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 $\mu g\ cm^{-2}$ 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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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., 2016; Lin et al., 2013b; Rohr, 2013). 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) (Krechmer et al., 2015; Lin et al., 2013b; Lin et al., 2012; Nguyen et al., 2015; Paulot et al., 2009; Surratt et al., 2010). The formation of SOA from these precursors is influenced by controllable anthropogenic emissions such as oxides of nitrogen (NO$_x$) and sulfur dioxide (SO$_2$). Atmospheric oxidation of SO$_2$ contributes to particle acidity, which enhances isoprene SOA formation through acid-catalyzed reactive uptake and multiphase chemistry of IEPOX and MAE (Gaston et al., 2014; Lin et al., 2012; Riedel et al., 2015; Surratt et al., 2010; Surratt et al., 2007), while NO$_x$ determines whether the oxidation pathway leading to IEPOX or MAE/HMML predominates (Lin et al., 2013b; Nguyen et al., 2015; Surratt et al., 2010). Isoprene SOA comprises a large portion of global atmospheric fine particles (PM$_{2.5}$, 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 (Gaston et al., 2014; Pye et al., 2013; Riedel et al., 2015; Xu et 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; Samet et al., 2000; Schwartz et al., 1993). $PM_{2.5}$,

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 α-

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 (Hawley et al., 2014a; Hawley et al., 2014b; Hawley and Volckens,

92   2013; Lichtveld et al., 2012; Volckens et al., 2009; Zavala et al., 2014). 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 (Hawley et al., 2014b; Lichtveld et al., 2012), but our study is the first to utilize the

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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 (Li et al., 2003; Reuter et al., 2010). 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 (Doyle et al., 2004; Doyle et al., 2007; Ebersviller et al., 2012a, b; Zavala et

110   al., 2014). 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 were attributable to particle-phase organic products.

116   **2.   Experimental Section**

117   **2.1 Generation of SOA in the Outdoor Chamber Facility.**   SOA were generated by

118   photochemically oxidizing a mixture of acidified sulfate seed aerosol, isoprene, and NO injected

119   into an outdoor smog chamber facility. The outdoor chamber is a 120-m^3 triangular cross-section

120   Teflon chamber located on the roof of the Gillings School of Global Public Health, University of

121   North Carolina at Chapel Hill. The chamber facility has been described in detail elsewhere by

122   Lichtveld et al. (2012). The outdoor chamber facility is equipped with sampling lines that allow

123   direct deposition exposure of cells, online chemical measurements, and filter collection for

124   offline chemical analysis. Sampling lines run from the underside of the chamber directly to the

125   chemistry lab below where online measurement instruments and the direct deposition exposure

126   device are located. Injection ports are also located on the underside of the chamber.

127   To generate isoprene-derived SOA, the chamber was operated on sunny days, under high

128   relative humidity, to allow natural sunlight to trigger photochemical reactions. Acidified sulfate

129   seed aerosols were generated by nebulizing an aqueous solution containing 0.06 M $MgSO_4$ +

130   0.06 M H_2SO_4 into the chamber to a particle concentration of approximately 170 μg m^{-3}, which

131   was allowed to stabilize for 30 min to ensure a well-mixed condition. After stabilization, 3.5

132   ppmv isoprene (Sigma-Aldrich, 99%) and 200 ppbv NO (AirGas, 1.00%) were injected into the

133   chamber. Photochemical aging was allowed for approximately one hour to reach the desired

134   exposure conditions of 30-40 μg m^{-3} growth of isoprene-derived SOA on the pre-existing 170 μg

135   m^{-3} of acidified sulfate aerosol. This chamber experiment was replicated on three separate sunny

136   days with temperatures ranging from 24.9°C to 26.8°C with a relative humidity of approximately

137   70% in the chamber.

138   **2.2 Control Chamber Experiments.** As a dark chamber control, to isolate the effect of SOA on

139   exposed cells, mixtures of isoprene, NO, and 170 μg m^{-3} of acidified sulfate seed aerosol were

140   injected into the chamber in the dark (after sunset). Conducting the chamber experiments in the

141   dark ensured no photochemical oxidation of isoprene. The dark control was replicated on three

142 different nights. Except for the absence of solar radiation (no SOA), all chamber operations and

143 exposure conditions were similarly maintained.

144   As an added control to ensure that the device itself and the cell handling had no

145 significant effect on cell cytotoxicity, cells were exposed in the EAVES to a clean chamber and

146 compared to unexposed cells kept in an incubator for the same duration as the exposure. The

147 cytotoxicity results ensured that there is no effect of chamber conditions and device operation on

148 the cells.

149 **2.3 Cell Culture.** Human bronchial epithelial (BEAS-2B) cells were maintained in keratinocyte

150 growth medium (KGM BulletKit; Lonza), a serum-free keratinocyte basal medium (KBM)

151 supplemented with 0.004% of bovine pituitary extract and 0.001% of human epidermal growth

152 factor, insulin, hydrocortisone, and GA-1000 (gentamicin, amphotericin B), and passaged

153 weekly. Passage number for both photochemical exposures and dark control exposures varied

154 between 52 and 60. Because BEAS-2B are an immortalized line of human bronchial epithelium,

155 there are limitations with its use such as it being genetically homogeneous, being a single cell

156 type, and being SV-40 transformed (Reddel et al., 1988). However, BEAS-2B is a stable,

157 proliferative cell line shown to be useful in airway inflammation studies such as ours (Devlin et

158 al., 1994).

159 **2.4 Direct Deposition Exposure.** In preparation for air-liquid interface exposures, cells were

160 seeded onto collagen-coated Millicell cell culture inserts (30 mm diameter, 0.4 μm pore size, 4.2

161 cm^2 filter area; Millipore, Cambridge, MA) at a density of 200,000 cells/well 24 hours prior to

162 exposure. At the time of exposure, cells reached ~80% confluence, confirmed through

163 microscopy. Immediately before exposure, cell medium was removed from the apical and

164 basolateral sides of 2 seeded Millicell cell culture inserts. One insert was transferred to a titanium

165   dish containing 1.5 mL of keratinocyte basal medium (KBM; Lonza), supplying cells with

166   nutrients from the basolateral side and constant moisture while allowing exposure to be

167   performed at an air-liquid interface. The other insert was transferred into a 6 well plate with 2

168   mL of KBM and placed in the incubator as an unexposed control.

169   Cells were exposed to chamber-generated isoprene SOA using the EAVES located in the

170   laboratory directly beneath the outdoor chamber (de Bruijne et al., 2009; Lichtveld et al., 2012).

171   The EAVES, located in an incubator at 37°C, sampled chamber air at 1 L min^{-1}. The target

172   relative humidity (RH) in the chamber during EAVES exposures was approximately 70%.

173   Exposure time was one hour commencing when target exposure conditions were achieved in the

174   outdoor chamber for both photochemical and dark control experiments. Detailed description of

175   the EAVES can be found in de Bruijne et al. (2009).

176   Following exposure, the cell culture insert was transferred to a 6-well tissue culture plate

177   containing 2 mL of fresh KBM. The control Millicell was also transferred to 2 mL of fresh

178   KBM. Nine hours post-exposure, extracellular medium was collected and total RNA was isolated

179   using Trizol (Life Technologies), consistent with past studies (de Bruijne et al., 2009).

180   Extracellular medium and the extracted RNA samples were stored at -20°C and -80°C,

181   respectively, until further analysis. For quality assurance purposes, the RNA concentration and

182   integrity were assessed using Nanodrop and Bioanalyzer over the period of storage. No changes

183   were observed under the given storage conditions.

184   **2.5 Filter Resuspension Exposure.** Chamber particles were collected, concurrently with

185   EAVES sampling, onto Teflon membrane filters (47 mm diameter, 1.0 μm pore size; Pall Life

186   Science) for photochemical (light) and dark chamber experiments to be used for chemical

187   analysis and resuspension exposures. The resuspension experiments served as a control for

188   possible effects of gaseous components such as ozone (O_3) and NO_x present in the direct

189   deposition experiments; however, prior studies have shown that gaseous components do not

190   yield cellular responses within the EAVES device (de Bruijne et al., 2009; Ebersviller et al.,

191   2012a, b). Mass loadings of SOA collected on the filters were calculated from sampling volumes

192   and average aerosol mass concentrations in the chamber during the sampling period. A density

193   correction of 1.6 g cm^{-3} (Riedel et al., 2016) and 1.25 g cm^{-3} (Kroll et al., 2006) was applied to

194   convert the measured volume concentrations to mass concentrations for the acidified sulfate seed

195   and SOA growth, respectively. The particles collected on Teflon filter membranes for

196   resuspension cell exposure were extracted by sonication in high-purity methanol (LC/MS

197   CHROMASOLV, Sigma-Aldrich). Filter samples from multiple experiments were combined and

198   the combined filter extract was dried under a gentle stream of nitrogen (N_2). KBM medium was

199   then added into the extraction vials to re-dissolve SOA constituents.

200   In preparation for filter resuspension exposures, cells were seeded in 24-well plates at a

201   density of 2.5×10^4 cells/well in 250 μL of KGM 2 days prior to exposure. At the time of

202   exposure when cells reached ~80% confluence, cells were washed twice with phosphate buffered

203   saline (PBS) buffer, and then exposed to KBM containing 0.01 and 0.1 mg mL^{-1} isoprene SOA

204   extract from photochemical experiment and seed particles from dark control experiments.

205   Following a 9-hour exposure, extracellular medium was collected and total RNA was

206   isolated using Trizol (Life Technologies) and stored alongside samples from direct deposition

207   exposures until further analysis.

208   **2.6 Chemical and Physical Characterization of Exposure**s.   Online and offline techniques

209   were used to characterize the SOA generated in the chamber. The online techniques measured

210   the gas-phase species NO, NO_x and O_3 and the physical properties of the aerosol continuously

211   throughout the chamber experiments. Offline techniques measured aerosol-phase species

212   collected onto Teflon membrane filters (47 mm diameter, 1.0 μm pore size; Pall Life Science)

213   from photochemical and dark chamber experiments. Filter samples were stored in 20 mL

214   scintillation vials protected from light at -20°C until analyses.

215   Real-time aerosol size distributions were measured using a Differential Mobility

216   Analyzer (DMA, Brechtel Manufacturing Inc.) coupled to a Mixing Condensation Particle

217   Counter (MCPC, Model 1710, Brechtel Manufacturing Inc.) located in the laboratory directly

218   underneath the chamber. O_3 and NO_x were measured with a ML 9811 series Ozone Photometer

219   (Teledyne Monitor Labs, Englewood, CO) and ML 9841 series NO_x Analyzer (American

220   Ecotech, Warren RI), respectively. Data were collected at one-minute intervals using a data

221   acquisition system (ChartScan/1400) interfaced to a computer. The presence of isoprene in the

222   chamber was confirmed and quantified using a Varian 3800 gas chromatograph (GC) equipped

223   with a flame ionization detector (FID).

224   Chemical characterization of SOA constituents was conducted offline from extracts of

225   filters collected from chamber experiments by gas chromatography interfaced with an electron

226   ionization quadrupole mass spectrometer (GC/EI-MS) or by ultra performance liquid

227   chromatography interfaced with a high-resolution quadrupole time-of-flight mass spectrometer

228   equipped with electrospray ionization (UPLC/ESI-HR-QTOFMS). Detailed operating conditions

229   for the GC/EI-MS and UPLC/ESI-HR-QTOFMS analyses as well as detailed filter extraction

230   protocols have been described previously by Lin et al. (2012). For GC/EI-MS analysis, filter

231   extracts were dried under a gentle stream of N_2 and trimethylsilylated by the addition of 100 μL

232   of BSTFA + TMCS (99:1 v/v, Supelco) and 50 μL of pyridine (anhydrous, 99.8%, Sigma-

233   Aldrich) and heated at 70 ºC for 1 h. For UPLC/ESI-HR-QTOFMS analysis, residues of filter

234 extracts were reconstituted with 150 µL of a 50:50 (v/v) solvent mixture of high-purity water and

235 methanol.

236   The isoprene-derived SOA markers: 2-methyltetrols, isomeric 3-methyltetrahydrofurans-

237 3,4-diols (3-MeTHF-3,4-diols), and 2-methylglyceric acid, synthesized according to the

238 published procedures (Lin et al., 2013b; Zhang et al., 2012), were available in-house as authentic

239 standards to quantify the major components of isoprene SOA. 2-Methyltetrol organosulfates,

240 synthesized as a mixture of tetrabutylammonium salts, were also available as a standard. Purity

241 was determined to be >99% by ^{1}H NMR and UPLC/ESI-QTOFMS analysis (Budisulistiorini et

242 al., 2015b). The C_5-alkene triols and IEPOX dimer were quantified using the response factor

243 obtained for the synthetic 2-methyltetrols.

244   A representative ambient $PM_{2.5}$ sample collected from the rural southeastern U.S.

245 (Yorkville, GA) (Lin et al., 2013a) during the summer of 2010 was analyzed in an identical

246 manner to confirm atmospheric relevance of the chamber-generated SOA constituents.

247 **2.7 Cytotoxicity Assay.**   Cytotoxicity was assessed through measurement of lactate

248 dehydrogenase (LDH) released into the extracellular medium from damaged cells using the LDH

249 cytotoxicity detection kit (Takara). To ensure that the EAVES device itself and operation

250 procedure had no effect on cytotoxicity, the LDH release from cells exposed to clean chamber air

251 was measured. LDH release by cells exposed via the EAVES to the photochemically aged (light)

252 and non-photochemically aged (dark) particles was compared to unexposed cells maintained in

253 the incubator for the same duration. For the resuspension exposures, LDH release by cells

254 exposed to SOA through resuspended extract of photochemically aged and non-photochemically

255 aged particles was compared to cells maintained in KBM only. Additionally, LDH release from

256   the light exposures, dark control, and resuspension exposures were compared to positive controls

257   exposed to 1% Triton X-100 to ensure that cell death would not affect gene expression results.

258   **2.8 Gene Expression Analysis.** We chose to measure the levels of the inflammation-related

259   mRNA in the BEAS-2B cells exposed to isoprene-derived SOA generated in our outdoor

260   chamber because various particle types are capable of sequestering cytokines (Seagrave, 2008).

261   Other direct deposition studies have also used mRNA transcripts as a proxy for cytokine

262   production (Hawley et al., 2014a; Hawley et al., 2014b; Hawley and Volckens, 2013; Lichtveld

263   et al., 2012; Volckens et al., 2009). Changes in *IL-8* and *COX-2* mRNA levels were measured

264   using QuantiTect SYBR Green RT-PCR Kit (Qiagen) and QuantiTect Primer Assays for

265   Hs_ACTB_1_SG (Catalog #QT00095431), Hs_PTGS2_1_SG (Catalog #QT00040586), and

266   Hs_CXCL8_1_SG (Catalog #QT00000322) for one-step RT-PCR analysis. All mRNA levels

267   were normalized against β-actin mRNA, which was used as a housekeeping gene. The relative

268   expression levels (i.e., fold change) of *IL-8* and *COX-2* were calculated using the comparative

269   cycle threshold ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001). For EAVES exposures, changes

270   in *IL-8* and *COX-2* from isoprene-derived SOA exposed cells were compared to cells exposed to

271   the dark controls. Similarly, for resuspension exposures changes in *IL-8* and *COX-2* from

272   isoprene-derived SOA exposed cells were compared to cells exposed to particles collected under

273   dark conditions.

274   **2.9 Statistical Analysis.** The software package GraphPad Prism 4 (GraphPad) was used for all

275   statistical analyses. All data were expressed as mean ± SEM (standard error of means).

276   Comparisons between data sets for cytotoxicity and gene expression analysis were made using

277   unpaired *t*-test with Welch's correction. Significance was defined as $p < 0.05$.

278   **3. Results and Discussion**

279   **3.1 Physical and Chemical Characterization of Exposure.** Figure 1 shows the change in

280   particle mass concentration and gas (O_3, NO, NO_x) concentration over time during typical

281   photochemical and dark control experiments. Under dark control conditions (Fig. 1a) there is no

282   increase in aerosol mass concentration following isoprene injection. Average total aerosol mass

283   concentration was 155.0±2.69 μg m^{-3} (1 standard deviation) with no particle mass attributable to

284   organic material.

285   In contrast, Fig. 1b shows an increase in aerosol mass concentration after 1 h post

286   isoprene injection, which can be attributed to the photochemical oxidation of isoprene and

287   subsequent production and reactive uptake of its oxidation products. The average increase in

288   aerosol mass concentration attributable to SOA formation for three daylight chamber

289   experiments conducted on separate days was 44.5±5.7 μg m^{-3}. Average total aerosol mass

290   concentration during particle exposure was 173.1± 4.2 μg m^{-3}.

291   O_3 and NO_x concentrations measured during EAVES exposure were approximately 270

292   ppb and 120 ppb for photochemical experiments. For dark control experiments (e.g., Fig. 1a), the

293   O_3 and NO_x concentrations were approximately 15 ppb and 180 ppb. Previous studies

294   characterizing the EAVES device show definitively that gas-phase products do not induce cell

295   response (de Bruijne et al., 2009). However, resuspension exposures were conducted in addition

296   to EAVES exposure to ensure that biological effects were attributable to only particle-phase

297   constituents and not gas-phase products such as O_3 and NO_x.

298   The chemical composition of aerosol, collected onto filters concurrently with cell

299   exposure and characterized by GC/EI-MS and UPLC/ESI-HR-QTOFMS, are shown in Fig. 2.

300   No isoprene-SOA tracers were observed in the filters collected from dark control experiments.

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301   The dominant particle-phase products of the isoprene-SOA collected from photochemical

302   experiments are derived from the low-NO channel, where IEPOX reactive uptake onto acidic

303   sulfate aerosol dominates, including 2-methyltetrols, C5-alkene triols, isomeric 3-MeTHF-3,4-

304   diols, IEPOX-derived dimers, and IEPOX-derived organosulfates. The sum of the IEPOX-

305   derived SOA constituents quantified by the available standards accounted for ~80% of the

306   observed SOA mass. The MAE-derived SOA constituents 2-methylglyceric acid and the

307   organosulfate derivative of MAE, derived from the high-NO channel, accounted for 1.4% of the

308   observed SOA mass, confirming that particle-phase products generated were predominantly

309   formed from the reactive uptake of IEPOX onto acidic sulfate aerosols. As demonstrated in

310   Figure 2, all the same particle-phase products are measured in the $PM_{2.5}$ sample collected in

311   Yorkville, GA (a typical low-NO region), demonstrating that the composition of the chamber-

312   generated SOA is atmospherically relevant. Recent SOA tracer measurements from the Southern

313   Oxidant and Aerosol Study (SOAS) campaign at Look Rock, TN, Centerville, AL, and

314   Birmingham, AL, also support the atmospheric relevance of IEPOX-derived SOA constituents

315   that dominate the isoprene SOA mass in summer in the southeastern U.S. (Budisulistiorini et al.,

316   2015a; Rattanavaraha et al., 2016).

317   **3.2 Cytotoxicity.** LDH release for cells exposed using the EAVES device is expressed as a fold-

318   change relative to the unexposed incubator control. For resuspension exposures, LDH release is

319   expressed as fold-change relative to cells exposed to KBM only. Results shown in Fig. 3a

320   confirm that there is no effect of chamber conditions and device operation on the cells when

321   comparing LDH release from cells exposed to a clean air chamber and cells unexposed in an

322   incubator. Additionally, LDH release from all exposure conditions in EAVES exposed cells (Fig.

323   3b) and resuspension exposed cells (Fig. 3c) is negligible relative to positive controls exposed to

324   1% Triton X-100, confirming that the exposure concentration of isoprene-derived SOA utilized

325   in this study was not cytotoxic. All cytotoxicity results ensured that exposure conditions were not

326   adversely affecting the cells nor their gene expression.

327   **3.3 Pro-inflammatory Gene Expression**.   Changes in the mRNA levels of *IL-8* and *COX-2*

328   from cells exposed to isoprene-derived SOA using the EAVES are shown as fold-changes

329   relative to dark controls in Fig. 4. This comparison, as well as the results of the resuspension

330   experiment discussed below, ensure that all effects seen in the cells are attributable to the

331   isoprene-derived SOA and no other factors. A one-hour exposure to a mass concentration of

332   approximately 45 µg m^{-3} of organic material was sufficient to significantly alter gene expression

333   of the inflammatory biomarkers in bronchial epithelial cells. Based on deposition efficiency

334   characterized by de Bruijne et al. (2009), the estimated dose was 0.29 µg cm^{-2} of total particle

335   mass with 23% attributable to organic material formed from isoprene photooxidation (0.067 µg

336   cm^{-2} of SOA).

337   Changes in the mRNA levels of *IL-8* and *COX-2* from cells exposed to resuspended

338   isoprene-derived SOA collected from photochemical experiments are shown as fold-changes

339   relative to cells exposed to resuspended particles from dark control experiments in Fig. 5. At a

340   low dose of 0.01 mg mL^{-1} of isoprene SOA extract there is no significant increase in *IL-8* and

341   *COX-2* mRNA expression. The isoprene SOA extract, however, induces a response at a dose of

342   0.1 mg mL^{-1}. The statistically significant increase in mRNA expression from the resuspension

343   exposure at 0.1 mg mL^{-1} confirms that similar fold changes observed for both *IL-8* and *COX- 2*

344   from the EAVES exposures are not attributable to gaseous photooxidation products, such as O$_3$,

345   and support the characterization of the EAVES as a particle exposure device (de Bruijne et al.,

346   2009).

347   The similar fold change observed in both the EAVES exposure and resuspension

348   exposure, in addition to confirming that the biological effects can be attributed to the particle-

349   phase photochemical products (isoprene-derived SOA), suggests that exposure by resuspension

350   is appropriate for isoprene-derived SOA and may yield results similar to direct deposition

351   exposures. Unlike diesel particulate extracts, which agglomerate during resuspension exposures,

352   isoprene-derived SOA constituents are water-soluble based on reverse-phase LC separations (Lin

353   et al., 2012; Surratt et al., 2006) and remain well mixed in the cell medium used for exposure.

354   Therefore, resuspension exposures do not appear to be a limitation for toxicological assessments

355   of isoprene SOA.

356   **3.4 Biological Implications**. The goal of this study was to initially identify potential biological

357   response associated with exposure to isoprene-derived SOA by using a direct exposure device as

358   a model that has both atmospheric and physiological relevance. With this model, a dose of 0.067

359    $\mu g\ cm^{-2}$ isoprene SOA, induced statistically significant increases in *IL-8* and *COX-2* mRNA

360   levels in exposed BEAS-2B cells. There are many ways to classify *in vitro* particle dosimetry

361   based on the various properties of particles (Paur et al., 2011). For this direct deposition study,

362   we chose to classify dose as SOA mass deposition per surface area of the exposed cells to mimic

363   lung deposition. Gangwal et al. (2011) used a multiple-path particle dosimetry (MPPD) model to

364   estimate that the lung deposition of ultrafine particles ranges from 0.006 to 0.02 $\mu g\ cm^{-2}$ for a 24-

365   hr exposure to a particle concentration of 0.1 mg m^{-3}. Based on this estimate, a dose of 0.067 μg

366   cm^{-2} of isoprene SOA in our study can be considered a prolonged exposure over the course of a

367   week. In fact, most other in vitro studies require dosing cells at a high concentration sometimes

368   close to a lifetime exposure to obtain a cellular response. Despite this limitation, in vitro

369   exposures serve as a necessary screening tool for toxicity (Paur et al., 2011).

370   Our findings are consistent with other studies showing that photochemical oxidation of

371 similar chemical mixtures increases toxicity in cell culture models and elevates expression of

372 inflammatory biomarker genes (Lichtveld et al., 2012; Rager et al., 2011). Previous *in vitro*

373 studies using a gas-phase only exposure system have shown that gas-phase products of isoprene

374 photooxidation significantly enhance cytotoxicity and *IL-8* expression (Doyle et al., 2004; Doyle

375 et al., 2007).

376   By choosing *IL-8* and *COX-2* as our genes of interest, we are able to compare our results

377 to other studies of known harmful particle exposures. In a similar study using the EAVES,

378 normal human bronchial epithelial (NHBE) cells exposed to 1.10 µg cm^{-2} diesel particulate

379 matter showed less than a 2-fold change over controls in both *IL-8* and *COX-2* mRNA

380 expression (Hawley et al., 2014b). In another study, A549 human lung epithelial cells were

381 exposed by direct deposition for 1 hour to photochemically-aged diesel exhaust particulates at a

382 dose of 2.65 µg cm^{-2} from a 1980 Mercedes or a 2006 Volkswagen (Lichtveld et al., 2012).

383 Exposure to aged Mercedes particulates induced a 4-fold change in IL-8 and ~2-fold change in

384 *COX-2* mRNA expression, while exposure to aged Volkswagen particulates induced a change of

385 ~1.5-fold in *IL-8* and 2-fold in *COX-2* mRNA expression (Lichtveld et al., 2012). Although the

386 differences in cell types preclude direct comparisons, the finding of significant increases in *COX-*

387 *2* and *IL-8* expression at doses much lower than reported for comparable increases in gene

388 expression levels induced by photochemically-aged diesel particulates is notable.

389   *IL-8* and *COX-2* are both linked to inflammation and oxidative stress (Kunkel et al.,

390 1991; Uchida, 2008). *IL-8* is a potent neutrophil chemotactic factor in the lung and its expression

391 by various cells plays a crucial role in neutrophil recruitment leading to lung inflammation

392 (Kunkel et al., 1991). *COX-2* is the inducible form of the cyclooxygenase enzyme, regulated by

393   cytokines and mitogens, and is responsible for prostaglandin synthesis associated with

394   inflammation (FitzGerald, 2003). Consistent with the reports that *IL-8* and *COX-2* play important

395   roles in lung inflammation (Li et al., 2013; Nocker et al., 1996), *in vivo* studies have shown that

396   isoprene oxidation products cause airflow limitation and sensory irritation in mice (Rohr et al.,

397   2003). In humans, the role of *IL-8* and *COX-2* in lung inflammation can be associated with

398   diseases such as chronic obstructive pulmonary disease and asthma (Fong et al., 2000; Nocker et

399   al., 1996; Peng et al., 2008).

400   The mechanism by which isoprene-SOA causes elevation of the inflammatory markers

401   *IL-8* and *COX-2* is not yet fully understood. However, recent work from our laboratory using the

402   acellular dithiothreitol (DTT) assay demonstrated that isoprene-derived SOA have equal or

403   greater ROS generation potential than diesel exhaust PM (Kramer et al., 2016; Rattanavaraha et

404   al., 2011). High levels of ROS in cells can overwhelm the antioxidant defense and lead to

405   cellular oxidative stress (Bowler and Crapo, 2002; Li et al., 2003; Sies, 1991). Following the

406   discovery of the potential importance of isoprene-SOA in generating ROS, Lin et al. (2016)

407   showed that isoprene-SOA formed from the reactive uptake of epoxides alters levels of oxidative

408   stress-associated genes, including *COX-2* in human lung cells. Oxidative stress caused by ROS

409   plays a major role in lung inflammation and the induction of oxidative stress can lead to *IL-8*

410   expression (Tao et al., 2003; Yan et al., 2015). Pathway analysis showed that gene expression of

411   the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway was induced in cells

412   exposed to isoprene-SOA (Lin et al., 2016) which has been reported to alter the expression of *IL-*

413   *8* through mRNA stabilization (Zhang et al., 2005). Therefore, isoprene-SOA may cause

414   increases in both *IL-8* and *COX-2* primarily through an oxidative stress response. Additionally,

415   the relationship between *IL-8* and *COX-2* can also explain the observed increase in *IL-8* gene

416   expression as the production of *IL*-8 can be stimulated through a *COX-2* dependent mechanism

417   in airway epithelial cells (Peng et al., 2008).

418   *In vitro* studies such as this one using a direct deposition model cannot fully elucidate

419   mechanisms of lung inflammation and potential pathogenesis but serve as a necessary part of

420   hazard characterization, particularly for a complex air mixture that has not been fully studied

421   (Hayashi, 2005; Paur et al., 2011). Therefore, further *in vitro* studies exploring the health

422   implication of the elevation of *IL-8* and *COX-2* due specifically to isoprene-SOA exposure are

423   necessary and may in turn justify further extension to *in vivo* work.

424   **4. Conclusions**

425   This study indicates that an atmospherically relevant composition of isoprene-derived

426   SOA is capable of increasing the expression of *IL-8* and *COX-2* in human bronchial epithelial

427   cells. The present study is an initial step in a long planned analysis of the biological impacts of

428   isoprene SOA exposure on lung cells. The SOA were generated as NO levels approached zero,

429   which represents conditions characteristic of urban locales downwind of rural isoprene sources.

430   As shown in Fig. 2, the aerosol generated for exposures in this study are chemically similar to

431   fine aerosol samples collected from the Southeastern U.S., which indicates that the chamber

432   exposures are representative of exposures that may be encountered by populations in regions

433   where isoprene emissions interact with anthropogenic pollutants. The same particle-phase

434   products found in our photochemical experiments have been measured in significant quantities

435   (accounting on average for 33% of fine organic aerosol mass) in ambient fine organic particles

436   collected in the Southeastern U.S. (Budisulistiorini et al., 2016; Budisulistiorini et al., 2013; Lin

437   et al., 2013b; Rattanavaraha et al., 2016) and in other isoprene-rich environments (Hu et al.,

438   2015). The results of this study show that, because of its abundance, isoprene SOA may be a

439   public health concern warranting further toxicological investigation through *in vitro* or *in vivo*

440   work.

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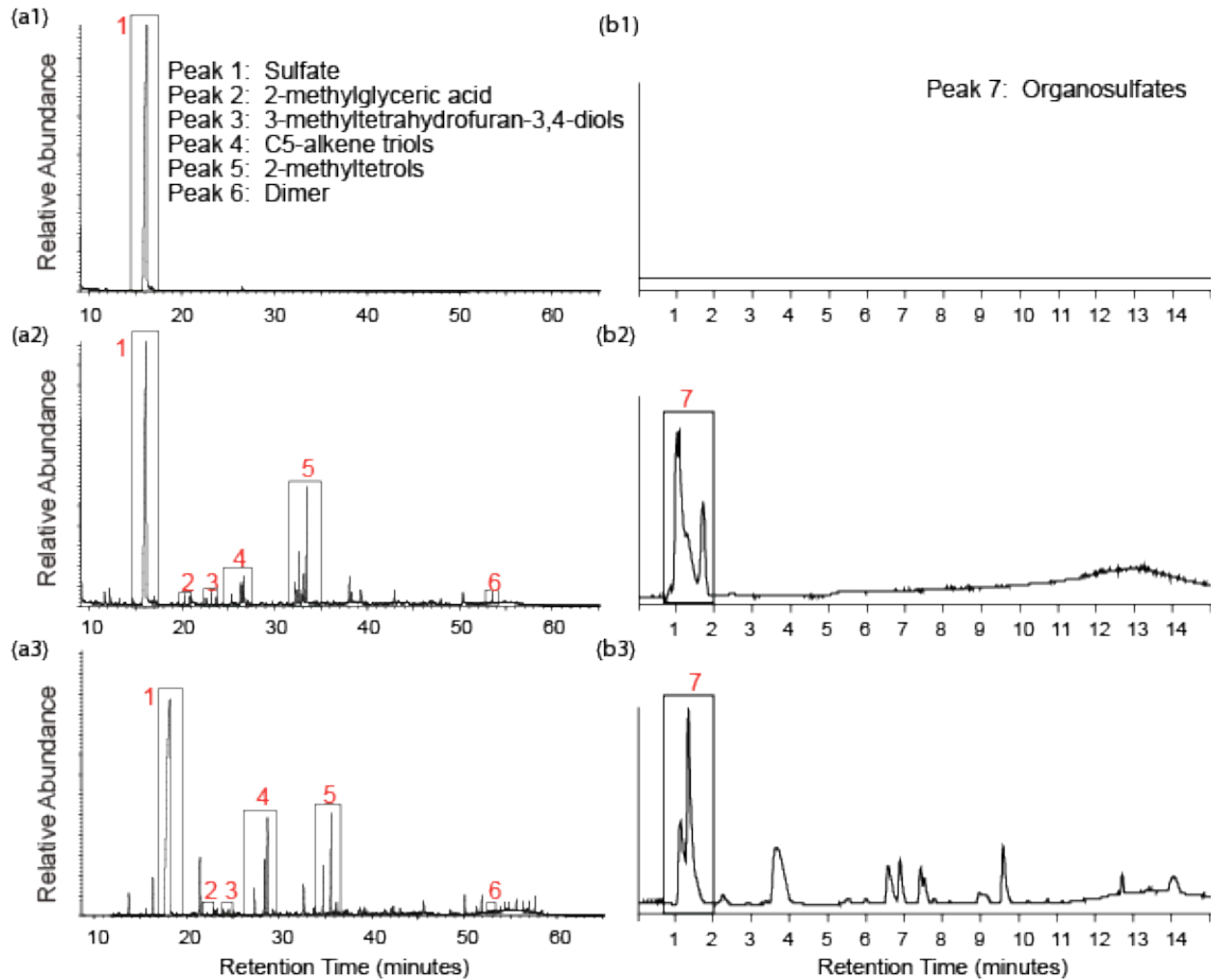733   Chemistry and Physics, 12, 8529-8535, 2012.

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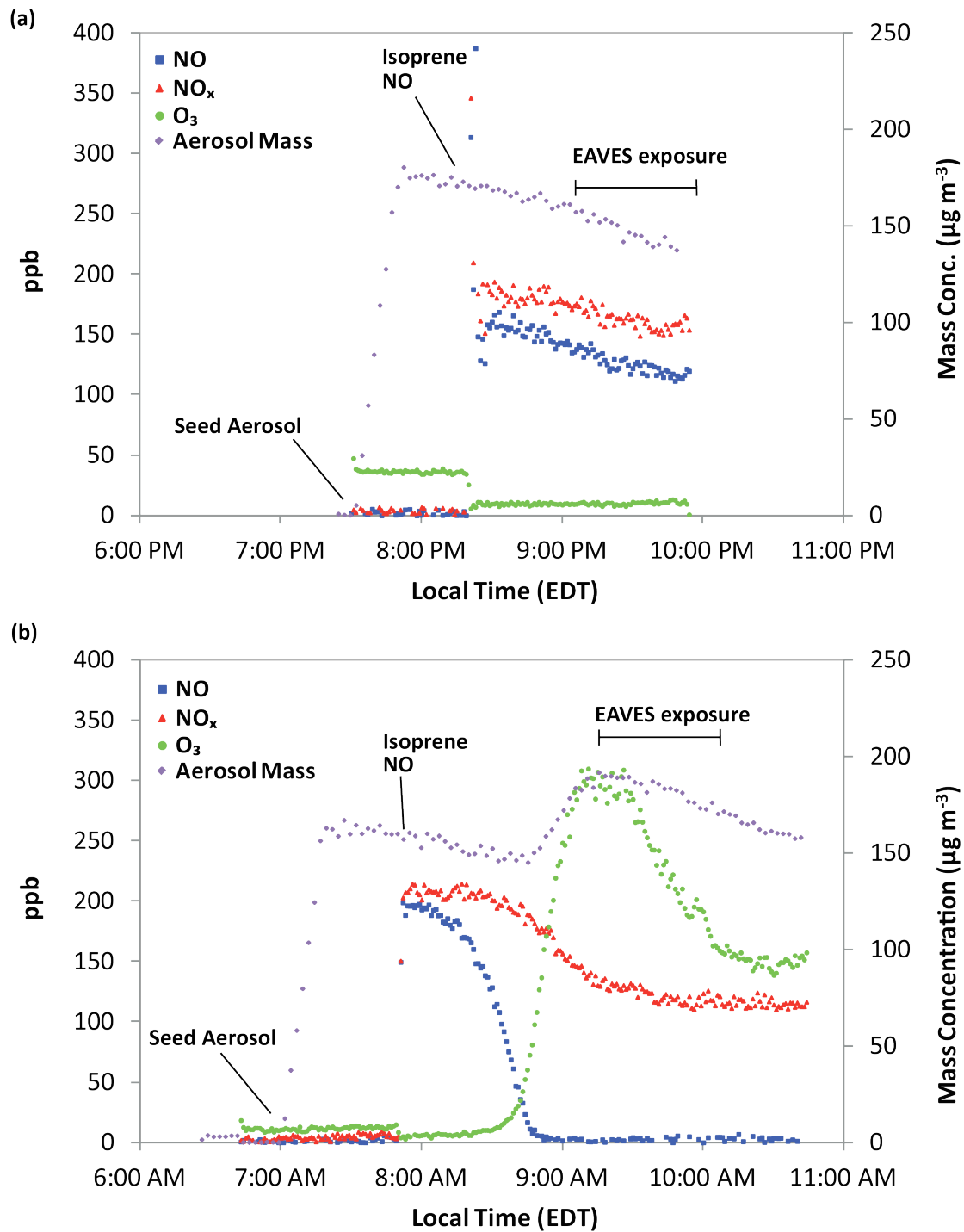
**Figure 1.** Aerosol mass concentration and gas-phase product concentrations over time for (a) dark control chamber experiment and (b) photochemically produced isoprene-derived SOA exposure chamber experiment.

739

Peak 1: Sulfate
Peak 2: 2-methylglyceric acid
Peak 3: 3-methyltetrahydrofuran-3,4-diols
Peak 4: C5-alkene triols
Peak 5: 2-methyltetrols
Peak 6: Dimer

Peak 7: Organosulfates

740

741 **Figure 2.** (a) GC/EI-MS total ion chromatograms (TICs) and (b) UPLC/ESI-HR-QTOFMS base

742 peak chromatograms (BPCs) from a (1) dark control chamber experiment, (2) isoprene-derived

743 SOA exposure chamber experiment, and (3) $PM_{2.5}$ sample collected from Yorkville, GA during
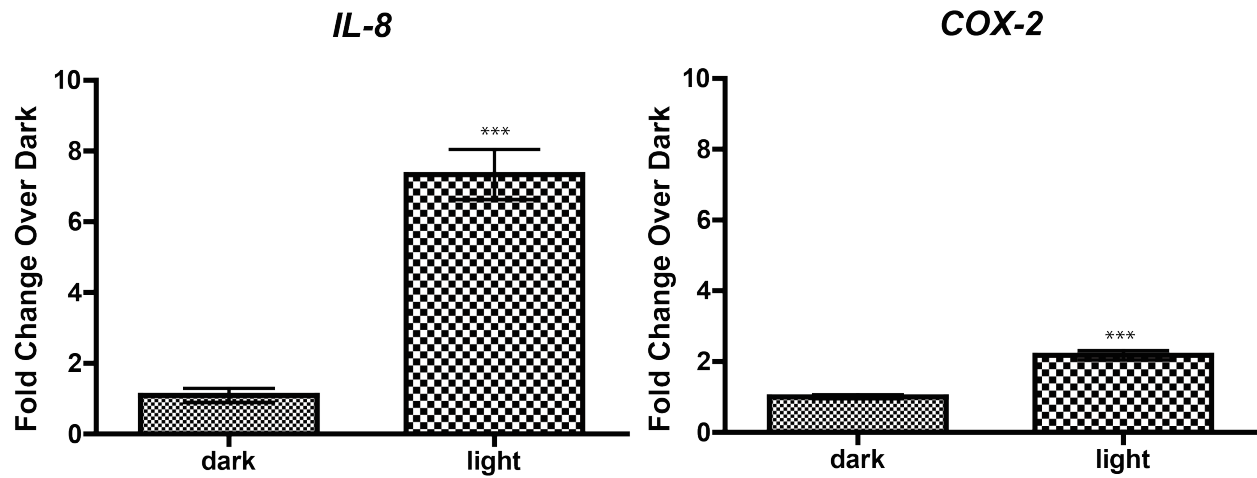
744 summer 2010.

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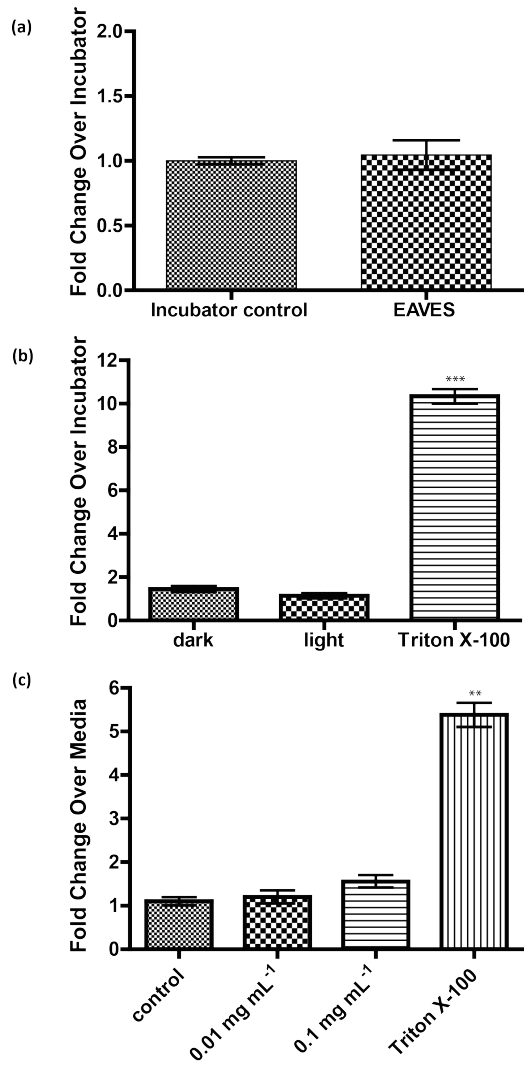
Figure 3. LDH release for (a) clean air controls, (b) EAVES exposures, normalized to incubator control, and (c) resuspension exposures, normalized to KBM only control. **p<0.005 and ***p<0.0005.
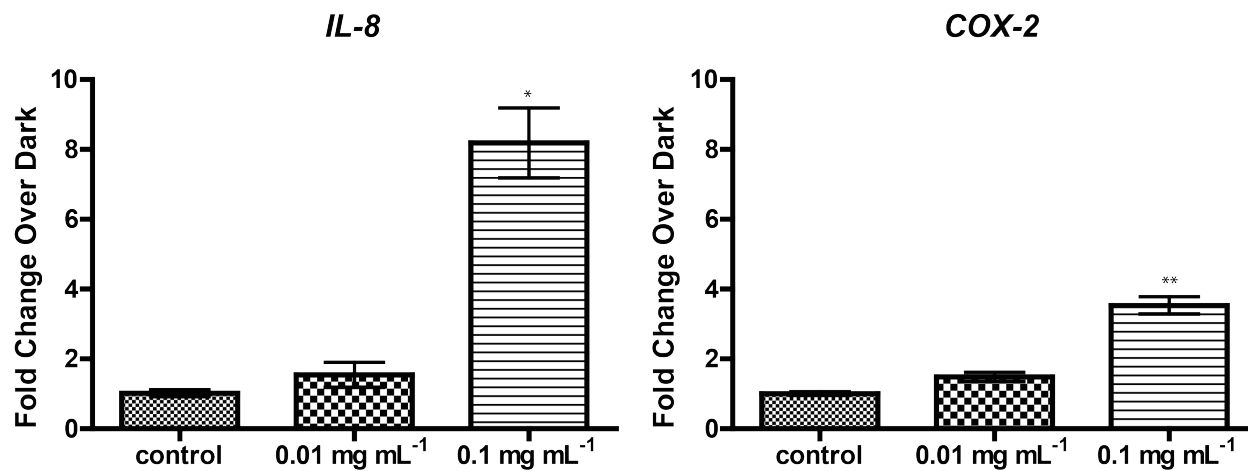
IL-8

COX-2

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750 **Figure 4.** *IL-8* and *COX-2* mRNA expression induced by exposure to isoprene-derived SOA

751 using EAVES device all normalized to dark control experiments and against housekeeping gene,

752 β-actin. All experiments conducted in triplicate. ***p<0.0005.

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IL-8

COX-2

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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.