewers' comments are then evaluated by members of the Review Committee, an independent panel of distinguished scientists who have no involvement in selecting or overseeing HEI studies. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, as necessary, to revise their report. The Critique reflects the information provided in the final version of the report.



# HEI STATEMENT

## Synopsis of Research Report 198

### Understanding the Early Biological Effects of Isoprene-Derived Particulate Matter Enhanced by Anthropogenic Pollutants

#### BACKGROUND

In this study Dr. Jason Surratt, who was a recipient of HEI's Walter A. Rosenblith New Investigator Award, and his colleagues characterized and compared the oxidative property and biological responses of laboratory-generated secondary organic aerosol (SOA) derived from the photochemical oxidation of isoprene in the presence of oxygen radicals or the condensation of key intermediates of isoprene oxidation. Isoprene was chosen because it is an abundant volatile organic compound derived from biogenic sources (i.e., certain types of vegetation) for which there is limited mechanistic information. Work has shown that the oxidation of isoprene follows two main pathways depending on the level of nitrogen oxides. At high levels, it leads to the formation of methacrolein (MACR), which is further oxidized to methacrylic acid epoxide (MAE). At low levels, isoprene is converted to isoprene hydroxyhydroperoxide (ISOPOOH) and subsequently to isoprene epoxydiols (IEPOX).

#### APPROACH

The aim of the study was to examine the oxidative potential and the effect on cellular toxicity and gene expression of SOA derived from either isoprene or one of its metabolites, which were synthesized in the author's laboratory. Oxidative potential was measured using the dithiothreitol (DTT) assay of extracts of SOA collected on filters, which assesses the ability of the SOA extracts to oxidize DTT in a test tube. Two types of cellular experiments were conducted: (1) exposure by resuspension to each SOA extract for 9 hours, at which time the extracellular medium was collected for analysis, and (2) a 1-hour direct exposure of the cells to the isoprene-derived SOA (in the aerosol state) followed by incubation in fresh medium for 9 hours, at which time the extracellular medium was collected for analysis.

The biological endpoints measured included:

- lactate dehydrogenase, used as a marker of cell toxicity;
- expression of specific inflammation- and oxidative stress-related genes (interleukin-8 [*IL-8*], prostaglandin-endoperoxide synthase 2 [*PTGS2*], and heme oxygenase 1; and
- expression of multiple genes using two separate panels of genes (a panel of 84 human oxidative stress-associated genes and a panel of 249 human inflammation-associated genes).

#### MAIN RESULTS AND INTERPRETATION

All SOA samples showed some DTT activity. ISOPOOH-derived SOA had the highest DTT response followed by MACR- and isoprene-derived SOA, which had similar oxidative potential.

#### What This Study Adds

- The study provides a thorough evaluation of oxidative potential and effects on gene expression of SOA derived from isoprene (an abundant organic compound that is released by vegetation), which contributes to ambient particulate matter levels.
- The results show that isoprene-derived SOA has some oxidative potential and induces genes that regulate antioxidant defenses, suggesting that this SOA leads to an increase in cellular oxidant burden.
- The study also tested SOAs derived from intermediate products of isoprene oxidation and showed that NO<sub>x</sub> levels can mediate SOA formation reaction pathways and may ultimately affect the SOA oxidative activity.

There was no increase in lactate dehydrogenase in any of the experiments using either direct SOA exposure or resuspension exposure.

Both direct exposure and resuspension exposure to isoprene-derived SOA increased the expression of PTGS2 and IL-8. Resuspension exposure to IEPOX- and MAE-derived SOA also increased the expression of PTGS2 (IL-8 was not measured), but exposure to ISOPOOH-derived SOA did not. MACR was not tested.

The expression profiles of the two gene panels showed that direct exposure to isoprene-derived SOA induced the differential expression of the largest number of genes (22 total) versus 2, 13, and 4 genes for IEPOX-, MAE-, and ISOPOOH-derived SOA, respectively. Given the different exposure protocols, it is not clear whether the results are a consequence of the different experimental conditions or of different activities of the SOA.

The investigators analyzed for enrichment of the affected genes within biological pathways and found that the pathway that appeared to be consistently enriched across the various experiments was

the one for Nrf2-mediated oxidative stress (i.e., nuclear factor [erythroid-derived 2]-like 2).

There was no clear relationship between DTT activity and the number of altered oxidative stress-related genes. Because the assays measure different oxidative pathways, they are not expected to be correlated.

### CONCLUSIONS

This project has shed some light on an important area of research on particulate matter and health, namely, that oxides of nitrogen can mediate SOA formation reactions and thereby affect the oxidative activity of the resulting SOA. In addition, the study has shown that direct exposure of cells to isoprene-derived SOA in the aerosol state increased the expression of genes in the Nrf2 pathway, which regulates antioxidant defenses, and suggests that this SOA can lead to an increase in cellular oxidant burden. Overall, the results indicate that the endpoints selected respond to chemical differences in the SOA precursors and suggest novel approaches for studying the effects of SOA.

## Understanding the Early Biological Effects of Isoprene-Derived Particulate Matter Enhanced by Anthropogenic Pollutants

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### ABSTRACT

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### INTRODUCTION

Airborne fine particulate matter (PM<sub>2.5</sub>; particulate matter  $\leq 2.5 \mu\text{m}$  in aerodynamic diameter\*) plays a key role in air quality, climate, and public health. Globally, the largest mass fraction of PM<sub>2.5</sub> is organic, dominated by secondary organic aerosol (SOA) formed from atmospheric oxidation of volatile organic compounds (VOCs). Isoprene from vegetation is the most abundant nonmethane VOC emitted into Earth's atmosphere. Isoprene has been recently recognized as one of the major sources of global SOA production that is enhanced by the presence of anthropogenic pollutants, such as acidic sulfate derived from sulfur dioxide (SO<sub>2</sub>), through multiphase chemistry of its oxidation products. Considering the abundance of isoprene-derived SOA in the atmosphere, understanding mechanisms of adverse health effects

through inhalation exposure is critical to mitigating its potential impact on public health. Although previous studies have examined the toxicological effects of certain isoprene-derived gas-phase oxidation products, to date, no systematic studies have examined the potential toxicological effects of isoprene-derived SOA, its constituents, or its SOA precursors on human lung cells.

### SPECIFIC AIMS

The overall objective of this study was to investigate the early biological effects of isoprene-derived SOA and its subtypes on BEAS-2B cells (a human bronchial epithelial cell line), with a particular focus on the alteration of oxidative stress- and inflammation-related genes. To achieve this objective, there were two specific aims:

1. Examine toxicity and early biological effects of SOA derived from the photochemical oxidation of isoprene, considering both urban and downwind-urban types of chemistry.
2. Examine toxicity and early biological effects of SOA derived directly from downstream oxidation products of isoprene (i.e., epoxides and hydroperoxides).

### METHODS

Isoprene-derived SOA was first generated by photooxidation of isoprene under natural sunlight in the presence of nitric oxide (NO) and acidified sulfate aerosols. Experiments were conducted in a 120-m<sup>3</sup> outdoor Teflon-film chamber located on the roof of the Gillings School of Global

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This Investigators' Report is one part of Health Effects Institute Research Report 198, which also includes a Critique by the Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Jason D. Surratt, 135 Dauer Drive, 164 Rosenau Hall, Campus Box # 7431; e-mail: [surratt@unc.edu](mailto:surratt@unc.edu).

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award CR-83467701 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

\* A list of abbreviations and other terms appears at the end of this volume.

Public Health, University of North Carolina at Chapel Hill (UNC–Chapel Hill). BEAS-2B cells were exposed to chamber-generated isoprene-derived SOA using the Electrostatic Aerosol in Vitro Exposure System (EAVES). This approach allowed us to generate atmospherically relevant compositions of isoprene-derived SOA and to examine its toxicity through in vitro exposures at an air–liquid interface, providing a more biologically relevant exposure model. Isoprene-derived SOA samples were also collected, concurrently with EAVES sampling, onto Teflon membrane filters for in vitro resuspension exposures and for analysis of aerosol chemical composition by gas chromatography/electron ionization-quadrupole mass spectrometry (GC/EI-MS) with prior trimethylsilylation and ultra-performance liquid-chromatography coupled to high-resolution quadrupole time-of-flight mass spectrometry equipped with electrospray ionization (UPLC/ESI-HR-QTOFMS). Isoprene-derived SOA samples were also analyzed by the dithiothreitol (DTT) assay in order to characterize their reactive oxygen species (ROS)-generation potential.

Organic synthesis of known isoprene-derived SOA precursors, which included isoprene epoxydiols (IEPOX), methacrylic acid epoxide (MAE), and isoprene-derived hydroxyhydroperoxides (ISOPOOH), was conducted in order to isolate major isoprene-derived SOA formation pathways from each other and to determine which of these pathways (or SOA types) is potentially more toxic. Since IEPOX and MAE produce SOA through multiphase chemistry onto acidic sulfate aerosol, dark reactive uptake experiments of IEPOX and MAE in the presence of acidic sulfate aerosol were performed in a 10-m<sup>3</sup> flexible Teflon indoor chamber at UNC–Chapel Hill. Since the generation of SOA from ISOPOOH (through a non-IEPOX route) requires a hydroxyl radical ( $\bullet$ OH)-initiated oxidation, ozonolysis of tetramethylethylene (TME) was used to form the needed  $\bullet$ OH radicals in the indoor chamber. The resultant low-volatility multifunctional hydroperoxides condensed onto nonacidified sulfate aerosol, yielding the ISOPOOH-derived SOA needed for exposures. Similar to the outdoor chamber SOAs, IEPOX-, MAE- and ISOPOOH-derived SOAs were collected onto Teflon membrane filters and were subsequently chemically characterized by GC/EI-MS and UPLC/ESI-HR-QTOFMS as well as for ROS-generation potential using the DTT assay. These filters were also used for resuspension in vitro exposures.

By conducting gene expression profiling, we provided mechanistic insights into the potential health effects of isoprene-derived SOA. First, gene expression profiling of 84 oxidative stress- and 249 inflammation-associated human genes was performed for cells exposed to isoprene-derived SOA generated in our outdoor chamber experiments in

EAVES or by resuspension. Two pathway-focused panels were utilized for this purpose: (1) nCounter GX Human Inflammation Kit comprised of 249 human genes (NanoString), and (2) Human Oxidative Stress Plus RT<sup>2</sup> Profiler PCR Array (Qiagen) comprised of 84 oxidative stress-associated genes. We compared the gene expression levels in cells exposed to SOA generated in an outdoor chamber from photochemical oxidation of isoprene in the presence of NO and acidified sulfate seed aerosol to cells exposed to a dark control mixture of isoprene, NO, and acidified sulfate seed aerosol to isolate the effects of the isoprene-derived SOA on the cells using the EAVES and resuspension exposure methods. Pathway-based analysis was performed for significantly altered genes using the ConsensusPathDB database, which is a database system for the integration of human gene functional interactions to provide biological pathway information for a gene set of interest. Pathway annotation was performed to provide biological pathway information for each gene set. The gene–gene interaction networks were constructed and visualized using the GeneMANIA Cytoscape app (version 3.4.1) to predict the putative function of altered genes. Lastly, isoprene-derived SOA collected onto filters was used in resuspension exposures to measure select inflammatory biomarkers, including interleukin 8 (*IL-8*) and prostaglandin-endoperoxide synthase 2 (*PTGS2*) genes, in BEAS-2B cells to ensure that effects observed from EAVES exposures were attributable to particle-phase organic products. Since EAVES and resuspension exposures compared well, gene expression profiling for IEPOX-, MAE- and ISOPOOH-derived SOA were conducted using only resuspension exposures.

## RESULTS AND CONCLUSIONS

Chemical characterization coupled with biological analyses show that atmospherically relevant compositions of isoprene-derived SOA alter the levels of 41 oxidative stress-related genes. Of the different composition types of isoprene-derived SOA, MAE- and ISOPOOH-derived SOA altered the greatest number of genes, suggesting that carbonyl and hydroperoxide functional groups are oxidative stress promoters. Taken together, the different composition types accounted for 34 of the genes altered by the total isoprene-derived SOA mixture, while 7 remained unique to the total mixture exposures, indicating that there is either a synergistic effect of the different isoprene-derived SOA components or an unaccounted component in the mixture.

The high-oxides of nitrogen (NO<sub>x</sub>) regime, which yielded MAE- and methacrolein (MACR)-derived SOA, had a higher ROS-generation potential (as measured by the DTT assay) than the low-NO<sub>x</sub> regime, which included

IEPOX- and isoprene-derived SOA. However, ISOPOOH-derived SOA, which also formed in the low-NO<sub>x</sub> regime, had the highest ROS-generation potential, similar to 1,4-naphthoquinone (1,4-NQ). This suggests that aerosol-phase organic peroxides contribute significantly to particulate matter (PM) oxidative potential. MAE- and MACR-derived SOA showed equal or greater ROS-generation potential than was reported in prior UNC–Chapel Hill studies on diesel exhaust PM, highlighting the importance of a comprehensive investigation of the toxicity of isoprene-derived SOA. Notably, ISOPOOH-derived SOA was one order of magnitude higher in ROS-generation potential than diesel exhaust particles previously examined at UNC–Chapel Hill. As an acellular assay, the DTT assay may not be predictive of oxidative stress; therefore, we also focused on the gene expression results from the cellular exposures.

We have demonstrated that the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and the redox-sensitive activation protein-1 (AP-1) transcription factor networks have been significantly altered upon exposure to isoprene-derived SOA. The identification of Nrf2 pathway in cells exposed to isoprene-derived SOA is in accordance with our findings using the DTT assay, which measures the thiol reactivity of PM samples as a surrogate for their ROS-generation potential. Specifically, our results point to the cysteine–thiol modifications within cells that lead to activation of Nrf2-related gene expression.

However, based on our gene expression results showing no clear relationship between DTT activity and the number of altered oxidative stress-related genes, the DTT activity of isoprene-derived SOA may not be directly indicative of toxicity relative to other SOA types. While activation of Nrf2-associated genes has been identified with responses to oxidative stress and linked to traffic-related air pollution exposure in both toxicological and epidemiological studies, their implicit involvement in this study suggests that activation of Nrf2-related gene expression may occur with exposures to all sorts of PM types.

By controlling the exposure time, method, and dose we demonstrated that among the SOA derived from previously identified individual precursors of isoprene-derived SOA, ISOPOOH-derived SOA alters more oxidative stress-related genes than does IEPOX-derived SOA, but fewer than MAE-derived SOA. This suggests that the composition of MAE-derived SOA may be the greatest contributor to alterations of oxidative stress-related gene expression observed due to isoprene-derived SOA exposure. Further study on induced levels of protein expression and specific toxicological endpoints is necessary to determine if the observed gene expression changes lead to adverse health

effects. In addition, such studies have implications for pollution-control strategies because NO<sub>x</sub> and SO<sub>2</sub> are controllable pollutants that can alter the composition of SOA, and in turn alter its effects on gene expression. The mass fraction of different components of atmospheric isoprene-derived SOA should be considered, but altering the fraction of high-NO<sub>x</sub> isoprene-derived SOA (e.g., MAE-derived SOA) may yield greater changes in gene expression than altering the fraction of low-NO<sub>x</sub> isoprene-derived SOA types (ISOPOOH- or IEPOX-derived SOA). Finally, this study confirms that total isoprene-derived SOA alters the expression of a greater number of genes than does SOA derived from the tested precursors. This warrants further work to determine the underlying explanation for this observation, which may be uncharacterized components of isoprene-derived SOA or the potential for synergism between the studied components.

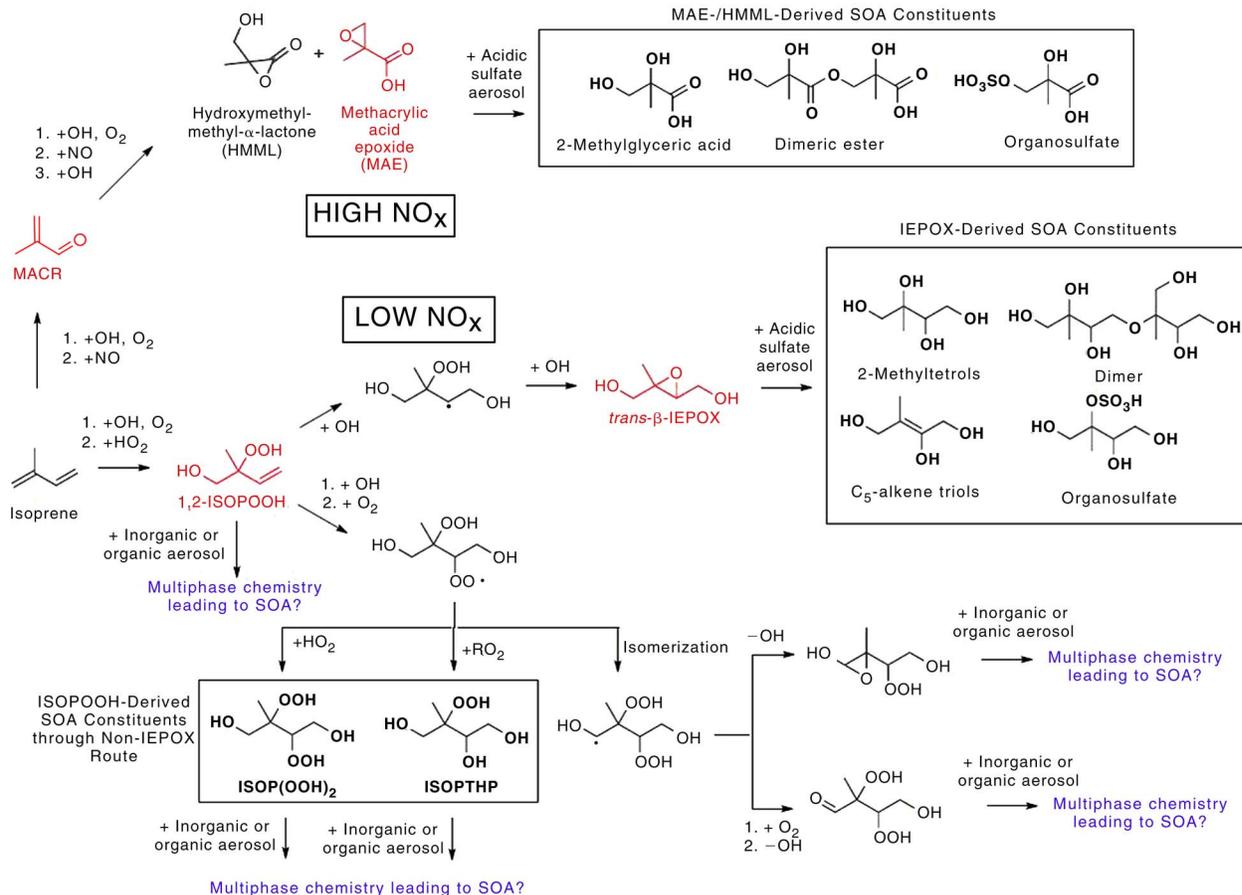
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## INTRODUCTION

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Airborne PM<sub>2.5</sub> plays a key role in air quality, climate, and public health. Globally, the largest mass fraction of PM<sub>2.5</sub> is organic, dominated by SOA formed from atmospheric oxidation of VOCs (Hallquist et al. 2009). Isoprene from vegetation (~600 Tg/yr) (Guenther et al. 2006) is the most abundant nonmethane hydrocarbon emitted into Earth's atmosphere and has been recently recognized as one of the major sources of global SOA production through multiphase chemistry of its oxidation products (Carlton et al. 2009; Pöschl and Shiraiwa 2015). Notably, isoprene is also generated during endogenous processes, especially isoprenoid biosynthesis, as it is one of the most abundant hydrocarbons exhaled by humans (Gelmont et al. 1981). The principal pathways of isoprene oxidation in the atmosphere are via reactions with atmospheric oxidants, such as •OH, nitrate radicals, and ozone (O<sub>3</sub>) (Atkinson and Arey 2003).

As shown in Figure 1, the •OH-initiated oxidation of isoprene represents a dominant pathway during daytime, and it has been recognized as an important source of SOA (Budisulistiorini et al. 2013; Claeys et al. 2004; Hu et al. 2015; Kroll et al. 2006; Surratt et al. 2006, 2010), generating a broad range of reactive intermediates that can further react to yield condensable species (Krechmer et al. 2015; Lin et al. 2012; Paulot et al. 2009; Riva et al. 2016; Surratt et al. 2010). The formation processes of isoprene-derived SOA are known to be greatly influenced by the presence of certain anthropogenic emissions, such as nitrogen oxides (NO<sub>x</sub> = NO + NO<sub>2</sub>), which determine the fate of organic peroxy radical (RO<sub>2</sub>•) transients, and acidic sulfate aerosol derived from the oxidation of SO<sub>2</sub> that



**Figure 1. Known chemical mechanisms leading to SOA formation from •OH-radical initiated oxidation of isoprene during daytime conditions.** MACR/MAE and ISOPOOH/IEPOX are known to be direct intermediate precursors to isoprene-derived SOA under high- and low-nitric oxide (NO) conditions, respectively (Krechmer et al. 2015; Lin et al. 2012, 2013b; Liu et al. 2016; Nguyen et al. 2015; Paulot et al. 2009; Riva et al. 2016; Surratt et al. 2010). IEPOX = isoprene epoxydiol; ISOPOOH = isoprene hydroxyhydroperoxide; MACR = methacrolein; MAE = methacrylic acid epoxide.

promotes acid-catalyzed reactive uptake (multiphase chemistry) of SOA precursors. As a result, these anthropogenic emissions can ultimately alter isoprene SOA composition and yields (Edney et al. 2005; Gaston et al. 2014; Kroll et al. 2006; Riedel et al. 2015; Surratt et al. 2006, 2007a,b, 2010). Isoprene-derived SOA is a major contributor to submicron organic aerosol mass measured in densely forested areas across the world (Hu et al. 2015). In the southeastern United States, where substantial biogenic VOC emissions and anthropogenic pollutants interact in summertime, isoprene-derived SOA has been shown to account

for up to 40% of organic submicron aerosol mass (Budisulistiorini et al. 2016; Xu et al. 2015). Considering the importance of isoprene emissions as a source of PM<sub>2.5</sub>, understanding the potential health effects of isoprene-derived SOA is crucial for addressing public health concerns stemming from community exposure to PM<sub>2.5</sub>. Furthermore, evaluating the health effects of SOA from isoprene oxidation is important from a public health perspective, not only because of its atmospheric abundance, but also because the anthropogenic contribution is the only component amenable to control (Budisulistiorini et al. 2017;

Gaston et al. 2014; Pye et al. 2013; Riedel et al. 2015; Xu et al. 2015).

Current understanding of isoprene-derived SOA formation is based on laboratory studies showing that gas-phase photooxidation (or  $\bullet\text{OH}$ -initiated oxidation) of isoprene generates key intermediate SOA precursors, including isomeric ISOPOOH (Krechmer et al. 2015; Liu et al. 2016; Riva et al. 2016) and IEPOX (Lin et al. 2012, 2014; Nguyen et al. 2014; Paulot et al. 2009; Surratt et al. 2010) under low- $\text{NO}_x$  conditions, and methacrolein (MACR) and MAE under high- $\text{NO}_x$  conditions (Lin et al. 2013a,b; Surratt et al. 2006). As shown in Figure 1, these highly reactive gaseous intermediates (shown in red) resulting from  $\bullet\text{OH}$ -initiated oxidation of isoprene can subsequently react with  $\bullet\text{OH}$  radicals or undergo acid-catalyzed multiphase chemistry that leads to SOA formation (Kjaergaard et al. 2012; Lin et al. 2012, 2013b; Nguyen et al. 2015; Riva et al. 2016; Surratt et al. 2010). Recent work has shown that isoprene-derived multifunctional hydroperoxides can undergo multiphase chemistry (Riva et al. 2016, 2017); however, as shown in Figure 1, more work is needed to understand these chemical processes.

Many studies have shown that  $\text{PM}_{2.5}$  is closely linked to health effects ranging from exacerbation of asthma symptoms to mortality associated with lung cancer and cardiopulmonary disease (Dockery et al. 1993; Samet et al. 2000; Schwartz et al. 1993).  $\text{PM}_{2.5}$ , in particular, has been linked to negative health outcomes with an estimated contribution of 3.2 million premature deaths worldwide as reported in the Global Burden of Disease Study 2010 (Lim et al. 2012). Despite evidence that particle composition affects toxicity, fewer studies focus on the link between chemical composition and health or other biological outcomes (Kelly and Fussell 2012). Prior work on complex air mixtures has shown that VOCs and their semivolatile oxidation products alter the composition and ultimately the toxicity of particles (e.g., Ebersviller et al. 2012a,b). SOA resulting from natural and anthropogenic gaseous precursors, such as  $\alpha$ -pinene and 1,3,5-trimethylbenzene, have been shown to affect cellular function (Gaschen et al. 2010; Jang et al. 2006); however, the exact adverse human health effects that might occur upon exposure to various SOA types remains unclear.

A few studies have investigated the potential health effects of isoprene oxidation products by *in vivo* or *in vitro* assays. Upper airway irritation has been reported in mice exposed to mixtures of isoprene and oxidant ( $\text{O}_3$  and nitrogen dioxide [ $\text{NO}_2$ ]) (Rohr et al. 2003; Wilkins et al. 2001). The observed effects were only partially explained by residual  $\text{O}_3$  and  $\text{NO}_2$  (known inducers of airway irritation) and the identified gaseous reaction products formalde-

hyde, formic acid, acetic acid, MACR, and methylvinyl ketone, implicating unidentified gaseous products as major contributing irritants.

We have previously exposed A549 human lung cells *in vitro* to a gas-phase mixture of first-generation products from photooxidation of isoprene and 1,3-butadiene in the presence of  $\text{NO}_x$  and reported that the irritant response, as measured by IL-8 protein secretion, could not be based solely on  $\text{O}_3$  generated by the systems (Doyle et al. 2004). These data suggested that effects of additional products, both known and yet to be identified, would be required to explain the observations. Wilkins and colleagues (2003) examined the relative humidity (RH)- and time-dependence of the irritant response of mice exposed to the ozonolysis products of isoprene by measuring respiration rate. Maximum irritation was observed for low-RH conditions and fresh mixtures, implying that over time these highly irritating intermediates react with water vapor or are lost to wet surfaces to produce mixtures that are less irritating. Recent studies on isoprene photooxidation have identified ISOPOOH (Krechmer et al. 2015; Liu et al. 2016; Riva et al. 2016), IEPOX (Lin et al. 2012; Surratt et al. 2010), MAE (Lin et al. 2013b), and HMML (Kjaergaard et al. 2012; Nguyen et al. 2015) as highly reactive gas-phase intermediates leading to SOA through multiphase chemistry or further oxidation to low-volatility condensable products. Formation of highly oxidized gaseous oligomeric hydroperoxides has also been recently reported from isoprene ozonolysis under low RH conditions (Riva et al. 2017), which together may provide a more complete explanation of cellular responses and respiratory effects previously observed from isoprene oxidation. To date, no systematic studies have examined the potential toxicological effects of isoprene-derived SOA, its constituents, or its SOA precursors on human lung cells.

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## SPECIFIC AIMS AND STUDY DESIGN

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The overall objective of this study was to investigate the early biological effects of isoprene-derived SOA, based on the current understanding of its formation through key precursors shown in Figure 1, on BEAS-2B cells with a particular focus on the alteration of oxidative stress- and inflammation-related genes. There were two specific aims in this study:

1. Examine toxicity and early biological effects of SOA derived from the photochemical oxidation of isoprene, considering both urban and downwind-urban types of chemistry.

2. Examine toxicity and early biological effects of SOA derived directly from downstream oxidation products of isoprene (i.e., epoxides and hydroperoxides, as well as specific SOA constituents that have authentic standards available).

To achieve the overall objective, we began by using the DTT assay to assess the potential of total isoprene-derived SOA, isoprene-derived epoxides and hydroperoxides, and their resultant SOA mixtures (Figure 1), for generating ROS. We then examined the toxicity of these aerosols through in vitro exposures. For our in vitro exposures, the overall isoprene-derived SOA (the sum of all pathways shown in Figure 1) was examined using both a direct SOA deposition (i.e., air–liquid interface) exposure device and resuspensions of SOA collected on filters in order to determine the effects of isoprene-derived SOA, generated through isoprene photooxidation experiments, on *PTGS2* and *IL-8* gene expression. We then explored the effect of isoprene-derived SOA exposure on a greater number of oxidative stress- and inflammation-related gene expressions using pathway-focused gene expression profiling, especially since differentially expressed genes are components of known biological pathways that provide mechanistic insights into the potential health effects of isoprene-derived SOA. Last, we systematically examined the effects of different types of isoprene SOA derived directly from MAE, IEPOX, and ISOPOOH on oxidative stress-related genes using the SOA resuspension methods.

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## METHODS

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### GENERATION AND CHEMICAL CHARACTERIZATION OF SOA FROM PHOTOCHEMICAL OXIDATION OF ISOPRENE AND MACR

#### Outdoor Chamber Experiments

Isoprene-derived SOA was generated by photooxidation of isoprene under natural sunlight in the presence of  $\text{NO}_x$  (initially NO) and acidified sulfate seed aerosols. Experiments were conducted in a 120-m<sup>3</sup> outdoor Teflon-film chamber located on the roof of the Gillings School of Global Public Health, UNC–Chapel Hill. The chamber facility has been described in detail previously (Ebersviller et al. 2012a; Lichetveld et al. 2012). Real-time aerosol size distributions and volume concentrations were monitored using a differential mobility analyzer (DMA; BMI model 2002) coupled to a mixing condensation particle counter (MCPC; BMI model 1710). The aerosol-sizing instrument was periodically calibrated and checked against National

Institute of Standards and Technology (NIST)-standard polystyrene latex sphere (PSL) particles. Prior to each experiment, the chamber was continuously flushed with at least five chamber-volumes of clean air. The background particle mass concentration was  $<1 \mu\text{g}/\text{m}^3$ . Acidified sulfate seed aerosols were atomized into the chamber (constant output atomizer, TSI 3076) from an aqueous solution containing 0.06 M magnesium sulfate ( $\text{MgSO}_4$ ) and 0.06 M sulfuric acid ( $\text{H}_2\text{SO}_4$ ) to provide a pre-existing aerosol surface for reactive uptake or partitioning of isoprene-derived SOA precursors. After seed aerosols reached the target mass concentration ( $\sim 100 \mu\text{g}/\text{m}^3$ , assuming unit density), 200 ppbv NO (Airgas, 1% NO/ $\text{N}_2$ ) and 3.5 ppmv isoprene (Sigma-Aldrich, 99%) were injected into the chamber under natural sunlight to initiate the photochemical oxidation. Isoprene mixing ratios were measured by a Varian CP-3800 Gas Chromatograph equipped with a flame ionization detector.  $\text{O}_3$  formation (from the chamber reactions) was measured with an ML9811 series  $\text{O}_3$  Photometer (American Ecotech, Warren, RI). NO disappearance and  $\text{NO}_x$  (i.e.,  $\text{NO}_2$  formed from chamber reactions) were measured with an ML9841 series  $\text{NO}_x$  Analyzer (American Ecotech, Warren, RI).  $\text{O}_3$  and  $\text{NO}_x$  meters were calibrated prior to experiments by gas-phase titration using a NIST-standard NO tank and stable  $\text{O}_3$  source. A typical experimental profile of isoprene-derived SOA generation in the outdoor chamber is shown in Figure 2. Experiments were conducted under humid conditions (50%–70% RH). Control experiments with the same concentrations of acidified sulfate seed aerosols and gaseous precursors were conducted in the absence of sunlight. After maximum SOA growth ( $\sim 35 \mu\text{g}/\text{m}^3$ ) was attained, aerosol samples were collected onto Teflon membrane filters (47 mm diameter, 1.0  $\mu\text{m}$  pore size; Pall Life Science) at a flow rate of 16–18 L/min for 1 hour for in vitro resuspension exposures and for analysis of aerosol chemical composition by GC/EI-MS with prior trimethylsilylation and UPLC/ESI-HR-QTOFMS, as described in detail elsewhere (Lin et al. 2012, 2014; Surratt et al. 2010). Mass loadings of SOA collected on the filters were calculated from sampling volumes and average aerosol mass concentrations in the chamber during the sampling period. A density correction of  $1.6 \text{ g}/\text{cm}^3$  (Riedel et al. 2016) and  $1.25 \text{ g}/\text{cm}^3$  (Kroll et al. 2006) was applied to convert the measured volume concentrations to mass concentrations for the acidified sulfate seed and SOA growth, respectively.

All filters from experiments in Table 1 were stored at  $-20^\circ\text{C}$  in the dark until extraction for chemical analysis and for the DTT and the cellular assays. The particles were extracted by sonication in high-purity methanol (LC/MS CHROMASOLV, Sigma-Aldrich) for the DTT assay. For the cellular assays, filter samples from multiple experiments

were combined, and the combined filter extract was dried under a gentle stream of nitrogen gas. Keratinocyte basal medium (KBM) was then added into the extraction vials to redissolve SOA constituents.

For typical urban-like chamber experiments (high-NO<sub>x</sub> condition,  $n = 3$ ), 1 ppmv MACR was used as the SOA precursor, and 100 ppb NO and 300 ppb NO<sub>2</sub> were injected in the presence of 160- $\mu\text{g}/\text{m}^3$  acidified seed aerosol. MACR is known to be the direct precursor of isoprene-derived SOA formation under high-NO<sub>2</sub>/NO ratio conditions (Surratt et al. 2006, 2010). As a result, these experiments were conducted in order to solely generate high-NO<sub>x</sub> isoprene-derived SOA. These were select experiments that were conducted for the DTT activity (oxidative potential) measurements discussed in the Results section. Similar to the isoprene-derived SOA experiments discussed earlier, filters were collected and stored at  $-20^\circ\text{C}$  in the dark until chemical and DTT analyses were performed and extracted using the same method, as described in detail elsewhere (Lin et al. 2012, 2014; Surratt et al. 2010). These experiments were only conducted for the DTT assay since MAE is directly produced from the oxidation of MACR (as shown in Figure 1). Thus, only MAE-derived SOA was used for assessing for genomic alterations. Table 2 summarizes all of the outdoor chamber experiments conducted in this study. All experiments were conducted in triplicate ( $n = 3$ ).

### Control Outdoor Chamber Experiments

As a dark chamber control, to isolate the effect of isoprene-derived SOA on exposed cells, mixtures of isoprene, NO, and 170  $\mu\text{g}/\text{m}^3$  of acidified sulfate seed aerosol were injected into the chamber in the dark (after sunset). Conducting the chamber experiments in the dark ensured no photochemical oxidation of isoprene. The dark control was replicated on three different nights. Except for the absence of solar radiation (no SOA), all chamber operations and exposure conditions were similarly maintained. These control outdoor chamber experiments are summarized in Table 1.

As an added control to ensure that the device itself and the cell handling had no significant effect on cell cytotoxicity, cells were exposed in the EAVES to a clean chamber and compared to unexposed cells kept in an incubator for the same duration as the exposure. The cytotoxicity results ensured that there is no effect of chamber conditions and device operation on the cells (see Results section).

### SYNTHESIS OF ISOPRENE-DERIVED SOA PRECURSORS: IEPOX, MAE, AND ISOPOOH

*Trans*- $\beta$ -IEPOX, MAE, and ISOPOOH were synthesized according to published synthetic procedures (Lin et al.

2013b; Riva et al. 2016; Zhang et al. 2012). Identity and purity (>99%) were confirmed by <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance spectroscopy, GC/EI-MS analysis with prior trimethylsilylation, or UPLC/ESI-HR-QTOFMS.

### SYNTHESIS OF ISOPRENE-DERIVED SOA CONSTITUENTS

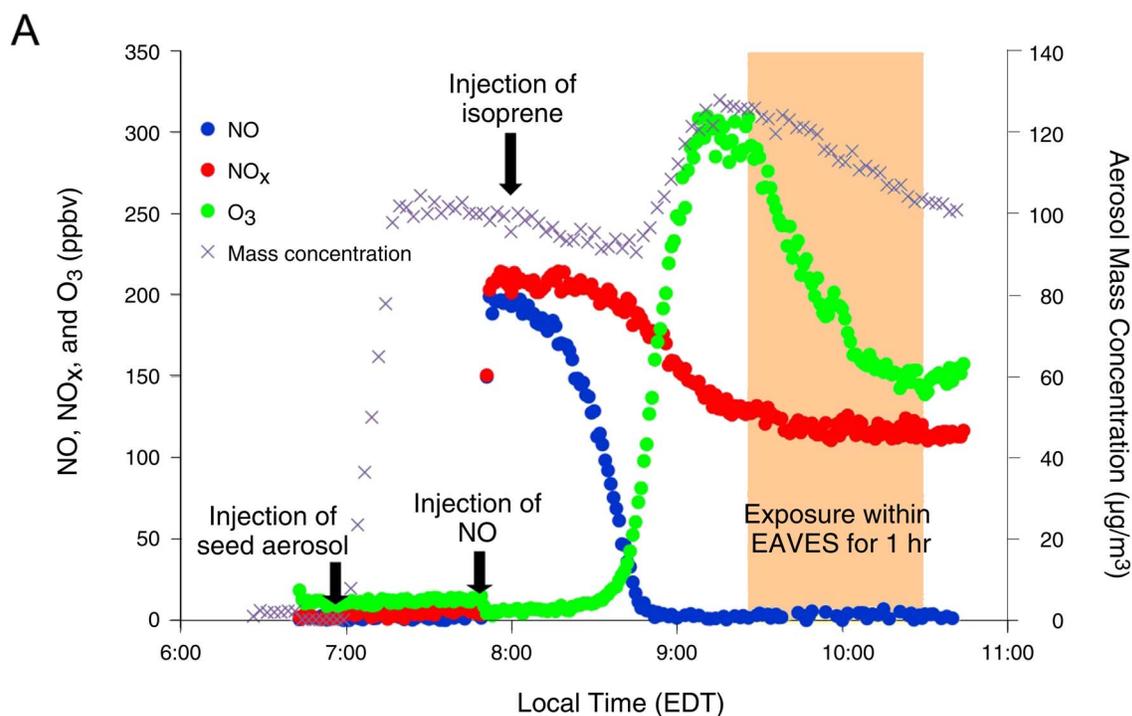
Authentic standards of certain IEPOX-derived SOA tracers (2-methyltetrols, 3-methyltetrahydrofuran-3,4-diols, and IEPOX-derived organosulfates) and MAE-derived SOA tracers (2-methylglyceric acid and MAE-derived organosulfates) were synthesized in house using procedures previously described by our group in order to quantify contributions of IEPOX- and MAE-derived SOA to the photochemically generated isoprene-derived SOA mass used in this study (Budisulistiorini et al. 2015; Zhang et al. 2012). In quantifying the total IEPOX-derived SOA contribution to the total isoprene-derived SOA mass, C<sub>5</sub>-alkene triols and oligomers of IEPOX were quantified by using the 2-methyltetrol standard as a surrogate. In quantifying the ISOPOOH-derived SOA contribution, the ISOPOOH-derived organosulfate (UPLC/ESI-HR-QTOFMS negative ion measured at  $m/z$  231.01801, C<sub>5</sub>H<sub>11</sub>O<sub>8</sub>S<sup>-</sup>) (Riva et al. 2016) was quantified using the IEPOX-derived organosulfate standard as a surrogate.

### GENERATION AND CHEMICAL CHARACTERIZATION OF IEPOX-, MAE-, AND ISOPOOH-DERIVED SOA

These experiments were performed in a 10-m<sup>3</sup> flexible Teflon indoor chamber (i.e., in the dark) at UNC-Chapel Hill in the absence of NO<sub>x</sub>.

### IEPOX- and MAE-Derived SOA

Reactive uptake experiments of IEPOX and MAE involved the injection of either authentic IEPOX or MAE standards into a dark chamber filled with pre-existing acidic sulfate aerosol. Prior work from our group has shown that this directly mimics atmospheric isoprene-derived SOA formation chemistry from these two precursors (Lin et al. 2012, 2013a). Operation of the chamber facility has been described in detail by Lin and colleagues (2012). Prior to each experiment, the chamber was flushed for at least 24 hours to replace at least five volumes of chamber air to ensure particle-free conditions. Particle size distributions were measured continuously using a DMA (BMI model 2002) coupled to an MCPC (BMI model 1710), or a scanning mobility particle sizer consisting of a DMA (TSI model 3081) coupled to a condensation particle counter (TSI model 3776). Acidified sulfate seed aerosol solutions containing 0.06 M MgSO<sub>4</sub> and 0.06 M H<sub>2</sub>SO<sub>4</sub>



**Figure 2. Experimental profile of the photochemical oxidation experiment of isoprene during the course of the experiment. A.** NO, NO<sub>x</sub>, and O<sub>3</sub> measured mixing ratios as well as aerosol mass concentrations; (figure continues next page) **B.** Decay of isoprene; **C.** Shift of aerosol size distribution to greater values following the photochemical oxidation of isoprene, consistent with condensational SOA growth.

were nebulized into the chamber to provide a pre-existing aerosol surface for reactive uptake of epoxides. Seed aerosols were atomized into the chamber until total aerosol mass concentrations of 78–92  $\mu\text{g}/\text{m}^3$  and 61–300  $\mu\text{g}/\text{m}^3$  were attained for experiments with IEPOX and MAE, respectively. Experiments were conducted under dry conditions (<10% RH) to minimize loss of gas-phase epoxides to chamber walls. Temperature and RH inside the chamber were continuously monitored using an OM-62 temperature RH data logger (OMEGA Engineering). A summary of the experimental conditions is given in Table 3.

Multiple experiments were performed to collect ~900  $\mu\text{g}$  SOA for each SOA source. Acidified sulfate seed aerosol-only experiments served as controls. For reactive uptake experiments, 600–1,200 ppbv of gas-phase *trans*- $\beta$ -IEPOX

or MAE was introduced into the indoor chamber by passing high-purity nitrogen gas at 2 L/min through a manifold heated to ~70°C for 2 hours. The concentrations were chosen to ensure formation of sufficient SOA mass for subsequent chemical and toxicological analyses. After 2 hours of reaction to allow maximum SOA growth and stabilization, aerosol samples were collected onto Teflon membrane filters (47 mm diameter, 1.0  $\mu\text{m}$  pore size; Pall Life Science) at a flow rate of 25 L/min for 3 hours. Mass loadings on the filters were calculated from total air volume sampled and average mass concentrations of aerosol during the sampling period. A density of 1.25  $\text{g}/\text{cm}^3$  for IEPOX-derived SOA and 1.35  $\text{g}/\text{cm}^3$  for MAE-derived SOA was applied to convert the measured volume concentrations to mass concentrations after SOA growth (Kroll et al. 2006).

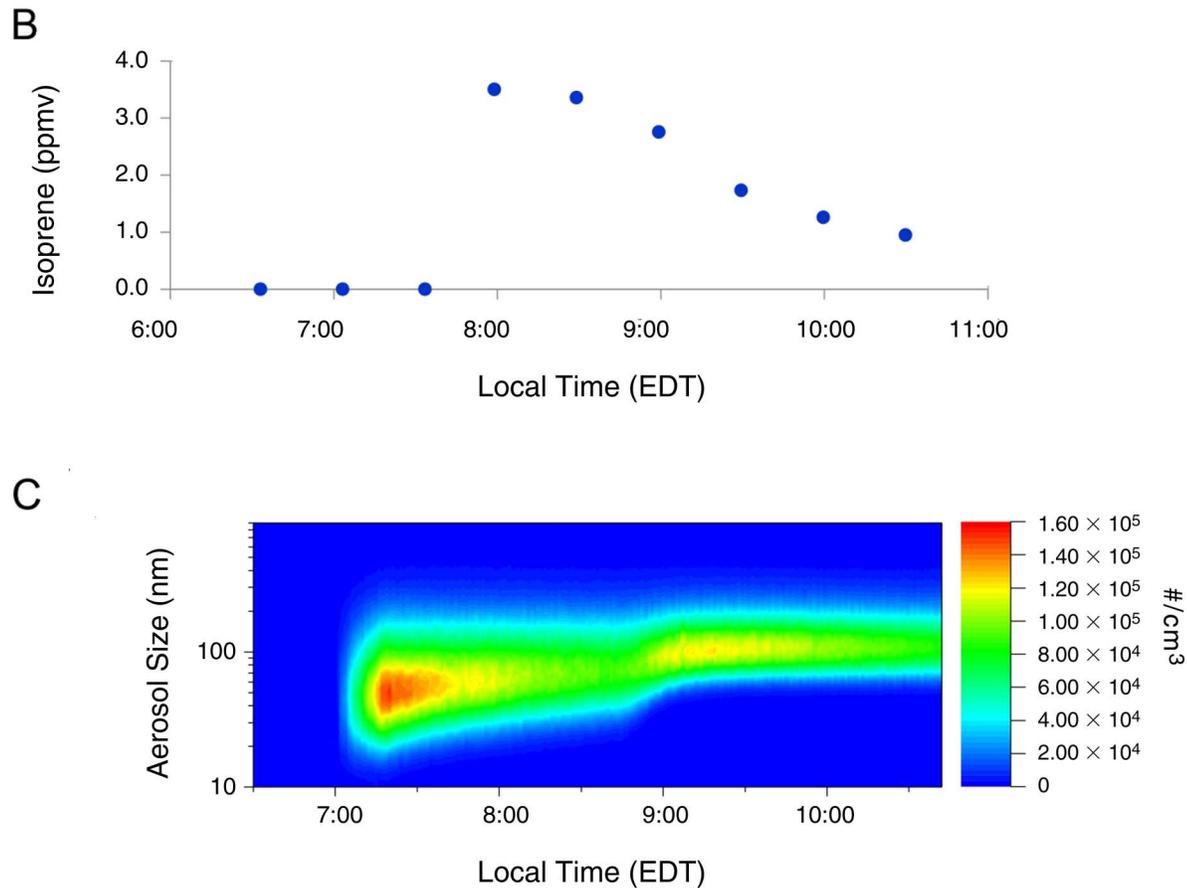


Figure 2. (Continued)

**Table 1.** Summary of Control Chamber Experiments for Cell Exposures within EAVES and by Filter Resuspension<sup>a,b</sup>

| Number | Experiment        | Isoprene | NO | Seed | Sunlight | SOA Formed |
|--------|-------------------|----------|----|------|----------|------------|
| 1      | Clean air control | –        | –  | –    | –        | –          |
| 2      | Seed only control | –        | –  | +    | –        | –          |
| 3      | Dark control      | +        | +  | +    | –        | –          |
|        | Photochemical run | +        | +  | +    | +        | +          |

<sup>a</sup> + and – represent presence or absence, respectively, of given parameters in each experiment.

<sup>b</sup> All experiments were conducted in triplicate ( $n = 3$ ).

**Table 2.** Summary of Outdoor Chamber Experimental Conditions<sup>a</sup>

| Date       | Precursor | Initial Conditions |          |                       |                           | Average During Filter Sampling |           |                       |                      | Collected Aerosol Mass (mg) |
|------------|-----------|--------------------|----------|-----------------------|---------------------------|--------------------------------|-----------|-----------------------|----------------------|-----------------------------|
|            |           | VOC (ppmv)         | NO (ppb) | NO <sub>2</sub> (ppb) | Seed (µg/m <sup>3</sup> ) | RH (%)                         | Temp (°C) | NO <sub>x</sub> (ppb) | O <sub>3</sub> (ppb) |                             |
| 08/22/2014 | Isoprene  | 5                  | 200      | 0                     | 103                       | 42                             | 36        | 180                   | 139                  | 0.827                       |
| 08/27/2014 | Isoprene  | 5                  | 200      | 0                     | 99                        | 48                             | 32        | 114                   | 95                   | 0.960                       |
| 09/30/2014 | Isoprene  | 5                  | 200      | 0                     | 102                       | 64                             | 24        | 152                   | 106                  | 1.000                       |
| 10/06/2014 | MACR      | 1                  | 100      | 300                   | 137                       | 35                             | 24        | 134                   | 345                  | 0.438                       |
| 10/08/2014 | MACR      | 1                  | 100      | 300                   | 143                       | 42                             | 28        | 142                   | 405                  | 0.368                       |
| 10/10/2014 | MACR      | 1                  | 100      | 300                   | 145                       | 50                             | 27        | 114                   | 318                  | 0.407                       |
| 07/09/2014 | Seed only | —                  | —        | —                     | 105                       | —                              | —         | —                     | —                    | 0.844                       |
| 07/15/2014 | Seed only | —                  | —        | —                     | 110                       | —                              | —         | —                     | —                    | 1.295                       |
| 07/18/2014 | Seed only | —                  | —        | —                     | 95                        | —                              | —         | —                     | —                    | 0.930                       |

<sup>a</sup> Seed aerosol was generated from nebulizing acidified sulfate seed aerosol solutions containing 0.06M MgSO<sub>4</sub> and 0.06M H<sub>2</sub>SO<sub>4</sub>. This served as sites for the condensation of isoprene- and MACR-derived oxidation products to produce SOA.

**Table 3.** Summary of Experimental Conditions used to Generate IEPOX- and MAE-Derived SOA<sup>a</sup>

| #  | Experiment      | Epoxide Precursor (ppbv) | Initial Seed (µg/m <sup>3</sup> ) | SOA Growth (µg/m <sup>3</sup> ) | Sampling Volume (m <sup>3</sup> ) | Mass Collected (µg) | Sum (µg) |
|----|-----------------|--------------------------|-----------------------------------|---------------------------------|-----------------------------------|---------------------|----------|
| 1  | Reactive uptake | IEPOX 600                | 92                                | 114.0                           | 3.06                              | 348.8               | 937.5    |
| 2  | Reactive uptake | IEPOX 600                | 84                                | 45.3                            | 3.06                              | 138.5               |          |
| 3  | Reactive uptake | IEPOX 600                | 78                                | 147.1                           | 3.06                              | 450.2               |          |
| 4  | Reactive uptake | MAE 600                  | 61                                | 21.6                            | 3.06                              | 66.1                | 854.6    |
| 5  | Reactive uptake | MAE 600                  | 62                                | 36.5                            | 3.06                              | 111.5               |          |
| 6  | Reactive uptake | MAE 600                  | 65                                | 32.4                            | 3.06                              | 99.1                |          |
| 7  | Reactive uptake | MAE 900                  | 227                               | 45.0                            | 3.91                              | 175.6               |          |
| 8  | Reactive uptake | MAE 900                  | 138                               | 24.9                            | 3.91                              | 97.1                |          |
| 9  | Reactive uptake | MAE 900                  | 236                               | 29.0                            | 3.90                              | 113.1               |          |
| 10 | Reactive uptake | MAE 1,200                | 300                               | 47.5                            | 4.04                              | 191.9               |          |
| 11 | Seed only       | —                        | 77                                | 42 <sup>b</sup>                 | 3.06                              | 182.1               | 903.5    |
| 12 | Seed only       | —                        | 328                               | 143 <sup>b</sup>                | 3.06                              | 721.4               |          |

<sup>a</sup> SOA Growth corresponds to the amount of SOA formed by the acid-catalyzed reactive uptake of IEPOX or MAE on the initial acidic sulfate seed aerosol injected into the chamber. Initial acidic sulfate seed aerosol was generated from nebulizing acidified sulfate seed aerosol solutions containing 0.06 M MgSO<sub>4</sub> and 0.06 M H<sub>2</sub>SO<sub>4</sub>. All experiments were conducted at 23°–25°C and <10% relative humidity.

<sup>b</sup> Final seed concentration.

After aerosol sample collection, filter samples were stored in 20 mL scintillation vials at  $-20^{\circ}\text{C}$  until analysis. The Teflon filter membranes were extracted by sonication in high-purity methanol (LC/MS CHROMASOLV, Sigma-Aldrich) for the DTT assay in the same manner as for chemical analysis. Multiple filter samples were combined to achieve the desired dose levels, and the combined filter extracts were dried under a gentle stream of nitrogen for the cellular assays. Growth factor-deprived KBM 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 for IEPOX- and MAE-derived SOA were extracted and reconstituted in the same manner.

Filter samples were chemically characterized by GC/EI-MS and UPLC/ESI-HR-QTOFMS. The removal efficiency of isoprene epoxide-derived SOA constituents from filters was estimated above 90%. Detailed sample preparation, column conditions, operating parameters for GC/EI-MS and UPLC/ESI-HR-QTOFMS have been published elsewhere (Zhang et al. 2011).

#### ISOPOOH-Derived SOA

ISOPOOH-derived SOA were also generated in a  $10\text{-m}^3$  flexible Teflon indoor chamber at UNC–Chapel Hill, as described by Riva and colleagues (2016). Experiments were performed at room temperature ( $25^{\circ}\text{C}$ ) under dark and low RH ( $<5\%$ ) conditions. Prior to each experiment, the chamber was flushed for at least 24 hours to replace at least five volumes of chamber air to ensure particle-free conditions, and  $\text{O}_3$  and VOC concentrations were below detection limits. Aerosol size distributions were measured continuously using a DMA (BMI model 2002) coupled to a MCPC (BMI model 1710).  $\text{O}_3$  concentration was monitored

over the course of the experiment using an ultraviolet photometric analyzer (Model 49P, Thermo-Environmental). Temperature and RH inside the chamber were continuously monitored using an OM-62 temperature RH data logger (OMEGA Engineering).

A nonacidified ammonium sulfate  $((\text{NH}_4)_2\text{SO}_4)$  seed aerosol solution containing 0.06 M  $(\text{NH}_4)_2\text{SO}_4$  was atomized into the chamber until the total aerosol mass concentration in the chamber was  $\sim 80\ \mu\text{g}/\text{m}^3$ . Because 90% of ISOPOOH +  $\bullet\text{OH}$  yields IEPOX, nonacidified  $(\text{NH}_4)_2\text{SO}_4$  seed was used to prevent the reactive uptake of IEPOX and allow the 10% of hydroperoxides to condense onto pre-existing aerosol (Berndt et al. 2016). After the aerosol injection, 300 ppb of 1,2-ISOPOOH, synthesized in house as described by Riva and colleagues (2016), was injected into the chamber by passing high-purity nitrogen gas through a manifold heated to  $\sim 70^{\circ}\text{C}$  at 2 L/min for 10 minutes then at 5 L/min for 80 minutes.  $\text{O}_3$  was introduced into the chamber using an  $\text{O}_3$  generator (model L21, Pacific Ozone) followed by a continuous injection of TME. The ozonolysis of TME formed the  $\bullet\text{OH}$  radicals needed for the oxidation of ISOPOOH. A summary of the experimental conditions is given in Table 4. Nonacidified  $(\text{NH}_4)_2\text{SO}_4$  seed-aerosol-only experiments served as controls. After the 1.5-hour injection of TME, aerosol samples were collected onto Teflon membrane filters (47 mm diameter, 1.0  $\mu\text{m}$  pore size; Pall Life Science). Mass loadings on the filters were calculated from total air volume sampled and average mass concentrations of aerosol during the sampling period. A density correction of  $1.6\ \text{g}/\text{cm}^3$  (Riedel et al. 2016) and  $1.25\ \text{g}/\text{cm}^3$  (Kroll et al. 2006) was applied to convert the measured volume concentrations to mass concentrations for the  $(\text{NH}_4)_2\text{SO}_4$  seed and the ISOPOOH-derived SOA.

**Table 4.** Summary of Experimental Conditions used in Generating ISOPOOH-Derived SOA<sup>a</sup>

| Experiment | Precursor Concentration (ppb) | Target $\text{O}_3$ (ppm) | TME <sup>b</sup> | Initial Seed ( $\mu\text{g}/\text{m}^3$ ) | SOA Growth ( $\mu\text{g}/\text{m}^3$ ) | Sampling Volume ( $\text{m}^3$ ) | Mass Collected ( $\mu\text{g}$ ) |
|------------|-------------------------------|---------------------------|------------------|---|---|----------------------------------|----------------------------------|
| ISOPOOH    | 300                           | 1.5                       | yes              | 76.45                                     | 47.76                                   | 2.80                             | 133.71                           |
| ISOPOOH    | 300                           | 1.5                       | yes              | 76.99                                     | 52.44                                   | 2.66                             | 139.76                           |
| ISOPOOH    | 300                           | 1.5                       | yes              | 77.28                                     | 54.34                                   | 3.04                             | 165.47                           |
| Seed only  | —                             | —                         | no               | 587.54                                    | —                                       | 0.45                             | 267                              |

<sup>a</sup> SOA Growth corresponds to the amount of SOA formed by the condensation of ISOPOOH-derived oxidation products on the initial nonacidified sulfate seed aerosol injected into the chamber. Initial nonacidified sulfate seed aerosol was generated from nebulizing acidified sulfate seed aerosol solutions containing 0.06 M  $(\text{NH}_4)_2\text{SO}_4$ . This seed aerosol was chosen to prevent any IEPOX from taking up onto these particles.

<sup>b</sup> TME = tetramethylethylene.

The Teflon filter membranes were extracted by sonication in high-purity methanol (LC/MS CHROMASOLV) for the DTT assay, in the same manner as for the other SOAs. For the cellular assays multiple filter samples were combined to achieve the desired dose levels, and the combined filter extracts were dried under a gentle stream of nitrogen. Growth factor-deprived KBM was then added to the extraction vials to redissolve ISOPOOH-derived SOA constituents for cell exposure. Control filters collected from nonacidified sulfate aerosol-only experiments were extracted and reconstituted in the same manner.

ISOPOOH-derived SOA formed during indoor oxidation experiments have been previously characterized by Riva and colleagues (2016) using GC/EI-MS, UPLC/ESI-HR-QTOFMS, and total aerosol peroxide analysis. The composition of filter samples collected in this study was validated by UPLC/ESI-HR-QTOFMS operated in the positive ion mode and were exactly the same as recently published work from our lab (Riva et al. 2016).

### ANALYSIS OF SOA COMPOSITION

Filter samples collected from all experiments were chemically characterized by both GC/EI-MS and UPLC/ESI-HR-QTOFMS. Detailed sample preparation, column conditions, operating parameters for GC/EI-MS and UPLC/ESI-HR-QTOFMS have been described elsewhere (Lin et al. 2012, 2016; Riva et al. 2016; Zhang et al. 2011).

### DTT ASSAY PROCEDURE FOR DETERMINING OXIDATIVE POTENTIAL

The prevailing view of the mode of action specific to  $PM_{2.5}$  is interaction with the airway epithelial cells and macrophages to generate ROS (Cho et al. 2005), triggering a cascade of events associated with inflammation and apoptosis. A commonly used method to quantify redox activity of a  $PM_{2.5}$  sample and its potential to generate ROS is the DTT assay (Li et al. 2009; Rattanavaraha et al. 2011; Verma et al. 2012, 2015). This assay measures the rate of DTT consumption, which is proportional to the concentration of the catalytically active redox active species in the sample (Rattanavaraha et al. 2011). Therefore, the DTT assay measures the potential of a  $PM_{2.5}$  sample to evoke oxidative stress. The DTT assay was utilized for measuring thiol reactivity (or oxidative potential) because it has been used extensively in past studies (e.g., Jiang et al. 2017; Li et al. 2009; Rattanavaraha et al. 2011; Verma et al. 2012, 2015); this allows comparison across studies. One major disadvantage with the DTT assay is that it does not directly measure production of ROS, but instead measures the thiol

reactivity. Thus, equating ROS production with thiol oxidation (which the DTT assay measures) has to be treated with care, especially since this may not directly mimic cellular responses. As a result, when using the DTT assay we reported the oxidative potential of the varying types of isoprene-derived SOA tested in this study. It is noted that this terminology (i.e., oxidative potential) has been extensively used in past studies (Li et al. 2009; Rattanavaraha et al. 2011; Verma et al. 2012, 2015) and thus is also utilized in the present study.

An aqueous buffer solution containing 0.05 mol/L potassium phosphate monobasic sodium hydroxide ( $KH_2PO_4$ , pH 7.4, Fisher Scientific) and 1 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) was prepared. To 10 mL of the buffer, 7.712 mg of the DTT standard (powder form) (Sigma-Aldrich) was added to prepare a fresh 5 mM DTT stock solution daily. A 0.5 mM working solution of DTT was prepared by mixing 100  $\mu$ L DTT stock with 900  $\mu$ L of buffer. A 1,4-NQ stock solution (1  $\mu$ g/ $\mu$ L), used as an external standard, was prepared by dissolving 0.5 mg of 1,4-NQ in 0.5 mL dimethyl sulfoxide (DMSO), and then mixing it with 990  $\mu$ L of buffer. A dithiobis-2-nitrobenzoic acid (DTNB) stock solution (10 mM) was prepared by dissolving 19.817 mg DTNB in 5 mL buffer. The 1 mM DTNB used for the assay was prepared by mixing 300  $\mu$ L DTNB with 2,700  $\mu$ L buffer.

Then 1,000  $\mu$ L of the aqueous buffer was added to an 8 mL clear borosilicate glass vial, followed by the addition of 50  $\mu$ L of 0.5 mM DTT (i.e., 25 nmol). Then varying types of isoprene-derived SOA extraction solutions (200  $\mu$ L), SOA constituent standard solutions (5–100  $\mu$ L) (i.e., IEPOX, 2-MT, 2MT-OS, MAE, 2-MG, 2-MG\_OS, and ISOPOOH), or the 1,4-NQ external standards (10–50  $\mu$ L) were added to the vial. Quality control tests revealed that the order of reagent addition did not affect assay results (Appendix Figure A.1, available on the HEI website). The reaction mixtures were incubated at 37°C for 30 minutes, and then the reactions were quenched by adding 100  $\mu$ L 1 mM DTNB (100 nmol) to titrate the remaining DTT in the reaction mixture. The absorbance of 5-thio-2-nitrobenzoic acid (TNB) formed by the oxidation of residual DTT with DTNB was measured at 412 nm using an ultraviolet–visible spectrophotometer (Hitachi U-3300 dual beam spectrophotometer) (Li et al. 2009; Rattanavaraha et al. 2011). The absorbance measurements were corrected for sample volume to account for dilution effects. The presence of methanol (200  $\mu$ L) in blank filter extraction solutions assayed in the same manner as chamber filter samples did not affect DTT consumption.

The calibration curves for the measured TNB absorbance versus nmol of DTT consumed in the reaction mixture, and

for various amounts of 1,4-NQ reacting with 25 nmol DTT, are shown in Appendix Figures A.2a and A.2b, respectively. DTT consumption with real PM samples could be back-calculated using the calibration curve based on titrating various amounts of DTT standards (0–25 nmol) with excess DTNB (100 nmol) to represent remaining DTT in the reactions. The effects of DTT consumption due to dissolved O<sub>2</sub> would be canceled out, because after the 30-minute reaction time, DTT was immediately quenched with DTNB in all experiments, and the calibration curve was prepared in the same manner. We have also tested blank filter extraction samples ( $n = 3$ ) and found that the average absorbance measurement of these blank samples was very close to the intercept of the DTT calibration curve using this approach. To examine whether DMSO could consume DTT, we spiked reagent blanks in aqueous DTT assay solutions with various amounts of DMSO, following the same procedure as for the 1,4-NQ standard. Consumption of DTT was negligible up to 30  $\mu$ L of DMSO. At the maximum volume of 50  $\mu$ L DMSO added in this investigation, absorbance at 412 nm decreased slightly (2%) (Appendix Figure A.3). Therefore, no more than 50  $\mu$ L 1,4-NQ working solution was added. H<sub>2</sub>SO<sub>4</sub> and MgSO<sub>4</sub> seed aerosols had no significant effects on the DTT assay (Appendix Figure A.4).

The measured ROS-generation potentials were expressed as DTT activity (nmol of DTT consumed/min/ $\mu$ g sample) and as the normalized index of oxidant generation (NIOG) for comparison with previously published studies. An index of oxidant generation (IOG) was calculated according to equation 1, where  $T$  is reaction time (min),  $M$  is sample mass ( $\mu$ g), and  $Abs_0$  and  $Abs'$  are initial absorbance and absorbance at time  $T$ , respectively. The NIOG calculation normalizes activity with respect to a 1,4-NQ standard (Li et al. 2009; Rattanavaraha et al. 2011).

$$IOG = \frac{Abs_0 - Abs'}{Abs_0} \times \frac{100}{T \times M} \quad (1)$$

$$NIOG_{\text{sample}} = \frac{IOG_{\text{sample}}}{IOG_{1,4-NQ}} \quad (2)$$

#### CELL CULTURE FOR DIRECT AIR-LIQUID INTERFACE EXPOSURE TO ISOPRENE-DERIVED SOA

BEAS-2B cells were cultured in keratinocyte growth medium (KGM, Lonza), containing serum-free KBM supplemented with 0.004% bovine pituitary extract, 0.001% of human epidermal growth factor, insulin, hydrocortisone,

and GA-1000 (gentamicin, amphotericin B) and passaged weekly. Passage number for the photochemical exposures and the dark control exposures varied between 52 and 60. Cells were grown at 37°C under an atmosphere containing 5% CO<sub>2</sub> in a humidified incubator. Because BEAS-2B is an immortalized line of human bronchial epithelium, there are limitations with its use such as it being genetically homogeneous, being a single cell type, and being SV-40-transformed (Reddel et al. 1988). However, BEAS-2B is a stable, proliferative cell line shown to be useful in airway inflammation studies such as ours (Devlin et al. 1994).

Prior to experiments, cells were subcultured on collagen-coated Millicell (Human VitroCol, Advanced BioMatrix) cell culture inserts (12 mm diameter, hydrophilic polytetrafluoroethylene, 0.4  $\mu$ m pore size, 0.69 cm<sup>2</sup> filter area, EMD Millipore) at a density of  $5 \times 10^4$  cells per insert for 24 hours. Cell culture inserts were housed in 12-well plates with growth medium (KGM) supplied. At the time of exposure when cells reached 60%–80% confluence, which was confirmed through microscopy, KGM was removed from the apical and basolateral cell surfaces. Cells were washed twice with phosphate buffered saline (PBS; Sigma) and placed in fresh media deprived of growth factor (KBM).

Air-liquid interface exposures were conducted using EAVES (de Bruijne et al. 2009) for direct deposition of isoprene-derived SOA on cultured BEAS-2B cells. The characteristics and applications of the EAVES device have been described in detail (de Bruijne et al. 2009; Lichtveld et al. 2012). Briefly, isoprene-derived SOA entrained in an air stream was charged by corona discharge and directly deposited onto the cells at the air-liquid interface by an electric field applied above the cells. The EAVES device was housed in an incubator maintained at 37°C, and air from the chamber passed over the cells at a flow rate of 1 L/min. At the time of exposure, the cells grown on Millicell cell culture inserts ( $n = 4$ ) were transferred to a sterile titanium dish containing 1.5 mL of fresh KBM. Cell culture inserts were placed above the medium to maintain cell viability and allow exposures to be performed at an air-liquid interface. Cells were exposed to chamber aerosol using the EAVES device for 1 hour to ensure that the cells exposed at the air-liquid interface were not stressed due to drying while maximizing deposited dose, which is consistent with prior UNC-Chapel Hill studies (de Bruijne et al. 2009; Lichtveld et al. 2012). A set of unexposed controls was maintained in the incubator. After exposure in the EAVES, cell culture inserts were transferred to new 12-well plates containing 1 mL fresh KBM per well. The extracellular medium was collected after incubation for 9 hours, based on prior time-course experiments monitoring

induction of oxidative stress and expression of inflammation-associated genes in BEAS-2B cells in response to isoprene-derived SOA exposure through filter resuspension (Arashiro et al. 2016). These time-course experiments are shown in Appendix Figure E.1. Total ribonucleic acid (RNA) was harvested using 200  $\mu$ L of Trizol (Life Technologies). Extracellular medium and the isolated RNA samples were stored at  $-20^{\circ}\text{C}$  and  $-80^{\circ}\text{C}$ , respectively, until further analysis. It is noted that the dose of isoprene-derived SOA was determined following size-dependence guidelines outlined in de Bruijne and colleagues (2009).

### CELL CULTURE FOR RESUSPENSION EXPOSURE TO ISOPRENE- AND MACR-DERIVED SOA

BEAS-2B cells were cultured as described for the direct exposure. Two days prior to exposure, cells were seeded in 24-well plates at a density of  $2.5 \times 10^4$  cells/well in 250  $\mu$ L of KGM. At the time of exposure when cells reached  $\sim 80\%$  confluence, cells were washed twice with PBS buffer, and then exposed to KBM containing 0.01 and 0.1 mg/mL isoprene-derived SOA extract from photochemical experiment and seed particles from dark control experiments. Outdoor chamber particles were collected from the chamber experiments generating isoprene- and MACR-derived SOA (Table 2), concurrently with EAVES sampling, onto Teflon membrane filters (47 mm diameter, 1.0  $\mu$ m pore size; Pall Life Science) for photochemical (light) and dark chamber experiments to be used for chemical analysis and resuspension exposures. The resuspension experiments served as a control for possible effects of gaseous components such as  $\text{O}_3$ ,  $\text{NO}_x$ , and organic oxidation products present in the direct deposition experiments; however, prior studies have shown that gaseous components, such as  $\text{NO}_x$ ,  $\text{O}_3$ , and organic oxidation products from diesel exhaust, do not yield cellular responses within the EAVES device (de Bruijne et al. 2009; Ebersviller et al. 2012a,b; Lichtveld et al. 2012); specifically, these EAVES characterization studies have demonstrated that cellular exposures to gases and particles derived from fresh or photochemically aged diesel exhaust when the EAVES charging and collection fields were turned off — and thus no particle deposition was occurring onto the cells — did not induce significant cell death or inflammatory mediator production, especially compared with incubator and clean air controls. Furthermore, it was also characterized in these studies that no cellular response was observed with the EAVES after exposure to toxic gases without PM present, suggesting that toxic gases are not able to readily partition to the air-liquid interface of the cells to induce an adverse exposure (Lichtveld et al. 2012).

The particles collected on Teflon filter membranes for resuspension cell exposure were extracted by sonication in high-purity methanol (LC/MS CHROMASOLV, Sigma-Aldrich). Filter samples from multiple experiments were combined, and the coiled filter extract was dried under a gentle stream of nitrogen gas. KBM was then added into the extraction vials to redissolve SOA constituents.

After a 9-hour exposure to the SOA filter extract, extracellular medium was collected. Total RNA was isolated using Trizol (Life Technologies) and stored alongside samples from direct deposition exposures at  $-20^{\circ}\text{C}$  and  $-80^{\circ}\text{C}$ , respectively, until further analysis.

### CELL CULTURE FOR RESUSPENSION EXPOSURES TO IEPOX-, MAE-, AND ISOPOOH-DERIVED SOA

Similar to the isoprene-derived SOA generated in the outdoor chamber experiments, BEAS-2B cells used for exposure to IEPOX-, MAE- and ISOPOOH-derived SOA were maintained and cultured in KGM (BulletKit; Lonza), which is serum-free KBM supplemented with 0.004% of bovine pituitary extract, 0.001% of human epidermal growth factor, insulin, hydrocortisone, and GA-1000 (gentamicin, amphotericin B). The cells were grown at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in a humidified incubator and passaged weekly.

In preparation for exposures, cells were seeded in 24-well plates at a density of  $2.5 \times 10^4$  cells/well in 250  $\mu$ L of KGM 2 days prior to exposure. At the time of exposure when cells reached 60%–70% confluence, cells were washed twice with the PBS buffer, and then exposed to KBM containing 1.0, 0.1, or 0.01 mg/mL SOA extract of chamber-generated aerosol samples for 24 hours. Experiments were conducted in triplicate per treatment group. Due to the high DTT activity (see Results section) of ISOPOOH-derived SOA, cells were exposed for 9 hours and 24 hours for single-gene analysis in order to capture potentially rapid changes in gene expression. Extracellular medium was collected, and total RNA was isolated using 350  $\mu$ L Trizol (Life Technologies) at the end of the exposure. Extracellular medium and the extracted RNA samples were stored at  $-20^{\circ}\text{C}$  and  $-80^{\circ}\text{C}$ , respectively, until further analysis.

### CYTOTOXICITY ANALYSIS

The lactate dehydrogenase (LDH) assay was utilized to assess cytotoxicity (cell viability), especially since this has been extensively used in past PM toxicity studies at UNC-Chapel Hill (de Bruijne et al. 2009; Lichtveld et al. 2012; Ebersviller et al. 2012a,b). LDH is a soluble and stable enzyme found inside living cells. When the cell membrane integrity is damaged, LDH is released into the extracellular

space. Therefore, we measured the presence of LDH in the culture medium as a marker of cell death. However, it should be noted that use of LDH as a marker of cellular viability has certain limitations. LDH activity may be affected by oxidants in the supernatant, leading to underestimation of cell death (Buchanan and Armstrong 1976; Anderson and Anderson 1987; Andersson et al. 2000; Kendig and Tarloff 2007; Maier et al. 1991; Panus et al. 1989; Postlethwait et al. 2000). Thus, use of additional viability assays, such as vital dyes (XTT or MTT assays), mitochondrial function, permeability indices, and pre- and post-exposure cell density counts per unit surface area (trypan blue exclusion methods), is recommended to provide a more refined assessment of cellular status for future studies.

The LDH release into the extracellular medium was measured using the LDH cytotoxicity detection kit (Takara) according to the manufacturer's protocol. Briefly, 50  $\mu$ L of each culture medium sample was mixed with 50  $\mu$ L of the assay mixture containing the catalyst and dye solution (iodotetrazolium chloride and sodium lactate) and was incubated at room temperature in the dark for 30 minutes. The reaction was stopped by adding 50  $\mu$ L of HCl (1N) to each well. The absorbance of samples was measured at 492 nm, against a reference wavelength of 620 nm. Low and high controls, which measure the levels of spontaneous LDH release from untreated cells and the maximum LDH activity that can be released from 100% killing of cells (treatment with 1% Triton X-100), were determined to calculate the percentage of induced cytotoxicity by isoprene-derived SOA exposure.

The LDH release from cells exposed to clean chamber air was measured to ensure that the device itself (used in the outdoor chamber experiments and operation procedure) had no effect on cytotoxicity. LDH release by cells exposed via the EAVES to the photochemically aged (light) and nonphotochemically aged (dark) particles was compared to release from unexposed cells maintained in the incubator for the same duration. For the filter resuspension exposures to isoprene-derived SOA, LDH release by cells exposed to SOA through resuspended extract of photochemically aged and nonphotochemically aged particles for 9 hours was compared to release by cells maintained in KBM only. Additionally, LDH release from the light exposures, dark control, and resuspension exposures was compared to release by positive control cells exposed to 1% Triton X-100 to ensure that cell death would not affect gene expression results.

Cytotoxicity was determined for the IEPOX-, MAE- and ISOPOOH-derived SOA exactly the same way as the total isoprene-derived SOA photochemically generated from the outdoor chamber experiments discussed earlier.

However, a few differences are noted here. After a 24-hour exposure, the supernatants were collected to assess LDH levels. Cells exposed to filter extracts from acidified sulfate aerosol-only or nonacidified sulfate aerosol-only experiments and cells maintained in KBM alone were treated as control groups.

## GENE EXPRESSION PROFILING

### Photochemically Generated Isoprene-Derived SOA — Expression of Inflammation-Related mRNA

We initially chose to measure the levels of the inflammation-related messenger RNA (mRNA) in the BEAS-2B cells exposed in the EAVES device to isoprene-derived SOA generated in our outdoor chamber because various particle types are capable of sequestering cytokines (Seagrave 2008). We also chose to examine the gene expression levels of *IL-8* and *PTGS2*, not only for their links to inflammation and oxidative stress (Kunkel et al. 1991; Uchida 2008), but because both have been examined in previous studies using the EAVES for fresh and aged diesel exhaust (Lichtveld et al. 2012). It is noted that *PTGS2*, also known as cyclooxygenase-2 or *COX-2* (i.e., *PTGS2* = *COX-2*), is an enzyme in humans that is encoded by the *PTGS2* genes. However, in the remainder of the text we refer to it as *PTGS2*. It is noted here that the same cell culture medium used for the LDH analyses was used for the gene expressions measurements of *PTGS2* and *IL-8*.

Other direct deposition studies have also used mRNA transcripts as a proxy for cytokine production (Hawley et al. 2014a,b; Hawley and Volckens 2013; Lichtveld et al. 2012; Volckens et al. 2009). Changes in *IL-8* and *PTGS2* mRNA levels were measured using QuantiTect SYBR Green RT-PCR Kit (Qiagen) and QuantiTect Primer Assays for Hs\_ACTB\_1\_SG (Catalog #QT00095431), Hs\_PTGS2\_1\_SG (Catalog #QT00040586), and Hs\_CXCL8\_1\_SG (Catalog #QT00000322) for one-step reverse transcription polymerase chain reaction (RT-PCR) analysis. All mRNA levels were normalized against  $\beta$ -actin (*ACTB*) mRNA, which was used as a housekeeping gene. The relative expression levels (i.e., fold change) of *IL-8* and *PTGS2* were calculated using the comparative cycle threshold ( $2^{-\Delta\Delta CT}$ ) method (Livak and Schmittgen 2001). For EAVES exposures, changes in *IL-8* and *PTGS2* from isoprene-derived SOA exposed cells were compared to cells exposed to the dark controls. Similarly, for resuspension exposures changes in *IL-8* and *PTGS2* from isoprene-derived SOA exposed cells were compared to cells exposed to particles collected under dark conditions. Based on the upregulation of *IL-8* and *PTGS2* we observed from these experiments (see Results section), we expanded

our analyses to a much larger set of oxidative stress- and inflammation-related genes. Isolated total RNA was further purified using Direct-zol RNA MiniPrep (Zymo Research) and then stored in nuclease-free water. The quality and quantity of RNA were assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies) and the NanoDrop 2000c spectrophotometer (Thermo Scientific). The 260 nm/280 nm absorbance ratios of all samples were determined to be >1.8. Gene expression analysis was performed according to the manufacturer's instructions using 50–100 ng of total purified RNA with two pathway-focused panels:

1. nCounter GX Human Inflammation Kit comprised of 249 human genes (NanoString), and
2. Human Oxidative Stress Plus RT<sup>2</sup> Profiler PCR Array (Qiagen, 96-well format, catalog no. PAHS-065Y) comprised of 84 oxidative stress-associated genes. The *PTGS2* gene is included in this gene array.

### IEPOX-, MAE- and ISOPOOH-Derived SOA Generated from Reactive Uptake of Authentic Standards

Oxidative stress-associated gene-expression analysis was conducted for RNA obtained from resuspension exposures to IEPOX-, MAE- and ISOPOOH-derived SOA generated in the dark indoor chamber facility. Cells were lysed with 350  $\mu$ L of Trizol Reagent (Life Technologies) at the end of exposure for total RNA isolation. Isolated RNA samples were further purified using the spin column-based Direct-zol RNA MiniPrep (Zymo Research). RNA quality and concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The absorbance ratios 260/280 nm of all samples were determined to be >1.8. An aliquot of RNA (100 ng) was copied into cDNA using a RT<sup>2</sup> First Strand Kit (Qiagen). The pathway-focused Human Oxidative Stress Plus RT<sup>2</sup> Profiler PCR Array (Qiagen, 96-well format, catalog #: PAHS-065Y) with 84 oxidative stress-associated genes (summarized in Appendix Table E.1.) was used to assess the exposure-induced differential gene expression with a Stratagene Mx3005P real time qPCR System (Agilent Technologies). Additionally, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assays (QuantiTect SYBR Green RT-PCR Kit, Qiagen) of selected individual genes, including *PTGS2* and *ACTB* (housekeeping gene), were also carried out for quality control. Experiments using the NanoString kit were not conducted.

## STATISTICAL METHODS AND DATA ANALYSIS

### Statistical Significance of DTT Results

One-way analysis of variance (ANOVA) followed by post hoc Fisher Least Significant Difference tests were applied to test the significance of differences between standards and outdoor and indoor chamber filter extracts;  $P < 0.05$  was considered statistically significant.

### Gene Expression Analysis

For isoprene-derived SOA generated in the outdoor chamber, analysis and normalization of the NanoString raw data was conducted using nSolver Analysis Software v2.5 (NanoString Technologies). Raw counts were normalized to a panel of 6 housekeeping genes: clathrin heavy chain (*CLTC*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta-glucuronidase (*GUSB*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), phosphoglycerate kinase 1 (*PGK1*), and tubulin beta class I (*TUBB*). The geometric mean of the six housekeeping genes was used to calculate normalization factors to minimize the noise from individual genes as well as to ensure that the calculations are not weighted toward the highest expression housekeeping targets. See Gene Expression Data Analysis Guidelines (MAN-C0011) from NanoString ([www.nanostring.com/support/data-analysis/nsolver-data-analysis-support](http://www.nanostring.com/support/data-analysis/nsolver-data-analysis-support)). Human Oxidative Stress Plus RT<sup>2</sup> Profiler PCR Array data were analyzed using RT<sup>2</sup> Profiler PCR Array Data Analysis software, version 3.5.

The differential gene expression analyses carried out using the RT<sup>2</sup> Profiler PCR Array Data Analysis software were based on the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen 2001). The selection of housekeeping genes was based on the available genes included in RT<sup>2</sup> Profiler PCR Array Human Oxidative Stress Pathway Plus (PAHS-065Y) and in nCounter GX Human Inflammation Kit, as shown in Appendix Tables E.1 and E.2, respectively. *ACTB* was used for quantitative real-time PCR-based analysis of gene expression throughout this study. As for the NanoString nCounter analysis platform for gene expression profiling that uses digital barcode technology for direct multiplexed measurement of analytes, *ACTB* was not available in the nCounter GX Human Inflammation Kit. Therefore, the geometric average of housekeeping gene expression level was used from a panel of 6 genes included in the kit. Their expression did not vary significantly. Calculations were performed using nSolver 2.5 ([www.nanostring.com/application/files/7715/1251/5220/Gene\\_Expression\\_Data\\_Analysis\\_Guidelines.pdf](http://www.nanostring.com/application/files/7715/1251/5220/Gene_Expression_Data_Analysis_Guidelines.pdf)). Fold changes of gene expression levels for exposure and control groups were calculated using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen 2001). Results with

fold-change cutoffs  $\geq 1.5$  and  $P < 0.05$  were considered significant (Fry et al. 2007). The false discovery rate (FDR)-adjusted  $P$  value was calculated based on the Benjamini-Hochberg method (Benjamini and Hochberg 1995).

For IEPOX-, MAE- and ISOPOOH-derived SOA generated in the dark indoor chamber, relative levels of gene expression for exposure and control groups, given as fold changes, were also calculated using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen 2001). Transcriptional changes in cells exposed to SOA constituents were compared to changes in cells exposed to the extracts from acidic sulfate aerosol controls for IEPOX- and MAE-derived SOA and from nonacidic sulfate aerosol controls for ISOPOOH-derived SOA to assess the effects induced solely by the extracted SOA constituents. Differentially expressed genes were identified using the Qiagen RT<sup>2</sup> Profiler Data Analysis Software v3.5, with significance defined as  $P < 0.05$ . The  $P$  value adjusted for FDR was estimated to be 0.0005 ( $\alpha/n$ ;  $\alpha = 0.05$ , and  $n = 84$  genes).

#### Pathway-Based Analysis Performed on Significantly Altered Genes to Provide Biological Pathway Information

Pathway-based analysis for isoprene-derived SOA generated in the outdoor chamber was performed for significantly altered genes using the ConsensusPathDB database (Kamburov et al. 2009, 2011, 2013), which is a database system for the integration of human gene functional interactions to provide biological pathway information for a gene set of interest. Over-representation analysis was used to interpret the function of altered genes. It is noted that over-representation analysis, also known as gene set enrichment analysis, detects enrichment of genes within biological categories. The significance ( $P$  value) of the observed overlap between the significantly altered gene set and the members of predefined pathways was calculated based on the hypergeometric distribution. Criteria of a minimum overlap of 2 genes between the input gene list and the pathway set at a  $P$  value cutoff of 0.01 were applied (Zhavoronkov et al. 2014). Pathway annotation was performed to provide biological pathway information for each gene set. The gene-gene interaction networks were constructed and visualized using the GeneMANIA Cytoscape app (version 3.4.1) to predict the putative function of altered genes (Shannon et al. 2003; Warde-Farley et al. 2010).

For IEPOX-, MAE- and ISOPOOH-derived SOA generated in the dark indoor chamber, network-based analysis to identify canonical pathways and transcription factors was carried out using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA). Gene networks representing enriched perturbed pathways were identified

through enrichment analysis carried out using the Fisher Exact test as detailed earlier (Rager et al. 2013).

## RESULTS

### CHARACTERIZATION OF EXPOSURE CONDITIONS

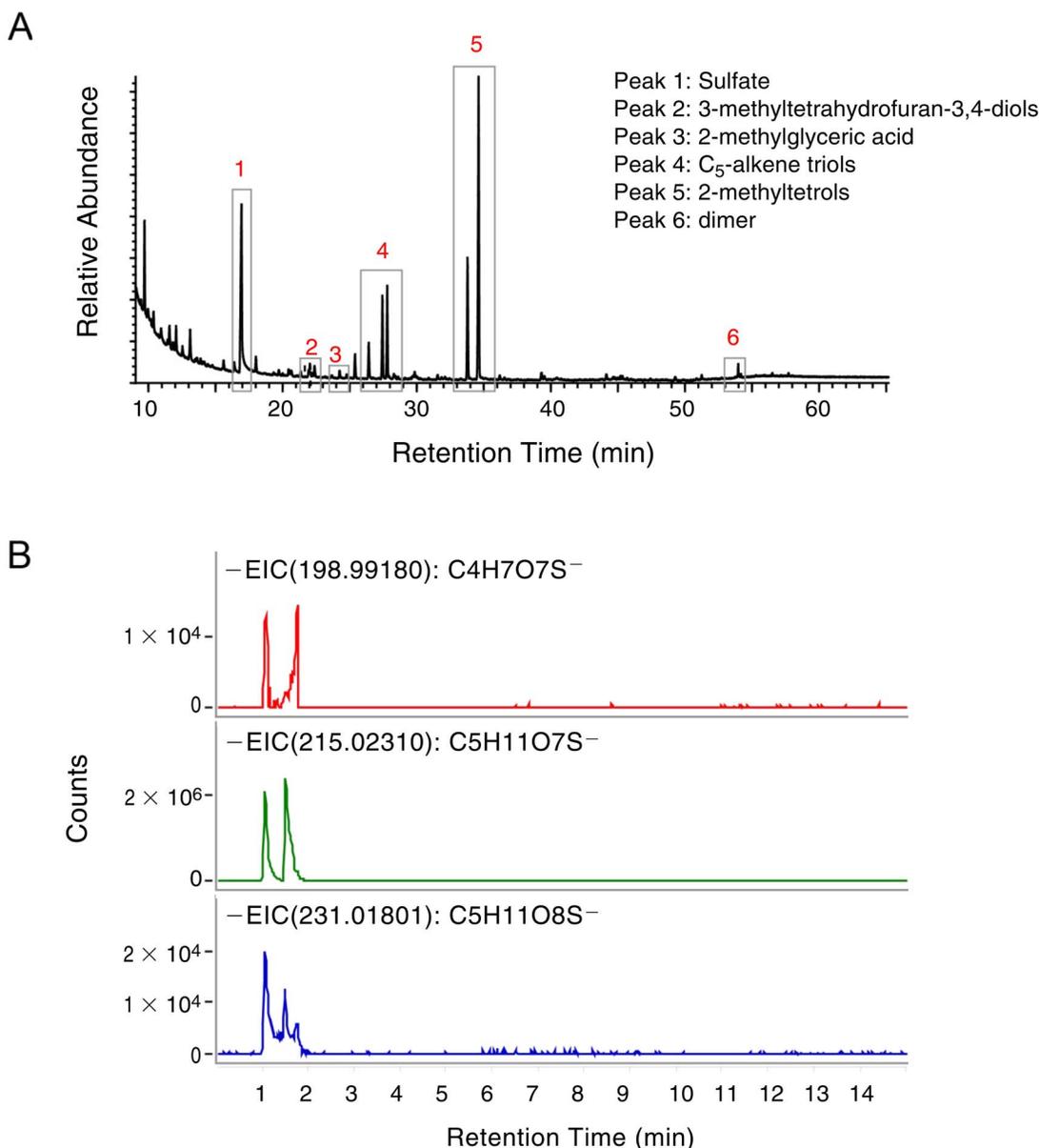
#### Photochemically Generated Isoprene-Derived SOA

We exposed BEAS-2B cells to the mixtures of isoprene-derived SOA generated from the photochemical oxidation of isoprene in the presence of acidified sulfate seed aerosols under natural sunlight, representing the downwind-urban atmospheric scenario in which isoprene is oxidized primarily under low-NO<sub>x</sub> (i.e., low-NO) conditions and subsequent SOA formation is influenced by mixing with anthropogenic emissions. Typical experimental profiles of real-time aerosol size distribution, levels of gaseous precursors (e.g., isoprene) and reaction products (NO<sub>x</sub>, and O<sub>3</sub>) are shown in Figure 2. On initiation of the photochemical experiment, acidified sulfate seed aerosol was atomized into the chamber using a constant output atomizer. Once the seed aerosol reached a target level of  $\sim 100 \mu\text{g}/\text{m}^3$  and had stabilized for 30 min, 200 ppbv of NO and 3.5 ppmv of isoprene were injected into the chamber. Figure 2A shows that isoprene-derived SOA formation commenced as the NO level approached zero through reaction with isoprene-derived RO<sub>2</sub>•, forming NO<sub>2</sub> and O<sub>3</sub> as secondary products. Figure 2B shows the rapid decay of isoprene precursor during the time course of the experiment. This observation is consistent with previous work by Kroll and colleagues (2006) that demonstrated RO<sub>2</sub>• + NO reactions suppressed SOA formation from isoprene due to the production of volatile products that do not yield condensable species. The SOA growth proceeds only when NO levels approach zero, because RO<sub>2</sub>• + HO<sub>2</sub>• reactions then dominate the oxidation chemistry of isoprene. This allows for the production of IEPOX and ISOPOOH, which are known as sources of isoprene-derived SOA under low-NO<sub>x</sub> conditions (Lin et al. 2012; Paulot et al. 2009; Riva et al. 2016; Surratt et al. 2010). It is noted that these experiments required the use of initial NO (200 ppbv) in order to photochemically generate sufficient levels of •OH radical needed to oxidize isoprene to yield SOA. SOA growth resulted in a mass concentration increase of  $\sim 35 \mu\text{g}/\text{m}^3$ . The mode of the aerosol size distribution shifted from  $\sim 70$  nm at the beginning of the experiment to  $\sim 150$  nm after injection of gaseous precursors, indicating growth from condensation of photochemical oxidation products to form SOA (Figure 2C). BEAS-2B cells were exposed to isoprene-derived SOA for 1 hour within the EAVES

device, when the SOA growth reached the maximum. The 1-hour exposure time was optimal for cell viability within the EAVES device based on prior studies (de Bruijne et al. 2009; Lichtveld et al. 2012). The isoprene-derived SOA dose to the cells was estimated to be 58 ng/cm<sup>2</sup> based on the deposition efficiency characterized by de Bruijne and colleagues (2009) using 198 nm fluorescent-labeled PSL particles. This size of PSL particle is close to the mode of

sulfate aerosols coated in isoprene-derived SOA observed in the current study.

Simultaneously with exposure, Teflon filters were collected for chemical analyses of particle-phase SOA constituents (Figure 3). The results in Figure 3 show that isoprene-derived SOA formation under the experimental conditions occurred predominantly via the channel of the reactive uptake of IEPOX onto pre-existing acidic sulfate seed



**Figure 3. Chemical characterization of chamber-generated isoprene SOA.** A. GC/EI-MS total ion chromatogram and B. UPLC/(-)ESI-HR-QTOFMS extracted ion chromatograms at *m/z* 198.99180, 215.02310, and 231.01801 corresponding to the MAE-, IEPOX-, and ISOPOOH-derived organosulfates, respectively.

aerosols, especially since the known IEPOX SOA tracers shown in Figure 1 were observed by GC/EI-MS and UPLC/ESI-HR-QTOFMS. By using authentic standards synthesized in-house, high concentrations of IEPOX-SOA tracers, including 3-methyltetrahydrofuran-3,4-diols, C<sub>5</sub>-alkene triols, 2-methyltetrols, dimers, and the IEPOX-derived organosulfates ( $m/z$  215.0231, C<sub>5</sub>H<sub>11</sub>O<sub>7</sub>S<sup>-</sup>), were measured that contributed to ~80% of the observed SOA mass. MAE-SOA tracers of isoprene oxidation via the high-NO<sub>x</sub> channel, including 2-methylglyceric acid and its organosulfate derivative ( $m/z$  198.9918, C<sub>4</sub>H<sub>7</sub>O<sub>7</sub>S<sup>-</sup>) were also observed and contributed ~1.4% of the total isoprene-derived SOA mass. A smaller contribution (~0.8%) was measured for the ISOPOOH-derived SOA tracer ( $m/z$  231.01801, C<sub>5</sub>H<sub>11</sub>O<sub>8</sub>S<sup>-</sup>) via the low-NO<sub>x</sub>, non-IEPOX route (Riva et al. 2016). These results are consistent with recent kinetics studies and field measurements showing efficient reactive uptake of IEPOX and a large IEPOX contribution to ambient SOA (Budisulistiorini et al. 2016; Lin et al. 2013a; Rattanavaraha et al. 2016; Riedel et al. 2015). Shown in Appendix Figure B.1 (available on the HEI website) are GC/EI-MS and UPLC/ESI-HR-QTOFMS total ion chromatograms from a dark control chamber experiment, an isoprene-derived SOA exposure chamber experiment, and a PM<sub>2.5</sub> sample from Yorkville, GA, during summer 2010. Notably, no isoprene-derived SOA tracers were observed in the filters collected from the dark control experiments. As demonstrated in Appendix Figure B.1, all the same particle-phase products are measured in the PM<sub>2.5</sub> collected in Yorkville, Georgia, demonstrating that the composition of the chamber-generated SOA is atmospherically relevant (Figure 3).

### IEPOX- and MAE-Derived SOA

Time profiles of aerosol mass concentrations measured during the reactive uptake experiments are shown in Appendix Figure B.2. SOA mass yields from reactive uptake of *trans*-β-IEPOX onto acidified sulfate aerosol are substantially larger than yields from reactive uptake of MAE under the same experimental conditions (<10% RH) and time scale (2-hour reaction time). These observations are consistent with recent flow tube studies of uptake kinetics that reported a higher reaction probability ( $\gamma$ ) for *trans*-β-IEPOX than for MAE (Gaston et al. 2014; Riedel et al. 2015), as well as with ambient measurements in the southeastern United States that found the sum of IEPOX-derived SOA tracers (642–1,225 ng/m<sup>3</sup>) to be substantially larger than that of MAE-derived SOA tracers (~20 ng/m<sup>3</sup>) (Lin et al. 2013a).

In Appendix Figure B.3, the GC/EI-MS total ion chromatograms of TMS-derivatized particle-phase reaction

products from reactive uptake of *trans*-β-IEPOX (panel A) and MAE (panel B) in the chamber experiments are compared to that of an ambient PM<sub>2.5</sub> sample (panel C) collected at a rural site in Yorkville, Georgia, downwind of a coal-burning power plant and experiencing high isoprene emissions during summer. The most abundant ion (Peak 1) is the bis(trimethylsilyl) sulfate derivative of extractable inorganic particle sulfate (Lin et al. 2013a). The isoprene-derived SOA tracers in chamber samples are identical to those in the field samples. In Appendix Figure B.4, total ion chromatograms from a 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<sub>5</sub>H<sub>11</sub>O<sub>7</sub>S<sup>-</sup>) (Appendix Figure B.4a) and the sulfate ester of 2-methylglyceric acid ( $m/z$  199; C<sub>4</sub>H<sub>7</sub>O<sub>7</sub>S<sup>-</sup>) (Appendix Figure B.4A) (Lin et al. 2013b; Surratt et al. 2010). Both ions are present in the extract of a typical ambient PM<sub>2.5</sub> sample (Appendix Figure B.4C). Consistent with previous studies (Lin et al. 2013a), the epoxide-derived SOA products represent the major organic aerosol constituents of the ambient PM<sub>2.5</sub> samples collected from the southeastern United States during summertime and support the validity of the chamber experiments as representative of ambient SOA composition.

### ISOPOOH-Derived SOA

Appendix Figure B.5 shows the change in particle mass concentration over time during an ISOPOOH oxidation experiment. There is no SOA growth observed during ISOPOOH injection into the chamber, but as soon as TME is introduced after the O<sub>3</sub> injection, •OH radicals are formed and proceed to oxidize ISOPOOH leading to SOA formation. Unlike the formation of IEPOX- and MAE-derived SOA in our chamber experiments discussed earlier, ISOPOOH-derived SOA formation is via oxidation and not via acid-catalyzed reactive uptake chemistry.

To verify the composition of our ISOPOOH-derived SOA to the ISOPOOH-derived SOA previously characterized (Riva et al. 2016), we used UPLC/ESI-HR-QTOFMS operated in the positive ion mode to detect SOA products due to its soft ionization and the availability of isoprene hydroxyhydroperoxide standards in house. Based on the accurate mass fitting and retention times, our UPLC/(+)ESI-HR-QTOFMS extracted ion chromatograms were exactly the same as in our previous study (Riva et al. 2016), indicating the strong presence of C<sub>5</sub>H<sub>12</sub>O<sub>5</sub> and C<sub>5</sub>H<sub>12</sub>O<sub>6</sub>, which are tentatively assigned to isoprene trihydroxyhydroperoxide (ISOPTHP) and isoprene dihydroxydihydroperoxide (ISOP(OOH)<sub>2</sub>) as shown in Figure 1. Both ISOPTHP and ISOP(OOH)<sub>2</sub> were also found in high abundance in ISOPOOH-derived SOA

formed under nonacidic conditions characterized by Riva and colleagues (2016). More importantly, ISOP<sub>2</sub>HP and ISOP(OOH)<sub>2</sub> have both been observed in a field study conducted in Centerville, Alabama (Lee et al. 2016; Riva et al. 2016), which highlights its atmospheric significance. Strong correlations have been observed between the mass spectra of laboratory-generated ISOPOOH-derived SOA and a positive matrix factorization (PMF) factor referred to as 91Fac from a field study conducted in Look Rock, Tennessee (Budisulistiorini et al. 2016; Riva et al. 2016).

Total aerosol peroxide measurements of ISOPOOH-derived SOA formed under nonacidic conditions show that approximately 40% of the organic mass is attributable to organic peroxides (Riva et al. 2016), which are strong

oxidants and may heavily influence the ROS-generation potential of ISOPOOH-derived SOA.

**DTT ACTIVITY OF TOTAL ISOPRENE- AND MACR-DERIVED SOA, ITS SUBTYPES, AND ITS INDIVIDUAL SOA CONSTITUENTS**

**Isoprene- and MACR-Derived SOA**

Figure 4 presents the NIOGs of the SOA generated from the outdoor and indoor chamber experiments. The DTT activities reported below are summarized in Table 5. The results suggest the oxidizing potential of MACR SOA is greater than that of isoprene-derived SOA, although the difference does not meet the criterion of statistical significance, most likely because of the small sample size. The high

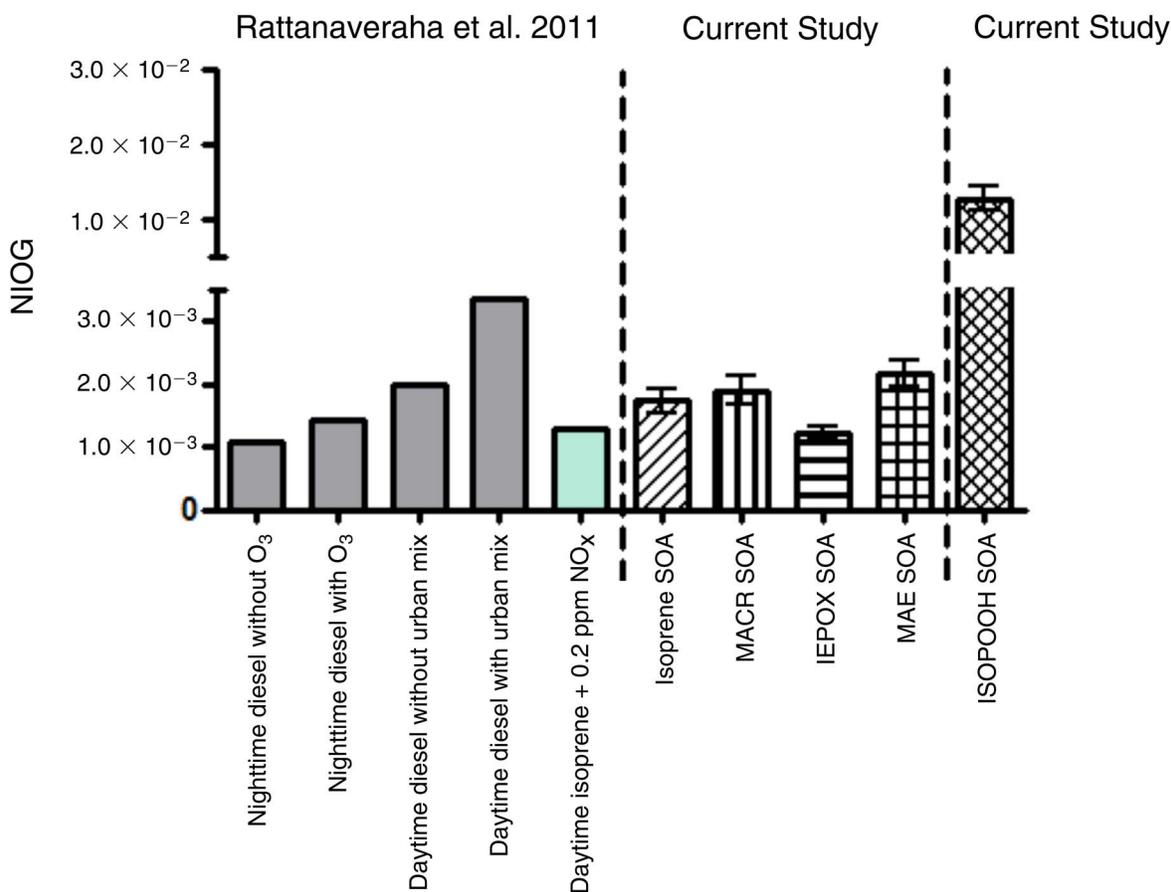


Figure 4. Comparison of NIOGs values derived from the DTT assay of isoprene-derived SOA and its subtypes generated in this study to those of chamber-generated PM samples in Rattanavaraha et al. (2011). The values shown are the mean ± standard error of the mean.

**Table 5.** DTT Activity of PM Constituents Measured in this Study<sup>a</sup>

| Sample                         | DTT Activity<br>(nmol of DTT<br>consumed/min/ $\mu$ g<br>sample) | NIOG<br>Values       |
|--------------------------------|--|----------------------|
| <b>Pure Compounds</b>          |  |                      |
| IEPOX                          | $7.00 \pm 1.39 \times 10^{-5}$                                   | $6.9 \times 10^{-5}$ |
| 2-MT                           | $4.44 \pm 0.92 \times 10^{-5}$                                   | $4.4 \times 10^{-5}$ |
| 2-MT_OS                        | $5.77 \pm 2.07 \times 10^{-5}$                                   | $5.5 \times 10^{-5}$ |
| MAE                            | $9.84 \pm 0.97 \times 10^{-5}$                                   | $9.7 \times 10^{-5}$ |
| 2-MG                           | $2.51 \pm 0.37 \times 10^{-4}$                                   | $2.4 \times 10^{-4}$ |
| 2-MG_OS                        | $7.58 \pm 1.14 \times 10^{-5}$                                   | $7.3 \times 10^{-5}$ |
| ISOPOOH                        | $4.90 \pm 2.20 \times 10^{-1}$                                   | $4.7 \times 10^{-1}$ |
| <b>Outdoor Chamber Samples</b> |  |                      |
| Isoprene SOA                   | $2.10 \pm 0.22 \times 10^{-3}$                                   | $1.7 \times 10^{-3}$ |
| MACR SOA                       | $2.30 \pm 0.27 \times 10^{-3}$                                   | $1.9 \times 10^{-3}$ |
| <b>Indoor Chamber Samples</b>  |  |                      |
| IEPOX-derived SOA              | $1.79 \pm 0.16 \times 10^{-3}$                                   | $1.2 \times 10^{-3}$ |
| MAE-derived SOA                | $3.13 \pm 0.30 \times 10^{-3}$                                   | $2.2 \times 10^{-3}$ |
| ISOPOOH-derived SOA            | $1.26 \pm 0.28 \times 10^{-2}$                                   | $2.4 \times 10^{-2}$ |

<sup>a</sup> Results in this study are expressed as mean  $\pm$  standard error of the mean;  
 $n = 3$  for each sample.

NIOG of 2-MG may contribute to the high NIOG of MACR SOA since 2-MG is a major product in MACR SOA (Chan et al. 2010; Surratt et al. 2006).

The NIOGs of isoprene-derived SOA ( $1.7 \times 10^{-3}$ ) and MACR SOA ( $1.9 \times 10^{-3}$ ) generated in our experiment are greater than the NIOG reported for aged diesel exhaust PM ( $1.6 \times 10^{-3}$ ) generated in an outdoor chamber reported by Li and colleagues (2009), although a note of caution in comparing NIOGs is that Li and colleagues (2009) extracted the outdoor chamber filters with water, while methanol was used in this study. The difference in protocol could alter the selectivity and efficiency of extraction, thus affecting the measurement of oxidative potential; however, any significance has not been determined. Although considerable attention has been focused on the oxidative potential of diesel exhaust PM (Biswas et al. 2009; Cheung et al. 2009; Geller et al. 2006; Li et al. 2009), the results of our DTT assay indicates that further investigation of the toxicological activity of isoprene and MACR SOA is warranted.

#### IEPOX-, MAE-, AND ISOPOOH-derived SOA

The NIOGs of the IEPOX- and MAE-derived SOA from the indoor chamber experiments are shown in Figure 4; the DTT activities are summarized in Table 5. The NIOG and the DTT activity of IEPOX SOA measured in the indoor chamber experiments are lower than the corresponding values of isoprene-derived SOA measured in the outdoor chamber experiments; however, as shown in Appendix Table C.2, our statistical test results using a one-way ANOVA with Fisher test for means comparisons indicate that this difference is not statistically significant if we set the threshold value to 0.05 ( $P$  value is 0.0921 when comparing IEPOX SOA to isoprene-derived SOA), which is likely due to the limitation of aerosol mass and sample size in our test. As discussed in subsequent sections of this report (i.e., Characterization of Altered Gene Expression), isoprene-derived SOA is found to induce significantly higher oxidative stress-associated gene expression in human lung cells compared to IEPOX SOA. This could be a result of redox-active components in the SOA from isoprene photooxidation that are not present in SOA from

pure IEPOX precursor. Organic peroxides, reported by Surratt and colleagues (2006) and Riva and colleagues (2016) to be present in substantial quantities in isoprene-derived SOA, could be a major contributor. We tested this possibility with the recently reported ISOPOOH isomer, 2-hydroperoxy-2-methylbut-3-en-1-ol (Krechmer et al. 2015) (which was also synthesized in our laboratory [Budisulistiorini et al. 2015]); the isomer was confirmed as an organic peroxide surrogate by  $^1\text{H}$  nuclear magnetic resonance spectroscopy and GC/EI-MS (>99%). DTT consumption (NIOG = 0.467, DTT activity = 0.490 nmol of DTT consumed/min/ $\mu\text{g}$  sample) was similar to that observed for 1,4-NQ, supporting a significant role of organic peroxides in PM oxidative potential. Recently, we have been able to generate ISOPOOH-derived SOA directly in our chamber (Riva et al. 2016). As demonstrated in Figure 4, ISOPOOH-derived SOA has a much higher oxidative potential than all other types of isoprene-derived SOA, including the total mixture of isoprene-derived SOA generated in the outdoor chamber. Notably, the NIOG for ISOPOOH-derived SOA was also higher than for aged diesel exhaust containing oxygenated polycyclic aromatic hydrocarbon (PAH) species as reported by Rattanavaraha and colleagues (2011).

Low redox activity of IEPOX-derived SOA (NIOG =  $1.2 \times 10^{-3}$ ) is expected based on the low NIOG of the major component 2-MT (NIOG =  $4.4 \times 10^{-5}$ ). The NIOGs of MAE-derived SOA (NIOG =  $2.2 \times 10^{-3}$ ) and MACR-derived SOA (NIOG =  $1.9 \times 10^{-3}$ ) are larger than the NIOGs of IEPOX and isoprene-derived SOA. This result is consistent with the presence of 2-MG (NIOG =  $2.4 \times 10^{-4}$ ) as a major component in extracts of both SOAs, and it supports more extensive investigation of the biological activity of SOA generated from these compounds *in vitro* and *in vivo*. Similar responses measured for blank filters and sulfate seed aerosol-only samples from the indoor and outdoor chamber experiments demonstrate the consistency of the DTT assay (Appendix Figure A.4).

In a recent study, the DTT assay was used to assess the ROS-generating ability of PM field samples taken from sites across the southeastern United States (Verma et al. 2015). Isoprene-derived SOA was resolved in ambient aerosol analyzed in Verma and colleagues (2015) by applying PMF to mass spectral data acquired using an aerosol mass spectrometer for source apportionment. The DTT activity of the water-soluble component of isoprene-derived SOA ( $8.8 \pm 21 \times 10^{-3}$  nmol/min/ $\mu\text{g}$ ) tested by Verma and colleagues (2015) is on the same order of magnitude as the DTT activity of isoprene-derived SOA ( $2.10 \pm 0.22 \times 10^{-3}$  nmol/min/ $\mu\text{g}$ ) generated in our outdoor chamber experiments. The similarity of these field

and laboratory results supports the atmospheric relevance of the outdoor chamber experiments, particularly in light of the consistency of the chemical composition of the chamber SOA measured in the southeastern United States (Budisulistiorini et al. 2015; Lin et al. 2013a).

### Individual SOA Constituents (2-MT, 2MT\_OS, 2-MG, and 2-MG\_OS)

The NIOGs of the isoprene-derived epoxides and their hydrolysis products are shown in Appendix Figure C.1, available on the HEI website. The 2-MT and organosulfate derivatives of 2-MT (2-MT\_OS) are major constituents of isoprene-derived SOA (Claeys et al. 2004; Edney et al. 2005; Surratt et al. 2006), while 2-MG and the organosulfate derivative of 2-MG (2-MG\_OS) are known constituents of MACR-derived SOA (Chan et al. 2010; Surratt et al. 2006). The NIOG of 2-MG is significantly greater ( $P < 0.001$ ) than the NIOGs of IEPOX, 2-MT, MAE, and 2-MG\_OS (Appendix Figure C.1), while IEPOX, 2-MT, MAE, and 2-MG\_OS are not statistically different from each other. In parallel with the NIOGs, 2-MG has the highest DTT activity relative to the hydrolysis products (Table 5). Notably, the authentic ISOPOOH standard (which also could serve as a surrogate for isoprene-derived peroxides likely in the SOA from ISOPOOH-derived SOA) and ISOPOOH-derived SOA had the highest NIOGs of all isoprene-derived SOA types and standards (Table 5 and Appendix Figure C.1).

### CYTOTOXICITY MEASUREMENTS

A series of control experiments were conducted to ensure that the observed cellular responses are attributable to isoprene-derived SOA (Table 1). Relative to cells maintained in the cell culture incubator, LDH assays indicate no significant cytotoxicity ( $P > 0.05$ ) to the BEAS-2B cells in the EAVES device exposed to clean air, acidified sulfate seed aerosol only, or to dark controls exposed to acidified sulfate seed aerosol and gaseous precursors simultaneously without sunlight. LDH release in the photochemical experiments was slightly enhanced compared to incubator controls (LDH fold increase = 1.19;  $P < 0.05$ ), but the overall cell death was estimated to be only 3%. Our prior filter resuspension work has also demonstrated that the cellular responses to gases — when observed from EAVES — were negligible, especially since both exposure methods yielded similar cellular responses and that Teflon filters utilized in that work only collected SOA particles. This allows us to conclude that the cellular responses in the EAVES are mostly attributable to the particle-phase products (Lichtveld et al. 2012; Arashiro et al. 2016). Additionally, LDH release from all exposure conditions in EAVES-exposed cells (Appendix Figure D.1, available on

the HEI website) and resuspension-exposed cells (Appendix Figure D.1) is negligible relative to positive controls exposed to 1% Triton X-100, confirming that the exposure concentration of isoprene-derived SOA utilized in this study was not cytotoxic and thus was appropriate for investigation of exposure-induced early genomic response.

For IEPOX- and MAE-derived SOA exposures, 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  $\leq 0.1$  mg/mL were not cytotoxic (cell death  $\leq 10\%$ ,  $P > 0.05$ ). Therefore, cells exposed to the dose level of 0.1 mg/mL were selected for gene expression analysis.

For ISOPOOH-derived SOA exposures, cytotoxicity due to 9- and 24-hour exposures is presented in Appendix Figure D.2 as the percentage of cell death, based on the LDH release for cells exposed to ISOPOOH-derived SOA and control nonacidified  $(\text{NH}_4)_2\text{SO}_4$  seed aerosol, compared to positive control cells exposed to 1% Triton X-100. Similar to our other exposures discussed above, low cytotoxicity ( $< 5\%$ ) ensured gene expression results were not affected by cell death.

## CHARACTERIZATION OF ALTERED GENE EXPRESSION

### Photochemically Generated Isoprene-Derived SOA

Proinflammatory gene expression (*IL-8* and *PTGS2*) were first assessed from isoprene-derived SOA exposures to screen for potential gene expression changes. Changes in the mRNA levels of *IL-8* and *PTGS2* from cells exposed to isoprene-derived SOA using EAVES are shown as fold changes relative to dark controls in Figure 5. This comparison, as well as the results of the filter resuspension experiment discussed later, ensures that all effects seen in the cells are solely attributable to the isoprene-derived SOA particles, especially since the filter resuspension experiment yields similar cellular changes, and that prior EAVES characterization studies have demonstrated that toxic gases do not induce changes in the cells since they are not efficiently deposited at the air-liquid interface as compared to the particles (de Bruijne et al. 2009; Lichtveld et al. 2012). A 1-hour exposure to a mass concentration of approximately  $45 \mu\text{g}/\text{m}^3$  of organic material was sufficient to significantly alter gene expression of the inflammatory biomarkers in bronchial epithelial cells. Based on deposition efficiency characterized by de Bruijne and colleagues (2009), the estimated dose was  $0.29 \mu\text{g}/\text{cm}^2$  of total particle mass with 23% attributable to organic material formed from isoprene photooxidation ( $0.067 \mu\text{g}/\text{cm}^2$  of SOA). For

this direct deposition study, we chose to classify dose as SOA mass deposition per surface area of the exposed cells to mimic lung deposition. Gangwal and colleagues (2011) used a multiple-path particle dosimetry model to estimate that the lung deposition of ultrafine particles ranges from  $0.006$  to  $0.02 \mu\text{g}/\text{cm}^2$  for a 24-hour exposure to a particle concentration of  $0.1 \text{ mg}/\text{m}^3$ . Based on this estimate, a dose of  $0.058 \mu\text{g}/\text{cm}^2$  of isoprene-derived SOA in our study can be considered a prolonged exposure over the course of a week. In fact, most other in vitro studies require dosing cells at a high concentration, sometimes close to a lifetime exposure, to obtain a cellular response. Despite this limitation, in vitro exposures serve as a necessary screening tool for toxicity (Paur et al. 2011).

Changes in the mRNA levels of *IL-8* and *PTGS2* from cells exposed to resuspended isoprene-derived SOA collected from photochemical experiments are shown as fold changes relative to cells exposed to resuspended particles from dark control experiments (Figure 6). At a low dose of  $0.01 \text{ mg}/\text{mL}$  of isoprene-derived SOA extract, there is no significant increase in *IL-8* and *PTGS2* mRNA expression. The isoprene-derived SOA extract, however, induces a response at a dose of  $0.1 \text{ mg}/\text{mL}$ . The statistically significant increase in mRNA expression from the resuspension exposure at  $0.1 \text{ mg}/\text{mL}$  confirms that similar fold changes observed for both *IL-8* and *PTGS2* from the EAVES exposures are not attributable to gaseous photooxidation products, such as  $\text{O}_3$ , and supports the characterization of the EAVES as a particle exposure device (de Bruijne et al. 2009).

By choosing *IL-8* and *PTGS2* as our initial genes of interest, we were able to compare our results to other studies of known harmful particle exposures. In a similar study using the EAVES, normal human bronchial epithelial cells exposed to  $1.10 \mu\text{g}/\text{cm}^2$  diesel PM showed less than a 2-fold change over controls in both *IL-8* and *PTGS2* mRNA expression (Hawley et al. 2014b). In another study, A549 human lung epithelial cells were exposed by direct deposition for 1 hour to photochemically aged diesel exhaust particulates at a dose of  $2.65 \mu\text{g}/\text{cm}^2$  from a 1980 Mercedes or a 2006 Volkswagen (Lichtveld et al. 2012). Exposure to aged Mercedes particulates induced a 4-fold change in *IL-8* and ~2-fold change in *PTGS2* mRNA expression, while exposure to aged Volkswagen particulates induced a change of ~1.5-fold in *IL-8* and 2-fold in *PTGS2* mRNA expression (Lichtveld et al. 2012). Although the differences in cell types preclude direct comparisons, it is notable that significant increases in *PTGS2* and *IL-8* expression were found at doses much lower than reported by others for comparable increases in gene expression levels induced by photochemically aged diesel particulates.

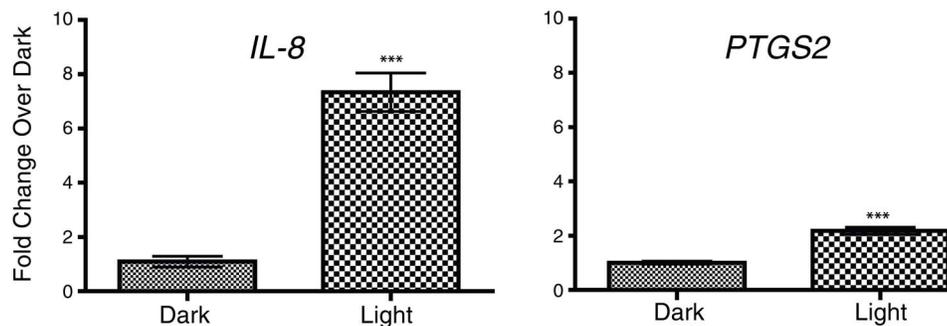


Figure 5. *IL-8* and *PTGS2* mRNA expression induced by exposure to isoprene-derived SOA using EAVES device all normalized to dark control experiments and against housekeeping gene, *ACTB*. All experiments conducted in triplicate ( $n = 3$ ). \*\*\* $P < 0.0005$ . The values shown in this figure are the mean  $\pm$  standard error of the mean. Raw data are shown in Appendix Figure E.2.

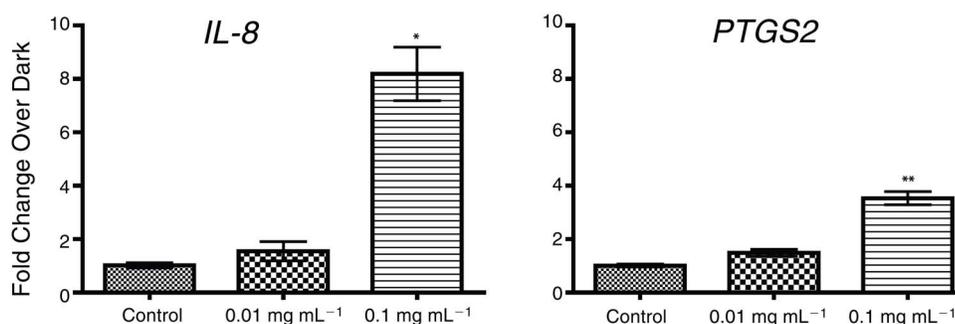


Figure 6. *IL-8* and *PTGS2* expression induced by exposure to isoprene-derived SOA using resuspension method all normalized to dark control experiments and against housekeeping gene, *ACTB*. All experiments conducted in triplicate ( $n = 3$ ). \* $P < 0.05$  and \*\* $P < 0.005$ . The values shown are the mean  $\pm$  standard error of the mean. Raw data are shown in Appendix Figure E.3.

The similar fold change observed in both the EAVES and resuspension exposures, in addition to confirming that the biological effects can be attributed to the particle-phase photochemical products (isoprene-derived SOA), suggests that exposure by resuspension is appropriate for isoprene-derived SOA and yields results similar to direct deposition exposures. However, it is noted here that the dose for EAVES experiments and resuspension methods should not be directly compared because the liquid height may affect the effective receiving dose and also toxicity for cells exposed to soluble isoprene-derived SOA constituents. In addition, the doses delivered are reported in different metrics ( $\mu\text{g}/\text{cm}^2$  versus  $\mu\text{g}/\text{mL}$ ). Unlike diesel particulate extracts, which agglomerate during resuspension exposures, isoprene-derived SOA constituents are water-soluble, based on reverse-phase liquid chromatography separations (Lin et al. 2012, 2013b; Surratt et al. 2006) and remain well

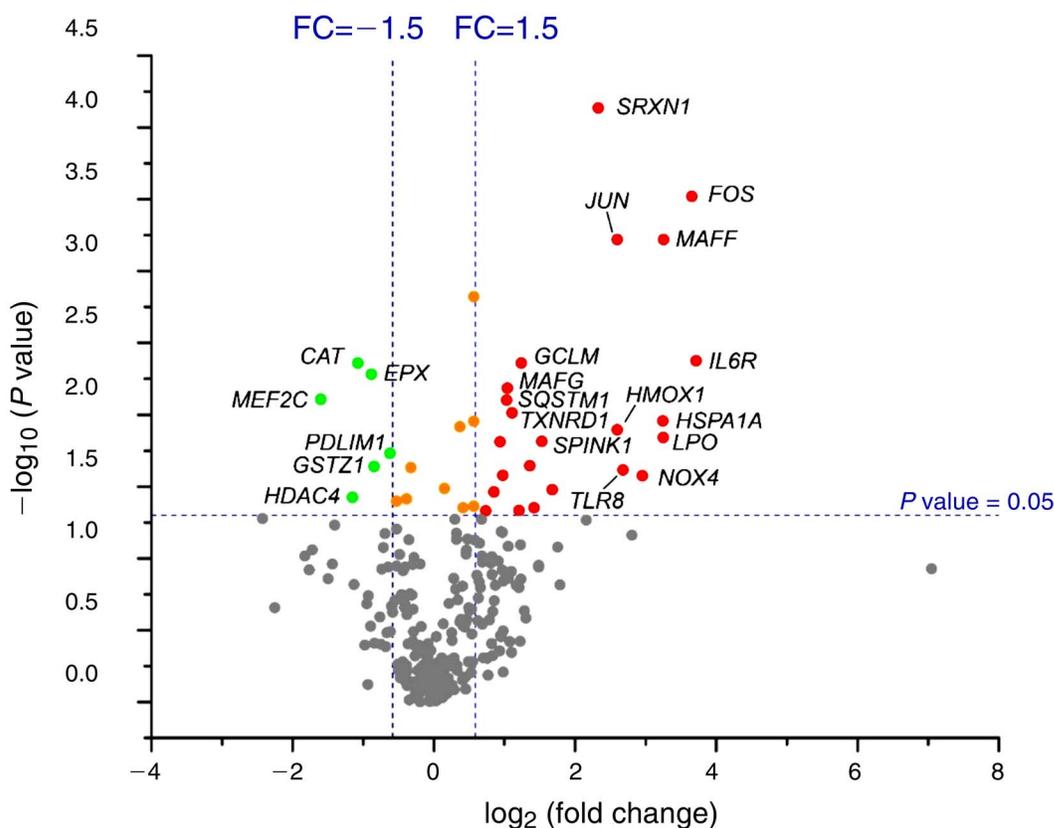
mixed in the cell medium used for exposure. Therefore, resuspension exposures do not appear to be a limitation for toxicological assessments of isoprene-derived SOA.

To identify differential expression of a larger set of genes (over 300 genes) in lung cells associated with exposure to isoprene-derived SOA, we extracted mRNA from dark controls and photochemical experiments. Appendix E, available on the HEI website, contains lists of the genes analyzed and gene expression data from our experiments. Gene symbols and full names of 84 oxidative stress-associated genes and housekeeping genes included in RT<sup>2</sup> Profiler PCR Array Human Oxidative Stress Pathway Plus (PAHS-065Y) are provided in Appendix Table E.1. Gene symbols and full names of 249 inflammation-associated genes and housekeeping genes included in nCounter GX Human Inflammation Kit (NanoString platform) are provided in Appendix Table E.2. The dark controls, which

take into account the effects of seed aerosol and gaseous precursors, were used to normalize response in photochemical experiments. Previous studies (as discussed earlier) in our laboratory demonstrated that the mass loading of sulfate seed aerosol used for the present study did not induce significant inflammatory gene expression in BEAS-2B cells over a 1-hour exposure time in the EAVES (Arashiro et al. 2016). BEAS-2B cells exposed to isoprene-derived SOA showed differential expression of 29 genes in total (Figure 7), of which 23 displayed increased expression and 6 displayed decreased expression. Table 6 shows the list of the 29 genes with significant changes (fold change cutoff =  $\pm 1.5$ ,  $P < 0.05$ ); 13 genes associated with inflammatory response were derived from the NanoString platform of 249 genes, and 16 genes involved

in oxidative stress were from the 84 genes on the RT<sup>2</sup> Profiler. After adjusting for multiple comparisons, 22 genes passed an FDR threshold of 0.3 (Chiarelli et al. 2016), and 4 genes passed a stringent FDR threshold of 0.05 (Appendix Figure E.5). The 22 differentially expressed genes in the gene sets exposed to isoprene-derived SOA with FDR  $< 0.3$  were analyzed for enrichment within biological pathways (Chiarelli et al. 2016). A Venn diagram showing the possible relations between the two gene expression platforms profiled in this study is provided in Appendix Figure E.4.

The WikiPathways-based analyses (Kutmon et al. 2016) revealed that the oxidative stress ( $P = 7.58 \times 10^{-12}$ ) and Nrf2 pathways ( $P = 5.77 \times 10^{-11}$ ) are the most significantly enriched — specifically, 6 of 16 genes (37.5 %) for inflammatory



**Figure 7. Volcano plot of differentially expressed genes in BEAS-2B cells upon exposure to isoprene SOA in the EAVES system.** Red dots indicate the significantly upregulated genes (23 genes). Green dots indicate the significantly downregulated genes (6 genes). Gray dots represent genes that did not significantly change in expression. Orange dots represent genes with fold changes below the threshold. Dashed lines indicate the  $-\log_{10} P$  value for  $P = 0.05$  and the  $\log_2$  values for fold changes of  $-1.5$  and  $1.5$ . A full list of differentially expressed genes can be found in Table 6. FC = fold change.

**Table 6.** Differentially Expressed Oxidative Stress- and Inflammation-Associated Genes in BEAS-2B Cells After Exposure to Isoprene SOA within the EAVES Device

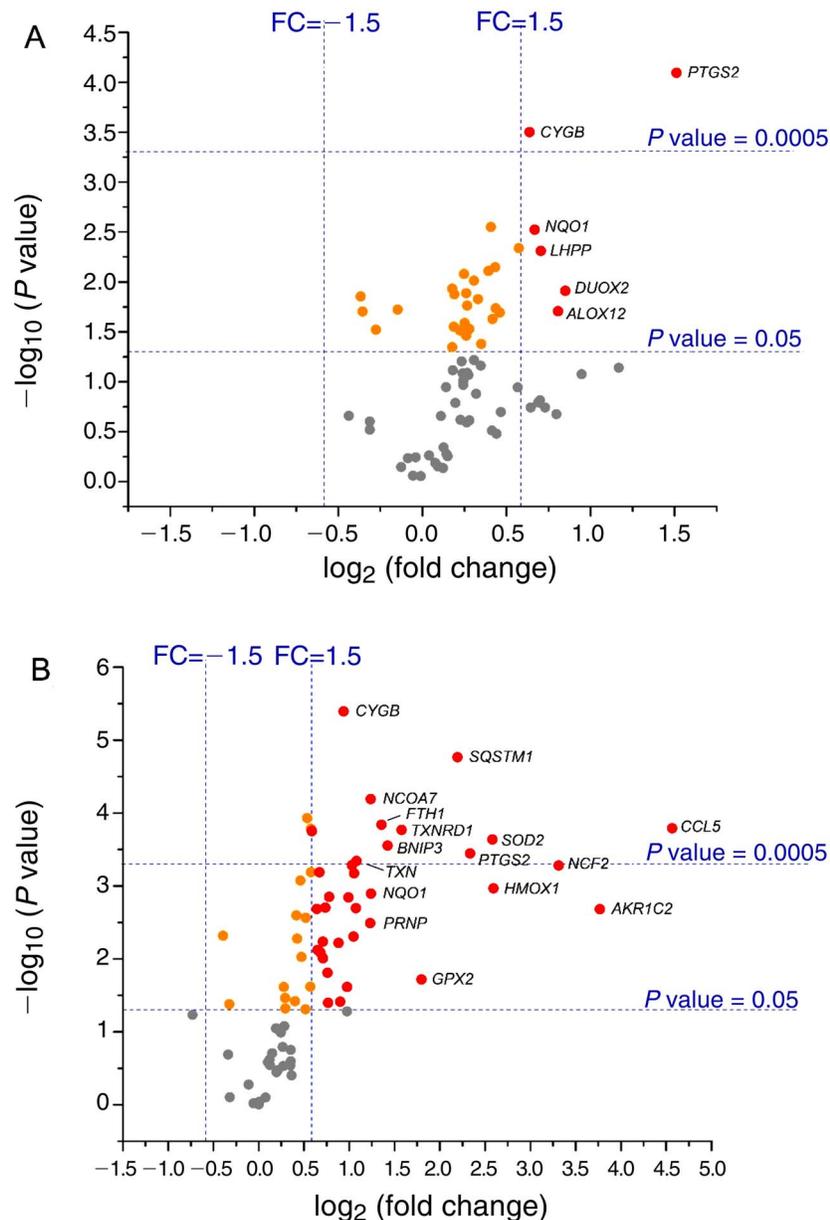
| Gene Name     | Fold Change | <i>P</i> Value | FDR-Adjusted <i>P</i> Value <sup>a</sup> | Panel                    |
|---------------|-------------|----------------|--|--------------------------|
| <i>AREG</i>   | 2.67        | 0.0443         | 0.4518                                   | NanoString               |
| <i>BCL6</i>   | 1.97        | 0.0263         | 0.4518                                   | NanoString               |
| <i>CD55</i>   | 3.22        | 0.0330         | 0.4518                                   | NanoString               |
| <i>FOS</i>    | 12.59       | 0.0003         | 0.0498                                   | NanoString               |
| <i>HDAC4</i>  | -2.22       | 0.0375         | 0.4518                                   | NanoString               |
| <i>IL23R</i>  | 2.57        | 0.0226         | 0.4518                                   | NanoString               |
| <i>IL6R</i>   | 13.11       | 0.0042         | 0.2092                                   | NanoString               |
| <i>JUN</i>    | 6.04        | 0.0006         | 0.0498                                   | NanoString               |
| <i>MAFF</i>   | 9.53        | 0.0006         | 0.0498                                   | NanoString               |
| <i>MAFG</i>   | 2.06        | 0.0065         | 0.2698                                   | NanoString               |
| <i>MAFK</i>   | 1.92        | 0.0154         | 0.4261                                   | NanoString               |
| <i>MEF2C</i>  | -3.03       | 0.0078         | 0.2775                                   | NanoString               |
| <i>TLR8</i>   | 6.40        | 0.0241         | 0.4518                                   | NanoString               |
| <i>BAG2</i>   | 1.67        | 0.0466         | 0.2059                                   | RT <sup>2</sup> Profiler |
| <i>CAT</i>    | -2.11       | 0.0044         | 0.1095                                   | RT <sup>2</sup> Profiler |
| <i>EPX</i>    | -1.84       | 0.0052         | 0.1095                                   | RT <sup>2</sup> Profiler |
| <i>GCLC</i>   | 2.31        | 0.0462         | 0.2059                                   | RT <sup>2</sup> Profiler |
| <i>GCLM</i>   | 2.36        | 0.0044         | 0.1095                                   | RT <sup>2</sup> Profiler |
| <i>GLA</i>    | 1.80        | 0.0343         | 0.1918                                   | RT <sup>2</sup> Profiler |
| <i>GSTZ1</i>  | -1.80       | 0.0228         | 0.1473                                   | RT <sup>2</sup> Profiler |
| <i>HMOX1</i>  | 6.05        | 0.0127         | 0.1165                                   | RT <sup>2</sup> Profiler |
| <i>HSPA1A</i> | 9.47        | 0.0110         | 0.1162                                   | RT <sup>2</sup> Profiler |
| <i>LPO</i>    | 9.51        | 0.0143         | 0.1165                                   | RT <sup>2</sup> Profiler |
| <i>NOX4</i>   | 7.73        | 0.0264         | 0.1585                                   | RT <sup>2</sup> Profiler |
| <i>PDLIM1</i> | -1.54       | 0.0185         | 0.1297                                   | RT <sup>2</sup> Profiler |
| <i>SPINK1</i> | 2.88        | 0.0153         | 0.1165                                   | RT <sup>2</sup> Profiler |
| <i>SQSTM1</i> | 2.04        | 0.0079         | 0.1162                                   | RT <sup>2</sup> Profiler |
| <i>SRXN1</i>  | 5.02        | 0.0001         | 0.0061                                   | RT <sup>2</sup> Profiler |
| <i>TXNRD1</i> | 2.16        | 0.0097         | 0.1162                                   | RT <sup>2</sup> Profiler |

<sup>a</sup> Benjamini-Hochberg method (Benjamini and Hochberg 1995).

markers (represented in the RT<sup>2</sup> Profiler platform) and 2 of 6 genes (33.3 %) for oxidative stress markers (represented in the NanoString platform). The BioCarta database (Nishimura 2001) also identified the pathway for oxidative stress-induced gene expression via Nrf2 ( $P = 1.12 \times 10^{-10}$ ) to be significantly enriched, with 1 of 16 genes (6.3%) represented in the RT<sup>2</sup> Profiler platform, and 4 of 6 genes (66.7%) represented in the NanoString platform. All pathways associated with isoprene-derived SOA exposure are listed in Appendix Table E.3.

### IEPOX- and MAE-Derived SOA

Volcano plots of differential gene expression (using the RT<sup>2</sup> Profiler platform) in BEAS-2B cells upon exposures to IEPOX-derived SOA and MAE-derived SOA are shown in Figure 8. A complete list of genes and *P* values is provided in Appendix Table E.4. 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



**Figure 8. Volcano plots of differential gene expression in BEAS-2B cells upon exposure by resuspension to: A. IEPOX-SOA and B. MAE-SOA.** Red dots indicate the significantly upregulated genes. Green dots indicate the significantly downregulated genes. Gray dots represent genes that did not significantly change in expression. Orange dots represent genes with fold changes between  $-1.5$  and  $1.5$ . Dashed lines indicate the  $-\log_{10} P$  values for  $P = 0.05$  and  $P = 0.0005$  and the  $\log_2$  values for fold changes of  $-1.5$  and  $1.5$ . A complete list of genes and  $P$  values is provided in Appendix Table E.4. FC = fold change.

changes in 2 genes from exposure to IEPOX-derived SOA extract and in 13 genes from exposure MAE-derived SOA extract remain significant.

Expression of *PTGS2* in the RT<sup>2</sup>-Profiler PCR Arrays was further confirmed with qRT-PCR analysis because exposure to both IEPOX- and MAE-derived SOA induced significant

fold changes in *PTGS2* expression. The comparison of fold change values between RT<sup>2</sup> Profiler PCR Arrays and qRT-PCR is shown in Appendix Figure E.6. 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 significantly higher level of *PTGS2* gene expression (7.09-fold;  $P = 0.01$ ) than does IEPOX-derived SOA (3.29-fold;  $P = 0.20$ ), consistent with RT<sup>2</sup> Profiler PCR array results (Appendix Table E.4) At low exposure concentrations (0.01 mg/mL), induction of *PTGS2* gene expression was not significant for either IEPOX- or MAE-derived SOA ( $P > 0.05$ ).

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 Nrf2-mediated oxidative stress response ( $P = 10^{-16}$ ) was enriched in both sets, with 1 of 6 genes (16%) represented in the IEPOX-SOA set and 13 of 36 genes (36%) represented in the MAE set (Appendix Table E.4), with NADPH dehydrogenase, quinone 1 (*NQO1*) being represented in both sets at  $P < 0.05$ . (While it didn't pass the stringent FDR, it does show differential expression via RT-PCR.)

### ISOPOOH-Derived SOA

The volcano plot of differential gene expression in BEAS-2B cells upon exposure to ISOPOOH-derived SOA for 24 hours is shown in Appendix Figure E.7. A complete list of significantly altered genes and  $P$  values is provided in Appendix Table E.5. With a fold-change cutoff value of 1.5, 32 oxidative stress-related genes were significantly altered ( $P < 0.05$ ). When FDR is considered, fold changes in 4 oxidative stress-associated genes remain significant.

The 32 differentially expressed genes from the ISOPOOH-derived SOA exposure were analyzed for enrichment within biological pathways. Similar to the isoprene-derived SOA exposure and the MAE- and IEPOX-derived SOA, the top canonical pathway for the ISOPOOH-derived SOA exposure was the Nrf2-mediated oxidative stress response ( $P = 6.12 \times 10^{-19}$ ), with 13 genes associated with the pathway. When adjusted for the FDR, only one gene remained associated with the pathway.

Single-gene analysis of *PTGS2* and heme oxygenase 1 (*HMOX1*) was conducted for quality control. Additionally, single-gene analysis was conducted for a 9- and 24-hour exposure to explore the effects of exposure time on gene expression changes. This was done as a result of ISOPOOH-derived SOA altering fewer genes than MAE-derived SOA for a 24-hour exposure, despite its higher oxidative potential measured by the DTT assay.

Appendix Figure E.8 shows the changes in mRNA levels of *PTGS2* and *HMOX1* for cells exposed to 0.1 mg/mL ISOPOOH-derived SOA for a 9- or 24-hour exposure. Fold changes are relative to the nonacidified (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> aerosol seed exposure (negative control). There is no change in the expression of *PTGS2* at 9- or 24-hour exposures, but

*HMOX1* is significantly altered at both exposure time points. The time course for the expression of *HMOX1* with a higher fold change after a 9-hour exposure compared with a 24-hour exposure is consistent with the differences in expression between 9- and 24-hour exposures for the isoprene-derived SOA.

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## DISCUSSION AND CONCLUSIONS

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### DTT ACTIVITY OF ISOPRENE-DERIVED SOA AND ITS COMPONENTS

The oxidative potential of the isoprene-derived SOA reported here is similar to that of daytime isoprene experiments in a study by Rattanavaraha and colleagues (2011) in which the oxidative potential of PM from aged diesel exhaust and other aged aerosol systems was systematically assessed in the presence and absence of a simulated urban hydrocarbon environment (UNC Mix) in an outdoor chamber facility. In the report by Rattanavaraha and colleagues (2011), filters were extracted with methanol, and NIOGs should be directly comparable to our study. Comparability is supported by the similarity of NIOGs determined in both studies for isoprene-derived SOA generated under similar conditions. The DTT activity of the outdoor chamber filter SOA is shown in Table 5. The DTT activities of MACR- and MAE-derived SOA in our experiments ( $2.3\text{--}3.1 \times 10^{-3}$  nmol DTT/min/ $\mu\text{g}$  PM) were similar to the DTT activity measured by Rattanavaraha and colleagues (2011) of aged diesel exhaust PM generated under clear natural sunlight without UNC VOC mixture ( $\sim 2.5 \times 10^{-3}$  nmol DTT/min/ $\mu\text{g}$  PM). This is the first report indicating that the ROS-generation potential of MACR SOA appears equivalent to that of daytime diesel exhaust PM. Relative to other strongly oxidizing PM reported in Rattanavaraha and colleagues (2011), the NIOGs of MACR- and isoprene-derived SOAs are lower than those of the PM generated during daytime by combined diesel exhaust, NO<sub>x</sub>, and UNC Mix (NIOG =  $3.3 \times 10^{-3}$ ) as well as daytime diesel exhaust, NO<sub>x</sub>, UNC Mix, toluene, and  $\alpha$ -pinene ( $3.9 \times 10^{-3}$ ). The similarities between MACR SOA and daytime diesel exhaust PM from Rattanavaraha and colleagues (2011) demonstrate the importance of characterizing the toxicity of SOA from this biogenic source enhanced by the presence of anthropogenic emissions. The NIOGs of ISOPOOH- and MACR-derived SOA were greater than that of nighttime diesel exhaust PM in the presence of O<sub>3</sub> and daytime diesel exhaust PM alone (Figure 4), with ISOPOOH-derived SOA having the greatest oxidative potential out of all isoprene-derived SOA types. Our findings suggest the need to further investigate ROS generation by isoprene and its

oxidation products. MACR-derived SOA, 2-MG, ISOPOOH (a surrogate for isoprene-derived organic peroxides in SOA), and ISOPOOH-derived SOA stand out as having a particularly high oxidative potential. While NIOGs of PM generated from aged diesel exhaust containing oxygenated PAH species reported in Rattanavaraha and colleagues (2011) are substantially higher than those reported from isoprene-derived SOA (except for ISOPOOH-derived SOA) reported in this study, the atmospheric abundance of isoprene-derived SOA and the enhanced yields on mixing with anthropogenic pollutants warrant further study of responses of biological systems to define potential health risks posed by exposure to this ubiquitous PM.

### BIOLOGICAL IMPLICATIONS — ACTIVATION OF Nrf2 AND AP-1 PATHWAYS

Exposure to PM<sub>2.5</sub> is associated with increased cardiovascular and pulmonary morbidity and mortality (Pope 2000). Generation of ROS in the respiratory system leading to oxidative stress and inflammation is considered to be strongly correlated with PM<sub>2.5</sub>-induced health effects (Reuter et al. 2010). As a result, our general hypothesis was that human lung cells exposed to isoprene-derived SOA will likely alter oxidative stress- and inflammation-related genes due to the chemical nature of isoprene-derived SOA constituents, such as those containing thiol reactive species like organic hydroxyhydroperoxides (ROOH) and carbonyls.

The chemical composition of isoprene-derived SOA to which cells were exposed in this work is formed predominantly through the low-NO<sub>x</sub> channel because of the high VOC/NO<sub>x</sub> ratio used for the experiments. Although no additional •OH source was added to the chamber, the photolysis of nitrous acid formed at the chamber walls in the early morning and photolysis of O<sub>3</sub> in the presence of water vapor yielded •OH radicals. Under such NO<sub>x</sub>-limited conditions, the majority of RO<sub>2</sub>• radicals from •OH-initiated oxidation react with hydroperoxyl radicals (HO<sub>2</sub>•) to yield ROOH and a wide array of multifunctional oxidation products that can contribute to SOA formation. The chamber-generated SOA has a molecular composition similar to ambient PM<sub>2.5</sub> observed during the summertime in the southeastern United States (Lin et al. 2013a; Rattanavaraha et al. 2016; Riva et al. 2016).

The acellular DTT assay demonstrated stronger oxidizing potential for total isoprene-derived SOA than for SOA derived from IEPOX, which is a major isoprene photooxidation product. Organic peroxides could be a major contributor to this difference, in light of the observation that isoprene-derived ROOH standards and ISOPOOH-derived SOA induced the strongest responses of the

assayed isoprene-derived SOA components. In addition, recent analysis of ROOH in a photochemically generated isoprene-derived SOA mixture by Jiang and colleagues (2017) also reveals a direct correlation of ROOH contents with DTT consumption. Exposure of cells to extractable SOA constituents from total isoprene-derived SOA and IEPOX-derived SOA also indicates more potent induction of oxidative stress response genes by isoprene-derived SOA extracts (Arashiro et al. 2016; Lin et al. 2016). These observations highlight the potential significance of ROOH constituents in total isoprene-derived SOA, as they would not have been present in the aerosols generated from pure IEPOX precursors. Although ISOPOOH- and MAE-derived SOA constituents account for only a small fraction of isoprene-derived SOA produced under chamber conditions in this study (<5% based on SOA tracers shown in Figure 1), they may be the major reactive components responsible for the induced cellular responses.

In this study, mRNA levels of 84 oxidative stress- and 249 inflammation-associated genes were profiled to gain insight into potential mechanisms leading to adverse health effects induced by isoprene-derived SOA. Expression of 29 genes from these two gene array platforms was found to be significantly modified ( $P < 0.05$ ), with 22 genes passing FDR after exposure to isoprene-derived SOA under conditions of minimal cytotoxicity (16/22 genes derived from the human oxidative stress RT<sup>2</sup> Profiler, and 6/22 genes derived from the NanoString human inflammation platform, respectively). Notably, while only minimal inflammatory responses were observed under the given exposure condition, the differentially expressed genes identified through the NanoString platform, including FBJ murine osteosarcoma viral oncogene homolog (*FOS*), jun proto-oncogene (*JUN*), v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (*MAFF*), v-maf musculoaponeurotic fibrosarcoma oncogene homolog G (*MAFG*), and v-maf musculoaponeurotic fibrosarcoma oncogene homolog K (*MAFK*) closely mirror the oxidative stress response via Nrf2 pathway (Figure 9A).

The identification of the Nrf2 pathway in cells exposed to isoprene-derived SOA is in accordance with our findings using the DTT assay, which measures the thiol reactivity of PM samples as a surrogate for their ROS-generation potential. The activity of Nrf2 is primarily regulated via its interaction with Keap1 (Kelch-like ECH-associated protein 1), where Keap1 binds to Nrf2 and tethers its nuclear translocation under basal conditions. The sulfhydryl groups within Keap1 act as sensors for oxidants and electrophiles. In the presence of ROS, critical cysteine residues (C151, C273, and C288) in Keap1 become oxidized and cause a conformational change of Keap1 (Bryan et al.

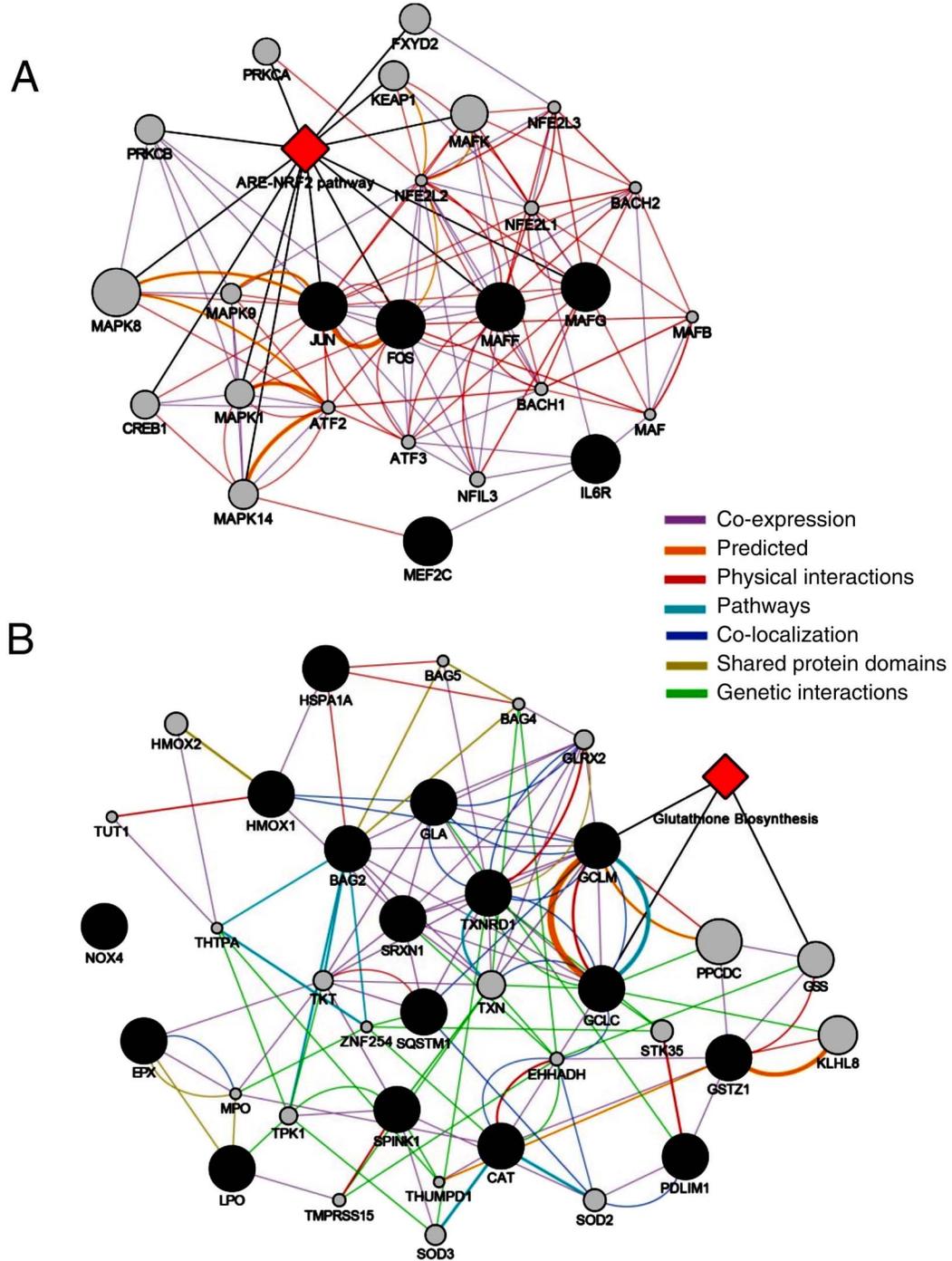


Figure 9. Networks of gene-gene functional interactions for differentially expressed genes (FDR < 0.3) derived from: A. NanoString human inflammation platform and B. human oxidative stress plus RT<sup>2</sup> Profiler. Black nodes represent the input genes, which were significantly altered upon isoprene SOA exposure, and gray nodes represent predicted related genes. Links represent networks. Red squares represent the top pathways significantly enriched. The interactive gene network construction was performed and visualized using the GeneMANIA Cytoscape app.

2013). As a consequence, Nrf2 is released from Keap1, and nuclear translocation of Nrf2 occurs. Thus, the results from the acellular DTT assay provide a plausible explanation to the cysteine–thiol modifications within cells that lead to the activation of Nrf2-related gene expression. Significant upregulation of both the glutamate-cysteine ligase catalytic subunit and the glutamate-cysteine ligase modifier subunit genes (*GCLC* and *GCLM*) identified via human oxidative stress RT<sup>2</sup> Profiler (Figure 9B) also points to the activation of cysteine metabolism and glutathione biosynthesis.

Expression of the small musculoaponeurotic fibrosarcoma (sMAF) transcription factors has been linked to stress response and detoxification pathways. The sMAF transcription factors are bZIP proteins that can form heterodimeric complexes with the bZIP protein Nrf2 and bind at the antioxidant response element (ARE) to initiate transcription of target antioxidant genes (Kannan et al. 2012; Ma 2013). Up-regulation of the three sMAF genes in this study agrees with our previous findings that isoprene-derived SOA constituents induce oxidative stress responses in BEAS-2B cells (Lin et al. 2016), and further provides mechanistic insights into the cellular response to isoprene-derived SOA exposure. Nrf2-sMAF complex has also been implicated in inflammasome activation (Ma 2013). These findings are consistent with exposure to isoprene-derived SOA leading to an increased burden of cellular oxidative stress, which induces a variety of cellular responses, including activation of redox-sensitive transcription factors and up-regulation of the expression of inflammatory response genes as observed here.

The induction of sulfiredoxin 1 (*SRXN1*) expression shown in this study is also in accord with the suggestion that the Nrf2 signaling is activated. *SRXN1* is a target of Nrf2 regulation coding for a protein functioning in antioxidant defense (Taylor et al. 2008) and regulated by the transcription factor AP-1 (Soriano et al. 2009). *FOS* and *JUN* are protooncogenes and members of the AP-1 family of bZIP transcription factors that function as heterodimers. They are transcribed in response to a wide variety of stimuli, including oxidative stress and inflammation (Park et al. 2009), and up-regulation observed here is consistent with the generation of ROS by isoprene-derived SOA. *JUN* is critical to maintenance of lung redox homeostasis, and deletion has been linked to progressive emphysema and consequent lung inflammation in mice exposed to cigarette smoke (Reddy et al. 2012).

In sum, this study characterizes differential gene expression in response to SOA derived from isoprene as a single source and applies genomics assessment as a tool to gain insight into potential response mechanisms. The work is

directly relevant to exposures in the southeastern United States, where isoprene is a major source of SOA during the summer months (Budisulistiorini et al. 2016; Lin et al. 2013a; Rattanavarha et al. 2016; Xu et al. 2015). Activation of Nrf2-associated genes (as observed here in isoprene-derived SOA and its SOA subtypes) has been identified with responses to oxidative stress and linked to traffic-related air pollution exposure in both toxicological and epidemiological studies (Pardo et al. 2015; Wittkopp et al. 2016), where repeated low-dose exposures are proposed to lead to lung oxidative damage and systemic inflammatory reactions in vivo (Pardo et al. 2016). Their implicit involvement in this study suggests biological significance with exposures to all sorts of PM types (Li et al. 2004; Pardo et al. 2015, 2016). Future studies are warranted to further understand its potential clinical significance and the link to health outcomes.

## GENERAL LIMITATIONS

First, the DTT assay utilized during this study is acellular and consequently does not simulate complex interactions involved in pathways of biological response. The potential of the SOA component of PM<sub>2.5</sub> to induce oxidative stress suggested by the results reported here strongly supports expanding research to investigate the responses in cell models (such as the BEAS-2B cells used in our present study), primary cells, and more complex organisms. Although the PM samples analyzed in this study are generally representative of ambient PM composition in the southeastern United States, they were generated in source-specific controlled chamber experiments. Furthermore, the chamber was operated as a batch reactor, and thus the experimental conditions are at best an approximation of ambient conditions. Although the experiments conducted during this study utilized ~ 600 ppbv of both IEPOX and MAE, ~ 300 ppbv of ISOPOOH, and 3.5 ppmv of isoprene, the chemical composition of the resultant SOA mimicked ambient SOA that is typically collected from isoprene-rich environments, including both rural and urban areas of the southeastern United States (Budisulistiorini et al. 2015; Lin et al. 2012, 2013a; Rattanavaraha et al. 2016). Despite that ambient concentrations of IEPOX, MAE, and ISOPOOH might only reach up to hundreds of pptv (possibly a few ppbv for IEPOX) (Paulot et al. 2009), and 10 ppbv for isoprene (Guenther et al. 2006), these experiments utilized higher concentrations in order to generate sufficient mass for both toxicological and chemical assessment. We acknowledge the fact that experiments were not randomized and there could be seasonal factors that could be possible sources of variability in photochemical reactions. However, we note that isoprene emissions are predominant

during warmer months (late spring and summer), and thus, many of our experiments overlapped with these time periods.

Comprehensive analysis of the chemical components of ambient SOA is necessary to identify additional constituents contributing to ROS generation by ambient PM. From the narrower perspective of NIOGs and DTT activity, a consideration in comparing the results reported here with those of other studies is sample preparation. In addition, selectivity of extraction solvents and solubility of aerosol components have been reported to affect redox activity determined by the DTT assay; specifically, in this study filters were extracted with methanol, which has been reported to extract redox-active organic components more efficiently from PM samples than the water used in other studies (McWhinney et al. 2013; Rattanavaraha et al. 2011; Verma et al. 2012; Yang et al. 2014).

Some caution is necessary in the extrapolation of our results to the *in vivo* situation. The lung is a complex organ. Lung lining fluid covering the respiratory epithelium contains proteins and antioxidants potentially mitigating the effects of inhaled chemicals (Cross et al. 1994). Such effects are not taken into consideration in this work. Furthermore, cells isolated from their local environment for use during *in vitro* studies may be more susceptible to damage and alteration (Devlin et al. 2005). In addition, the BEAS-2B cell line is immortalized, and some biological functions may have been altered relative to primary cell lines. Nevertheless, reproducible results from the BEAS-2B cell line are critical to gaining initial insights into cellular effects of isoprene-derived SOA exposure and have been shown to be useful in airway inflammation studies such as ours (Devlin et al. 1994).

Although we began our studies using a direct deposition device to conduct our *in vitro* exposures to isoprene-derived SOA at an air–liquid interface in order to mimic a more biologically relevant exposure, we shifted to using resuspension exposure methods, especially for the IEPOX-, MAE-, and ISOPOOH-specific subtypes of isoprene-derived SOA. Importantly, we found that either direct deposition or resuspension exposures yielded the same conclusions about oxidative stress through the Nrf2 pathways playing a role in the cellular responses. We recognize that the resuspension exposure model, which potentially alters shape, size, and composition of a particulate exposure, may not be as sensitive to particulate exposure. However, we decided resuspension exposures were better for controlled exposures at various time points and for exposures to IEPOX- and MAE-derived SOA generated by uptake experiments, and ISOPOOH-derived SOA

generated by oxidation experiments, which were done under very dry conditions not conducive to cell viability.

As an initial exploration of the potential biological effects resulting from human lung cells exposed to isoprene-derived SOA, the biological endpoint assessed in this work was pathway-focused gene expression, which does not necessarily translate to cellular functions which are regulated at the protein level, nor does it indicate health outcomes measured at the tissue or organ system level. However, we can use the gene expression data to hypothesize likely outcomes to design future studies on the downstream effects of gene alteration, including protein production and physiological effects. Because gene expression is continuous, even two exposure time points at 9 and 24 hours are not enough to analyze changes over time. We may be missing the expression of certain genes that are altered much earlier or much later than the chosen time points. With its strong ROS-generating potential, ISOPOOH-derived SOA may induce a significant change in *HMOX1* after a 1-hour exposure that could decrease by 9 or 24 hours. This may explain why we see fewer oxidative stress-related genes affected by a 24-hour exposure to ISOPOOH-derived SOA than to MAE-derived SOA, despite ISOPOOH-derived SOA having greater oxidative potential as measured by the DTT assay.

Additionally, measures of oxidative potential through the DTT assay may not be directly relatable to all oxidative stress response observed in a biological system. As a chemical assay, the DTT assay is a measure of the ability of a compound to oxidize DTT. In relation to oxidative stress, the DTT assay may mimic the compound's ability to modify the cysteine residues of Keap1 leading to Nrf2 activation through a measure of its electrophilicity (or thiol reactivity). As expected, we demonstrated that the Nrf2 pathway was activated by all types of isoprene-derived SOA, which we determined had ROS-generating potential through the DTT assay. We are limited to drawing any further conclusions about oxidative stress, including relative gene expression levels, in a biological system — aside from the potential to activate the Nrf2 pathway. The DTT assay can serve as an initial screening for the potential of a compound to affect oxidative stress response in cells, but biological assays are needed to verify actual biological response in a cellular model.

Finally, even though we measured significant gene expression changes due to isoprene-derived SOA exposure, a limitation of the present research is the lack of direct comparison to known toxic compounds (positive controls). There are no existing studies conducted in a similar manner using the same exposure conditions and biological endpoint with a positive control. Our findings

could have been strengthened by conducting tandem resuspension exposures with a known air toxicant, such as diesel exhaust, at the same dose and exposure time to serve as a positive control. Importantly, our use of the DTT assay did allow us to make comparisons to other types of PM generated in our smog chamber previously (Rattanavara et al. 2011), including different forms of fresh and aged diesel PM types. It was interesting to observe that isoprene-derived SOA and its subtypes were able to generate DTT activity equal to or greater than these diesel PM types previously examined in our chamber facility.

## IMPLICATIONS OF FINDINGS

The results presented in this study suggest that exposure to isoprene-derived SOA extracts of all types increase levels of oxidative stress responses in BEAS-2B cells. Oxidative stress can occur as a result of damage to cellular proteins, lipids, membranes, and DNA due to ROS generated by particle uptake in target cells such as airway epithelial cells (Nel 2005). The exact mechanism in which isoprene-derived SOA is capable of generating ROS in human lung cells is still unknown, but we can conclude that the isoprene-derived SOA leads to oxidative stress as seen by the activation of the Nrf2 and AP-1 pathways as an antioxidant response. If the Nrf2 and AP-1 pathways

cannot restore oxidative balance, continued oxidative stress can lead to the activation and recruitment of cytokines and chemokines that cause localized inflammation of lung tissue and systemic inflammation (Nel 2005).

Oxidative stress is associated with chronic pulmonary inflammation and contributes to respiratory and cardiovascular health outcomes (Donaldson et al. 2001; Kirkham and Barnes 2013; Rahman and Adcock 2006) and cancer (Reuter et al. 2010). However, the enrichment of the Nrf2 network does not necessarily suggest definite health hazards associated with isoprene-derived SOA, as it may just mean increased antioxidant defense capabilities in response to isoprene-derived SOA exposure. For diesel exhaust, Nrf2 serves as a key transcription factor protecting against proinflammatory effects of particulate pollutants through its regulation of antioxidant defense (Li et al. 2004). However, our results showed elevations of *IL-8* gene expression in BEAS-2B exposed to isoprene-derived SOA, which is an indication of the proinflammatory effects of isoprene-derived SOA. Further studies exploring additional inflammation-associated genes and proteins may be needed to posit that oxidative stress caused from isoprene-derived SOA exposure can cause localized lung tissue inflammation.

Figure 10 highlights gene expression changes observed during this study that are unique to each component type

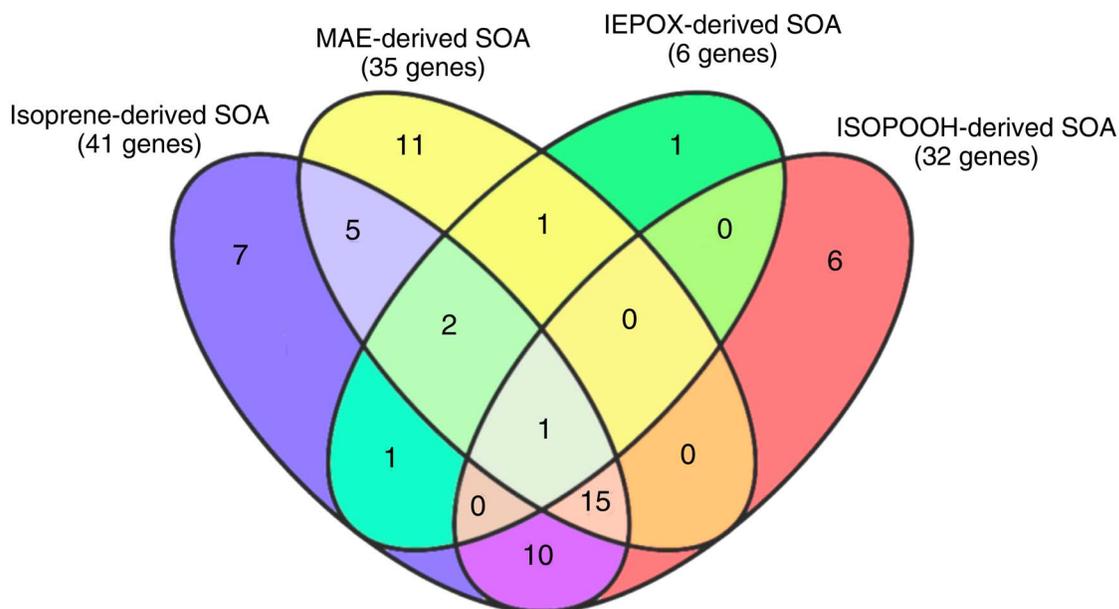


Figure 10. Venn diagram of gene expression changes ( $P < 0.05$ ) common among isoprene-derived SOA, MAE-derived SOA, IEPOX-derived SOA, and ISOPOOH-derived SOA exposure.

of isoprene-derived SOA, with MAE- and ISOPOOH-derived SOA having the greatest number of uniquely altered genes as well as the greatest number of altered genes. Despite all being components of isoprene-derived SOA, the constituents of MAE-, IEPOX-, and ISOPOOH-derived SOA vary, primarily through functional groups specific to the pathway of formation. The functional groups contributing to the differences in MAE-, IEPOX-, and ISOPOOH-derived SOA constituents, which are carboxylic acids, alcohols, and ROOHs, respectively, likely lead to the differences in genes altered by each component. Our findings suggest that the carboxylic acid and ROOH functional groups may be the dominant contributors to observed gene expression alterations in isoprene-derived SOA, as MAE- and ISOPOOH-derived SOA altered the greatest number of genes. The single gene common to all exposure types was *NQO1*, a cytoprotective protein whose primary function is the detoxification of quinones (Dinkova-Kostova and Talalay 2010). *NQO1* is induced by oxidative stress, dioxin, and PAHs (Dinkova-Kostova and Talalay 2010). Isoprene-derived SOA, despite not being chemically similar to quinones, elicits the expression of *NQO1* in all its forms. The expression of *NQO1* is mediated through the Keap1/Nrf2/ARE pathway (Dinkova-Kostova and Talalay 2010), which is consistent with the enrichment of the Nrf2 pathway in all our exposures. *NQO1* is cited as being protective against the toxic and carcinogenic effects of numerous carcinogens and oxidative stress, with polymorphisms in *NQO1* being associated with increased risk for disease such as cancer (Dinkova-Kostova and Talalay 2010).

Although *HMOX1* promoter polymorphisms are rare, they leave certain populations at risk of respiratory diseases due to decreased defenses against oxidative stress (Fredenburgh et al. 2007; Yamada et al. 2000). Additionally, polymorphisms in *NQO1*, the gene common to all tested types of isoprene-derived SOA exposure, may increase lung cancer risk (Saldivar et al. 2005). With significant fold changes of *HMOX1* associated with isoprene-derived SOA and fold changes of *NQO1* seen in all exposures, there may be a subset of the population that could be susceptible to disease resulting from high levels of oxidative stress due to isoprene-derived SOA exposure.

Aside from the biological implications of this work, there are numerous atmospheric implications as well. The results of this study showed that of all the isoprene-derived SOA types formed from various precursors, MAE- and ISOPOOH-derived SOA had the greatest effect on oxidative stress-related gene expression. Although MAE- and ISOPOOH-derived types of SOA account for a smaller fraction of isoprene-derived SOA compared to IEPOX-derived SOA (as it did in our total isoprene-derived SOA mixture), they may be responsible for more biological effects even at

their smaller fractions. In areas with high-NO<sub>x</sub> concentrations, MAE-derived SOA comprises a greater fraction of isoprene SOA-derived mass; however, recent work has shown that IEPOX- and ISOPOOH-derived types of SOA account for the largest fraction of isoprene-derived SOA mass (Budisulistiorini et al. 2016; Hu et al. 2015; Riva et al. 2016). This has implications on pollution control because NO<sub>x</sub> is a controllable pollutant, that if controlled can potentially alter the composition of isoprene-derived SOA, which we have shown has differing effects on gene expression.

Because isoprene-derived SOA comprises a large portion of global PM<sub>2.5</sub> (Carlton et al. 2009; Hallquist et al. 2009; Henze et al. 2008; Hu et al. 2015), there is a large potential global public health impact of this research. There are direct impacts to areas such as the southeastern United States where particle-phase products found in our photochemical experiments have been measured in significant quantities (accounting on average for 40% of fine organic aerosol mass) in ambient fine organic aerosol particles (Budisulistiorini et al. 2013, 2016; Lin et al. 2013a; Rattanavaraha et al. 2016). Even in areas of the world not directly affected by isoprene-derived SOA, this research has major implications, as it has demonstrated that the functional groups associated with the different isoprene-derived SOA constituents likely affect the alterations of oxidative stress-related gene expression. Specifically, the carboxylic acid and ROOH functional groups may be more influential in altering gene expression. This may aid in predicting the gene-expression-altering capability of SOAs derived from other biogenic (non-isoprene) or anthropogenic VOCs specific to a location.

Finally, we have shown that the total isoprene-derived SOA mixture significantly affects a large number of oxidative stress-related genes, some at very high levels, which could not be explained by the individual SOAs from isoprene or by their sum. This highlights the importance of treating air pollution of all types as a mixture, because exposures are rarely isolated to specific compounds and could have synergistic effects.

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### MATERIALS AVAILABLE ON THE HEI WEBSITE

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Additional Materials, available on the HEI website at [www.healtheffects.org/publications](http://www.healtheffects.org/publications), comprise Appendices A, B, C, D, and E, which contain supplemental material not included in the printed report.

Appendix A. Quality Assurance and Calibrations for DTT Assay

Appendix B. Additional Characterization Results of Exposure Conditions

Appendix C. Additional Results from the DTT Assay

Appendix D. Cytotoxicity Measurements of Exposure Conditions

Appendix E. Genes Analyzed and Additional Gene Expression Data for Exposures

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 ABOUT THE AUTHORS
 

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**Jason D. Surratt** received his Ph.D. in chemistry from the California Institute of Technology. Surratt is currently a professor in the Department of Environmental Sciences and Engineering located within the Gillings School of Global Public Health at UNC–Chapel Hill. His research interests are in understanding the fundamental atmospheric chemistry that occurs in both the gas and condensed phases that yields SOA; this helps to improve our understanding of how SOA present in PM<sub>2.5</sub> adversely affects air quality and human health, as well as revealing potential mechanisms to mitigate the production of SOA.

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 OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH
 

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Arashiro M, Lin YH, Zhang Z, Sexton KG, Gold A, Jasper IJ, et al. 2018. Effect of secondary organic aerosol from isoprene-derived hydroxyhydroperoxides on the expression of oxidative stress response genes in human bronchial epithelial cells. *Environ Sci Processes Impacts* 20(2):332–339.

Lin YH, Arashiro M, Clapp PW, Cui T, Sexton KG, Vizuete W, et al. 2017. Gene expression profiling in human lung cells exposed to isoprene-derived secondary organic aerosol. *Environ Sci Technol* 51(14):8166–8175.

Arashiro M, Lin YH, Sexton KG, Zhang Z, Jaspers I, Fry RC, et al. 2016. In vitro exposure to isoprene-derived secondary organic aerosol by direct deposition and its effects on COX-2 and IL-8 gene expression. *Atmos Chem Phys* 16(22):14079–14090.

Kramer AJ, Rattanavaraha W, Zhang Z, Gold A, Surratt JD, Lin YH. 2016. Assessing the oxidative potential of isoprene-derived epoxides and secondary organic aerosol. *Atmos Environ* 130:211–218.

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Research Report 198, *Understanding the Early Biological Effects of Isoprene-Derived Particulate Matter Enhanced by Anthropogenic Pollutants*, J.D. Surratt et al.

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## INTRODUCTION

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A large number of epidemiological studies have reported associations between increases in exposure to ambient particulate matter (PM\*) and higher rates of mortality and hospitalization, especially in health-compromised individuals. It has been hypothesized that the common pathophysiological mechanism underlying the effects of PM is through reactive oxygen species (ROS) that are present on the particle surfaces reacting with human cells and tissues. These reactions could lead to oxidative stress, which in turn could trigger inflammatory responses in both the lung and the circulatory system. In vitro studies have been used as proof-of-concept to evaluate the reactivity of particles from different sources and of different composition and to evaluate possible mechanistic pathways affected by particle exposure. However, a clear link between in vitro responses to PM-generated ROS and in vivo effects has not been established.

In June 2012, Dr. Jason Surratt of the University of North Carolina at Chapel Hill submitted a proposal, titled “Understanding the health effects of isoprene-derived particulate matter enhanced by anthropogenic pollutants,” in response to RFA 11-2: Walter A. Rosenblith New Investigator Award. In his application Dr. Surratt proposed to examine the early biological responses to exposure to PM derived from chemical reactions of isoprene in the air, which result in the production of secondary organic aerosol (SOA), in cultured human bronchial epithelial cells with a focus on the expression of genes involved in oxidative stress and inflammation. A minor goal of the study was to evaluate the oxidative potential of the same SOA. Isoprene was chosen as the SOA precursor because it is an abundant

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Dr. Jason D. Surratt’s 3-year study, “Understanding the Health Effects of Isoprene-Derived Particulate Matter Enhanced by Anthropogenic Emissions,” began in May 2013. Total expenditures were \$450,000. The draft Investigators’ Report from Surratt and colleagues was received for review in April 2017. A revised report, received in January 2018, was accepted for publication in February 2018. During the review process, the HEI Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators’ Report and the Review Committee’s Critique.

This document has not been reviewed by public or private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views of these parties, and no endorsements by them should be inferred.

\* A list of abbreviations and other terms appears at the end of this volume.

compound derived from biogenic sources (i.e., certain types of vegetation) for which there is limited mechanistic information. The HEI Research Committee recommended the study for funding because it was innovative and interdisciplinary, combining atmospheric chemistry and toxicology, and would evaluate isoprene oxidation products. The Committee noted that the study was relevant to HEI since isoprene has a similar chemical structure to that of butadiene, a compound emitted in vehicle exhausts, and since levels of isoprene are expected to increase in the future due to climate change.

This Critique provides the HEI Review Committee’s evaluation of the study. It is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the study and by placing the Investigators’ Report into a broader scientific perspective.

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## SCIENTIFIC BACKGROUND

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### SOA FORMATION FROM ISOPRENE

Pollutants in ambient air derive from both anthropogenic and biogenic sources and are present in the gas- and particle-phase. Volatile organic compounds (VOCs) are important components because they also react with atmospheric oxidants, such as hydroxyl radicals ( $\bullet\text{OH}$ ) to form SOA. SOA levels are not regulated under the National Ambient Air Quality Standards, but they account for a large fraction of  $\text{PM} \leq 2.5 \mu\text{m}$  in aerodynamic diameter ( $\text{PM}_{2.5}$ ) in many areas of the United States and influence climate and human health (Xu et al. 2015).

ROS are free oxygen radicals, meaning they have lost an electron due to oxidation (so that they have one unpaired electron on the oxygen atom); as such, radicals are very reactive and initiate the transformation of organic compounds either by extracting hydrogen or by adding a double bond. The initial step in the formation of SOA is the oxidation of VOCs by the hydroxyl radical ( $\bullet\text{OH}$ ) or by other species such as the nitrate radical ( $\text{NO}_3\bullet$ ) and ozone ( $\text{O}_3$ ) (Hallquist et al 2009; Kroll and Seinfeld 2008). From this reaction a number of reactive intermediates are formed, which can further react with other ROS (such as the hydroperoxyl radical,  $\text{HO}_2\bullet$ ) and ultimately lead to the

formation of lower volatility species that partition to the particle phase (i.e., SOA) (Kroll and Seinfeld 2008).

Isoprene is a biogenic VOC and a key precursor of global SOA due to its abundance and high reactivity with oxygen radicals (Xu et al. 2015). Anthropogenic pollutants, in particular mixtures of nitrogen oxides ( $\text{NO}_x$ ), have been shown to greatly enhance isoprene as a source of SOA (Lin et al. 2013; Xu et al. 2015), as was found for SOA from other VOCs (Kroll and Seinfeld 2008). Specifically, the oxidation of isoprene follows two main pathways depending on the  $\text{NO}_x$  level. At high  $\text{NO}_x$ , it leads to the formation of methacrolein (MACR), which is further oxidized to methacrylic acid epoxide (MAE). At low  $\text{NO}_x$ , isoprene is converted to isoprene hydroxyhydroperoxide (ISOPOOH) and subsequently to isoprene epoxydiols (IEPOX). These reactions are schematically summarized in the Critique Figure. In this study, both isoprene and its known intermediate products of atmospheric oxidation — MACR, MAE, ISOPOOH, and IEPOX (highlighted in grey in the figure) — were used as precursors of SOA.

#### CHEMICAL AND BIOLOGICAL ASSAYS

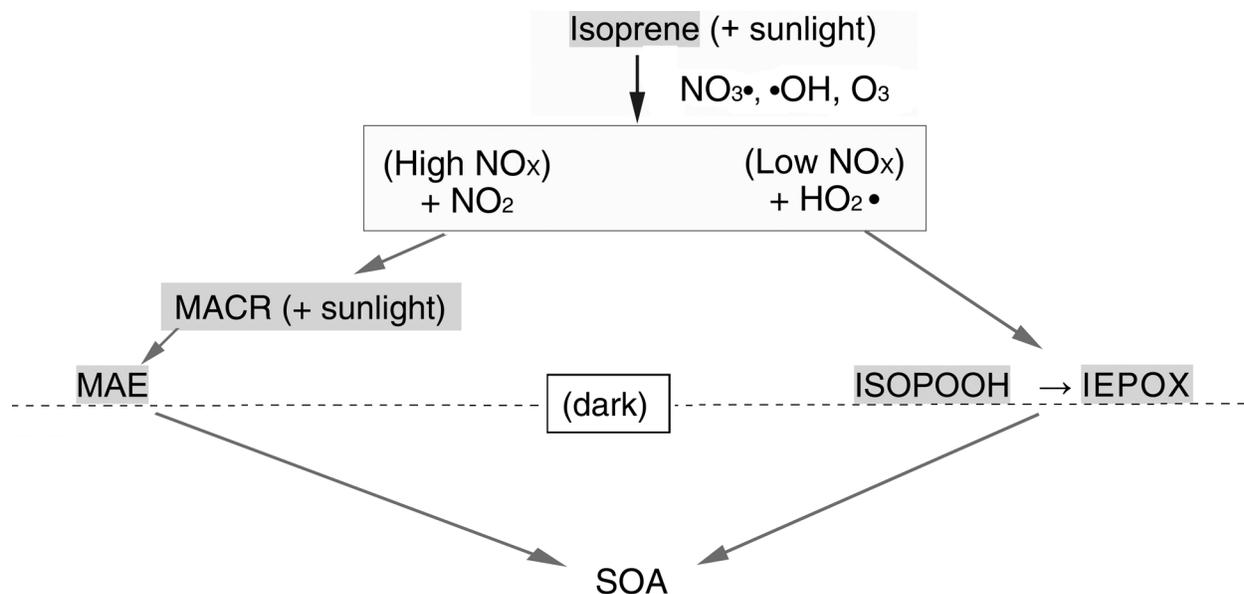
Oxidative stress is hypothesized to be an important underlying mechanism by which exposure to PM may lead to the production of pro-inflammatory mediators and the progression of some diseases; it occurs when ROS overwhelm the natural antioxidant defenses (Ghio et al 2012;

Delfino et al 2013; Miller et al. 2012). Ambient aerosols may lead to oxidative stress by transporting ROS on particles into the respiratory system or introducing aerosol components (such as metals and organic compounds) that are capable of stimulating cellular generation of ROS (Ghio et al. 2012; Miller et al. 2012).

ROS initially would trigger compensatory changes in the cell antioxidant systems; however, if a biochemical imbalance occurs and the generation of ROS exceeds the natural antioxidant capacity of the cells, oxidative stress occurs (Nel 2005).

Several chemical and biological assays are used to evaluate the oxidative activity of particles (for a recent review, see Hedayat et al. 2015) and the pathophysiological pathways that are affected by PM exposures. The ability of PM to generate ROS and chemically oxidize target molecules in noncellular systems is referred to as *oxidative potential*. A commonly used chemical assay to quantify the oxidative potential of PM is the dithiothreitol (DTT) assay, which is based on the oxidation of DTT to its disulfide form. The rate of DTT consumption is used as a measure of the concentration of ROS in the extract (see for example Charrier et al. 2015 and Samara 2017).

The capacity of PM to induce cells to generate ROS intracellularly also contributes to *oxidative stress* (Ghio et al. 2012; Li et al. 2003). Assays to measure oxidative stress have included measuring the fluorescence increase upon oxidation of a dye, oxidation of ascorbate, as well as the



**Critique Figure.** A simplified schematic showing the chemistry of isoprene forming SOA in the presence of oxygen radicals and sunlight under high or low  $\text{NO}_x$  conditions (Krechmer et al. 2015; Lin et al. 2012, 2013).

level or expression of pro-inflammatory molecules such as interleukin-6 and tumor necrosis factor-alpha (Miller et al. 2012) or of anti-inflammatory enzymes such as heme oxygenase 1 (HMOX1) (Li et al. 2003), or of various oxidative stress- and inflammation-associated genes (Sijan et al 2015; Volckens et al. 2009). These assays have primarily used cell lines of lung macrophages or epithelial cells derived from humans or rodents. In some studies, cultures of human bronchial epithelial cells obtained from biopsies were used (e.g., Volckens et al. 2009).

The most common approach for delivering particles to cell cultures has been to use soluble extracts of particles collected on filters. This approach, which is known as *particle resuspension exposure*, has limitations because the extracts do not include many components present in ambient aerosols and because the method of PM delivery does not reflect human inhalation exposures. On the other hand, it has been widely considered a practical approach to test mechanistic hypotheses. To overcome the limitations of the aqueous extract, some researchers have used an Electrostatic Aerosol in Vitro Exposure System (EAVES) (de Bruijne et al. 2009; Volckens et al. 2009), which removes particles from the air flow by electrically charging them and causing them to precipitate onto the cells. In this study, Surratt and colleagues used both particle exposure methods.

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## SPECIFIC AIMS

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The overall objective of this study was to examine the oxidative potential of SOAs generated from isoprene and their effect on gene expression. The specific aims were to:

1. examine toxicity and early biological effects of SOA derived from the photochemical oxidation of isoprene, and
2. examine toxicity and early biological effects of SOA derived directly from downstream oxidation products of isoprene (i.e., epoxides and hydroperoxides).

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## STUDY DESIGN AND METHODS

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The investigators generated SOAs in a chamber and tested them using two types of assay: a DTT chemical assay and a cellular assay using a human bronchial epithelium cell line (BEAS-2B).

## SOA GENERATION

The investigators generated two types of SOA: (1) SOA derived from the photo-oxidation of isoprene in an outdoor environmental chamber; and (2) SOA derived from the oxidation of key intermediates derived from the photo-oxidation of isoprene (shown in the Critique Figure) in a dark indoor chamber. The latter compounds were synthesized by the authors in the laboratory. In all experiments, some form of sulfate was added as a seed to the chamber to act as a substrate onto which the semivolatile species could condense. The schematic representation of isoprene chemistry and the major chemical species generated can be seen in Figure 1 of the Investigators' Report.

The SOA precursors used in the study and the chamber conditions are listed below. For all precursors a combination of magnesium sulfate and sulfuric acid (referred to as acidified sulfate) was used as a seed, with the exception of ISOPOOH, for which ammonium sulfate was used.

- Isoprene — added to an outdoor chamber in the presence of low NO<sub>x</sub> (200 ppb nitric oxide [NO]);
- MACR — added to an outdoor chamber in the presence of high NO<sub>x</sub> (100 ppb NO and 300 ppb nitrogen dioxide [NO<sub>2</sub>]);
- MAE, a product of isoprene oxidation at high NO<sub>x</sub> — added to an indoor (dark) chamber (in the absence of NO<sub>x</sub>);
- ISOPOOH, a product of isoprene oxidation at low NO<sub>x</sub> — added to an indoor (dark) chamber together with an oxidant, tetramethylethylene (in the absence of NO<sub>x</sub>); and
- IEPOX, a product of isoprene oxidation at low NO<sub>x</sub> — added to an indoor (dark) chamber (in the absence of NO<sub>x</sub>).

SOA was collected on filters and stored frozen until needed for the experiments. At that point the SOA was extracted by sonication in methanol, and then was dried and resuspended in keratinocyte basal medium, a serum-free medium. For the photochemically generated SOA, SOA generated in the dark was used as a control; for the SOA generated in the dark from the intermediate precursors, SOA generated in the presence of the seed alone was used as control.

## DTT ASSAY

All SOAs were tested in the DTT assay. For this assay, particle extracts were added to the DTT solution; DTT consumption was measured over a period of 30 minutes. The DTT activity (i.e., oxidative potential) was reported as nmoles of DTT consumed per minute per µg of PM and

also as the normalized index of oxidant generation (NIOG), which takes into account various reaction parameters and is normalized relative to the activity of a 1,4-naphthoquinone standard (Li et al. 2009; Rattanavaraha et al. 2011).

## CELLULAR EXPERIMENTS

For these experiments, the investigators used a cell line derived from human bronchial epithelium. Two types of cellular experiments were conducted:

- a 1-hour direct exposure in the EAVES system followed by incubation in fresh medium for 9 hours, at which time the extracellular medium was collected for analysis — isoprene-derived SOA only, and
- resuspension exposure for 9 hours and, in some experiments, 24 hours, at which time the extracellular medium was collected for analysis — all types of SOA. A similar range of doses was used for all SOAs.

The biological endpoints measured and methods used included:

- lactate dehydrogenase (LDH), used as a marker of cytotoxicity (i.e., cell viability) release in the extracellular liquid;
- expression (i.e., messenger ribonucleic acid [mRNA] level) of inflammation- and oxidative stress-related genes: interleukin-8 (*IL-8*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), and *HMOX1* measured by the polymerase chain reaction (PCR) using specific gene primers. In some experiments *PTGS2* was quantified using the gene panel described below;
- expression (mRNA level) using two separate panels of genes measured by PCR of total mRNA:
  - a panel of 84 human oxidative stress-associated genes (referred to as RT<sup>2</sup> Profiler), which includes the *PTGS2* gene, and
  - a panel of 249 human inflammation-associated genes (referred as NanoString GX Human Inflammation).

## STATISTICAL ANALYSES

One-way analysis of variance was used to test the significance of differences in the DTT results between different SOA extracts. Differential gene expression of the gene panels was carried out using the data analysis software provided for the specific panel. Results were normalized using  $\beta$ -*actin* expression for the RT<sup>2</sup> Profiler panel and using the geometric average of the expression of six housekeeping genes included in the NanoString gene panel kit. Results with a fold-change cutoff  $\geq 1.5$  and  $P < 0.05$  were

considered significant. The results were corrected for the false discovery rate to adjust for multiple comparisons.

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## KEY RESULTS

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### ISOPRENE-DERIVED SOA CHARACTERISTICS

The mode of the size distribution of isoprene-derived SOA generated in the outdoor chamber shifted from circa 70 nm at the beginning of the experiment to circa 150 nm after the injection of the gaseous oxidants (NO and NO<sub>2</sub>). The cells were exposed when the concentration of SOA in the reaction chamber had reached its maximum at approximately 35  $\mu\text{g}/\text{m}^3$ . Compositional analysis revealed the presence of the three major photo-oxidation products mentioned earlier. The size distribution modes of the other SOA were not reported. The chamber profiles and composition of SOA derived from the intermediate precursors are shown in Appendix C of the report (available on the HEI website).

### DTT ASSAY

All SOA samples showed some DTT activity. ISOPOOH-derived SOA had the highest DTT response followed by MACR- and isoprene-derived SOA, which had similar oxidative potential ( $1.9 \times 10^{-3}$  and  $1.7 \times 10^{-3}$  NIOG, respectively).

### LDH

There was no increase in LDH in any of the experiments using either direct SOA exposure or resuspension exposure.

### GENE EXPRESSION

#### Single Genes (*IL-8* and *PTGS2*)

The investigators found that direct exposure to isoprene-derived SOA induced an increase in the production of *PTGS2* and *IL-8* mRNA. Experiments using resuspended particles (from the same aerosol) confirmed these findings and also showed that the response was dose dependent.

Resuspension exposure of cells to IEPOX- and MAE-derived SOA also increased the expression of *PTGS2* mRNA (*IL-8* was not measured), but exposure to ISOPOOH-derived SOA did not (see in Appendix E of the Investigators' Report, available on the HEI website).

### Gene Panels

The main results of the experiments on mRNA expression profiles based on the two selected gene panels were the following:

- isoprene-derived SOA (EAVES exposure) induced the differential expression of 22 genes (after adjusting for multiple comparisons), more than those affected by SOA derived from the tested precursors; and
- IEPOX-derived SOA, MAE-derived SOA, and ISO-POOH-derived SOA (9-hr resuspension exposure) induced the differential expression of 2, 13, and 4 genes, respectively.

The Critique Table summarizes the types of SOAs studied, the DTT and gene expression assays conducted for each SOA type, and the key results. The LDH results are not included.

The investigators analyzed for enrichment of the affected genes within biological pathways. The pathway

that appeared to be consistently enriched across the various experiments was the Nrf2-mediated oxidative stress pathway (i.e., nuclear factor [erythroid-derived 2]-like 2).

### CORRELATION BETWEEN DTT AND GENE EXPRESSION

The authors concluded that there was “no clear relationship between DTT activity and the number of altered oxidative stress-related genes.”

### REVIEW COMMITTEE EVALUATION

In its independent review of the study, the HEI Review Committee concluded that Surratt and colleagues conducted a systematic study addressing an important question, namely, whether SOA from isoprene (a biogenic organic compound) or from some of the intermediate products formed from atmospheric chemical reactions of

**Critique Table.** Summary of SOA Precursors Tested and Results of Assays Conducted for Each SOA<sup>a,b</sup>

| SOA Precursor | Generation Conditions  | DTT assay (NIOG) | Gene Expression (mRNA) Endpoints |                    |                       |       |                       |                       |  |
|---------------|------------------------|------------------|----------------------------------|--------------------|-----------------------|-------|-----------------------|-----------------------|--|
|               |                        |                  | IL-8, PTGS2, and HMOX1           |                    | Gene panels           |       |                       |                       |  |
|               |                        |                  | Resuspension                     | EAVES              | Resuspension          | EAVES | Resuspension          |                       |  |
| Isoprene      | acidified sulfate seed | ↑↑               | ↑↑↑ IL-8<br>↑ PTGS2              | ↑↑ IL-8<br>↑ PTGS2 | 22 <sup>d</sup> genes |       |                       |                       |  |
| MACR          | acidified sulfate seed | ↑↑               |                                  |                    |                       |       |                       |                       |  |
| MAE           | acidified sulfate seed | ↑↑               |                                  |                    |                       |       | ↑↑ PTGS2 <sup>c</sup> | 13 genes <sup>d</sup> |  |
| IEPOX         | acidified sulfate seed | ↑                |                                  |                    |                       |       | ↑ PTGS2 <sup>c</sup>  | 2 genes <sup>d</sup>  |  |
| ISOPOOH       | ammonium sulfate       | ↑↑↑              |                                  |                    |                       |       | = PTGS2<br>↑ HMOX1    | 4 genes <sup>d</sup>  |  |

<sup>a</sup> Gray cells indicate endpoint not measured; number of arrows provides estimation of size of the effects relative to control; = indicates no change relative to control.

<sup>b</sup> Results can be found in the Investigators' Report as follows: for the DTT assay NIOG, see Figure 4; for gene expression endpoints, see Figure 5 (IL-8 and PTGS2 — isoprene SOA/EAVES), Figure 6 (IL-8 and PTGS2 — isoprene SOA/resuspension), and Figure E.6 (PTGS2 — MAE and IEPOX SOA) and Figure E.7 (PTGS2 and HMOX1 — ISOPOOH) of Appendix E (available on the HEI website).

<sup>c</sup> Only PTGS2 was measured using the two gene panels.

<sup>d</sup> After correction for the false discovery rate.

isoprene has oxidative potential (as measured in the DTT assay) that can cause gene expression changes in epithelial cell cultures.

Isoprene is an important biogenic source of SOA in the atmosphere and thus understanding whether these SOAs have effects on cells is of considerable interest. The Committee concluded that this multidisciplinary study was carefully conducted. In particular, the generation and characterization of the aerosols were considered to be very good. However, the Review Committee noted that season of the year varied for the outdoor environmental chamber experiments (i.e., experiments using isoprene or seed only were conducted in the summer, while experiments using MACR were conducted in the fall). Although this is an accepted practice, it is not known how the different experimental conditions (such as amount of daylight and ambient temperature) may have affected the chamber reactions and consequently the results of the study.

A limitation of the study is the reliance on the DTT assay to determine the ability of particles to trigger oxidative reactions. Because the DTT assay is a chemical assay, it provides only an indication of the concentration of reactive oxidant species in the particle extract. Moreover, it may not represent the oxidant kinetics that occur when ambient PM/aerosols come into contact with respiratory tract lining fluid natural antioxidants. Thus, a correlation between the results of this assay and the biological assays should not necessarily be expected. Having a cellular measure of oxidative stress would have strengthened the results of the transcriptional assays. Another limitation of the DTT assay as conducted was the use of methanol to extract PM from the filter, because methanol has been shown to increase the DTT activity relative to water extraction (see Rattanavaraha et al. 2011; Yang et al. 2014). The investigators used this method because it was used in earlier studies conducted in the laboratory.

The use of the BEAS-2B airway cell line was considered appropriate in this context, even though it has limitations because this is a so-called immortal cell line, and the cells' responses may not be representative of normal (primary) human cells. The Committee also thought that using both the EAVES system and particle resuspension for delivering particles to the cells was a strength of the study. However, the exposure protocols and durations were very different between the two methods (1 hour followed by a 9-hour recovery with EAVES versus 9 hours in the presence of the particles resuspended in solution). These differences were not explained or justified, and thus a comparison of the results between the two methods is difficult. Given this concern, it was encouraging to observe similar production of IL-8 and PTGS2 mRNA in the two systems. A similar

comparison of the exposure methods for the gene panel studies was not conducted. The Committee noted that only one dose of the extract was used in the biological assay, and the choice of the dose was not justified.

The Review Committee thought that the LDH assay, which was used as a measure of cytotoxicity, was a weak surrogate for this endpoint because it can be affected by oxidants in the medium, leading to underestimation of cell death. Although the investigators concluded that the concentrations the SOAs used were not cytotoxic, cell viability could be a confounder in the gene expression studies. The Committee thought that the use of a direct viability assay (for example, vital dyes, permeability indices, or cell density counts) would have provided a more reliable assessment of cellular status than LDH levels. On the other hand, the Committee noted that the gene expression and pathway analyses are commendable aspects of the study.

The Committee agreed with the investigators' conclusion that all isoprene- and intermediate precursor-derived SOAs showed DTT activity, with the highest activity associated with the SOA from the ISOPOOH intermediate. However, it is hard to interpret the significance of this result to real-world conditions where the level of the intermediate precursor in the isoprene oxidation pathway differs depending on the NO<sub>x</sub> concentrations.

Results on the transcription activity of the SOAs from the three intermediate precursors show that the latter are less active than isoprene-derived SOA measured in the EAVES system. Given the different protocols (EAVES versus resuspension), it is not clear whether the results are a consequence of the different exposures or of different activities of the SOA. The Committee also agreed that there was no clear relationship between the results of the DTT assay and gene expression. It also noted that this is not a limitation because the assays measure different oxidative pathways and are not expected to be correlated.

The Committee also agreed that exposure of cells to isoprene-derived SOA in the aerosol state (i.e., EAVES exposure) increased the expression of genes in the Nrf2 pathway, which regulates antioxidant defenses (for a review of the multiple roles of this pathway see Bryan et al. 2013) and suggests that this SOA leads to an increase in cellular oxidant burden. This finding is consistent with results of the study by Li and colleagues (2004) who found that diesel exhaust particles also activated this pathway.

In summary, this project has shed some light on an important area of PM health research, namely, that NO<sub>x</sub> mediates the SOA formation reactions and may affect the oxidative activity of the resulting SOA. In this regard, tests of SOA derived from isoprene under different NO<sub>x</sub>

conditions would have been useful. Overall, the results indicate that the endpoints selected respond to chemical differences in the SOA precursors and suggest novel approaches for studying the effects of SOA.

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## ABBREVIATIONS AND OTHER TERMS

|                                |   |   |  |
|--------------------------------|---|---|--|
| 1,4-NQ                         | 1,4-naphthoquinone  | MAFG  | v-maf musculoaponeurotic fibrosarcoma oncogene homolog G               |
| $2^{-\Delta\Delta CT}$         | comparative cycle threshold   | MAFK  | v-maf musculoaponeurotic fibrosarcoma oncogene homolog K               |
| <i>ACTB</i>                    | $\beta$ -actin  | MCPC  | mixing condensation particle counter                                   |
| ANOVA                          | analysis of variance  | MgSO <sub>4</sub>                               | magnesium sulfate  |
| AP-1                           | activation protein-1  | mRNA  | messenger ribonucleic acid   |
| ARE                            | antioxidant response element  | NIOG  | normalized index of oxidant generation                                 |
| BEAS-2B                        | a human bronchial epithelial cell line                              | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | ammonium sulfate   |
| DMA                            | differential mobility analyzer                                      | NIOG  | normalized index of oxidant generation                                 |
| DMSO                           | dimethyl sulfoxide  | NIST  | National Institute of Standards and Technology                         |
| DTNB                           | dithiobis-2-nitrobenzoic acid                                       | NO  | nitric oxide   |
| DTT                            | dithiothreitol  | NO <sub>2</sub>                                 | nitrogen dioxide   |
| EAVES                          | Electrostatic Aerosol in Vitro Exposure System                      | NO <sub>3</sub> •                               | nitrate radical  |
| FDR                            | false discovery rate  | NO <sub>x</sub>                                 | oxides of nitrogen   |
| <i>FOS</i>                     | FBJ murine osteosarcoma viral oncogene homolog                      | NQO1  | NADPH dehydrogenase, quinone 1   |
| GC/MS                          | gas chromatography mass spectrometry                                | Nrf2  | nuclear factor (erythroid-derived 2)-like 2                            |
| GC/EI-MS                       | gas chromatography/electron ionization-quadrupole mass spectrometry | O <sub>3</sub>                                  | ozone  |
| H <sub>2</sub> SO <sub>4</sub> | sulfuric acid   | •OH   | hydroxyl radical   |
| HMML                           | hydroxymethyl-methyl- $\alpha$ -lactone                             | PAH   | polycyclic aromatic hydrocarbon  |
| <i>HMOX1</i>                   | heme oxygenase 1  | PBS   | phosphate buffer saline  |
| HO <sub>2</sub> •              | hydroperoxyl radical  | PCR   | polymerase chain reaction  |
| IEPOX                          | isoprene epoxydiol  | PM  | particulate matter   |
| <i>IL-8</i>                    | interleukin 8   | PM <sub>2.5</sub>                               | particulate matter $\leq 2.5 \mu\text{m}$ in aerodynamic diameter      |
| ISOPOOH                        | isoprene hydroxyhydroperoxide                                       | PMF   | positive matrix factorization  |
| ISOPTHP                        | isoprene trihydroxyhydroperoxide                                    | PSL   | polystyrene latex sphere   |
| ISOP(OOH) <sub>2</sub>         | isoprene dihydroxydihydroperoxide                                   | <i>PTGS2</i>                                    | prostaglandin-endoperoxide synthase 2                                  |
| <i>JUN</i>                     | jun proto-oncogene  | qRT-PCR   | quantitative real-time reverse transcription polymerase chain reaction |
| KBM                            | keratinocyte basal medium   | RH  | relative humidity  |
| Keap1                          | Kelch-like ECH-associated protein 1                                 | RNA   | ribonucleic acid   |
| KGM                            | keratinocyte growth medium  | RO <sub>2</sub> •                               | organic peroxy radical   |
| LDH                            | lactate dehydrogenase   | ROOH  | organic hydroxyhydroperoxide   |
| MACR                           | methacrolein  | ROS   | reactive oxygen species  |
| MAE                            | methacrylic acid epoxide  |   |  |
| <i>MAFF</i>                    | v-maf musculoaponeurotic fibrosarcoma oncogene homolog F            |   |  |

(Continued next page)

## ABBREVIATIONS AND OTHER TERMS

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*(Continued)*

|                 |   |                    |   |
|-----------------|---|--------------------|---|
| RT-PCR          | reverse transcription polymerase chain reaction | TNB                | 5-thio-2-nitrobenzoic acid  |
| sMAF            | small musculoaponeurotic fibrosarcoma           | UPLC/ESI-HR-QTOFMS | ultra-performance liquid-chromatography electrospray ionization-high resolution-quadrupole time-of-flight mass spectrometry |
| SO <sub>2</sub> | sulfur dioxide                                  | VOC                | volatile organic compound   |
| SOA             | secondary organic aerosol                       |                    |   |
| TME             | tetramethylethylene                             |                    |   |

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