

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 know. 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 is 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 portion, 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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1 ***In Vitro* Exposure to Isoprene-Derived Secondary Organic Aerosol by Direct Deposition**
2 **and its Effects on COX-2 and IL-8 Gene Expression**

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30 **Abstract**

31 Atmospheric oxidation of isoprene, the most abundant non-methane hydrocarbon emitted into
32 Earth's atmosphere primarily from terrestrial vegetation, is now recognized as a major
33 contributor to the global secondary organic aerosol (SOA) burden. Anthropogenic pollutants
34 significantly enhance isoprene SOA formation through acid-catalyzed heterogeneous chemistry
35 of epoxide products. Since isoprene SOA formation as a source of fine aerosol is a relatively
36 recent discovery, research is lacking on evaluating its potential adverse effects on human health.
37 The objective of this study was to examine the effect of isoprene-derived SOA on inflammation-
38 associated gene expression in human lung cells using a direct deposition exposure method. We
39 assessed altered expression of inflammation-related genes in human bronchial epithelial cells
40 (BEAS-2B) exposed to isoprene-derived SOA generated in an outdoor chamber facility.
41 Measurements of gene expression of known inflammatory biomarkers interleukin 8 (IL-8) and
42 cyclooxygenase 2 (COX-2) in exposed cells, together with complementary chemical
43 measurements, showed that a dose of $0.067 \mu\text{g cm}^{-2}$ of SOA from isoprene photooxidation leads
44 to statistically significant increases in *IL-8* and *COX-2* mRNA levels. Resuspension exposures
45 using aerosol filter extracts corroborated these findings, supporting the conclusion that isoprene-
46 derived SOA constituents induce the observed changes in mRNA levels. The present study is an
47 attempt to examine the early biological responses of isoprene SOA exposure in human lung cells.

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52 **1. Introduction**

53 Recent work has shown that isoprene (2-methyl-1,3-butadiene) is an important precursor
54 of secondary organic aerosol (SOA), which has potential impacts on climate change and public
55 health (Lin et al., 2013b; Rohr, 2013; Lin et al., 2016). Current understanding of isoprene SOA
56 formation is based on laboratory studies showing that gas-phase photooxidation of isoprene
57 generates key SOA precursors, including isomeric isoprene epoxydiols (IEPOX), methacrylic
58 acid epoxide (MAE), hydroxymethyl-methyl- lactone (HMML), and isoprene
59 hydroxyhydroperoxides (ISOPOOH) (Paulot et al., 2009; Surratt et al., 2010; Lin et al., 2012;
60 Lin et al., 2013b; Nguyen et al., 2015; Krechmer et al., 2015). The formation of SOA from these
61 precursors is influenced by controllable anthropogenic emissions such as oxides of nitrogen
62 (NO_x) and sulfur dioxide (SO_2). Atmospheric oxidation of SO_2 contributes to particle acidity,
63 which enhances isoprene SOA formation through acid-catalyzed reactive uptake and multiphase
64 chemistry of IEPOX and MAE (Surratt et al., 2007; Surratt et al., 2010; Lin et al., 2012; Gaston
65 et al., 2014; Riedel et al., 2015), while NO_x determines whether the oxidation pathway leading to
66 IEPOX or MAE/HMML predominates (Lin et al., 2013b; Surratt et al., 2010; Nguyen et al.,
67 2015). Isoprene SOA comprises a large portion of global atmospheric fine particles ($\text{PM}_{2.5}$,
68 aerosol with aerodynamic diameters $\leq 2.5 \mu\text{m}$) (Carlton et al., 2009; Henze et al., 2008) but few
69 studies have focused on its health implications (Lin et al., 2016). Evaluating the health effects of
70 SOA from isoprene oxidation is important from a public health perspective, not only because of
71 its atmospheric abundance, but also because the anthropogenic contribution is the only
72 component amenable to control (Pye et al., 2013; Gaston et al., 2014; Xu et al., 2015; Riedel et
73 al., 2015).

74 Many studies have shown that particulate matter is closely linked to health effects
75 ranging from exacerbation of asthma symptoms to mortality associated with lung cancer and
76 cardiopulmonary disease (Dockery et al., 1993; Schwartz et al., 1993; Samet et al., 2000). PM_{2.5},
77 in particular, has been linked to negative health outcomes with an estimated contribution of 3.2
78 million premature deaths worldwide as reported in the Global Burden of Disease Study 2010
79 (Lim et al., 2012). Despite evidence that particle composition affects toxicity, fewer studies
80 focus on the link between chemical composition and health/biological outcomes (Kelly and
81 Fussell, 2012). Prior work on complex air mixtures has shown that gaseous volatile organic
82 compounds (VOCs) alter the composition and ultimately the toxicity of particles (Ebersviller et
83 al., 2012a, b). SOA resulting from natural and anthropogenic gaseous precursors, such as -
84 pinene and 1,3,5-trimethylbenzene, have been shown to affect cellular function (Gaschen et al.,
85 2010; Jang et al., 2006) and recently isoprene-SOA formed from the reactive uptake of epoxides
86 has been shown to induce the expression of oxidative stress genes (Lin et al., 2016).

87 The objective of this study is to generate atmospherically relevant isoprene-derived SOA
88 and examine its toxicity through *in vitro* exposures using a direct deposition device. Compared to
89 exposure of cells in culture media to resuspended particles, direct particle deposition likely
90 provides a more biologically relevant exposure model and enhances sensitivity of cells to air
91 pollution particle exposures (Volckens et al., 2009; Lichtveld et al., 2012; Hawley et al., 2014a;
92 Hawley et al., 2014b; Zavala et al., 2014; Hawley and Volckens, 2013). The Electrostatic
93 Aerosol *in vitro* Exposure System (EAVES) used in this study deposits particles generated in our
94 outdoor photochemical chamber directly onto lung cells by electrostatic precipitation (de Bruijne
95 et al., 2009). Similar techniques and devices have been used to expose cells to diesel exhaust
96 particles (Lichtveld et al., 2012; Hawley et al., 2014b), but our study is the first to utilize the

97 EAVES to explore the potential adverse effects of isoprene SOA on human lung cells.
98 Additionally, for a more atmospherically relevant exposure, isoprene-SOA was photochemically
99 generated in an outdoor chamber to mimic its formation in the atmosphere.

100 We have recently demonstrated through a chemical assay that isoprene-derived SOA has
101 the potential for inducing reactive oxygen species (ROS) (Kramer et al., 2016), which are linked
102 to oxidative stress and inflammation (Reuter et al., 2010; Li et al., 2003). An *in vitro* study that
103 followed supported the potential for isoprene-SOA to affect the levels of oxidative stress genes
104 (Lin et al., 2016). In this study we chose to examine the gene expression levels of interleukin-8
105 (*IL-8*) and cyclooxygenase-2 (*COX-2*), not only for their links to inflammation and oxidative
106 stress (Kunkel et al., 1991; Uchida, 2008), but because both have been examined in previous
107 studies using the EAVES for fresh and aged diesel exhaust (Lichtveld et al., 2012). Other studies
108 on air pollution mixtures have also examined *IL-8* as a biological endpoint due to its involvement
109 with inflammation (Zavala et al., 2014; Ebersviller et al., 2012a, b; Doyle et al., 2004; Doyle et
110 al., 2007). We compared the gene expression levels in cells exposed to SOA generated in an
111 outdoor chamber from photochemical oxidation of isoprene in the presence of NO and acidified
112 sulfate seed aerosol to cells exposed to a dark control mixture of isoprene, NO, and acidified
113 sulfate seed aerosol to isolate the effects of the isoprene-derived SOA on the cells using the
114 EAVES. In addition, we collected SOA onto filters for subsequent resuspension exposure to
115 ensure that effects observed from EAVES exposures were attributable to particle-phase organic
116 products.

117 **2. Experimental Section**

118 **2.1 Generation of SOA in the Outdoor Chamber Facility.** SOA were generated by
119 photochemically oxidizing a mixture of acidified sulfate seed aerosol, isoprene, and NO injected

120 into an outdoor smog chamber facility. The outdoor chamber is a 120-m³ triangular cross-section
121 Teflon chamber located on the roof of the Gillings School of Global Public Health, University of
122 North Carolina at Chapel Hill. The chamber facility has been described in detail elsewhere by
123 Lichtveld et al. (2012). The outdoor chamber facility is equipped with sampling lines that allow
124 direct deposition exposure of cells, online chemical measurements, and filter collection for
125 offline chemical analysis. Sampling lines run from the underside of the chamber directly to the
126 chemistry lab below where online measurement instruments and the direct deposition exposure
127 device are located. Injection ports are also located on the underside of the chamber.

128 To generate isoprene-derived SOA, the chamber was operated on sunny days, under high
129 relative humidity, to allow natural sunlight to trigger photochemical reactions. Acidified sulfate
130 seed aerosols were generated by nebulizing an aqueous solution containing 0.06 M MgSO₄ +
131 0.06 M H₂SO₄ into the chamber to a particle concentration of approximately 170 µg m⁻³, which
132 was allowed to stabilize for 30 min to ensure a well-mixed condition. After stabilization, 3.5
133 ppmv isoprene (Sigma-Aldrich, 99%) and 200 ppbv NO (AirGas, 1.00%) were injected into the
134 chamber. Photochemical aging was allowed for approximately one hour to reach the desired
135 exposure conditions of 30-40 µg m⁻³ growth of isoprene-derived SOA on the pre-existing 170 µg
136 m⁻³ of acidified sulfate aerosol. This chamber experiment was replicated on three separate sunny
137 days with temperatures ranging from 24.9°C to 26.8°C with a relative humidity of approximately
138 70% in the chamber.

139 **2.2 Control Chamber Experiments.** As a dark chamber control, to isolate the effect of SOA on
140 exposed cells, mixtures of isoprene, NO, and 170 µg m⁻³ of acidified sulfate seed aerosol were
141 injected into the chamber in the dark (after sunset). Conducting the chamber experiments in the
142 dark ensured no photochemical oxidation of isoprene. The dark control was replicated on three

143 different nights. Except for the absence of solar radiation (no SOA), all chamber operations and
144 exposure conditions were similarly maintained.

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

150 **2.3 Cell Culture.** Human bronchial epithelial (BEAS-2B) cells were maintained in keratinocyte
151 growth medium (KGM BulletKit; Lonza), a serum-free keratinocyte basal medium (KBM)
152 supplemented with 0.004% of bovine pituitary extract and 0.001% of human epidermal growth
153 factor, insulin, hydrocortisone, and GA-1000 (gentamicin, amphotericin B), and passaged
154 weekly. Passage number for photochemical exposures and dark control exposures varied
155 between 52 and 60. Because BEAS-2B are an immortalized line of human bronchial epithelium,
156 there are limitations with its use such as it being genetically homogeneous, being a single cell
157 type, and being SV-40 transformed (Reddel et al., 1988). However, BEAS-2B is a stable,
158 proliferative cell line shown to be useful in airway inflammation studies such as ours (Devlin et
159 al., 1994).

160 **2.4 Direct Deposition Exposure.** In preparation for air-liquid interface exposures, cells were
161 seeded onto collagen-coated Millicell cell culture inserts (30 mm diameter, 0.4 μm pore size, 4.2
162 cm^2 filter area; Millipore, Cambridge, MA) at a density of 200,000 cells/well 24 hours prior to
163 exposure. At the time of exposure, cells reached ~80% confluence, confirmed through
164 microscopy. Immediately before exposure, cell medium was removed from the apical and
165 basolateral sides of 2 seeded Millicell cell culture inserts. One insert was transferred to a titanium

166 dish containing 1.5 mL of keratinocyte basal medium (KBM; Lonza), supplying cells with
167 nutrients from the basolateral side and constant moisture while allowing exposure to be
168 performed at an air-liquid interface. The other insert was transferred into a 6 well plate with 2
169 mL of KBM and placed in the incubator as an unexposed control.

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

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

185 **2.5 Filter Resuspension Exposure.** Chamber particles were collected, concurrently with
186 EAVES sampling, onto Teflon membrane filters (47 mm diameter, 1.0 µm pore size; Pall Life
187 Science) for photochemical (light) and dark chamber experiments to be used for chemical
188 analysis and resuspension exposures. The resuspension experiments served as a control for

189 possible effects of gaseous components such as ozone (O₃) and NO_x present in the direct
190 deposition experiments; however, prior studies have shown that gaseous components do not
191 yield cellular responses within the EAVES device (de Bruijne et al., 2009; Ebersviller et al.,
192 2012a, b). Mass loadings of SOA collected on the filters were calculated from sampling volumes
193 and average aerosol mass concentrations in the chamber during the sampling period. A density
194 correction of 1.6 g cm⁻³ (Riedel et al., 2016) and 1.25 g cm⁻³ (Kroll et al., 2006) was applied to
195 convert the measured volume concentrations to mass concentrations for the acidified sulfate seed
196 and SOA growth, respectively. The particles collected on Teflon filter membranes for
197 resuspension cell exposure were extracted by sonication in high-purity methanol (LC/MS
198 CHROMASOLV, Sigma-Aldrich). Filter samples from multiple experiments were combined and
199 the combined filter extract was dried under a gentle stream of nitrogen (N₂). KBM medium was
200 then added into the extraction vials to re-dissolve SOA constituents.

201 In preparation for filter resuspension exposures, cells were seeded in 24-well plates at a
202 density of 2.5×10⁴ cells/well in 250 μL of KGM 2 days prior to exposure. At the time of
203 exposure when cells reached ~80% confluence, cells were washed twice with phosphate buffered
204 saline (PBS) buffer, and then exposed to KBM containing 0.01 and 0.1 mg mL⁻¹ isoprene SOA
205 extract from photochemical experiment and seed particles from dark control experiments.

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

209 **2.6 Chemical and Physical Characterization of Exposures.** Online and offline techniques
210 were used to characterize the SOA generated in the chamber. The online techniques measured
211 the gas-phase species NO, NO_x and O₃ and the physical properties of the aerosol continuously

212 throughout the chamber experiments. Offline techniques measured aerosol-phase species
213 collected onto Teflon membrane filters (47 mm diameter, 1.0 μm pore size; Pall Life Science)
214 from photochemical and dark chamber experiments. Filter samples were stored in 20 mL
215 scintillation vials protected from light at -20°C until analyses.

216 Real-time aerosol size distributions were measured using a Differential Mobility
217 Analyzer (DMA, Brechtel Manufacturing Inc.) coupled to a Mixing Condensation Particle
218 Counter (MCPC, Model 1710, Brechtel Manufacturing Inc.) located in the laboratory directly
219 underneath the chamber. O_3 and NO_x were measured with a ML 9811 series Ozone Photometer
220 (Teledyne Monitor Labs, Englewood, CO) and ML 9841 series NO_x Analyzer (American
221 Ecotech, Warren RI), respectively. Data were collected at one-minute intervals using a data
222 acquisition system (ChartScan/1400) interfaced to a computer. The presence of isoprene in the
223 chamber was confirmed and quantified using a Varian 3800 gas chromatograph (GC) equipped
224 with a flame ionization detector (FID).

225 Chemical characterization of SOA constituents was conducted offline from extracts of
226 filters collected from chamber experiments by gas chromatography interfaced with an electron
227 ionization quadrupole mass spectrometer (GC/EI-MS) or by ultra performance liquid
228 chromatography interfaced with a high-resolution quadrupole time-of-flight mass spectrometer
229 equipped with electrospray ionization (UPLC/ESI-HR-QTOFMS). Detailed operating conditions
230 for the GC/EI-MS and UPLC/ESI-HR-QTOFMS analyses as well as detailed filter extraction
231 protocols have been described previously by Lin et al. (2012). For GC/EI-MS analysis, filter
232 extracts were dried under a gentle stream of N_2 and trimethylsilylated by the addition of 100 μL
233 of BSTFA + TMCS (99:1 v/v, Supelco) and 50 μL of pyridine (anhydrous, 99.8%, Sigma-
234 Aldrich) and heated at 70°C for 1 h. For UPLC/ESI-HR-QTOFMS analysis, residues of filter

235 extracts were reconstituted with 150 μ L of a 50:50 (v/v) solvent mixture of high-purity water and
236 methanol.

237 The isoprene-derived SOA markers: 2-methyltetrols, isomeric 3-methyltetrahydrofurans-
238 3,4-diols (3-MeTHF-3,4-diols), and 2-methylglyceric acid, synthesized according to the
239 published procedures (Lin et al., 2013b; Zhang et al., 2012), were available in-house as authentic
240 standards to quantify the major components of isoprene SOA. 2-Methyltetrol organosulfates,
241 synthesized as a mixture of tetrabutylammonium salts, were also available as a standard. Purity
242 was determined to be >99% by ^1H NMR and UPLC/ESI-QTOFMS analysis (Budisulistiorini et
243 al., 2015b). The C_5 -alkene triols and IEPOX dimer were quantified using the response factor
244 obtained for the synthetic 2-methyltetrols.

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

248 **2.7 Cytotoxicity Assay.** Cytotoxicity was assessed through measurement of lactate
249 dehydrogenase (LDH) released into the extracellular medium from damaged cells using the LDH
250 cytotoxicity detection kit (Takara). To ensure that the EAVES device itself and operation
251 procedure had no effect on cytotoxicity, the LDH release from cells exposed to clean chamber air
252 was measured. LDH release by cells exposed via the EAVES to the photochemically aged (light)
253 and non-photochemically aged (dark) particles was compared to release from unexposed cells
254 maintained in the incubator for the same duration. For the resuspension exposures, LDH release
255 by cells exposed to SOA through resuspended extract of photochemically aged and non-
256 photochemically aged particles was compared to release by cells maintained in KBM only.
257 Additionally, LDH release from the light exposures, dark control, and resuspension exposures

258 was compared to release by positive control cells exposed to 1% Triton X-100 to ensure that cell
259 death would not affect gene expression results.

260 **2.8 Gene Expression Analysis.** We chose to measure the levels of the inflammation-related
261 mRNA in the BEAS-2B cells exposed to isoprene-derived SOA generated in our outdoor
262 chamber because various particle types are capable of sequestering cytokines (Seagrave, 2008).
263 Other direct deposition studies have also used mRNA transcripts as a proxy for cytokine
264 production (Hawley et al., 2014a; Hawley et al., 2014b; Hawley and Volckens, 2013; Volckens
265 et al., 2009; Lichtveld et al., 2012). Changes in *IL-8* and *COX-2* mRNA levels were measured
266 using QuantiTect SYBR Green RT-PCR Kit (Qiagen) and QuantiTect Primer Assays for
267 Hs_ACTB_1_SG (Catalog #QT00095431), Hs_PTGS2_1_SG (Catalog #QT00040586), and
268 Hs_CXCL8_1_SG (Catalog #QT00000322) for one-step RT-PCR analysis. All mRNA levels
269 were normalized against β -actin mRNA, which was used as a housekeeping gene. The relative
270 expression levels (i.e., fold change) of *IL-8* and *COX-2* were calculated using the comparative
271 cycle threshold (2^{-CT}) method (Livak and Schmittgen, 2001). For EAVES exposures, changes
272 in *IL-8* and *COX-2* from isoprene-derived SOA exposed cells were compared to cells exposed to
273 the dark controls. Similarly, for resuspension exposures changes in *IL-8* and *COX-2* from
274 isoprene-derived SOA exposed cells were compared to cells exposed to particles collected under
275 dark conditions.

276 **2.9 Statistical Analysis.** The software package GraphPad Prism 4 (GraphPad) was used for all
277 statistical analyses. All data were expressed as mean \pm SEM (standard error of means).
278 Comparisons between data sets for cytotoxicity and gene expression analysis were made using
279 unpaired *t*-test with Welch's correction. Significance was defined as $p < 0.05$.

280 3. Results and Discussion

281 **3.1 Physical and Chemical Characterization of Exposure.** Figure 1 shows the change in
282 particle mass concentration and gas (O_3 , NO, NO_x) concentration over time during typical
283 photochemical and dark control experiments. Under dark control conditions (Fig. 1a) there is no
284 increase in aerosol mass concentration following isoprene injection. Average total aerosol mass
285 concentration was $155.0 \pm 2.69 \mu\text{g m}^{-3}$ (1 standard deviation) with no particle mass attributable to
286 organic material.

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

293 O_3 and NO_x concentrations measured during EAVES exposure were approximately 270
294 ppb and 120 ppb for photochemical experiments. For dark control experiments (e.g., Fig. 1a), the
295 O_3 and NO_x concentrations were approximately 15 ppb and 180 ppb. Previous studies
296 characterizing the EAVES device show definitively that gas-phase products do not induce cell
297 response (de Bruijne et al., 2009). However, resuspension exposures were conducted in addition
298 to EAVES exposure to ensure that biological effects were attributable to only particle-phase
299 constituents and not gas-phase products such as O_3 and NO_x .

300 The chemical composition of aerosol, collected onto filters concurrently with cell
301 exposure and characterized by GC/EI-MS and UPLC/ESI-HR-QTOFMS, are shown in Fig. 2.
302 No isoprene-SOA tracers were observed in the filters collected from dark control experiments.

303 The dominant particle-phase products of the isoprene-SOA collected from photochemical
304 experiments are derived from the low-NO channel, where IEPOX reactive uptake onto acidic
305 sulfate aerosol dominates, including 2-methyltetrols, C5-alkene triols, isomeric 3-MeTHF-3,4-
306 diols, IEPOX-derived dimers, and IEPOX-derived organosulfates. The sum of the IEPOX-
307 derived SOA constituents quantified by the available standards accounted for ~80% of the
308 observed SOA mass. The MAE-derived SOA constituents 2-methylglyceric acid and the
309 organosulfate derivative of MAE, derived from the high-NO channel, accounted for 1.4% of the
310 observed SOA mass, confirming that particle-phase products generated were predominantly
311 formed from the reactive uptake of IEPOX onto acidic sulfate aerosols. As demonstrated in
312 Figure 2, all the same particle-phase products are measured in the PM_{2.5} sample collected in
313 Yorkville, GA (a typical low-NO region), demonstrating that the composition of the chamber-
314 generated SOA is atmospherically relevant. Recent SOA tracer measurements from the Southern
315 Oxidant and Aerosol Study (SOAS) campaign at Look Rock, TN, Centerville, AL, and
316 Birmingham, AL, also support the atmospheric relevance of IEPOX-derived SOA constituents
317 that dominate the isoprene SOA mass in summer in the southeastern U.S. (Budisulistiorini et al.,
318 2015a; Rattanavaraha et al., 2016).

319 **3.2 Cytotoxicity.** LDH release for cells exposed using the EAVES device is expressed as a fold-
320 change relative to the unexposed incubator control. For resuspension exposures, LDH release is
321 expressed as fold-change relative to cells exposed to KBM only. Results shown in Fig. 3a
322 confirm that there is no effect of chamber conditions and device operation on the cells when
323 comparing LDH release from cells exposed to a clean air chamber and cells unexposed in an
324 incubator. Additionally, LDH release from all exposure conditions in EAVES exposed cells (Fig.
325 3b) and resuspension exposed cells (Fig. 3c) is negligible relative to positive controls exposed to

326 1% Triton X-100, confirming that the exposure concentration of isoprene-derived SOA utilized
327 in this study was not cytotoxic. All cytotoxicity results ensured that exposure conditions were not
328 adversely affecting the cells nor their gene expression.

329 **3.3 Pro-inflammatory Gene Expression.** Changes in the mRNA levels of *IL-8* and *COX-2*
330 from cells exposed to isoprene-derived SOA using the EAVES are shown as fold-changes
331 relative to dark controls in Fig. 4. This comparison, as well as the results of the resuspension
332 experiment discussed below, ensure that all effects seen in the cells are attributable to the
333 isoprene-derived SOA and no other factors. A one-hour exposure to a mass concentration of
334 approximately $45 \mu\text{g m}^{-3}$ of organic material was sufficient to significantly alter gene expression
335 of the inflammatory biomarkers in bronchial epithelial cells. Based on deposition efficiency
336 characterized by de Bruijne et al. (2009), the estimated dose was $0.29 \mu\text{g cm}^{-2}$ of total particle
337 mass with 23% attributable to organic material formed from isoprene photooxidation ($0.067 \mu\text{g}$
338 cm^{-2} of SOA).

339 Changes in the mRNA levels of *IL-8* and *COX-2* from cells exposed to resuspended
340 isoprene-derived SOA collected from photochemical experiments are shown as fold-changes
341 relative to cells exposed to resuspended particles from dark control experiments in Fig. 5. At a
342 low dose of 0.01 mg mL^{-1} of isoprene SOA extract there is no significant increase in *IL-8* and
343 *COX-2* mRNA expression. The isoprene SOA extract, however, induces a response at a dose of
344 0.1 mg mL^{-1} . The statistically significant increase in mRNA expression from the resuspension
345 exposure at 0.1 mg mL^{-1} confirms that similar fold changes observed for both *IL-8* and *COX-2*
346 from the EAVES exposures are not attributable to gaseous photooxidation products, such as O_3 ,
347 and support the characterization of the EAVES as a particle exposure device (de Bruijne et al.,
348 2009).

349 The similar fold change observed in both the EAVES exposure and resuspension
350 exposure, in addition to confirming that the biological effects can be attributed to the particle-
351 phase photochemical products (isoprene-derived SOA), suggests that exposure by resuspension
352 is appropriate for isoprene-derived SOA and may yield results similar to direct deposition
353 exposures. Unlike diesel particulate extracts, which agglomerate during resuspension exposures,
354 isoprene-derived SOA constituents are water-soluble based on reverse-phase LC separations
355 (Surratt et al., 2006; Lin et al., 2012) and remain well mixed in the cell medium used for
356 exposure. Therefore, resuspension exposures do not appear to be a limitation for toxicological
357 assessments of isoprene SOA.

358 **3.4 Biological Implications.** The goal of this study was to initially identify potential biological
359 response associated with exposure to isoprene-derived SOA by using a direct exposure device as
360 a model that has both atmospheric and physiological relevance. With this model, a dose of 0.067
361 $\mu\text{g cm}^{-2}$ of isoprene SOA, induced statistically significant increases in *IL-8* and *COX-2* mRNA
362 levels in exposed BEAS-2B cells. There are many ways to classify in vitro particle dosimetry
363 based on the various properties of particles (Paur et al., 2011). For this direct deposition study,
364 we chose to classify dose as SOA mass deposition per surface area of the exposed cells to mimic
365 lung deposition. Gangwal et al. (2011) used a multiple-path particle dosimetry (MPPD) model to
366 estimate that the lung deposition of ultrafine particles ranges from 0.006 to 0.02 $\mu\text{g cm}^{-2}$ for a 24-
367 hr exposure to a particle concentration of 0.1 mg m^{-3} . Based on this estimate, a dose of 0.067 μg
368 cm^{-2} of isoprene SOA in our study can be considered a prolonged exposure over the course of a
369 week. In fact, most other in vitro studies require dosing cells at a high concentration sometimes
370 close to a lifetime exposure to obtain a cellular response. Despite this limitation, in vitro
371 exposures serve as a necessary screening tool for toxicity (Paur et al., 2011).

372 Our findings are consistent with other studies showing that photochemical oxidation of
373 similar chemical mixtures increases toxicity in cell culture models and elevates expression of
374 inflammatory biomarker genes (Lichtveld et al., 2012; Rager et al., 2011). Previous *in vitro*
375 studies using a gas-phase only exposure system have shown that gas-phase products of isoprene
376 photooxidation significantly enhance cytotoxicity and *IL-8* expression (Doyle et al., 2004; Doyle
377 et al., 2007).

378 By choosing *IL-8* and *COX-2* as our genes of interest, we are able to compare our results
379 to other studies of known harmful particle exposures. In a similar study using the EAVES,
380 normal human bronchial epithelial (NHBE) cells exposed to 1.10 $\mu\text{g cm}^{-2}$ diesel particulate
381 matter showed less than a 2-fold change over controls in both *IL-8* and *COX-2* mRNA
382 expression (Hawley et al., 2014b). In another study, A549 human lung epithelial cells were
383 exposed by direct deposition for 1 hour to photochemically-aged diesel exhaust particulates at a
384 dose of 2.65 $\mu\text{g cm}^{-2}$ from a 1980 Mercedes or a 2006 Volkswagen (Lichtveld et al., 2012).
385 Exposure to aged Mercedes particulates induced a 4-fold change in *IL-8* and ~2-fold change in
386 *COX-2* mRNA expression, while exposure to aged Volkswagen particulates induced a change of
387 ~1.5-fold in *IL-8* and 2-fold in *COX-2* mRNA expression (Lichtveld et al., 2012). Although the
388 differences in cell types preclude direct comparisons, the finding of significant increases in *COX-*
389 *2* and *IL-8* expression at doses much lower than reported for comparable increases in gene
390 expression levels induced by photochemically-aged diesel particulates is notable.

391 *IL-8* and *COX-2* are both linked to inflammation and oxidative stress (Kunkel et al.,
392 1991; Uchida, 2008). *IL-8* is a potent neutrophil chemotactic factor in the lung and its expression
393 by various cells plays a crucial role in neutrophil recruitment leading to lung inflammation
394 (Kunkel et al., 1991). *COX-2* is the inducible form of the cyclooxygenase enzyme, regulated by

395 cytokines and mitogens, and is responsible for prostaglandin synthesis associated with
396 inflammation (FitzGerald, 2003). Consistent with the reports that *IL-8* and *COX-2* play important
397 roles in lung inflammation (Nocker et al., 1996; Li et al., 2013), *in vivo* studies have shown that
398 isoprene oxidation products cause airflow limitation and sensory irritation in mice (Rohr et al.,
399 2003). In humans, the role of *IL-8* and *COX-2* in lung inflammation can be associated with
400 diseases such as chronic obstructive pulmonary disease and asthma (Nocker et al., 1996; Peng et
401 al., 2008; Fong et al., 2000).

402 The mechanism by which isoprene-SOA causes elevation of the inflammatory markers
403 *IL-8* and *COX-2* is not yet fully understood. However, recent work from our laboratory using the
404 acellular dithiothreitol (DTT) assay demonstrated that isoprene-derived SOA has significant
405 ROS generation have equal or greater ROS generation potential than diesel exhaust PM (Kramer
406 et al., 2016). High levels of ROS in cells can overwhelm the antioxidant defense and lead to
407 cellular oxidative stress (Sies, 1991; Bowler and Crapo, 2002; Li et al., 2003). Following the
408 discovery of the potential importance of isoprene-SOA in generating ROS, Lin et al. (2016)
409 showed that isoprene-SOA formed from the reactive uptake of epoxides alters levels of oxidative
410 stress-associated genes, including *COX-2* in human lung cells. Oxidative stress caused by ROS
411 plays a major role in lung inflammation and the induction of oxidative stress can lead to *IL-8*
412 expression (Tao et al., 2003; Yan et al., 2015). Specifically, oxidants can activate the
413 transcription factor NF- B, which regulates a wide range of inflammatory genes including *IL-8*
414 and *COX-2* (Barnes and Adcock, 1997; Schreck et al., 1992). Pathway analysis showed that gene
415 expression of the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway was
416 induced in cells exposed to isoprene SOA (Lin et al., 2016) which has been reported to alter the
417 expression of *IL-8* through mRNA stabilization (Zhang et al., 2005). Therefore, isoprene-SOA

418 may cause increases in both *IL-8* and *COX-2* primarily through an oxidative stress response.
419 Additionally, the relationship between *IL-8* and *COX-2* can also explain the observed increase in
420 *IL-8* gene expression as the production of *IL-8* can be stimulated through a *COX-2* dependent
421 mechanism in airway epithelial cells (Peng et al., 2008).

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

432 **4. Conclusions**

433 This study indicates that an atmospherically relevant composition of isoprene-derived
434 SOA is capable of increasing the expression of *IL-8* and *COX-2* in human bronchial epithelial
435 cells. The present study is an initial step in a long planned analysis of the biological impacts of
436 isoprene SOA exposure on lung cells. The SOA were generated as NO levels approached zero,
437 which represents conditions characteristic of urban locales downwind of rural isoprene sources.
438 As shown in Fig. 2, the aerosol generated for exposures in this study are chemically similar to
439 fine aerosol samples collected from the Southeastern U.S., which indicates that the chamber
440 exposures are representative of exposures that may be encountered by populations in regions

441 where isoprene emissions interact with anthropogenic pollutants. The same particle-phase
442 products found in our photochemical experiments have been measured in significant quantities
443 (accounting on average for 33% of fine organic aerosol mass) in ambient fine organic particles
444 collected in the Southeastern U.S. (Lin et al., 2013b; Budisulistiorini et al., 2013; Rattanavaraha
445 et al., 2016; Budisulistiorini et al., 2016) and in other isoprene-rich environments (Hu et al.,
446 2015). The results of this study show that, because of its abundance, isoprene SOA may be a
447 public health concern warranting further toxicological investigation through *in vitro* or *in vivo*
448 work.

449

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460

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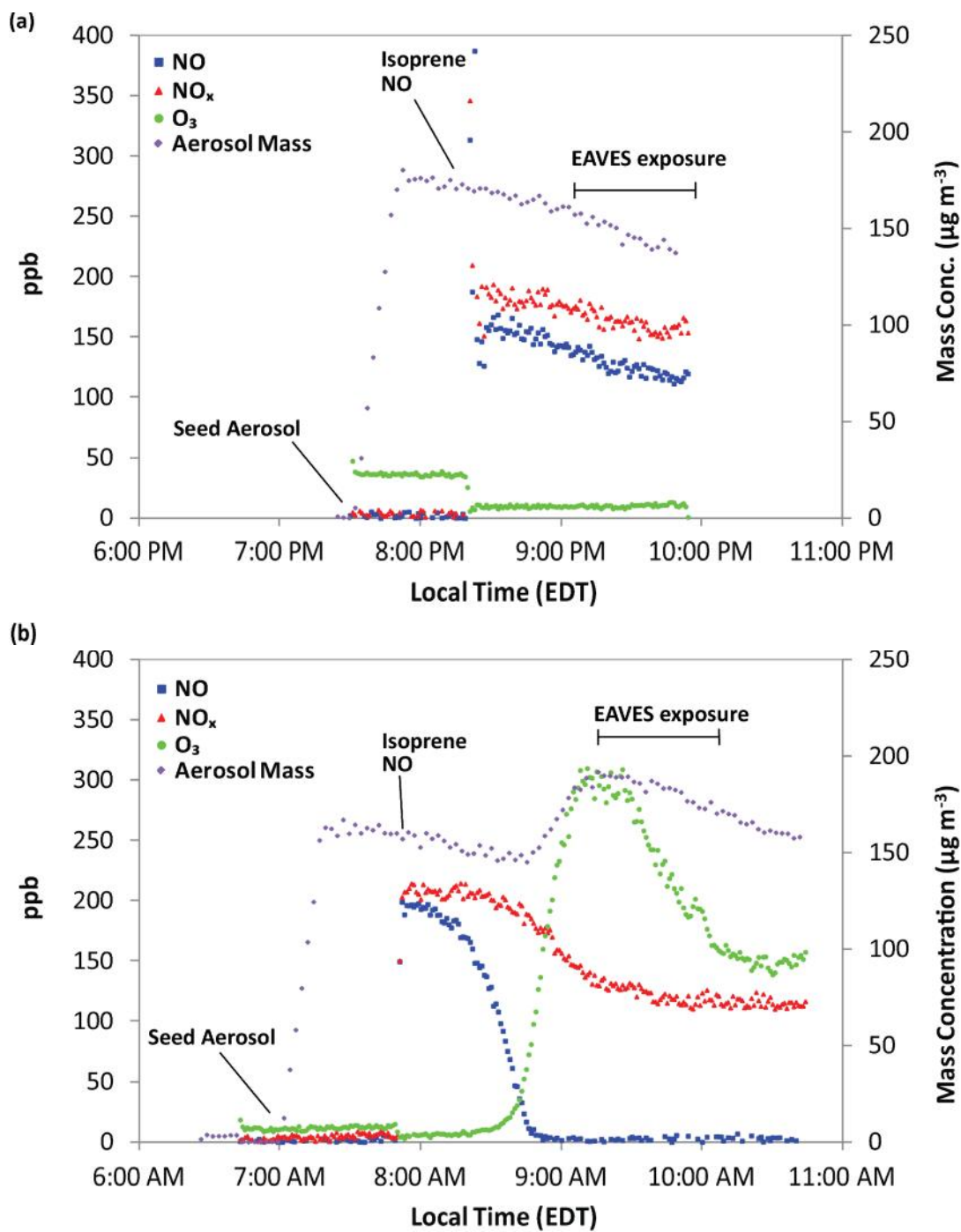
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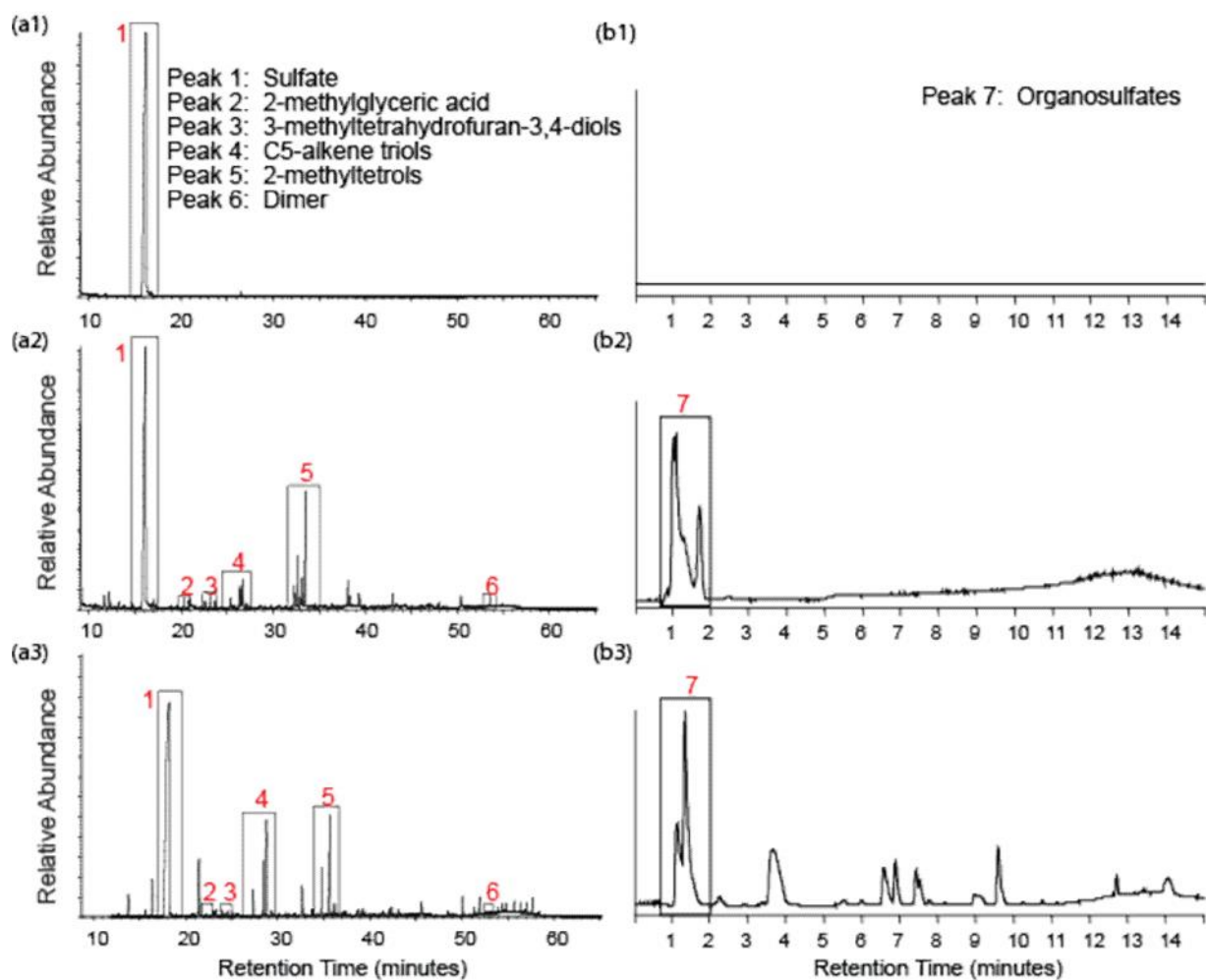
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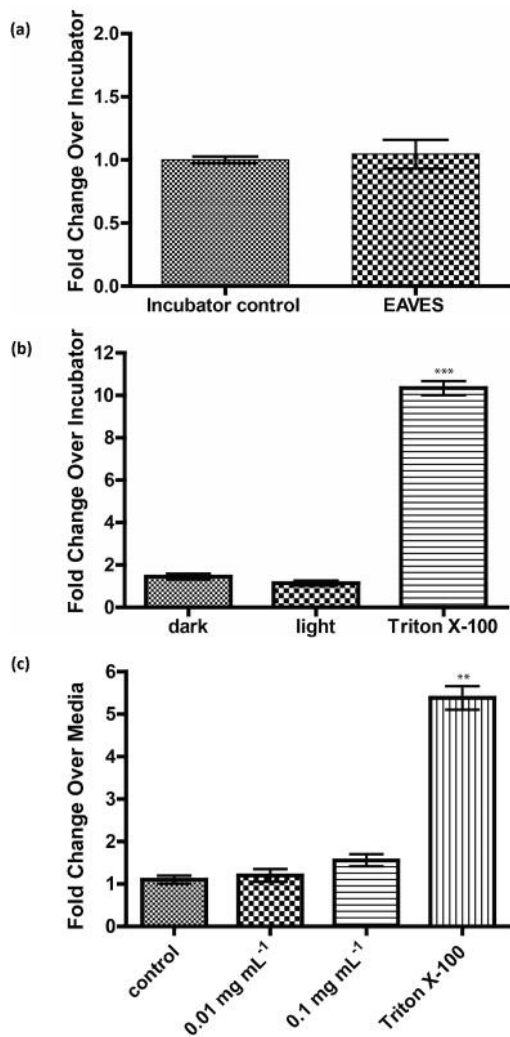


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 758 **Figure 1.** Aerosol mass concentration and gas-phase product concentrations over time for (a)
 759 dark control chamber experiment and (b) photochemically produced isoprene-derived SOA
 760 exposure chamber experiment.



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763 **Figure 2.** (a) GC/EI-MS total ion chromatograms (TICs) and (b) UPLC/ESI-HR-QTOFMS base
 764 peak chromatograms (BPCs) from a (1) dark control chamber experiment, (2) isoprene-derived
 765 SOA exposure chamber experiment, and (3) PM_{2.5} sample collected from Yorkville, GA during
 766 summer 2010.

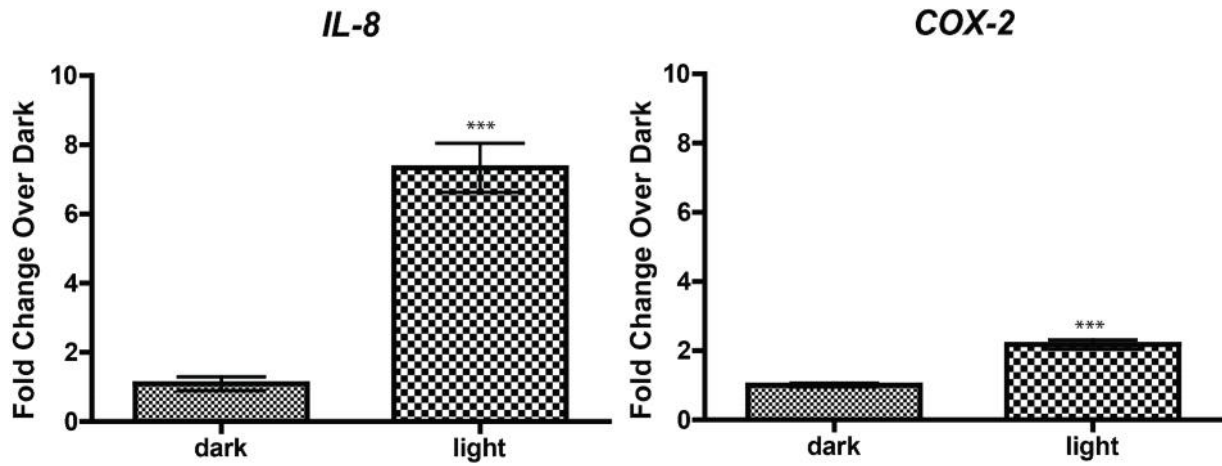


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768 **Figure 3.** LDH release for (a) clean air controls, (b) EAVES exposures, normalized to incubator

769 control, and (c) resuspension exposures, normalized to KBM only control. **p<0.005 and

770 ***p<0.0005.



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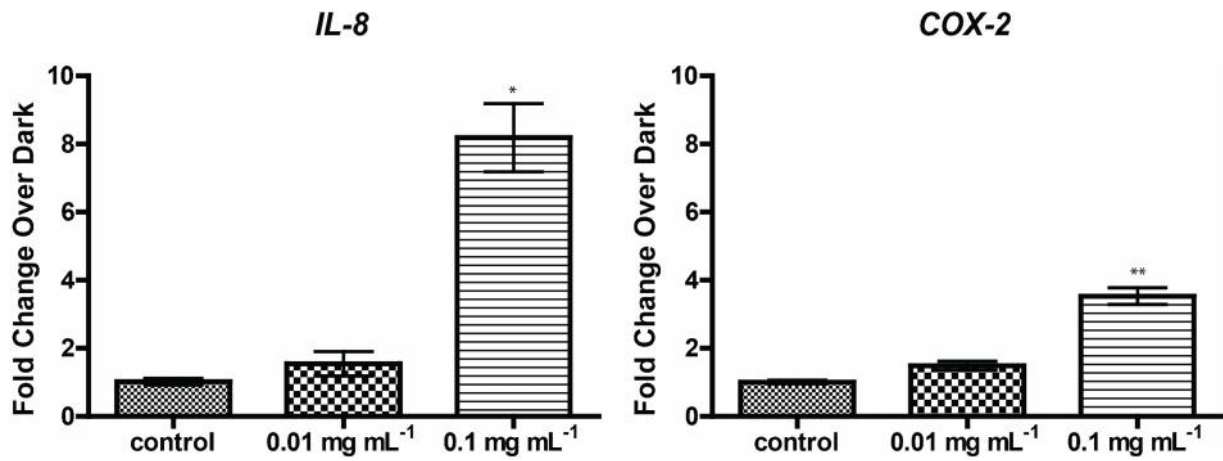
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Figure 4. *IL-8* and *COX-2* mRNA expression induced by exposure to isoprene-derived SOA using EAVES device all normalized to dark control experiments and against housekeeping gene, *-actin*. All experiments conducted in triplicate. *** $p < 0.0005$.



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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 ** $p < 0.005$.