Comparison of Ethylene- and Ozone-Induced Nasal Toxicity: A Similar Mode of Action?

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Understanding the Health Risks of Lower Olefins, 11/06/14, Austin, TX

Slides Prepared for the 2014 Symposium on Understanding the Health Risks of Lower Olefins
Outline

• Comparative nasal anatomy

• Nasal toxicity of ethylene and ozone

• Mode of action of ozone-induced nasal pathology

• Inhalation study to understand the mode of action of ethylene-induced nasal pathology

• Summary and questions
### Comparative Nasal Airway Structure and Function

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Monkey</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Volume (cm³)</strong></td>
<td>16</td>
<td>8</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Turbinate Anatomy</strong></td>
<td>Simple</td>
<td>Simple</td>
<td>Complex</td>
</tr>
<tr>
<td><strong>Olfactory Epithelial Surface Area</strong></td>
<td>Small &lt;10%</td>
<td>Moderate 20-30%</td>
<td>Large 50%</td>
</tr>
<tr>
<td><strong>Breathing</strong></td>
<td>Oronasal</td>
<td>Oronasal</td>
<td>Nasal</td>
</tr>
</tbody>
</table>
Rodent Nasal Airway Epithelium and Tissue Selection

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Ethylene-Induced Nasal Pathology

1. Eosinophilic Rhinitis
2. Increased Epithelial Mucus
3. Epithelial Hyalinosis (Ym1/Ym2 Protein)
Distribution of Ethylene-Induced Nasal Inflammatory and Epithelial Lesions

T1

Mid-Septum

T2

T3

Nasopharyngeal Meatus

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Ethylene-Induced Eosinophilic Rhinitis in F344 Rats

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Ethylene-Induced Increase in Nasal Epithelial Mucosubstances in F344 Rats

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# Comparison of Nasal Pathology in F344 Rats Exposed to Inhaled Toxicants

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>Rhinitis with Eosinophils</th>
<th>Increase in Epithelial Mucus</th>
<th>Epithelial Hyalinosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylene</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>(10,000 ppm, 20 days)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propylene</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>(10,000 ppm, 20 days; Pottenger et al., 2007)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ozone</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>(0.5 ppm, 20 mo; Harkema et al., 1997)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorine</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>(2.5 ppm, 2 yr; Ibanes et al., 1996)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Ozone ($O_3$)

- One of the most reactive chemicals
- Secondary gaseous air pollutant in photochemical smog
- 131 million people (45% of the U.S. population) live in communities where average ambient concentrations exceed the NAAQS
- Respiratory toxicant causing airway inflammation and remodeling
- Long-term exposure causes an increase in mortality
Comparative Nasal Toxicity of Ozone


O3-Induced Remodeling of Maxilloturbinate in Rat

Filtered Air (0 ppm O3)  13 wks, 5 days/wk  O3 (0.5 ppm)

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Eosinophilic Rhinitis and Nasal Epithelial Remodeling in Mice Exposed to Ozone

Inhalation Exposure

Nasal Epithelium

Neutrophils

Epithelial Repair & Remodeling

Eosinophils

Day 1 of Exposure

Day 24 of Exposure

Injury → Necrosis → Neutrophils → Eosinophils & Mucous Cell Metaplasia
Temporal Cell and Cytokine Responses in the Nasal Airway of Mice Exposed to Ozone

- C57BL/6 male mice
- 0 or 0.5 ppm ozone exposure
- 4h/day for 1, 2, 4, 9 or 24 consecutive weekdays
- Nasal histopathology and morphometry
- RT-PCR (epithelial and inflammatory gene expression)
- Clustering of gene expression and phenotype changes with time of exposure

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Ozone-Induced Nasal Pathology Restricted to the Proximal Lateral Wall

- Filtered Air (0ppm O3)
  - Normal Nasal Transitional Epithelium
- 0.5 ppm O3, 24 days
  - Hyperplastic Epithelium with Hyalinosis
Major Basic Protein-laden Eosinophils
Nasal Epithelial Remodeling in Mice Exposed to Ozone

24-day Air Exposure 24-day Ozone Exposure
Nasal Mucosa (Hyalinosis, arrows)

Intraepithelial Mucosubstances

Ym1/2 Chitinase-Like Proteins

Intraepithelial Mucosubstances per Basal Lamina 24 weekdays of Ozone Exposure

Epithelial Ym1/2 per Basal Lamina 24 weekdays of Ozone Exposure
Temporal Changes in Granulocytic Influx with Repeated Ozone Exposure (1-9 days; 4h/day)

Density of Granulocytes in Nasal Mucosa
Temporal Changes in Nasal Epithelium with Repeated Ozone Exposure (1-9 days; 4h/day)

Epithelial Mucosubstances

Epithelial Ym1/Ym2 Protein

[Images and graphs showing changes in mucosubstances and Ym1/Ym2 proteins over time]
Eosinophilic Rhinitis and Nasal Epithelial Remodeling in Mice Exposed to Ozone

Inhalation Exposure

Nasal Epithelium

Day 1 of Exposure

Neutrophils

Epithelial Repair & Remodeling

Eosinophils

Day 9 of Exposure

Injury → Necrosis → Neutrophils → **Eosinophils & Mucous Cell Metaplasia**
Selected nasal mucosal tissues for gene expression analyses (PCR-arrays, qRT-PCR)

- Mice sacrificed at designated times post-exposure
- Nasal cavities split in half
- Two halves immediately immersed in RNAlater® solution
- Nasal mucosal tissues microdissected from airways
- RNA extracted from site-selected nasal mucosal tissues
- Conducted PCR-arrays (pooled cDNA by group) and qRT-PCR assays (cDNA from individual rats)
Unsupervised Hierarchical Clustering of Temporal Changes in O3-Induced Gene Expression and Morphometric *Phenotypes in the Nasal Mucosa

Th1 Inflammatory Profile

Th2 Inflammatory Profile
Temporal Fold Changes in Th1 and Th2 Cytokine Gene Expression

**IL-1β**

<table>
<thead>
<tr>
<th>Exposure Days (Hours post-exposure)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (2)</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td>1 (24)</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>2 (24)</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>4 (24)</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>9 (24)</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>

**IL-5**

<table>
<thead>
<tr>
<th>Exposure Days (Hours post-exposure)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (2)</td>
<td>30.0 ± 1.5</td>
</tr>
<tr>
<td>1 (24)</td>
<td>25.5 ± 1.2</td>
</tr>
<tr>
<td>2 (24)</td>
<td>20.0 ± 0.8</td>
</tr>
<tr>
<td>4 (24)</td>
<td>15.0 ± 0.5</td>
</tr>
<tr>
<td>9 (24)</td>
<td>10.0 ± 0.3</td>
</tr>
</tbody>
</table>

**IL-6**

<table>
<thead>
<tr>
<th>Exposure Days (Hours post-exposure)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (2)</td>
<td>15.0 ± 0.5</td>
</tr>
<tr>
<td>1 (24)</td>
<td>12.0 ± 0.4</td>
</tr>
<tr>
<td>2 (24)</td>
<td>10.0 ± 0.3</td>
</tr>
<tr>
<td>4 (24)</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>9 (24)</td>
<td>2.5 ± 0.1</td>
</tr>
</tbody>
</table>

**IL-13**

<table>
<thead>
<tr>
<th>Exposure Days (Hours post-exposure)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (2)</td>
<td>25.0 ± 1.5</td>
</tr>
<tr>
<td>1 (24)</td>
<td>20.0 ± 1.2</td>
</tr>
<tr>
<td>2 (24)</td>
<td>15.0 ± 0.8</td>
</tr>
<tr>
<td>4 (24)</td>
<td>10.0 ± 0.5</td>
</tr>
<tr>
<td>9 (24)</td>
<td>5.0 ± 0.3</td>
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*Significant change from baseline.
Repeated, episodic ozone exposures in mice induce Th2 cytokine overexpression, eosinophilic rhinitis and increased epithelial chitinase-like proteins (Ym1/Ym2).

These results suggest an etiologic role of ambient ozone in the development of nonallergic eosinophilic rhinitis.

Our animal study gives biologic plausibility to epidemiologic associations between ozone exposure and eosinophilic rhinitis and systemic eosinophil cationic proteins in children (Frischer et al. 2001, 1993)
What is the Role of Lymphoid Cells in Eosinophilic Rhinitis & Nasal Epithelial Remodeling in Mice Exposed to Ozone?
Rag 2-/- Gamma C-/- Mice

• Cross of common cytokine receptor gamma chain (γc) KO mouse with Rag2 (recombinase activating gene 2)-deficient mice

• Double KO mice lack T cells, B cells, NK cells, and type 2 innate lymphoid cells (ILCs) – Lymphoid cell-deficient animals

• Mice are useful in combination with parental Rag2 KO mice for sorting out the role of ILCs
Rag 2-/- Mice

• Contains a disruption of the recombination activating gene 2 (Rag2)

• Homozygous mice exhibit total inability to initiate V(D)J rearrangement and fail to generate mature T or B lymphocytes

• Do have type 2 innate lymphoid cells
Ozone-Induced Eosinophilic Rhinitis is Dependent on Innate Lymphoid Cells

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Filtered Air</th>
<th>0.8 ppm O3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Rag2(-/-)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Rag2(-/-)γc(-/-)</td>
<td>6</td>
<td>6</td>
</tr>
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### Density of Eosinophils in the Nasal Mucosa

- **Air Exposed**
  - T, B & ILCs Sufficient
  - C57BL/6
- **O3 Exposed**
  - T, B, & ILCs Sufficient
    - C57BL/6

- **O3 Exposed**
  - T & B Cells Depleted
    - ILCs Sufficient
      - Rag2(-/-)
  - **O3 Exposed**
  - T, B, & ILCs Depleted
    - Rag2(-/-)γc(-/-)
Ozone-Induced Mucous Cell Metaplasia is Dependent on Innate Lymphoid Cells

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Rag2(-/-)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Rag2(-/-)γc(-/-)</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Volume Density of Epithelial Mucosubstances

![Graph showing volume density of epithelial mucosubstances](image)

* P < 0.05
Ozone-Induced Hyalinosis (Ym1/Ym2) is Dependent on Innate Lymphoid Cells

<table>
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<tr>
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<tbody>
<tr>
<td>C57BL/6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Rag2(-/-)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Rag2(-/-)γc(-/-)</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Volume Density of Epithelial Ym1/Ym2 Protein

Air Exposed T, B & ILCs Sufficient

C57BL/6

O3 Exposed T, B, & ILCs Sufficient

C57BL/6

O3 Exposed T & B Cells Depleted

ILCs Sufficient

Rag2(-/-)γc(-/-)

O3 Exposed T, B, & ILCs Depleted

Rag2(-/-)γc(-/-)

* P < 0.05
## Study Summary and Conclusion

O3-induced eosinophilic rhinitis and nasal epithelial remodeling are mediated by ILCs.

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>T &amp; B cells</th>
<th>ILCs</th>
<th>O3-induced lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rag2(-/-)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rag2(-/-)γc(-/-)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Multifaceted Role of Type 2 Innate Lymphoid Cells (ILC2) in Airway Inflammation

IL-33 (alarmin) Immunohistochemistry
C57BL/6 Mice, Nasal Epithelium

Filtered Air Control
0 ppm Ozone

9-day Ozone Exposure
0.5 ppm, 4h/day
Questions to be answered with Current MOA Study

• Will a 12-wk episodic exposure to ethylene followed by a one day ethylene challenge cause nasal lesions that resemble those of a known respiratory sensitizer (ortho-phthalaldehyde; OPA) or a common respiratory irritant (ozone; O3)?

• Do the nasal inflammatory and epithelial lesions caused by inhaled ethylene persist after 2 wks postexposure in filtered air?
Questions to be answered with Current MOA Study

- Will episodic exposures to ethylene cause an increase in the severity of nasal toxicity with an increase in the number of exposures?

- Does the mode of action for the ethylene-induced nasal lesions resemble that of ozone-induced eosinophilic rhinitis and nasal epithelial remodeling or OPA-induced eosinophilic inflammation?
Current Mode of Action Study

• **Purpose**: To determine if ethylene is a respiratory irritant or sensitizer (Study 1)? Does the mode of action of ethylene resemble that of ozone (Study 2)?

• **Animals**: F344/DuCrI rats (Study 1); C57BL/6 mice, Rag2-/-, and Rag2-/-yc-/- (Study 2)

• **Episodic exposure**: 2wk ethylene (5d/wk; 10,000 ppm) → 2wk filtered air (0 ppm) → 2wk ethylene → 2wk filtered air → 2wk ethylene → 2wk filtered air → 1-day ethylene challenge

• **Endpoints**: Nasal histopathology; morphometry; RT-PCR (mRNA expression of inflammatory cytokines and epithelial proteins (e.g., secretory); pulmonary function (rat)
Initial Results: Epithelial Mucosubstances in Rat Nasopharyngeal Meatus (2wk-Ethylene Exposure and 2wk Postexposure)

* Significantly different, $p < 0.05$

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Initial Results: Epithelial Mucosubstances in Mouse Nasopharyngeal Meatus (2wk-Ethylene Exposure and 2wk Postexposure)

* Significantly different, $p < 0.05$
Summary

✓ Comparative nasal anatomy

✓ Nasal toxicity of ethylene and ozone

✓ Mode of action of ozone-induced nasal pathology

✓ Inhalation study to understand the mode of action of ethylene-induced nasal pathology
Acknowledgments

- American Chemistry Council’s Olefins Panel (Dr. Marcy Banton, E/P Workgroup Chair)
- Michigan State University (Drs. Harkema, Wagner, Brandenberger, and Kumagai)
- EPA Great Lakes Air Center for Integrated Environmental Research

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Questions?

Thank you!

Caricature by David Levine, New York Review of Books