

Notes to Editor:

1. We have highlighted in red all of the changes made for the final submission throughout the main text. These include the changes suggested by the Reviewers.
2. Below we provide a point-by-point response to the Editor and the reviewers.

Anonymous Referee #3

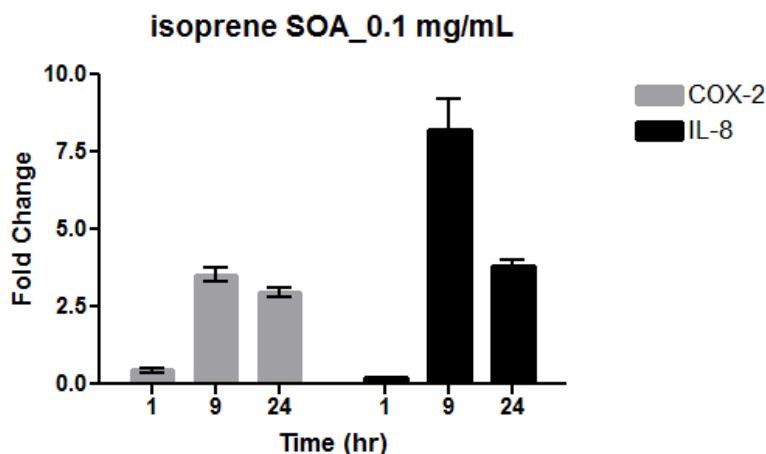
Received and published: 16 June 2016

In this paper, the toxicity of isoprene-derived secondary organic aerosol (SOA) was examined using the electrostatic aerosol in vitro exposure system (EAVES). The toxicity was evaluated by the lactate dehydrogenase (LDH) assay and also by probing the increase in the inflammatory genes *il-8* and *cox*. Exposures were performed in the light and the dark, for induction of isoprene SOA. The SOA obtained from the EAVES was also compared to PM_{2.5} collected in Yorkville. Cells maintained in the EAVES system were also compared to cells maintained in regular incubator.

The study is very interesting and provides a new comprehensive approach in understanding the activity of different aerosol components. Overall, the experiments are well described and documented. However, some evidence and logic to explain several issues are still lacking.

Major issues: 1. The authors have used 1 hour exposure time. How the setting of 1 hour exposure was chosen? Have different time been measured e.g. longer or shorter than 1 hour?

We were limited by EAVES operating conditions. We chose a 1 hour exposure time to ensure that the cells exposed at the air liquid interface were not stressed due to drying while maximizing deposited dose. However, exposure to the deposited particles would have continued over the 9 hour post-collection period. The nine hour post-collection time point was chosen to be consistent with Lichtveld et al. (2012) who used the same exposure device on this outdoor chamber facility. There will be changes to the levels of mRNA expressed at any given point of collection and gene expression does have a time profile. Because of the nature of the chamber exposures, one post-collection time point was chosen and the isoprene-SOA exposures were compared to the dark control exposure for that specific time. However, a time course analysis was conducted using resuspension techniques to show that *COX-2* and *IL-8* are maximized at 9 hours as shown in the graph below.



2. "Photochemical aging was allowed for approximately one hour to reach the desired exposure conditions of 30-40 $\mu\text{g m}^{-3}$ growth of isoprene-derived SOA on the pre-existing 170 $\mu\text{g m}^{-3}$ of acidified sulfate aerosol" How was this calculation performed? Is this number relevant to real exposure to isoprene SOA? Please also relate to 0.067 $\mu\text{g cm}^{-2}$.

A Differential Mobility Analyzer (DMA, Brechtel Manufacturing Inc.) coupled to a Mixing Condensation Particle Counter (MCPC, Model 1710, Brechtel Manufacturing Inc.) was used to measure the particle mass concentration in the chamber throughout the experiment.

As stated on page 9, lines 192-195: "A density correction of 1.6 g cm^{-3} (Riedel et al., 2016) and 1.25 g cm^{-3} (Kroll et al., 2006) was applied to convert the measured volume concentrations to mass concentrations for the acidified sulfate seed and SOA growth, respectively".

The isoprene-SOA growth was measured by taking the difference of the particle mass concentration before isoprene and NO injection and the mass concentration measured once the reaction stabilized and the mass concentration in the chamber peaked.

To put the dose of 0.067 $\mu\text{g cm}^{-2}$ in context of an exposure, new text has been added on page 16, lines 360-369 as follows:

"There are many ways to classify in vitro particle dosimetry based on the various properties of particles (Paur et al., 2011). For this direct deposition study, we chose to classify dose as SOA mass deposition per surface area of the exposed cells to mimic lung deposition. Gangwal et al. (2011) used a multiple-path particle dosimetry (MPPD) model to estimate that the lung deposition of ultrafine particles ranges from 0.006 to 0.02 $\mu\text{g cm}^{-2}$ for a 24-hr exposure to a particle concentration of 0.1 mg m^{-3} . Based on this estimate, a dose of 0.067 $\mu\text{g cm}^{-2}$ of isoprene SOA in our study can be considered a prolonged exposure over the course of a week. In fact, most other in vitro studies require dosing cells at a high concentration, sometimes close to a lifetime exposure to obtain a cellular response. Despite this limitation, in vitro exposures serve as a necessary screening tool for toxicity (Paur et al., 2011)."

3. Cytotoxicity measured by LDH is not sufficient for concluding that the isoprene secondary organic aerosol is not toxic. Another assay with a different principal should be performed, such as Hoechst (that interferes with DNA replication and not based on the activity of lactate dehydrogenase enzyme). In addition it would be useful to have an image of the cells before and after exposure?

Cytotoxicity measured through LDH was not an endpoint of interest and was not intended to be used to determine if isoprene-SOA was toxic or non-toxic to cells. Instead, we used LDH as an initial measure of cytotoxicity to ensure that our exposures were not overly toxic to interfere with subsequent gene expression measurements. Additionally, no observed morphological changes and similar RNA concentration and RNA integrity (260/280 and 260/230 values) in all samples, measured using Nanodrop, helped us assess that our exposures did not affect our subsequent gene expression measurements.

No morphological changes were observed after exposure so images were not collected.

4. Triton-X 1% ruptures the cell's membrane, causing leakage of the inner content of the cells. Therefore, its use as positive control is not be appropriate. It is better to use other cytotoxic agents that are known to cause cell death.

Triton-X 1% was used to rupture the cell membrane for a positive control as per the LDH protocol from the manufacturer. The LDH is within the cell membrane and Triton-X 1% serves as a positive control by releasing LDH into the supernatant.

5. What is the biological significance of the increase expression of il-8 and cox genes? Please describe its relevance to a signaling mechanisms that is relevant to isoprene exposure.

We included statements about the role of *IL-8* and *COX-2* in inflammation and diseases such as chronic obstructive pulmonary disease and asthma but did not want to make overreaching statements about what the elevations of *IL-8* and *COX-2* from our study mean in terms of human health. The mechanism by which isoprene-SOA increases the expression of *IL-8* and *COX-2* is not yet understood but some further discussion about mechanism relating exposure to expression of the two inflammation genes has been added on pages 17-19, lines 389-423 as follows:

"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 (Li et al., 2013; Nocker et al., 1996), 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 (Fong et al., 2000; Nocker et al., 1996; Peng et al., 2008).

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). Pathway analysis showed that gene expression of the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway was induced in cells exposed to isoprene-SOA (Lin et al., 2016) which has been reported to alter the expression of IL-8 through mRNA stabilization (Zhang et al., 2005). Therefore, isoprene-SOA may cause increases in both IL-8 and COX-2 primarily through an oxidative stress response. Additionally, the relationship between IL-8 and COX-2 can also explain the observed increase in IL-8 gene expression as the production of IL-8 can be stimulated through a COX-2 dependent mechanism in airway epithelial cells (Peng et al., 2008).

In vitro studies such as this one using a direct deposition model cannot fully elucidate mechanisms of lung inflammation and potential pathogenesis but serve as a necessary part of hazard characterization, particularly for a complex air mixture that has not been fully studied (Hayashi, 2005; Paur et al., 2011). Therefore, further in vitro studies exploring the health implication of the elevation of

IL-8 and COX-2 due specifically to isoprene-SOA exposure are necessary and may in turn justify further extension to in vivo work.”

Minor issues:

6. Materials and methods: 2.3 section should contain the concentration of all the components in the medium, including antibiotics.

The concentrations of all components in the medium have now been reported in the text on page 7, lines 149-153.

“Human bronchial epithelial (BEAS-2B) cells were maintained in keratinocyte growth medium (KGM BulletKit; Lonza), a serum-free keratinocyte basal medium (KBM) supplemented with 0.004% of bovine pituitary extract and 0.001% of human epidermal growth factor, insulin, hydrocortisone, and GA-1000 (gentamicin, amphotericin B), and passaged weekly”

Sterile cell culture techniques were employed and, to prevent low level contamination in the cell culture medium, antibiotics were not added.

7. Section 2.7: add the formation of cDNA using RT (kit, company etc.)

We utilized one-step RT-PCR using the QuantiTect SYBR Green RT-PCR Kit which combines the reverse transcription reaction with the PCR reaction. This has been clarified on page 12, lines 263-266 as follows:

“Changes in IL-8 and COX-2 mRNA levels were measured in BEAS-2B cells exposed to isoprene-derived SOA generated in our outdoor chamber facility using QuantiTect SYBR Green RT-PCR Kit (Qiagen) and QuantiTect Primer Assays for Hs_ACTB_1_SG (Catalog #QT00095431), Hs_PTGS2_1_SG (Catalog #QT00040586), and Hs_CXCL8_1_SG (Catalog #QT00000322) for one-step RT-PCR analysis”

8. Section 2.7: add the primers sequence for both gene tested.

According to Qiagen technical services, the primer sequences are proprietary and confidential. For additional information, the primers' unique catalog number has been added to the text on page 12, lines 263-266 as follows:

“Changes in IL-8 and COX-2 mRNA levels were measured in BEAS-2B cells exposed to isoprene-derived SOA generated in our outdoor chamber facility using QuantiTect SYBR Green RT-PCR Kit (Qiagen) and QuantiTect Primer Assays for Hs_ACTB_1_SG (Catalog #QT00095431), Hs_PTGS2_1_SG (Catalog #QT00040586), and Hs_CXCL8_1_SG (Catalog #QT00000322) for one-step RT-PCR analysis”

9. There is no reference to Figure 5 in the text.

Figure 5 was mistakenly referred to as Figure 4 in the text. This has been corrected on page 15, Lines 337-339:

“Changes in the mRNA levels of IL-8 and COX-2 from cells exposed to resuspended isoprene-derived SOA collected from photochemical experiments are shown as fold-changes relative to cells exposed to resuspended particles from dark control experiments in Fig. 5”

10. When relating to genes, please use small italics letters (il-8, cox)

We have followed the guidelines for human gene nomenclature as listed in Wain (2002) by using upper-case letters.

11. In figure 2 the a3 graph (on the right panel) the line is in red. This is probably a mistake. If not please add the purpose for the red line in the legend 12. In the graphs indicating fold change, it would be better to write compared to what in the Y axis and not just the legend. Also add information about the normalizing gene in the legend.

The line color has been changed from red to black. The y-axis for the graphs indicating fold change now includes what the fold change is compared against. The information about the normalizing gene has been added to the legend.

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