Reviewer 3 comments:

The lack of context of this work remains largely unaddressed. The authors have modified the paper slightly, now asserting that this work is more exploratory in nature and that it shows more research in isoprene SOA toxicity is warranted. All three reviewers, in some form or other, have raised the issue questioning how isoprene SOA causes a potentially adverse health response, or put another way, what is the significance of increases in IL-8 and COX-2 genes when exposed to isoprene SOA. The author’s answer to this question is essentially based on their other reported research. The logic put forward in this paper is summarized in lines 400 to 410 (copied below, next paragraph).

"The mechanism by which isoprene-SOA causes elevation of the inflammatory markers IL-8 and COX-2 is not yet fully understood. However, recent work from our laboratory using the acellular dithiothreitol (DTT) assay demonstrated that isoprene-derived SOA have equal or greater ROS generation potential than diesel exhaust PM (Kramer et al., 2016; Rattanavaraha et al., 2011). High levels of ROS in cells can overwhelm the antioxidant defense and lead to cellular oxidative stress (Bowler and Crapo, 2002; Li et al., 2003; Sies, 1991). Following the discovery of the potential importance of isoprene-SOA in generating ROS, Lin et al. (2016) showed that isoprene-SOA formed from the reactive uptake of epoxides alters levels of oxidative stress-associated genes, including COX-2 in human lung cells. Oxidative stress caused by ROS plays a major role in lung inflammation and the induction of oxidative stress can lead to IL-8 expression (Tao et al., 2003; Yan et al., 2015)."

The logic is: Diesel is a known toxic aerosol component (implicitly assumed), and ROS (as measured by the DTT assay) is linked to oxidative stress, which is linked to various adverse health endpoints. Since isoprene SOA has equal or higher DTT per mass activities than diesel, and DTT assay is a measure of aerosol oxidative potential, isoprene may be toxic through an oxidative stress route. The problem with this logic is that it all hinges on isoprene SOA being more toxic than diesel, as reported by (Kramer et al., 2016; Rattanavaraha et al., 2011). These papers indeed show this, but the comparison in these two papers is flawed because, for some reason, the diesel DTT per mass reported in Kramer et al., and Rattanavaraha et al, (note, that Rattanavaraha et al appears to simply reuses Kramer et al data, so it all comes down to Kramer) is vastly lower (by about a factor of 10) than what others have reported in both chamber and ambient studies [Bates et al., 2015; Charrier et al., 2015; McWhinney et al., 2013]. (Note, more papers on ambient DTT per mass activities in locations highly impacted by vehicle emissions also exist, but I have not included them here).

The response of the authors to this issue raised in the first review was that Bates et al isoprene DTT per mass was based on AMS PMF data and so the isoprene SOA was a different form than the isoprene SOA in this experiment. But that is not the issue. Bates also gives low DTT per mass activities for Other OA (ie, expected to be mainly biogenic SOA). The issue is not DTT per mass for isoprene, there is actually some agreement on this, the issue is why is Rattanavaraha DTT per mass for diesel so low relative to other studies? By only citing their own work and ignoring other published data the authors have made a substantial flaw in reasoning and also give a biased interpretation. The line, "our laboratory using the acellular dithiothreitol (DTT) assay demonstrated that isoprene-derived SOA have equal or greater ROS generation potential than diesel exhaust PM." is factually correct since the citation is limited to their work, but is known to be much more complicated than they knowledge. It seems to be defined narrowly here simply to support the premise of this paper. This issue must be rectified before publication.
Response to Reviewer 3 comments:

Our intention was not to use the previous DTT assay work to frame the entirety of our work. We cited the work because it is one of the few that has been done on the isoprene-SOA system. Because the DTT assay is an acellular assay it is not an effective way to assess or compare biological effects of isoprene-SOA and diesel exhaust. Instead, we highlight the work of Lichtveld et al. (2012) and Hawley et al. (2014) to better put our results into context of the highly studied system of diesel exhaust and draw comparisons between the effect on IL-8 and COX-2 from isoprene-SOA and diesel exhaust as stated in line 378-390.

Despite the use of a different cell line, these studies are more comparable to our study because they are also in vitro studies utilizing a direct deposition exposure method to measure the changes in gene expression unlike the acellular chemical based DTT assay measuring redox potential. However, in response to the reviewer’s comment, we have revised the statement in lines 402-405 to read:

“The mechanism by which isoprene-SOA causes elevation of the inflammatory markers IL-8 and COX-2 is not yet fully understood. However, recent work from our laboratory using the acellular dithiothreitol (DTT) assay demonstrated that isoprene-derived SOA has significant ROS generation potential (Kramer et al., 2016).”

Although the DTT assay in not a focus of this paper, in this response we try to address the reviewer’s concerns about the discrepancy in the comparison of ROS generating potential of isoprene-SOA to diesel exhaust. First, Rattanavaraha et al. (2011) does not reuse data from Kramer et al. (2016) but graphical data from Rattanavaraha et al. (2011) is presented in Kramer et al. (2016). Kramer et al. (2016) found that isoprene-SOA show equal or greater ROS generation potential than reported in the study on diesel exhaust conducted by Rattanavaraha et al. (2011) for nighttime diesel with and without ozone and daytime diesel without urban mix. The numbers reported for Rattanavaraha et al. (2011) is likely lower in comparison to the other studies cited by the reviewer because EDTA was used in the DTT assay to remove any interference from transition metals in order to focus specifically on the effects of different organic systems. In Charrier et al. (2015), they conclude that copper and manganese largely account for all of the DTT response from nighttime sources and “daytime sources are also generally dominated by metals, but these samples also have a large (up to 50%) contribution from unknown compounds.” In McWhinney et al. (2013) they state that “the redox activity of DEP is not extractable by moderately polar (methanol) and nonpolar (dichloromethane) organic solvents.” They report “only 2-11% of the redox activity was in the water-soluble potion, while the remainder occurred at the black carbon surface.” There may have been lower DTT activity reported in Rattanavaraha et al. (2011) for nighttime diesel particles because they utilized a methanol extraction to compare soluble organic components, but found that oxidized PAH products enhanced DTT activity in daytime diesel samples. As stated in the previous response, the DTT assay in (Bates et al., 2015) is not comparable to Kramer et al. (2016) or Rattanavaraha et al. (2011).

Despite the DTT activity of isoprene-SOA being lower in comparison to the DTT activity measured due to transition metals in ambient samples, the abundance of isoprene-SOA in the atmosphere is not insignificant and its ROS generation potential cannot be discounted. Regardless, our suggested reasoning for increased expressions of IL-8 and COX-2 via an oxidative stress pathway hinges more on the finding by the cited Lin et al. (2016), showing altered expression of oxidative stress genes via an in vitro study, than Kramer et al. (2016).

In regards to the reviewers comment that “All three reviewers, in some form or other, have raised the issue questioning how isoprene SOA causes a potentially adverse health response, or put
another way, what is the significance of increases in il-8 and cox-2 genes when exposed to isoprene SOA”, we had addressed this issue in our previous edit by including the following (line 391-417):

“IL-8 and COX-2 are both linked to inflammation and oxidative stress (Kunkel et al., 1991; Uchida, 2008). IL-8 is a potent neutrophil chemotactic factor in the lung and its expression by various cells plays a crucial role in neutrophil recruitment leading to lung inflammation (Kunkel et al., 1991). COX-2 is the inducible form of the cyclooxygenase enzyme, regulated by cytokines and mitogens, and is responsible for prostaglandin synthesis associated with inflammation (FitzGerald, 2003). Consistent with the reports that IL-8 and COX-2 play important roles in lung inflammation (Nocker et al., 1996; Li et al., 2013), in vivo studies have shown that isoprene oxidation products cause airflow limitation and sensory irritation in mice (Rohr et al., 2003). In humans, the role of IL-8 and COX-2 in lung inflammation can be associated with diseases such as chronic obstructive pulmonary disease and asthma (Nocker et al., 1996; Peng et al., 2008; Fong et al., 2000).”

The mechanism by which isoprene-SOA causes elevation of the inflammatory markers IL-8 and COX-2 is not yet fully understood. However, recent work from our laboratory using the acellular dithiothreitol (DTT) assay demonstrated that isoprene-derived SOA has significant ROS generation potential (Kramer et al., 2016). High levels of ROS in cells can overwhelm the antioxidant defense and lead to cellular oxidative stress (Sies, 1991; Bowler and Crapo, 2002; Li et al., 2003). Following the discovery of the potential importance of isoprene-SOA in generating ROS, Lin et al. (2016) showed that isoprene-SOA formed from the reactive uptake of epoxides alters levels of oxidative stress-associated genes, including COX-2 in human lung cells. Oxidative stress caused by ROS plays a major role in lung inflammation and the induction of oxidative stress can lead to IL-8 expression (Tao et al., 2003; Yan et al., 2015). Specifically, oxidants can activate the transcription factor NF-κB, which regulates a wide range of inflammatory genes including IL-8 and COX-2 (Barnes and Adcock, 1997; Schreck et al., 1992) Therefore, isoprene-SOA may cause increases in IL-8 and COX-2 primarily through an oxidative stress response. Additionally, the relationship between IL-8 and COX-2 can also explain the observed increase in IL-8 gene expression as its production can be stimulated through a COX-2 dependent mechanism in airway epithelial cells (Peng et al., 2008).”

Because the gene expression of COX-2 and IL-8 can be regulated at various levels and have varying biological effects, we remain cautious about making any conclusive statements about the mechanism in which both were elevated and the resulting health effects from the elevations based on this study alone. However, to provide even further evidence about the importance of in vitro work (separate from acellular techniques such as DTT) we have added a citation from Hatch et al. (2014) on page 19 line 418-427 as follows:

“In vitro studies such as this one using a direct deposition model cannot fully elucidate mechanisms of lung inflammation and potential pathogenesis but serve as a necessary part of hazard characterization, particularly for a complex air mixture that has not been fully studied (Hayashi, 2005; Paur et al., 2011). Ozone exposure studies have shown that comparable dose and effect measurements for IL-8 and COX-2 can be found between in vivo and in vitro exposures which add promise to extrapolating effects seen in vitro to effects in vivo (Hatch et al., 2014). In vivo effects associated with isoprene-SOA exposure in vitro cannot be inferred as it is a different system from ozone, so further in vitro studies exploring the health implication of the elevation of IL-8 and COX-2 due specifically to isoprene-SOA exposure are necessary and may in turn justify further extension to in vivo work.”
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In Vitro Exposure to Isoprene-Derived Secondary Organic Aerosol by Direct Deposition and its Effects on COX-2 and IL-8 Gene Expression

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Abstract

Atmospheric oxidation of isoprene, the most abundant non-methane hydrocarbon emitted into Earth’s atmosphere primarily from terrestrial vegetation, is now recognized as a major contributor to the global secondary organic aerosol (SOA) burden. Anthropogenic pollutants significantly enhance isoprene SOA formation through acid-catalyzed heterogeneous chemistry of epoxide products. Since isoprene SOA formation as a source of fine aerosol is a relatively recent discovery, research is lacking on evaluating its potential adverse effects on human health.

The objective of this study was to examine the effect of isoprene-derived SOA on inflammation-associated gene expression in human lung cells using a direct deposition exposure method. We assessed altered expression of inflammation-related genes in human bronchial epithelial cells (BEAS-2B) exposed to isoprene-derived SOA generated in an outdoor chamber facility. Measurements of gene expression of known inflammatory biomarkers interleukin 8 (IL-8) and cyclooxygenase 2 (COX-2) in exposed cells, together with complementary chemical measurements, showed that a dose of 0.067 µg cm\(^{-2}\) of SOA from isoprene photooxidation leads to statistically significant increases in IL-8 and COX-2 mRNA levels. Resuspension exposures using aerosol filter extracts corroborated these findings, supporting the conclusion that isoprene-derived SOA constituents induce the observed changes in mRNA levels. The present study is an attempt to examine the early biological responses of isoprene SOA exposure in human lung cells.
1. Introduction

Recent work has shown that isoprene (2-methyl-1,3-butadiene) is an important precursor of secondary organic aerosol (SOA), which has potential impacts on climate change and public health (Lin et al., 2013b; Rohr, 2013; Lin et al., 2016). Current understanding of isoprene SOA formation is based on laboratory studies showing that gas-phase photooxidation of isoprene generates key SOA precursors, including isomeric isoprene epoxydiols (IEPOX), methacrylic acid epoxide (MAE), hydroxymethyl-methyl-α-lactone (HMML), and isoprene hydroxyhydroperoxides (ISOPOOH) (Paulot et al., 2009; Surratt et al., 2010; Lin et al., 2012; Lin et al., 2013b; Nguyen et al., 2015; Krechmer et al., 2015). The formation of SOA from these precursors is influenced by controllable anthropogenic emissions such as oxides of nitrogen (NOx) and sulfur dioxide (SO2). Atmospheric oxidation of SO2 contributes to particle acidity, which enhances isoprene SOA formation through acid-catalyzed reactive uptake and multiphase chemistry of IEPOX and MAE (Surratt et al., 2007; Surratt et al., 2010; Lin et al., 2012; Gaston et al., 2014; Riedel et al., 2015), while NOx determines whether the oxidation pathway leading to IEPOX or MAE/HMML predominates (Lin et al., 2013b; Surratt et al., 2010; Nguyen et al., 2015). Isoprene SOA comprises a large portion of global atmospheric fine particles (PM2.5, aerosol with aerodynamic diameters ≤2.5 μm) (Carlton et al., 2009; Henze et al., 2008) but few studies have focused on its health implications (Lin et al., 2016). 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 (Pye et al., 2013; Gaston et al., 2014; Xu et al., 2015; Riedel et al., 2015).
Many studies have shown that particulate matter 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; Schwartz et al., 1993; Samet et al., 2000). 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/biological outcomes (Kelly and Fussell, 2012). Prior work on complex air mixtures has shown that gaseous volatile organic compounds (VOCs) alter the composition and ultimately the toxicity of particles (Ebersviller et al., 2012a, b). SOA resulting from natural and anthropogenic gaseous precursors, such as α-pinene and 1,3,5-trimethylbenzene, have been shown to affect cellular function (Gaschen et al., 2010; Jang et al., 2006) and recently isoprene-SOA formed from the reactive uptake of epoxides has been shown to induce the expression of oxidative stress genes (Lin et al., 2016).

The objective of this study is to generate atmospherically relevant isoprene-derived SOA and examine its toxicity through \textit{in vitro} exposures using a direct deposition device. Compared to exposure of cells in culture media to resuspended particles, direct particle deposition likely provides a more biologically relevant exposure model and enhances sensitivity of cells to air pollution particle exposures (Volckens et al., 2009; Lichtveld et al., 2012; Hawley et al., 2014a; Hawley et al., 2014b; Zavala et al., 2014; Hawley and Volckens, 2013). The Electrostatic Aerosol \textit{in vitro} Exposure System (EAVES) used in this study deposits particles generated in our outdoor photochemical chamber directly onto lung cells by electrostatic precipitation (de Bruijne et al., 2009). Similar techniques and devices have been used to expose cells to diesel exhaust particles (Lichtveld et al., 2012; Hawley et al., 2014b), but our study is the first to utilize the
EAVES to explore the potential adverse effects of isoprene SOA on human lung cells. Additionally, for a more atmospherically relevant exposure, isoprene-SOA was photochemically generated in an outdoor chamber to mimic its formation in the atmosphere.

We have recently demonstrated through a chemical assay that isoprene-derived SOA has the potential for inducing reactive oxygen species (ROS) (Kramer et al., 2016), which are linked to oxidative stress and inflammation (Reuter et al., 2010; Li et al., 2003). An in vitro study that followed supported the potential for isoprene-SOA to affect the levels of oxidative stress genes (Lin et al., 2016). In this study we chose to examine the gene expression levels of interleukin-8 (IL-8) and cyclooxygenase-2 (COX-2), 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). Other studies on air pollution mixtures have also examined IL-8 as a biological endpoint due to its involvement with inflammation (Zavala et al., 2014; Ebersviller et al., 2012a, b; Doyle et al., 2004; Doyle et al., 2007). 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. In addition, we collected SOA onto filters for subsequent resuspension exposure to ensure that effects observed from EAVES exposures were attributable to particle-phase organic products.

2. Experimental Section

2.1 Generation of SOA in the Outdoor Chamber Facility. SOA were generated by photochemically oxidizing a mixture of acidified sulfate seed aerosol, isoprene, and NO injected
into an outdoor smog chamber facility. The outdoor chamber is a 120-m³ triangular cross-section Teflon chamber located on the roof of the Gillings School of Global Public Health, University of North Carolina at Chapel Hill. The chamber facility has been described in detail elsewhere by Lichtveld et al. (2012). The outdoor chamber facility is equipped with sampling lines that allow direct deposition exposure of cells, online chemical measurements, and filter collection for offline chemical analysis. Sampling lines run from the underside of the chamber directly to the chemistry lab below where online measurement instruments and the direct deposition exposure device are located. Injection ports are also located on the underside of the chamber.

To generate isoprene-derived SOA, the chamber was operated on sunny days, under high relative humidity, to allow natural sunlight to trigger photochemical reactions. Acidified sulfate seed aerosols were generated by nebulizing an aqueous solution containing 0.06 M MgSO₄ + 0.06 M H₂SO₄ into the chamber to a particle concentration of approximately 170 μg m⁻³, which was allowed to stabilize for 30 min to ensure a well-mixed condition. After stabilization, 3.5 ppmv isoprene (Sigma-Aldrich, 99%) and 200 ppbv NO (AirGas, 1.00%) were injected into the chamber. Photochemical aging was allowed for approximately one hour to reach the desired exposure conditions of 30-40 g m⁻³ growth of isoprene-derived SOA on the pre-existing 170 g m⁻³ of acidified sulfate aerosol. This chamber experiment was replicated on three separate sunny days with temperatures ranging from 24.9°C to 26.8°C with a relative humidity of approximately 70% in the chamber.

2.2 Control Chamber Experiments. As a dark chamber control, to isolate the effect of SOA on exposed cells, mixtures of isoprene, NO, and 170 g 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.

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.

2.3 Cell Culture. Human bronchial epithelial (BEAS-2B) cells were maintained in keratinocyte growth medium (KGM BulletKit; Lonza), a serum-free keratinocyte basal medium (KBM) supplemented with 0.004% of bovine pituitary extract and 0.001% of human epidermal growth factor, insulin, hydrocortisone, and GA-1000 (gentamicin, amphotericin B), and passaged weekly. Passage number for photochemical exposures and dark control exposures varied between 52 and 60. Because BEAS-2B are 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).

2.4 Direct Deposition Exposure. In preparation for air-liquid interface exposures, cells were seeded onto collagen-coated Millicell cell culture inserts (30 mm diameter, 0.4 μm pore size, 4.2 cm² filter area; Millipore, Cambridge, MA) at a density of 200,000 cells/well 24 hours prior to exposure. At the time of exposure, cells reached ~80% confluence, confirmed through microscopy. Immediately before exposure, cell medium was removed from the apical and basolateral sides of 2 seeded Millicell cell culture inserts. One insert was transferred to a titanium
dish containing 1.5 mL of keratinocyte basal medium (KBM; Lonza), supplying cells with nutrients from the basolateral side and constant moisture while allowing exposure to be performed at an air-liquid interface. The other insert was transferred into a 6 well plate with 2 mL of KBM and placed in the incubator as an unexposed control.

Cells were exposed to chamber-generated isoprene SOA using the EAVES located in the laboratory directly beneath the outdoor chamber (de Bruijne et al., 2009; Lichtveld et al., 2012). The EAVES, located in an incubator at 37°C, sampled chamber air at 1 L min⁻¹. The target relative humidity (RH) in the chamber during EAVES exposures was approximately 70%. Exposure time was one hour commencing when target exposure conditions were achieved in the outdoor chamber for both photochemical and dark control experiments. Detailed description of the EAVES can be found in de Bruijne et al. (2009).

Following exposure, the cell culture insert was transferred to a 6-well tissue culture plate containing 2 mL of fresh KBM. The control Millicell was also transferred to 2 mL of fresh KBM. Nine hours post-exposure, extracellular medium was collected and total RNA was isolated using Trizol (Life Technologies), consistent with past studies (de Bruijne et al., 2009). Extracellular medium and the extracted RNA samples were stored at -20°C and -80°C, respectively, until further analysis. For quality assurance purposes, the RNA concentration and integrity were assessed using Nanodrop and Bioanalyzer over the period of storage. No changes were observed under the given storage conditions.

2.5 Filter Resuspension Exposure. Chamber particles were collected, concurrently with EAVES sampling, onto Teflon membrane filters (47 mm diameter, 1.0 µm 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 ozone (O$_3$) and NO$_x$ present in the direct
deposition experiments; however, prior studies have shown that gaseous components do not
yield cellular responses within the EAVES device (de Bruijne et al., 2009; Ebersviller et al.,
2012a, b). 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 g cm$^{-3}$ (Riedel et al., 2016) and 1.25 g 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. 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 combined filter extract was dried under a gentle stream of nitrogen (N$_2$). KBM medium was
then added into the extraction vials to re-dissolve SOA constituents.

In preparation for filter resuspension exposures, cells were seeded in 24-well plates at a
density of 2.5×10$^4$ cells/well in 250 µL of KGM 2 days prior to exposure. At the time of
exposure when cells reached ~80% confluence, cells were washed twice with phosphate buffered
saline (PBS) buffer, and then exposed to KBM containing 0.01 and 0.1 mg mL$^{-1}$ isoprene SOA
extract from photochemical experiment and seed particles from dark control experiments.

Following a 9-hour exposure, extracellular medium was collected and total RNA was
isolated using Trizol (Life Technologies) and stored alongside samples from direct deposition
exposures until further analysis.

2.6 Chemical and Physical Characterization of Exposures. Online and offline techniques
were used to characterize the SOA generated in the chamber. The online techniques measured
the gas-phase species NO, NO$_x$ and O$_3$ and the physical properties of the aerosol continuously
throughout the chamber experiments. Offline techniques measured aerosol-phase species collected onto Teflon membrane filters (47 mm diameter, 1.0 µm pore size; Pall Life Science) from photochemical and dark chamber experiments. Filter samples were stored in 20 mL scintillation vials protected from light at -20°C until analyses.

Real-time aerosol size distributions were measured using a Differential Mobility Analyzer (DMA, Brechtel Manufacturing Inc.) coupled to a Mixing Condensation Particle Counter (MCPC, Model 1710, Brechtel Manufacturing Inc.) located in the laboratory directly underneath the chamber. O₃ and NOₓ were measured with a ML 9811 series Ozone Photometer (Teledyne Monitor Labs, Englewood, CO) and ML 9841 series NOₓ Analyzer (American Ecotech, Warren RI), respectively. Data were collected at one-minute intervals using a data acquisition system (ChartScan/1400) interfaced to a computer. The presence of isoprene in the chamber was confirmed and quantified using a Varian 3800 gas chromatograph (GC) equipped with a flame ionization detector (FID).

Chemical characterization of SOA constituents was conducted offline from extracts of filters collected from chamber experiments by gas chromatography interfaced with an electron ionization quadrupole mass spectrometer (GC/EI-MS) or by ultra performance liquid chromatography interfaced with a high-resolution quadrupole time-of-flight mass spectrometer equipped with electrospray ionization (UPLC/ESI-HR-QTOFMS). Detailed operating conditions for the GC/EI-MS and UPLC/ESI-HR-QTOFMS analyses as well as detailed filter extraction protocols have been described previously by Lin et al. (2012). For GC/EI-MS analysis, filter extracts were dried under a gentle stream of N₂ and trimethylsilylated by the addition of 100 µL of BSTFA + TMCS (99:1 v/v, Supelco) and 50 µL of pyridine (anhydrous, 99.8%, Sigma-Aldrich) and heated at 70 °C for 1 h. For UPLC/ESI-HR-QTOFMS analysis, residues of filter
extracts were reconstituted with 150 µL of a 50:50 (v/v) solvent mixture of high-purity water and methanol.

The isoprene-derived SOA markers: 2-methyltetrols, isomeric 3-methyltetrahydrofurans-3,4-diols (3-MeTHF-3,4-diols), and 2-methylglyceric acid, synthesized according to the published procedures (Lin et al., 2013b; Zhang et al., 2012), were available in-house as authentic standards to quantify the major components of isoprene SOA. 2-Methyltetrol organosulfates, synthesized as a mixture of tetrabutylammonium salts, were also available as a standard. Purity was determined to be >99% by $^1$H NMR and UPLC/ESI-QTOFMS analysis (Budisulistiorini et al., 2015b). The C$_5$-alkene triols and IEPOX dimer were quantified using the response factor obtained for the synthetic 2-methyltetrols.

A representative ambient PM$_{2.5}$ sample collected from the rural southeastern U.S. (Yorkville, GA) (Lin et al., 2013a) during the summer of 2010 was analyzed in an identical manner to confirm atmospheric relevance of the chamber-generated SOA constituents.

2.7 Cytotoxicity Assay. Cytotoxicity was assessed through measurement of lactate dehydrogenase (LDH) released into the extracellular medium from damaged cells using the LDH cytotoxicity detection kit (Takara). To ensure that the EAVES device itself and operation procedure had no effect on cytotoxicity, the LDH release from cells exposed to clean chamber air was measured. LDH release by cells exposed via the EAVES to the photochemically aged (light) and non-photochemically aged (dark) particles was compared to release from unexposed cells maintained in the incubator for the same duration. For the resuspension exposures, LDH release by cells exposed to SOA through resuspended extract of photochemically aged and non-photochemically aged particles 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.

2.8 Gene Expression Analysis. We chose to measure the levels of the inflammation-related
mRNA in the BEAS-2B cells exposed to isoprene-derived SOA generated in our outdoor
chamber because various particle types are capable of sequestering cytokines (Seagrave, 2008).
Other direct deposition studies have also used mRNA transcripts as a proxy for cytokine
production (Hawley et al., 2014a; Hawley et al., 2014b; Hawley and Volckens, 2013; Volckens
et al., 2009; Lichtveld et al., 2012). Changes in IL-8 and COX-2 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 RT-PCR analysis. All mRNA levels
were normalized against β-actin mRNA, which was used as a housekeeping gene. The relative
expression levels (i.e., fold change) of IL-8 and COX-2 were calculated using the comparative
cycle threshold (2^(-ΔΔCT)) method (Livak and Schmittgen, 2001). For EAVES exposures, changes
in IL-8 and COX-2 from isoprene-derived SOA exposed cells were compared to cells exposed to
the dark controls. Similarly, for resuspension exposures changes in IL-8 and COX-2 from
isoprene-derived SOA exposed cells were compared to cells exposed to particles collected under
dark conditions.

2.9 Statistical Analysis. The software package GraphPad Prism 4 (GraphPad) was used for all
statistical analyses. All data were expressed as mean ± SEM (standard error of means).
Comparisons between data sets for cytotoxicity and gene expression analysis were made using
unpaired t-test with Welch’s correction. Significance was defined as p < 0.05.
3. Results and Discussion

3.1 Physical and Chemical Characterization of Exposure. Figure 1 shows the change in particle mass concentration and gas (O$_3$, NO, NO$_x$) concentration over time during typical photochemical and dark control experiments. Under dark control conditions (Fig. 1a) there is no increase in aerosol mass concentration following isoprene injection. Average total aerosol mass concentration was 155.0±2.69 µg m$^{-3}$ (1 standard deviation) with no particle mass attributable to organic material.

In contrast, Fig. 1b shows an increase in aerosol mass concentration after 1 h post isoprene injection, which can be attributed to the photochemical oxidation of isoprene and subsequent production and reactive uptake of its oxidation products. The average increase in aerosol mass concentration attributable to SOA formation for three daylight chamber experiments conducted on separate days was 44.5±5.7 µg m$^{-3}$. Average total aerosol mass concentration during particle exposure was 173.1± 4.2 µg m$^{-3}$.

O$_3$ and NO$_x$ concentrations measured during EAVES exposure were approximately 270 ppb and 120 ppb for photochemical experiments. For dark control experiments (e.g., Fig. 1a), the O$_3$ and NO$_x$ concentrations were approximately 15 ppb and 180 ppb. Previous studies characterizing the EAVES device show definitively that gas-phase products do not induce cell response (de Bruijne et al., 2009). However, resuspension exposures were conducted in addition to EAVES exposure to ensure that biological effects were attributable to only particle-phase constituents and not gas-phase products such as O$_3$ and NO$_x$.

The chemical composition of aerosol, collected onto filters concurrently with cell exposure and characterized by GC/EI-MS and UPLC/ESI-HR-QTOFMS, are shown in Fig. 2. No isoprene-SOA tracers were observed in the filters collected from dark control experiments.
The dominant particle-phase products of the isoprene-SOA collected from photochemical experiments are derived from the low-NO channel, where IEPOX reactive uptake onto acidic sulfate aerosol dominates, including 2-methyltetrols, C5-alkene triols, isomeric 3-MeTHF-3,4-diols, IEPOX-derived dimers, and IEPOX-derived organosulfates. The sum of the IEPOX-derived SOA constituents quantified by the available standards accounted for ~80% of the observed SOA mass. The MAE-derived SOA constituents 2-methylglyceric acid and the organosulfate derivative of MAE, derived from the high-NO channel, accounted for 1.4% of the observed SOA mass, confirming that particle-phase products generated were predominantly formed from the reactive uptake of IEPOX onto acidic sulfate aerosols. As demonstrated in Figure 2, all the same particle-phase products are measured in the PM$_{2.5}$ sample collected in Yorkville, GA (a typical low-NO region), demonstrating that the composition of the chamber-generated SOA is atmospherically relevant. Recent SOA tracer measurements from the Southern Oxidant and Aerosol Study (SOAS) campaign at Look Rock, TN, Centerville, AL, and Birmingham, AL, also support the atmospheric relevance of IEPOX-derived SOA constituents that dominate the isoprene SOA mass in summer in the southeastern U.S. (Budisulistiorini et al., 2015a; Rattanavaraha et al., 2016).

3.2 Cytotoxicity. LDH release for cells exposed using the EAVES device is expressed as a fold-change relative to the unexposed incubator control. For resuspension exposures, LDH release is expressed as fold-change relative to cells exposed to KBM only. Results shown in Fig. 3a confirm that there is no effect of chamber conditions and device operation on the cells when comparing LDH release from cells exposed to a clean air chamber and cells unexposed in an incubator. Additionally, LDH release from all exposure conditions in EAVES exposed cells (Fig. 3b) and resuspension exposed cells (Fig. 3c) 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. All cytotoxicity results ensured that exposure conditions were not adversely affecting the cells nor their gene expression.

3.3 Pro-inflammatory Gene Expression. Changes in the mRNA levels of *IL-8* and *COX-2* from cells exposed to isoprene-derived SOA using the EAVES are shown as fold-changes relative to dark controls in Fig. 4. This comparison, as well as the results of the resuspension experiment discussed below, ensure that all effects seen in the cells are attributable to the isoprene-derived SOA and no other factors. A one-hour exposure to a mass concentration of approximately 45 µg 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 et al. (2009), the estimated dose was 0.29 µg cm$^{-2}$ of total particle mass with 23% attributable to organic material formed from isoprene photooxidation (0.067 µg cm$^{-2}$ of SOA).

Changes in the mRNA levels of *IL-8* and *COX-2* 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 in Fig. 5. At a low dose of 0.01 mg mL$^{-1}$ of isoprene SOA extract there is no significant increase in *IL-8* and *COX-2* mRNA expression. The isoprene SOA extract, however, induces a response at a dose of 0.1 mg mL$^{-1}$. The statistically significant increase in mRNA expression from the resuspension exposure at 0.1 mg mL$^{-1}$ confirms that similar fold changes observed for both *IL-8* and *COX-2* from the EAVES exposures are not attributable to gaseous photooxidation products, such as O$_3$, and support the characterization of the EAVES as a particle exposure device (de Bruijne et al., 2009).
The similar fold change observed in both the EAVES exposure and resuspension exposure, 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 may yield results similar to direct deposition exposures. Unlike diesel particulate extracts, which agglomerate during resuspension exposures, isoprene-derived SOA constituents are water-soluble based on reverse-phase LC separations (Surratt et al., 2006; Lin et al., 2012) 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 SOA.

3.4 Biological Implications. The goal of this study was to initially identify potential biological response associated with exposure to isoprene-derived SOA by using a direct exposure device as a model that has both atmospheric and physiological relevance. With this model, a dose of 0.067 µg cm$^{-2}$ of isoprene SOA, induced statistically significant increases in IL-8 and COX-2 mRNA levels in exposed BEAS-2B cells. There are many ways to classify in vitro particle dosimetry based on the various properties of particles (Paur et al., 2011). 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 et al. (2011) used a multiple-path particle dosimetry (MPPD) model to estimate that the lung deposition of ultrafine particles ranges from 0.006 to 0.02 µg cm$^{-2}$ for a 24-hr exposure to a particle concentration of 0.1 mg m$^{-3}$. Based on this estimate, a dose of 0.067 µg cm$^{-2}$ of isoprene 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).
Our findings are consistent with other studies showing that photochemical oxidation of similar chemical mixtures increases toxicity in cell culture models and elevates expression of inflammatory biomarker genes (Lichtveld et al., 2012; Rager et al., 2011). Previous in vitro studies using a gas-phase only exposure system have shown that gas-phase products of isoprene photooxidation significantly enhance cytotoxicity and IL-8 expression (Doyle et al., 2004; Doyle et al., 2007).

By choosing IL-8 and COX-2 as our genes of interest, we are able to compare our results to other studies of known harmful particle exposures. In a similar study using the EAVES, normal human bronchial epithelial (NHBE) cells exposed to 1.10 µg cm\(^{-2}\) diesel particulate matter showed less than a 2-fold change over controls in both IL-8 and COX-2 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 µg 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 COX-2 mRNA expression, while exposure to aged Volkswagen particulates induced a change of ~1.5-fold in IL-8 and 2-fold in COX-2 mRNA expression (Lichtveld et al., 2012). Although the differences in cell types preclude direct comparisons, the finding of significant increases in COX-2 and IL-8 expression at doses much lower than reported for comparable increases in gene expression levels induced by photochemically-aged diesel particulates is notable.

IL-8 and COX-2 are both linked to inflammation and oxidative stress (Kunkel et al., 1991; Uchida, 2008). IL-8 is a potent neutrophil chemotactic factor in the lung and its expression by various cells plays a crucial role in neutrophil recruitment leading to lung inflammation (Kunkel et al., 1991). COX-2 is the inducible form of the cyclooxygenase enzyme, regulated by
cytokines and mitogens, and is responsible for prostaglandin synthesis associated with inflammation (FitzGerald, 2003). Consistent with the reports that IL-8 and COX-2 play important roles in lung inflammation (Nocker et al., 1996; Li et al., 2013), in vivo studies have shown that isoprene oxidation products cause airflow limitation and sensory irritation in mice (Rohr et al., 2003). In humans, the role of IL-8 and COX-2 in lung inflammation can be associated with diseases such as chronic obstructive pulmonary disease and asthma (Nocker et al., 1996; Peng et al., 2008; Fong et al., 2000).

The mechanism by which isoprene-SOA causes elevation of the inflammatory markers IL-8 and COX-2 is not yet fully understood. However, recent work from our laboratory using the acellular dithiothreitol (DTT) assay demonstrated that isoprene-derived SOA has significant ROS generation have equal or greater ROS generation potential than diesel exhaust PM (Kramer et al., 2016). High levels of ROS in cells can overwhelm the antioxidant defense and lead to cellular oxidative stress (Sies, 1991; Bowler and Crapo, 2002; Li et al., 2003). Following the discovery of the potential importance of isoprene-SOA in generating ROS, Lin et al. (2016) showed that isoprene-SOA formed from the reactive uptake of epoxides alters levels of oxidative stress-associated genes, including COX-2 in human lung cells. Oxidative stress caused by ROS plays a major role in lung inflammation and the induction of oxidative stress can lead to IL-8 expression (Tao et al., 2003; Yan et al., 2015). Specifically, oxidants can activate the transcription factor NF-κB, which regulates a wide range of inflammatory genes including IL-8 and COX-2 (Barnes and Adcock, 1997; Schreck et al., 1992). Pathway analysis showed that gene expression of the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway was induced in cells exposed to isoprene-SOA (Lin et al., 2016) which has been reported to alter the expression of IL-8 through mRNA stabilization (Zhang et al., 2005). Therefore, isoprene-SOA
may cause increases in both IL-8 and COX-2 primarily through an oxidative stress response. Additionally, the relationship between IL-8 and COX-2 can also explain the observed increase in IL-8 gene expression as the production of IL-8 can be stimulated through a COX-2 dependent mechanism in airway epithelial cells (Peng et al., 2008).

*In vitro* studies such as this one using a direct deposition model cannot fully elucidate mechanisms of lung inflammation and potential pathogenesis but serve as a necessary part of hazard characterization, particularly for a complex air mixture that has not been fully studied (Hayashi, 2005; Paur et al., 2011). Ozone exposure studies have shown that comparable dose and effect measurements for IL-8 and COX-2 can be found between *in vivo* and *in vitro* exposures which add promise to extrapolating effects seen *in vitro* to effects *in vivo* (Hatch et al., 2014). *In vivo* effects associated with isoprene-SOA exposure *in vitro* cannot be inferred as it is a different system from ozone, so therefore, further *in vitro* studies exploring the health implication of the elevation of IL-8 and COX-2 due specifically to isoprene-SOA exposure are necessary and may in turn justify further extension to *in vivo* work.

**4. Conclusions**

This study indicates that an atmospherically relevant composition of isoprene-derived SOA is capable of increasing the expression of IL-8 and COX-2 in human bronchial epithelial cells. The present study is an initial step in a long planned analysis of the biological impacts of isoprene SOA exposure on lung cells. The SOA were generated as NO levels approached zero, which represents conditions characteristic of urban locales downwind of rural isoprene sources. As shown in Fig. 2, the aerosol generated for exposures in this study are chemically similar to fine aerosol samples collected from the Southeastern U.S., which indicates that the chamber exposures are representative of exposures that may be encountered by populations in regions
where isoprene emissions interact with anthropogenic pollutants. The same particle-phase products found in our photochemical experiments have been measured in significant quantities (accounting on average for 33% of fine organic aerosol mass) in ambient fine organic particles collected in the Southeastern U.S. (Lin et al., 2013b; Budisulistiorini et al., 2013; Rattanavaraha et al., 2016; Budisulistiorini et al., 2016) and in other isoprene-rich environments (Hu et al., 2015). The results of this study show that, because of its abundance, isoprene SOA may be a public health concern warranting further toxicological investigation through in vitro or in vivo work.

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Figure 1. Aerosol mass concentration and gas-phase product concentrations over time for (a) dark control chamber experiment and (b) photochemically produced isoprene-derived SOA exposure chamber experiment.
Figure 2. (a) GC/EI-MS total ion chromatograms (TICs) and (b) UPLC/ESI-HR-QTOFMS base peak chromatograms (BPCs) from a (1) dark control chamber experiment, (2) isoprene-derived SOA exposure chamber experiment, and (3) PM$_{2.5}$ sample collected from Yorkville, GA during summer 2010.
**Figure 3.** LDH release for (a) clean air controls, (b) EAVES exposures, normalized to incubator control, and (c) resuspension exposures, normalized to KBM only control. **p<0.005 and ***p<0.0005.
**Figure 4.** *IL*-8 and *COX*-2 mRNA expression induced by exposure to isoprene-derived SOA using EAVES device all normalized to dark control experiments and against housekeeping gene, β-actin. All experiments conducted in triplicate. ***p<0.0005.
Figure 5. IL-8 and COX-2 expression induced by exposure to isoprene-derived SOA using resuspension method all normalized to dark control experiments and against housekeeping gene, β-actin. All experiments conducted in triplicate. *p<0.05 and **p<0.005.