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 μg 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., 2013b; Rohr, 2013; Lin et al., 2016). Current understanding of isoprene SOA formation is based on laboratory studies showing that gas-phase photooxidation of isoprene generates key SOA precursors, including isomeric isoprene epoxydiols (IEPOX), methacrylic acid epoxide (MAE), hydroxymethyl-methyl-α-lactone (HMML), and isoprene hydroxyhydroperoxides (ISOPOOH) (Paulot et al., 2009; Surratt et al., 2010; Lin et al., 2012; Lin et al., 2013b; Nguyen et al., 2015; Krechmer et al., 2015). The formation of SOA from these precursors is influenced by controllable anthropogenic emissions such as oxides of nitrogen (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 (Surratt et al., 2007; Surratt et al., 2010; Lin et al., 2012; Gaston et al., 2014; Riedel et al., 2015), while NO_x determines whether the oxidation pathway leading to IEPOX or MAE/HMML predominates (Lin et al., 2013b; Surratt et al., 2010; Nguyen et al., 2015). Isoprene SOA comprises a large portion of global atmospheric fine particles ($PM_{2.5}$, 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 (Pye et al., 2013; Gaston et al., 2014; Xu et al., 2015; Riedel 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; 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 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 (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-m^3 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 MgSO$_4$ +

131   0.06 M H$_2$SO$_4$ 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 (O_3) 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 (N_2). 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 2.5×10^4 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 Exposure**s. 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 O_3 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 ºC for 1 h. For UPLC/ESI-HR-QTOFMS analysis, residues of filter

235   extracts were reconstituted with 150 µL 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 ^{1}H 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 $PM_{2.5}$ 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 ($2^{-\Delta\Delta CT}$) 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 ± 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±2.69 µg 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±5.7 µg m^{-3}. Average total aerosol mass

292 concentration during particle exposure was 173.1± 4.2 µg 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 µg 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 µg cm^{-2} of total particle

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

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   μg 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 μg 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 μg 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 μg 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 potential (Kramer et al., 2016). High levels of ROS in cells can overwhelm the

406   antioxidant defense and lead to cellular oxidative stress (Sies, 1991; Bowler and Crapo, 2002; Li

407   et al., 2003). Following the discovery of the potential importance of isoprene-SOA in generating

408   ROS, Lin et al. (2016) showed that isoprene-SOA formed from the reactive uptake of epoxides

409   alters levels of oxidative stress-associated genes, including *COX-2* in human lung cells.

410   Oxidative stress caused by ROS plays a major role in lung inflammation and the induction of

411   oxidative stress can lead to *IL-8* expression (Tao et al., 2003; Yan et al., 2015). Specifically,

412   oxidants can activate the transcription factor NF-κB, which regulates a wide range of

413   inflammatory genes including *IL-8* and *COX-2* (Barnes and Adcock, 1997; Schreck et al., 1992).

414   Therefore, isoprene-SOA may cause increases in both *IL-8* and *COX-2* primarily through an

415   oxidative stress response. Additionally, the relationship between *IL-8* and *COX-2* can also

416   explain the observed increase in *IL-8* gene expression as the production of *IL*-8 can be stimulated

417   through a *COX-2* dependent mechanism 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). Ozone exposure studies have shown that comparable dose and

422   effect measurements for *IL-8* and *COX-2* can be found between *in vivo* and *in vitro* exposures

423   which add promise to extrapolating effects seen *in vitro* to effects *in vivo* (Hatch et al., 2014). *In*

424   *vivo* effects associated with isoprene-SOA exposure *in vitro* cannot be inferred as it is a different

425   system from ozone, so further *in vitro* studies exploring the health implication of the elevation of

426   *IL-8* and *COX-2* due specifically to isoprene-SOA exposure are necessary and may in turn justify

427   further extension to *in vivo* work.

428   **4. Conclusions**

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

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

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

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

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

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

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

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

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

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

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

440   collected in the Southeastern U.S. (Lin et al., 2013b; Budisulistiorini et al., 2013; Rattanavaraha

441 et al., 2016; Budisulistiorini et al., 2016) and in other isoprene-rich environments (Hu et al.,

442 2015). The results of this study show that, because of its abundance, isoprene SOA may be a

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

444 work.

445

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456

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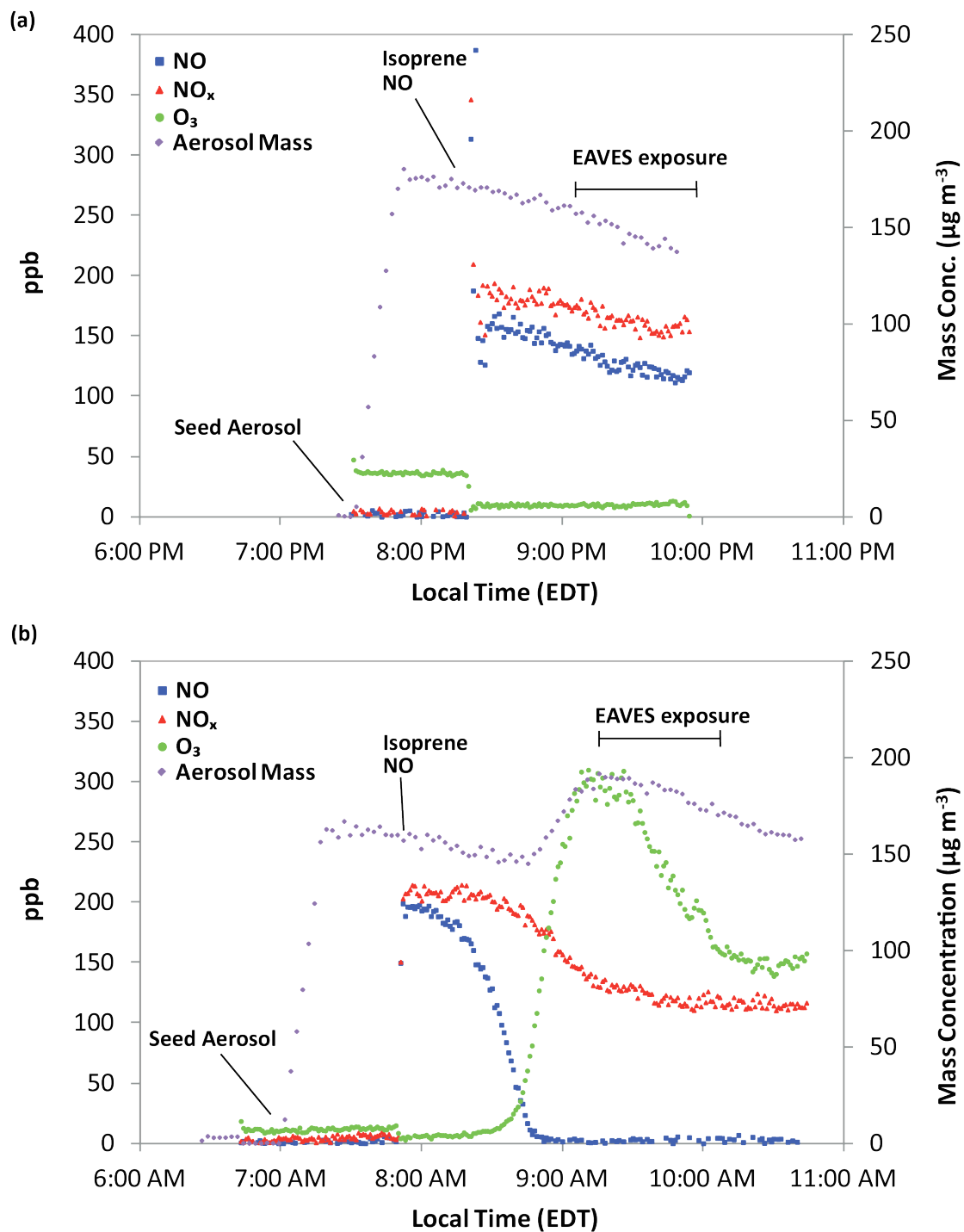
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754   **Figure 1.** Aerosol mass concentration and gas-phase product concentrations over time for (a)

755   dark control chamber experiment and (b) photochemically produced isoprene-derived SOA

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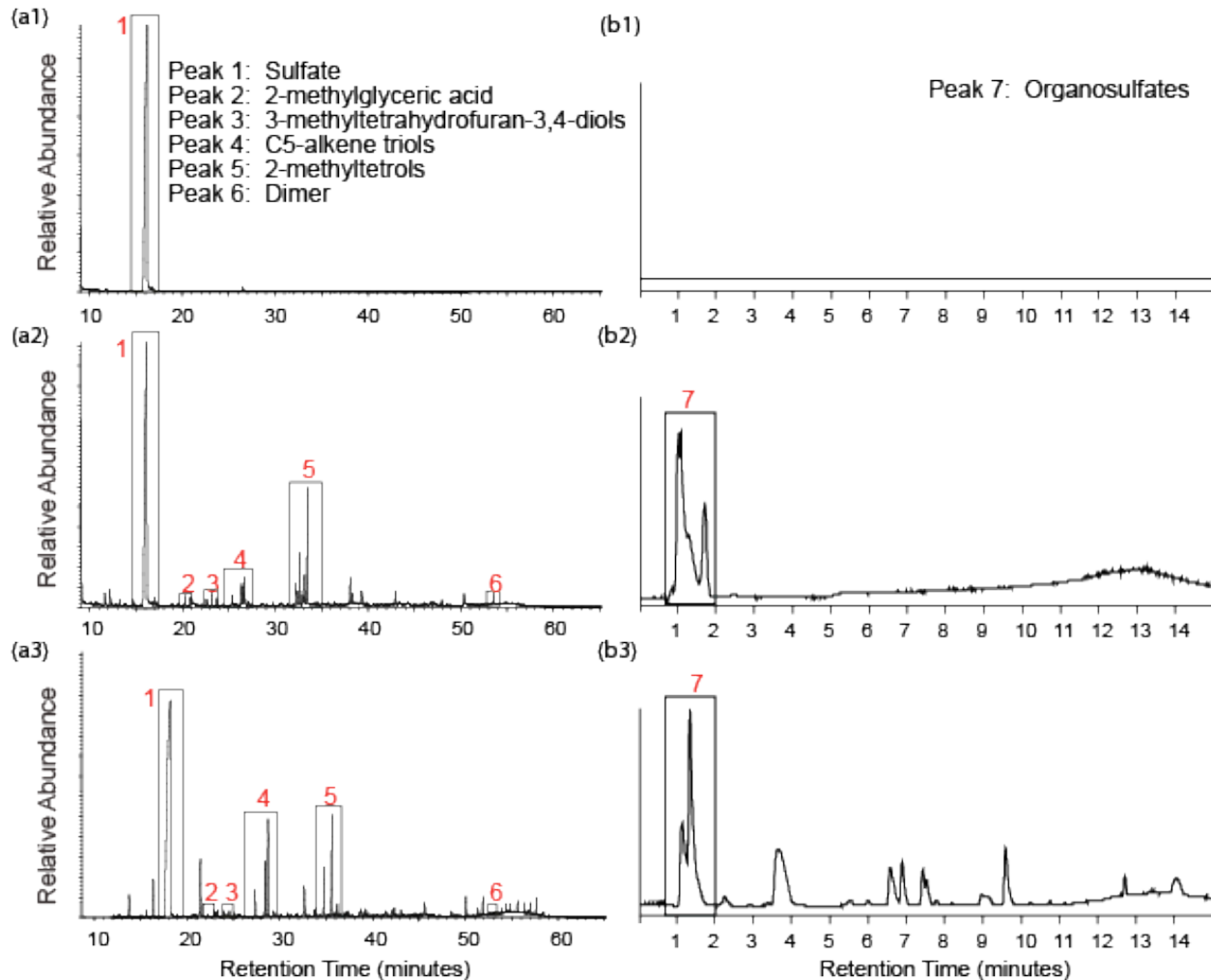
Figure 2. (a) GC/EI-MS total ion chromatograms (TICs) and (b) UPLC/ESI-HR-QTOFMS base peak chromatograms (BPCs) from a (1) dark control chamber experiment, (2) isoprene-derived SOA exposure chamber experiment, and (3) PM$_{2.5}$ sample collected from Yorkville, GA during summer 2010.

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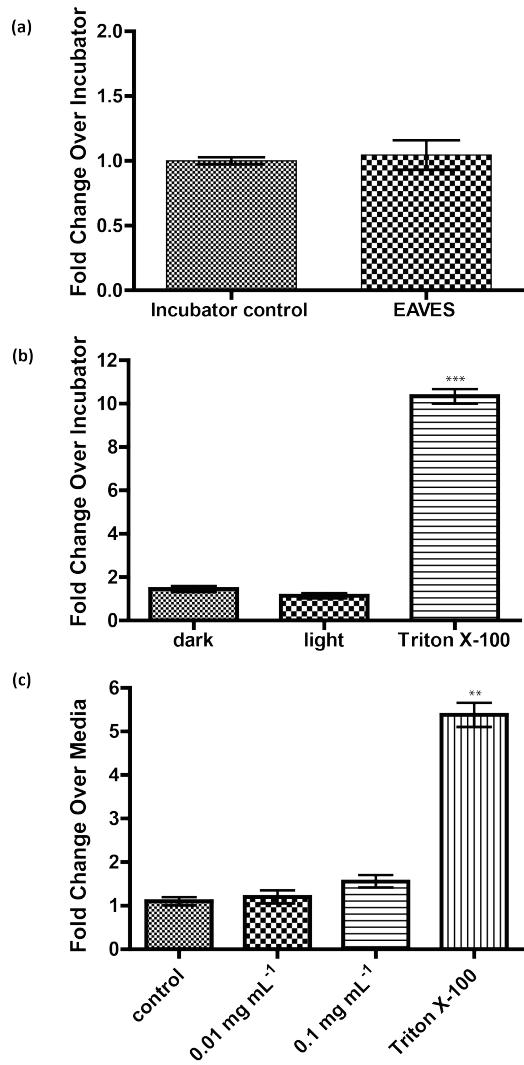
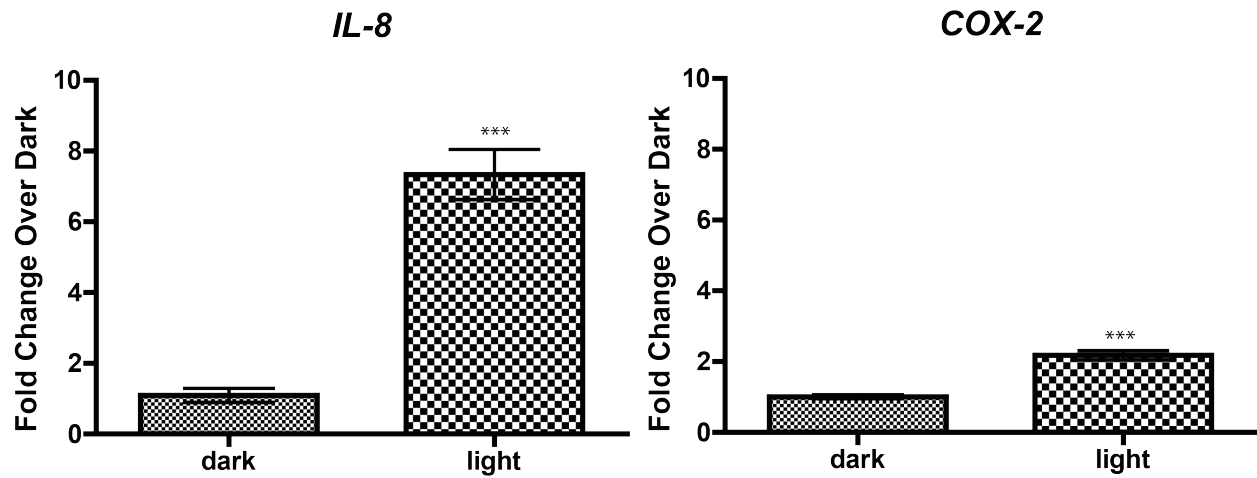


Figure 3. LDH release for (a) clean air controls, (b) EAVES exposures, normalized to incubator control, and (c) resuspension exposures, normalized to KBM only control. **p<0.005 and ***p<0.0005.

767

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

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

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

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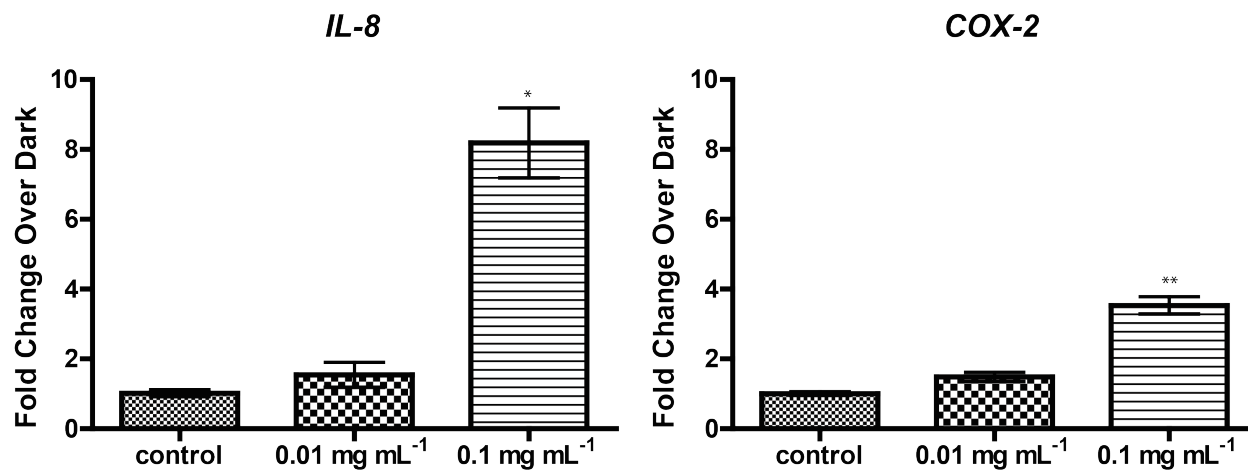
Figure 5. *IL-8* and *COX-2* expression induced by exposure to isoprene-derived SOA using

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.