

## Innovative Experimental Techniques to Help Understand Exposure to Volatile Organic Air Toxics

Harvey E. Jeffries<sup>1</sup>, Kenneth G. Sexton<sup>1</sup>, and Ilona Jaspers<sup>2</sup>. <sup>1</sup>*School of Public Health.* <sup>2</sup>*School of Medicine University of North Carolina at Chapel Hill.*

The overall goal of this project was to develop and demonstrate new experimental techniques and methodologies drawn from human health effects and atmospheric chemistry practices, which can be used to advance and prioritize the study of the risks of realistic atmospheric mixtures and their chemical reactions. These mixtures will include volatile organic air toxics and their transformation products, a significant subset of hazardous air pollutants (HAPs). Simultaneously, we tested for their true toxicological effects on cultured human lung cells.

We advanced our knowledge of the fundamental mechanisms that determine the atmospheric fate and secondary formation of HAPs. This contributed to more accurately describing the environmental transport and fate of complex mixtures in risk assessment calculations and simulations. We showed the advantage of combining two experimental methods—smog chambers used to generate test atmospheres, interfaced directly with gas-phase *in vitro* techniques—to investigate what gaseous mixtures, components, and reaction products within these mixtures are most potent in causing toxic effects. We demonstrated that lung cells can be effectively exposed to reacting urban-like gas mixtures from our outdoor “smog” chamber. We have shown in this project that experimentally-produced photochemical products of primary pollutant mixtures, such as 1,3-butadiene, isoprene, methanol, or toluene with nitrogen oxides, representing atmospheric transformations, can be more toxic than the precursor pollutants (as measured as inflammation, interleukin-8 [IL-8], interleukin-6 [IL-6], and cytotoxicity or cell death). IL-8 is the same endpoint measured in both healthy humans and those with asthma and chronic obstructive pulmonary diseases (COPD) after exposure to air pollutants (Bossen, 2003, Gerritsen, 2005, Holz, 1999, Holgate, 2003, Devlin, 2005). We have shown that for compounds like isoprene, the major photochemical products produced are methacrolein and methylvinylketone, which contribute to the toxicity of the transformation mixture beyond the effects induced by ozone alone. In contrast, when methanol is photochemically transformed in the atmosphere to primarily formaldehyde, this product does not contribute to inflammation or cytotoxicity beyond the ozone produced. In addition, small differences in molecular structure (e.g., one methyl group) between similar compounds, such as 1,3-butadiene and 2-methyl-1,3-butadiene (isoprene), can result in surprising differences in relative cytotoxicity and inflammation in both the original compounds and their photochemical products.

Furthermore, we showed that by conducting experimental exposures to mixtures of identified transformation products, subsequent confirmation and accounting tests can be performed. The confirmation experiments, which examine the effects of identified photochemical transformation products, have demonstrated that the major products that were formed during photochemical reactions of the chemicals significantly contribute to the cytotoxicity and inflammation measured, beyond the effects induced by the ozone formed during these reactions. As the confirmation experiments were performed with the same concentrations observed in the full photochemical system, they can account for how much these major products contribute to the full effect observed from the complete photochemical system. In some cases (comparing compounds and endpoints considered), our data suggest that there are clearly effects caused by other photochemical products not directly tested.

Along with this system, a new *in vitro* exposure protocol was developed to better demonstrate how lung cells of relatively healthy individuals, once exposed to low levels of ozone on a daily basis, may respond to acute exposures to HAPs. Results demonstrate that pre-exposing cells repeatedly to ozone caused a sensitization effect. Therefore, the enhanced responses (i.e., increases in cytotoxicity and inflammatory cytokine release) caused by subsequent challenges to primary products generated from isoprene and

butadiene photochemical mixtures have potential implications for susceptible, but otherwise healthy, humans and those with asthma and COPD.

In addition to the experiments evaluating how photochemical transformations affect the toxicity of reactive atmospheric pollutants (i.e., isoprene, 1,3-butadiene, methanol, toluene) and experiments to evaluate which of the products influence the toxicity the most (i.e., product confirmation experiments with isoprene, 1,3-butadiene and methanol), new methods were used to evaluate if glutathione (GSH)-mediated oxidative stress was the mechanism behind the response.

Antioxidants provide cellular defenses against reactive oxygen species. Among all the antioxidants found within the lung, GSH is considered to be a main antioxidant molecule; therefore we chose to study this pathway of response. Buthionine sulphoximine (BSO) is a potent and selective inhibitor of GSH synthesis and has been used to deplete intracellular GSH levels during previous studies. GSH reduced ethyl ester (GSH-ET) increases the intracellular level of GSH within samples, thus creating a protective environment against some oxidative stress pathways. GSH-ET and BSO are commonly used with *in vivo* and *in vitro* methods to study the mechanism of response induced by pollutant exposures.

A549 cells were used as the exposure model combined with GSH-ET- or BSO- modified serum-free media during exposures to ozone, formaldehyde, a synthetic product mixture containing methacrolein and methyl vinyl ketone, and photochemically generated products of isoprene or 1,3-butadiene and nitric oxide mixtures.

Overall, these results imply that production of cytokines by human respiratory epithelial cells is affected by photochemical oxidant-induced oxidative stress (as indicated by changes in IL-8 production), and that GSH in these cells may or may not modulate this production. These data suggest that although this pathway was not the main response inducer, it cannot be used to infer what other mechanism is causing the response. The response may or may not be due to another oxidative stress pathway such as lipid peroxides or direct oxidative damage on the cellular surface. In summary, the upregulation of inflammatory cytokine production and cytotoxicity via lactate dehydrogenase release are not entirely mediated by oxidative stress.

In addition, chemical and toxicological experiments were conducted with d-limonene and a commercial cleaning product containing d-limonene, and compared with similar matching experiments with a similar cleaning product using 2-butoxy ethanol (a glycol ether) as the active solvent. These experiments were conducted to investigate important questions raised in potentially conflicting control strategies for outdoor ozone and indoor air pollutants (e.g., cleaning agents). The butoxy ethanol is less reactive in the atmosphere and would be preferred in regards to controlling ambient ozone. However, our results show that d-limonene and cleaning product containing d-limonene, in the absence of ozone, are less toxic (as measured by inflammation indicated by IL-8) than the glycol ether-based cleaning product. Toxic products from d-limonene are generated from mixing with ozone, including formaldehyde and aerosols. D-limonene contributions to outdoor ozone due to leaks from indoor use are estimated to be very small, but regulators suggest controlling d-limonene and other terpene use indoors, which would encourage the use of less reactive but potentially more inflammatory products. Also, it appears that the other components of the commercial cleaner containing d-limonene affect the formation of these toxic products, and we recommend that any future toxicity studies performed on commercial products use the actual products (rather than using solely the active agent).

**Implications:** Our findings suggest that standard risk assessments based on traditional methods such as emissions release estimates or ambient monitoring of air toxics may be significantly underestimated. Further, epidemiology studies may falsely find associations between chemicals of interest which are actually surrogates for other secondary toxic species formed from other chemicals. This is based on our

findings that the familiar chemicals and mixtures we have examined react in the atmosphere, contributing to photochemical reactions producing ambient ozone and secondary HAPs, that contribute significantly to inflammation (the same IL-8 endpoint measured in both healthy humans and those with asthma and COPD after exposure to air pollutants) or cytotoxicity. Therefore, the smog-chamber-*in-vitro*-toxicology system and experimental design provides a more realistic approach to estimating the toxicity of ambient air pollutants, by using dynamic mixtures directly in the gas phase with all products formed, both known and unidentified. Furthermore, by conducting toxicity exposures to mixtures of identified transformation products, subsequent confirmation and accounting tests can be performed. The confirmation experiments, which examine the effects of identified photochemical transformation products, have demonstrated that the major products that were formed during photochemical reactions of the chemicals significantly contribute to the cytotoxicity and inflammation measured, beyond the effects induced by the ozone formed during these reactions. In some cases (comparing compounds and endpoints considered), our data suggest there are clearly effects caused by other photochemical products not directly tested or measured. This is a clear advantage over many other techniques which require collection, identification, and testing with a specific compound that may not be available. Furthermore, these techniques provide for dynamic direct exposure in the original gas phase in real time without any collection techniques, which prevents potential modification of the toxic compounds being studied. Perhaps most importantly, having been demonstrated and proven, these techniques can be used to screen for the full toxic potential of atmospheric pollutants from new commercial products without releasing them into the atmosphere. Further tests with identified degradation products can be helpful in identifying other long range potential risks. The power and utility of these new methods could be expanded to include additional types of cells and/or toxicological endpoints to be indicators of other types of potential health effects and risks.

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**Presentations:**

Doyle M., Sexton K.G., Jaspers I., Bridge K., and Jeffries H.E. (2004). Effects of 1,3-Butadiene, Isoprene, and their Photochemical Degradation Products on Human Lung Cells. Poster presented at Annual Meeting of The Society of Toxicology, Baltimore, Maryland, March 21-25, 2004.

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Ebersviller S., Naess E., Sexton K.G., and Jeffries H.E. (2006). A Comparison of the Oxidation Products and Secondary Organic Aerosol Formation Potential of d-Limonene and a Household Cleaning Product Containing d-Limonene. Presentation at the Conference on Indoor Environmental Quality: Problems, Research and Solutions, Durham, NC, July 17-19, 2006.

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