The Potentiating Effects of Acetaminophen on Oxidant Air Pollutant Sensory Irritation and the Onset of Asthma

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Acknowledgements

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I also would like to extend my great thanks to both Greg Smith as well as Joe Cichocki. Without their constant support and knowledgebase, none of this would have been possible. Thank you for all of your personalized guidance over the years.

Finally, thank you to my family and friends for always pushing me to be my best in everything I choose to pursue, whether great or small.
Abstract

Through its toxic metabolites, acetaminophen can cause oxidative injury in the liver. This damage has not yet been investigated in the respiratory tract. If acetaminophen also causes oxidative stress and injury here, this widely used antipyretic could potentiate the adverse effects of oxidant air pollutants. Thus, the primary goal of this project is to determine if low non-hepatotoxic doses of APAP is correlated with an increase of oxidative stress in the airways, possibly linking APAP to the onset of asthma. Using data that reflected murine breathing patterns, the addition of acetaminophen greatly increased the reflex irritant response to ETS through the potentiation of the oxidant sensory irritant, most likely caused by acetaminophen's metabolite, NAPQI.
Introduction

As defined by the National Heart, Lung, and Blood Institute, “Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role: in particular, mast cells, eosinophils, T lymphocytes, macrophages, neutrophils, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an increase in the existing bronchial hyperresponsiveness to a variety of stimuli. Reversibility of airflow limitation may be incomplete in some patients with asthma.” (National Asthma Education and Prevention Program 2007). In other words, asthma is a chronic, lifetime condition that plagues people of all ages. Asthma severity and response to treatment can vary from person to person and both depend on the conjunction of the previously mentioned factors.

One major feature of asthma is the inflammation of the airways. This inflammation is brought about by the exposure to allergens and irritants, such as tobacco smoke, which trigger an IgE-mediated event, resulting in the release of histamine, leukotrienes, and prostaglandins from mast cells. This inflammation is accompanied by bronchoconstriction and narrowing of the patient’s airways. These events are mainly in relation to bronchial smooth muscle contraction. Although exposure to irritants is a common factor associated with this constriction, other events can precipitate the same effects, including stress, exercise, and exposure to cold air. In particular, sensitized mast cells may be activated by osmotic stimuli in addition to irritants and allergens. Osmotic
stimuli are believed to mediate bronchoconstriction in exercise-induced asthma (National Asthma Education and Prevention Program 2007). In fact, osmotic/exercise-induced mast cell activation leads to the same mediator release and bronchoconstriction of similar magnitude as allergens. These mechanisms of non-allergic events are less well defined but they still contribute to the overall spectrum of asthmatic diseases (Myers and Tomasio 2011).

The prevalence of asthma worldwide is growing at an alarming rate. As of recent, approximately 235 million people across the globe currently battle with asthma, and approximately 250,000 people die from it each year. The incidence of American children being diagnosed with the disease each year is about 9.5%, contrasted with the same data point some 30 years ago of approximately 3.6% (National Asthma Education and Prevention Program 2007). How has asthma integrated so quickly into today’s human population? Everything from host factors to environmental precipitants has been proposed to play some part in this modern day epidemic.

Utilizing serum IgE concentrations as a surrogate endpoint, recent studies have shown that those with first-degree relatives affected by asthma have a 20-25% risk of contracting the disease, contrasted with approximately 4-8% in a normal population. More specifically, those children with a mother who presents with atopic, or allergic, asthma, as discussed previously, have a higher risk of contracting asthma than those with an atopic father. With these data in mind, a genetic association seems to be apparent. However, recent studies centered on genetic associations have yet to confirm any such lineage. Recent data have only been able to suggest a possibility of association or confirm
a genetic link to only one phenotypic characteristic of the overarching disease (National Asthma Education and Prevention Program 2007).

Worldwide, about 250,000 people die from asthma each year. Approximately 80% of this mortality stems from low or middle income countries. The other 20% of deaths may occur in higher standing countries, but the majority of the deaths are found to occur to people with lower socioeconomic standing. This association between economic standing and the onset of asthma suggests some correlation with socioeconomic status and disease. Further, this association could possibly be due to the increased amounts of irritants that lower income families are exposed to, triggering more atopic reactions to take place, or due to a lack in accessing healthcare. Another common difference present throughout families of varying economic standings is diet. Thus, one’s diet may influence the development of asthma, further justifying the possibility of environmental causation.

At the forefront of the environmental component of asthma lies the hygiene hypothesis. This hypothesis revolves around an overabundance of thymus helper type 2 lymphocytes (TH2) cells over TH1 thymus helper type 1 lymphocyte (TH1) cells. This higher concentration of TH2 cells can be caused by a variety of correlating factors, including the widespread use of antibiotics, a sensitization to house-dust mites and cockroaches, an urban environment, and a Western lifestyle. On the other hand, those having older siblings, an early exposure to day care, tuberculosis, measles, or hepatitis A infection, or a rural home setting are more likely to develop an abundance of TH1 cells. Thus, those with a larger amount of TH1 cells are less likely to develop asthma. This hypothesis is highly reliant on the assumption that the immune system of a newborn is skewed towards the TH2 cell over the TH1 cell. Thus, lack of those stimuli that promotes
the development of TH1 cells seems to be the ultimate causative factor. Further, the production of TH2 cells promotes signal cascades that decrease the production of TH1 cells and vice versa, providing further conceptual support for this hypothesis (Myers and Tomasio 2011). However, is there a genetic indication that promotes the development of TH2 cells over TH1 cells that coincides with this hypothesis? This question proposes some sort of balance between the two contributing factors.

With the hygiene hypothesis in mind, one of the most obvious environmental contributors to the development of asthma is the presence of allergens in the environment. Although the role of allergens has not been fully defined, the activation of an IgE mediated reaction generally leads to inflammation and a TH2 response. Long-term exposure to allergens can cause a dominant TH2 presence as well as continuous allergic inflammation, leading to the onset of asthma. Among the most common allergens shown to have an association with the development of asthma are dog and cat dander. However, at the same time, some current research has shown that early exposure to these allergens could help prevent the onset of asthma, leaving many questions unanswered. Also, exposure to the cockroach, mouse, or dust mite allergens may play a large role in the development of asthma in urban settings (Kazani and Israel 2012).

Correlations also exist that tie air pollution, smoking, and a poor diet to the development of asthma. However, up to this point, the involvement of irritants and allergens have likely only painted half of the picture. More specifically, in the past few years, both an increase in the use of acetaminophen containing products in children and an increase in asthma prevalence have been observed. Thus, an association between acetaminophen and asthma has been suggested (Eccles 2006).
Today, acetaminophen, also known as Tylenol and referred to herein as APAP (\(n\)-acetyl-\(para\)-aminophenol), is the most commonly used antipyretic and analgesic in the United States. The medication is indicated as monotherapy for both fever and mild to moderate pain as well as adjunct therapy for moderate to severe pain. The drug has also been utilized as prophylactic treatment for many conditions, including headaches, as well as for osteoarthritis pain. APAP is available as both brand and generic over the counter. However, the drug is also found in a variety of prescription and over the counter combination products, including Percocet, Vicodin, and Fioricet. The use of APAP combined with other medications in recent history has greatly contributed to the increased intake in today’s population. Although these combination products have proven to be very beneficial in some cases, their use has also been shown to increase one’s risk of overdosing on APAP as many do not account for the additional APAP when taking the product (Eccles 2006).

An oral APAP overdose for an adult is defined as taking over 4 grams of the product per day for several weeks or taking over approximately 10 grams in less than 8 hours. Fatalities occur with acute doses of over 15 grams. For infants, an acute overdose of 150mg/kg or higher has been associated with adverse effects. The recommended monotherapy dose is approximately 15 mg/kg/dose every 4-6 hours, not to exceed 4 grams per day if taking the medication over the counter. The drug is also available as an IV formulation with a similar dosing schedule and toxicity range. It has also been noted that infants under the age of 2 should not receive APAP over the counter. However, the oral drug can be prescribed down to the age of 3 months (Eccles 2006).
APAP exerts its analgesic and antipyretic effects centrally, with minimal if any anti-inflammatory activity. Although its mechanism of action is still under investigation, it has been proposed to involve inhibition of the cyclooxygenase (COX) enzymes. More specifically, it has been proposed that the drug inhibits a COX-3 variant more specifically than COX-1 or COX-2 (DRUGDEX® System). This inhibition has been shown to contribute to an elevation in the overall pain threshold and reduction in pyretic activity. Due to the inconclusive evidence for the actual mechanism of APAP, other pathways have been suggested, including one involving the activation of cannabinoid receptors and another inducing hypoalgesia via substance P. Along with the inhibition of the COX enzymes, the antipyretic effects may also be potentiated by inhibiting endogenous pyrogens in the hypothalamic thermoregulatory center (DRUGDEX® System).

The metabolism of APAP takes place primarily in the liver when administered orally and involves both toxic and non-toxic metabolites. The main metabolic pathway has been shown to be glucuronidation, closely followed by sulfation and N-hydroxylation and rearrangement. The toxicity does not stem from the final products of these three pathways. Instead, the toxicity of APAP derives from a free radical intermediate, N-acetyl-P-benzoquinone-imine (NAPQI), formed from the N-hydroxylation and rearrangement pathway. NAPQI is highly reactive, causes cellular oxidative stress, and covalently binds to cellular macromolecules. Cytochrome P450 plays a major role in this pathway; polymorphisms found in this cytochrome’s three main isoenzymes, CYP 2D6, CYP 1A2, and CYP 2E1, can account for the varying toxicities found across the population. In most cases, the production of NAPQI is followed by its detoxification via glutathione conjugation; however, toxicity can occur when this pathway becomes
saturated. Saturation can be prompted when one overdoses on APAP or when one’s metabolism of the drug is increased, as seen in rapid metabolizers. The overproduction of NAPQI cannot be counterbalanced by the amount of glutathione available. Eventually, glutathione stores are depleted and the toxic metabolite is available to react with tissue macromolecules (Borne 1995).

The main target organ of APAP toxicity is the liver. Because the drug is metabolized through the liver and produces NAPQI in some instances, this toxic metabolite covalently binds to the liver protein, resulting in the loss of function and possible cytotoxicity. More specifically, James has hypothesized that the primary target of the toxic metabolite are the mitochondrial proteins of the cell, thus limiting energy production and leading to cell death. Of the many enzymes affected by NAPQI, N-10-formyltetrahydrofolate dehydrogenase has been identified as a biological target for NAPQI adduction. More specifically, this enzyme is responsible for oxidizing formaldehyde to carbon dioxide. With the inactivation of this enzyme, liver injury is imminent. A second enzyme revealed by James is the 50-kDa mitochondrial protein responsible for the dehydrogenation of glutamate. Although only partially inactivated by the covalent binding of NAPQI, both of these enzymes are greatly slowed, which can exert a large amount of toxicity on the liver (James et al. 2003).

Asthma has been shown to be associated with general APAP consumption (less than once a month use for an average of 4-5 days). In contrast to the effects on the liver, patients who have been involved in the proposed link between asthma and APAP have taken therapeutic doses of APAP, instead of an overdose. More specifically, a retrospective study dating back to 1994 has shown that as the sale of APAP in English
speaking countries has increased, so has the prevalence of asthma. Many have hypothesized that this increase in prevalence is directly correlated with the increased consumption of APAP. This possible link is supported by the glutathione depleting effects of APAP in the liver. If a similar depletion in the airways occurs alongside that that takes place in the liver, this decrease in the antioxidant, glutathione, leaves the individual more susceptible to oxidative stress brought about by other irritants, even at the proper dosing schedule. Increases in inflammatory mediators as well as oxidative stress have been suggested to correlation with the onset of asthma. Thus, this decrease in airway antioxidant potential could possibly be linked to the increase in atopic asthma symptoms (Newson et al. 2000). Studies of the effects of low doses of APAP on the respiratory tract are largely absent. A study that investigates glutathione loss following APAP in the airways is necessary to determine whether this is a possible mechanism for the increase in disease in the last 30 years. Thus, the primary goal of this project is to determine if low non-hepatotoxic doses of APAP is correlated with an increase of oxidative stress in the airways, possibly linking APAP to the onset of asthma.

Overall, asthma has been shown to be a multifactorial disease that is a culmination of both genetic and environmental causative factors. Although much work has been done thus far to determine the ultimate causes and potential therapies to combat the disease state, much work is still to come. Of the many environmental factors that remain unclear, the use of acetaminophen and its correlation with asthma may show some promise in future studies. Also, as mentioned before, the exposure of youth to irritants has shown a correlation with the onset of asthma. Thus, the exposure to environmental tobacco smoke also shows a correlation with the onset of asthma; however, a definitive
mechanism has yet to surface. Hopefully, by exposing these and other existing correlations, the etiology of the complex and multifactorial disease that is asthma will begin to become clearer, opening up new avenues for therapeutic intervention.

The current study is focused on determining if APAP induces oxidative stress in respiratory tissues and whether the respiratory irritation effects of exposure to acrolein, a primary chemical component of environmental tobacco smoke (ETS), is enhanced by the stress. Acrolein was selected as it allows for a more mechanistic understanding of the pro-irritant capacities of ETS, which has been shown to contribute to the increased prevalence of asthma. Acrolein is a known pro-oxidant that interacts with the transient receptor potential cation channel, subfamily A, member 1 (TRPA1) to produce the respiratory irritant-induced sensory irritation response.

**Materials and Methods**

*Experimental Approaches.* The hypothesis that the metabolites of APAP act as pro-oxidants in the airways and increases the body’s response to acrolein was tested in a mouse model. Towards this end, animals were euthanized 0-3 hrs after 100 mg/kg APAP administration (ip). The role of oxidative stress in inducing the irritant reflex response was examined by multiple approaches. First, the effects of APAP on the responses to the pro-oxidant irritant acrolein, and the non-oxidant irritant cyclohexanone were examined to confirm any effects of APAP were oxidant specific rather than generalized in nature. Cyclohexanone activates chemosensory nerves by the transient receptor potential vanillin 1 (TRPV) receptor (Saunders et al. 2013; Willis et al. 2011). Second, the effect of APAP was examined in animals pretreated with 5-phenyl-1-pentyne (5-PP) to inhibit nasal CYP
metabolism (Roberts et al. 1998; Morris and Buckpitt 2009; Morris 2013) and bioactivation of APAP. (Bioactivation of APAP is necessary for its pro-oxidant effects.) Third, the glutathione depleting agent, diethylmaleate (DEM), was administered to determine if modulation of nasal antioxidant status could replicate the effects of APAP. This agent is conjugated with glutathione via glutathione-S-transferases, resulting in depletion of tissue glutathione levels (Phimister et al. 2004; Boyland and Chasseaud 1967).

Animal procedures. Female C57Bl/6J mice were used for all experiments. Mice were obtained from Jackson Laboratories (Woods Hole, MA, USA) and housed in American Association for Accreditation of Laboratory Animal Care-accredited facilities at the University of Connecticut under standard environmental conditions (12-h light-dark cycle at 23°C). Mice were housed over hardwood shavings (Sani-Chip Dry, P. J. Murphy Forest Products, Montville, New Jersey). Food (Lab Diet; PMI Nutrition International, Brentwood, Missouri) and tap water were provided ad libitum. Animals were 7 weeks of age on arrival, were acclimated for at least 10 days prior to use and were used within 10 weeks of arrival. All animal procedures were approved by the University of Connecticut Institutional Animal Care and Use Committee.

APAP, dissolved in warm 37°C saline (10 mg/ml), was administered via ip injection at a dose of 100 mg/kg. When administered, the cytochrome P450 inhibitor 5-PP was given ip at a dose of 100 mg/kg (10 mg/ml in olive oil) 1hr prior to APAP treatment (Morris 2013). Diethylmaleate was administered at a dose of 250 mg/kg (0.5 M solution in corn oil, ip, Phimster et al. 2005). Control animals received vehicle injections. Mice were exposed to airborne irritants as described below; irritant exposure
concentrations were selected to produce demonstrable, but submaximal irritation. For euthanasia and tissue collection, mice were anesthetized with urethane (1.3 g/kg) followed by exsanguination (Cichocki et al. 2014a).

**Breathing Pattern Analysis.** Mice were held in a double plethysmograph (Buxco, Inc, Sharon, Connecticut) connected to a directed airflow nose-only inhalation chamber (CH Technologies, Westwood, New Jersey) for irritant exposure to allow monitoring of breathing parameters during the exposure. Animals were placed in the plethysmograph for a 15-min acclimatization, 5-min baseline, and then a 10 minute exposure to irritant. Stimulation of nasal trigeminal nerves induces the reflex sensory irritation response that is characterized by a pause at the onset of each expiration (due to glottal closure), termed braking, and is quantitated by measuring the duration of the braking (Willis et al. 2011). Breathing patterns were monitored continuously during the baseline and exposure periods using Emka Technologies (Falls Church, Virginia) Iox 2 software.

**Respiratory Irritant Exposures.** Mice were exposed to ETS for 10 minutes or to the irritant vapors, acrolein or cyclohexanone, for the same duration. Mice were continuously exposed to constant levels of irritant to allow for the most precise estimation of irritant- or APAP- induced changes in breathing. For exposure clean- or irritant-laden air was drawn into the headspace of the double plethysmograph at a flow rate of 1L/min.

Acrolein (nominal concentration 2 ppm) atmospheres were generated by flash evaporation; cyclohexanone (nominal concentration, 1500 ppm) atmospheres were generated by passing filtered air through liquid cyclohexanone in a gas washing bottle;
airborne vapor concentrations were monitored by gas chromatography using a Varian 3800 gas chromatograph as described previously (Willis et al. 2011).

Statistical Analysis. Numbers of animals per group were selected to detect a 25% difference between groups based on our previous experience with the methodologies. Data were analyzed by XLSTAT (Addinsoft, New York, New York). Individual data values were excluded \textit{a priori} if they deviated from the mean by more than 3 standard deviations. Data are reported as mean $\pm$ SE unless otherwise indicated. Data were compared by an Unpaired T-Test or ANOVA followed, as appropriate, by Newman-Keuls test. When appropriate data were log transformed to correct for heteroscedasticity. The sensory irritation response was assessed by monitoring duration of braking throughout the irritant exposure; for time course studies these data were analyzed by repeated-measures ANOVA followed by Newman-Keuls test. A p-value less than 0.05 was required for statistical significance.

Results

As illustrated in Figure 1, mice were exposed to acrolein and APAP (n = 8) or acrolein alone (n = 16), following the aforementioned procedure. The mice

![Figure 1: Effects of APAP on Acrolein Toxicity](image)

\textbf{Figure 1}: The above figure shows the effect that APAP has on enhancing acrolein induced sensory irritations, reflected in the mice's duration of breaking.
that were exposed to acrolein alone showed a moderate sensory irritation response as indicated by an increase in braking over the exposed interval \((t = 0 - 10 \text{ min})\). To increase validity, data used to represent acrolein alone were pooled from three groups: acrolein with saline, acrolein with 5-PP, and acrolein with corn oil due to the results illustrated in figure 3, as statistical analyses revealed that the response to acrolein was not altered by any of these vehicle treatments. A reported experimental time interval of \(t = 10 \text{ min}\) was utilized due to increased variation beyond this interval, as seen in the following figures as well. While APAP alone did not produce the irritation response, animals pretreated with APAP demonstrated a much greater irritation response to acrolein. Utilizing a two-tailed student’s t-test, the difference between the average response to acrolein alone and the average response to acrolein with APAP pretreatment were found to be significant with a p value of <0.0007 (ave. of t= 0 to t = 10). Tables 1 and 2 further elaborate on the data set forth in figure 1, showing the additional analyses of average minute ventilation (MV), tidal volume (TV), and frequency (f) of the specified mice in the time interval noted. Both tables show that the minute ventilation decreased, tidal volume increased, and frequency decreased in response to the addition of acrolein when compared to baseline.

### Table 1: Total Acrolein

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>MV (ml/min)</th>
<th>TV (ml)</th>
<th>f (breaths per min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-5 – 0 min</td>
<td>69.37</td>
<td>0.263</td>
<td>272.28</td>
</tr>
<tr>
<td>1 – 10 min</td>
<td>56.98</td>
<td>0.301</td>
<td>208.05</td>
</tr>
</tbody>
</table>

### Table 2: Acrolein and APAP

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>MV (ml/min)</th>
<th>TV (ml)</th>
<th>f (breaths per min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-5 – 0 min</td>
<td>72.09</td>
<td>0.290</td>
<td>255.97</td>
</tr>
<tr>
<td>1 – 10 min</td>
<td>56.59</td>
<td>0.342</td>
<td>181.64</td>
</tr>
</tbody>
</table>
Additionally, when pretreated with APAP, the mice tended to breathe at a lower frequency and, thus, higher tidal volume when being exposed to acrolein to take in the same (or relatively same) amount of air.

Figure 2 shows the results of mice being exposed to both cyclohexanone alone (n = 7) as well as cyclohexanone combined with APAP (n = 4). As seen in the figure, very little differences were observed between the two study groups. This lack of significant difference was confirmed by a comparison of the two groups, utilizing a two-tailed student’s t-test (p = 0.953). Tables 3 and 4 also show very little difference in trends between pre-exposure and exposure time periods.

**Figure 2:** The above figure shows the effect that APAP has on the sensory irritation of cyclohexanone, reflected in the mice’s duration of breaking.

<table>
<thead>
<tr>
<th>TABLE 3: Cyclohexanone</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV (ml/min)</td>
</tr>
<tr>
<td>-5 – 0 min</td>
</tr>
<tr>
<td>1 – 10 min</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 4: Cyclohexanone and APAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV (ml/min)</td>
</tr>
<tr>
<td>-5 – 0 min</td>
</tr>
<tr>
<td>1 – 10 min</td>
</tr>
</tbody>
</table>
Figure 3 illustrates the average durations of breaking of the following vehicles: acrolein plus 5-PP (n = 2), acrolein plus saline (n = 7), acrolein plus corn oil (n = 3), acrolein plus olive oil (n = 2) and acrolein alone (n = 2). More specifically, these numbers represent the average duration of breaking of mice exposed to the specified vehicles during the

![Figure 3: Comparison of Selected Vehicles](image)

**Figure 3:** The above figure represents the average irritation response of the specified population of mice from t = 0 min to t = 10 min.

<table>
<thead>
<tr>
<th>TABLE 5: Acrolein Alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV (ml/min)</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>-5 – 0 min</td>
</tr>
<tr>
<td>1 – 10 min</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 6: Acrolein and 5-PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV (ml/min)</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>-5 – 0 min</td>
</tr>
<tr>
<td>1 – 10 min</td>
</tr>
</tbody>
</table>
interval of 2-10 minutes. As mentioned previously, this figure shows that little differences exist between the five selected vehicles, with a p value greater than 0.05 for each calculated using the 1-Way ANOVA test, followed by a Newman-Keuls test for multiple comparisons, from minutes 2-10. Tables 1, 5, 6, 7, and 8 further illustrate this non-significant difference.

Figure 4 shows the effect of 5-PP administration 1 hour prior to APAP on the acrolein response. (n = 5). As seen before, acrolein produced a moderate irritation response; this response was greatly enhanced in animals pretreated with 5-PP.

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**Figure 4:** The above figure shows the effect that 5-PP has on the toxicity of APAP and acrolein, reflected in the mice’s duration of breaking.
with APAP. This effect of APAP was absent in mice treated with the CYP inhibitor 5-PP. Thus, the response in 5-PP closely resembled that of acrolein alone (n = 16), supported by a p value of greater than 0.05 for the 5-PP and APAP as well as the acrolein alone groups, obtained using a 1-Way ANOVA Test and a Newman-Keuls Test. Table 9 also shows a very similar trend to that seen in table 1, which supports the non-significant difference between the two vehicles.

Figure 5 shows the effects of DEM, a non-toxic glutathione depleter, on the acrolein response. Similar to APAP, DEM greatly increased the response to acrolein.

**TABLE 9: Acrolein, 5-PP, and APAP**

<table>
<thead>
<tr>
<th>Time</th>
<th>MV (ml/min)</th>
<th>TV (ml)</th>
<th>f (breaths per min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-5 – 0 min</td>
<td>53.13</td>
<td>0.223</td>
<td>238.72</td>
</tr>
<tr>
<td>1 – 10 min</td>
<td>54.07</td>
<td>0.277</td>
<td>204.79</td>
</tr>
</tbody>
</table>

**Figure 5: Effect of DEM on Acrolein Response**

**Figure 5:** The above figure shows the effect that DEM has on enhancing acrolein induced sensory irritations, reflected in the mice's duration of breaking.

**TABLE 10: Acrolein and DEM**

<table>
<thead>
<tr>
<th>Time</th>
<th>MV (ml/min)</th>
<th>TV (ml)</th>
<th>f (breaths per min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-5 – 0 min</td>
<td>59.23</td>
<td>0.250</td>
<td>240.16</td>
</tr>
<tr>
<td>1 – 10 min</td>
<td>45.68</td>
<td>0.294</td>
<td>166.31</td>
</tr>
</tbody>
</table>
With a p value of 0.0014, obtained using a two-tailed student’s t-test, the differences between the breathing patterns produced by mice when exposed to acrolein alone (n = 16) versus acrolein plus DEM (n = 6) prove to be significant. Further, the values and trend represented in table 10 resembles that shown in table 2.

Finally, figure 6 shows the average duration of breaking of the mice exposed to the specified vehicle over the course of 0-10 min of exposure. As supported by the shown p values, obtained using a 1-Way ANOVA Test, little differences existed between acrolein alone (n = 16) and acrolein combined with 5-PP and APAP (n = 5), as mentioned in figure 4 as well. The same principle held true when comparing acrolein combined with APAP (n = 8) and acrolein combined with DEM (n = 6). However, when contrasting the two aforementioned groups, a significant difference existed, as shown by the p values under 0.05, suggested greater durations of breaking in those mice exposed to acrolein and APAP as well as those exposed to acrolein and DEM.
Discussion

The present study demonstrates that APAP, at near therapeutic doses, modulates respiratory responses to acrolein, the primary oxidant sensory irritant in tobacco smoke. More specifically, the common analgesic APAP enhances one’s response to oxidant air pollutants, a concept that has not been investigated in the past. As mentioned previously, this study utilized APAP doses of 100 mg/kg, contrasted to a recommended dose in humans of 15 mg/kg. It has been shown that therapeutic levels of APAP fall between 5-20 ug/ml, and hepatotoxicity occurs at blood levels of around 150 ug/mL (Rumack and Matthew 1975). Although the dosing in this study falls above the recommended dosing for a therapeutic response, the peak blood levels of APAP were approximately 35 ug/mL, following a 100 mg/kg dose (Morris Lab, Unpublished Data), which falls well below the threshold for hepatotoxicity. Previous studies by other groups have also shown that APAP blood levels in a mouse model are approximately 40 ug/mL at 15 minutes post-injection at the 100 mg/kg dose. These levels then fall to therapeutic levels, approximately 35 ug/mL, within 1 hour post-injection, validating the previous point (Gu et al. 2005).

Previous studies have shown that APAP, at overtly toxic doses, depletes nasal glutathione stores (Gu et al. 2005). This depletion likely leads to an oxidative stress response in the airways (Cichocki et al. 2014a, b). These events have not been investigated at therapeutic doses of APAP in the airways. However, the current study suggests that therapeutic doses of approximately 100 mg/kg in a mouse model result in local activation of APAP, leading to enhanced oxidant sensitivity caused by its metabolite, NAPQI. The results in this study do not rule out other possibilities, such as
hepatic events (escape of activated APAP or depletion of blood glutathione), as contributing factors to the oxidative stress response that affects the respiratory tract (Phimister et al. 2005; Gu et al. 2005). Nevertheless, our results demonstrate that APAP causes a significant modulation of airway sensitivity to oxidant chemicals.

The first experiments were aimed at exploring the oxidant sensory irritant properties of both acrolein and APAP. As figure 1 illustrates, APAP clearly potentiated the sensory irritation response to acrolein. Alone, acrolein only produces a moderate braking response in the mouse’s breathing, suggesting moderate irritation. As shown above, APAP alone does not increase the mouse’s irritation response (Morris Labs, Unpublished Data). However, when pretreated with APAP one hour prior to acrolein exposure, the braking response is potentiated. As mentioned previously, this additional braking response is most likely a result of glutathione depletion in the airways, brought about via NAPQI (Gu et al. 2004). Normally, glutathione is utilized to detoxify acrolein, leaving the airways unharmed. Thus, by reducing the amount available, increased oxidant sensory irritation is imminent. The results of this figure are strengthened by those found when the mice were pretreated with DEM, a known glutathione depleting agent, one hour prior to acrolein exposure. The similar trends in both the irritation response as well as the values shown in table 10 (comparing them to table 2) suggest that APAP at therapeutic doses had a glutathione depleting effect, leading to enhanced irritation.

The potentiation observed when administering APAP prior to acrolein exposure suggests that APAP can alter complex integrated airway responses via pro-oxidant mechanisms. More specifically, the addition of APAP to acrolein caused prolonged breaking in the mouse’s breathing pattern, which stems from the stimulation of
chemosensory nerves via the oxidant sensitive TRPA1 receptor (Andre et al. 2008). In addition to adding APAP to a TRPA1 agonist, the effects of APAP on a known TRPV1 agonist, cyclohexanone, were also observed to confirm or refute the specific pro-oxidant nature of APAP (figure 2). Because cyclohexanone is not an oxidant, the results from these experiments showed that little difference exists between cyclohexanone alone and cyclohexanone combined with APAP, thus confirming the specific pro-oxidant toxicity of APAP.

Because it is known that APAP is activated and metabolized via CYP enzymes in the respiratory tract, independent of the metabolism that occurs within the liver, DEM treatment was utilized to imitate the glutathione depletion that APAP is responsible for (Gu et al. 2005). Given at a dose that produced similar amounts of glutathione depletion to APAP, DEM showed similar trends when combined with acrolein to those shown by APAP and acrolein, suggesting that this depletion of glutathione may be the major pathway in APAP’s enhancement of acrolein sensory irritation.

After confirming that the effects of APAP were pro-oxidant in nature, this study utilized a known cytochrome P450 inhibitor to confirm or refute that APAP’s effects on respiratory responses to oxidants stemmed from a metabolite, likely NAPQI, produced via CYP enzymes, rather than the parent compound. As seen in figure 4 and figure 1, when combined with acrolein, APAP has a synergistic effect. However, when 5-PP is added to the previously mentioned vehicles, the trend resembles that set by acrolein alone. This decrease in braking brought on by the addition of 5-PP suggests that APAP metabolites are not being formed due to CYP inhibition, leading to less toxicity in the airways at this dose. This also confirms the notion that an APAP metabolite, likely
NAPQI, is the causative species in this potentiation scenario. The above conclusions are summarized in figure 6, which shows the similarities between acrolein alone and the aforementioned 5-PP combination as well as those between the DEM/acrolein combination and the APAP/acrolein combination.

The current study focuses primarily on the acute respiratory response to ETS and other oxidant air pollutants, represented by acrolein. As mentioned previously, with the “APAP hypothesis” regarding the increased in asthma prevalence in mind, air pollutants and airway oxidative stress are major factors when looking at the onset of asthma in today’s population due mainly to the increase of pro-inflammatory factors upon exposure (Reidl and Nel 2008; Holguin 2013). Because this study demonstrates that APAP increases murine respiratory responses to pro-oxidant irritation, it suggests that APAP may have a role in increasing one’s likelihood of developing asthma. Because this study was conducted in the acute setting, further investigational studies may be implicated for more long term trends between therapeutic levels of APAP and the onset of asthma. Regardless, this study confirms that even at therapeutic doses, APAP can elicit a toxic response.

**Conclusion**

This study shows that when APAP is given at therapeutic doses, pro-oxidant toxicities are present in a mouse model. These toxicities potentiate the acute airway response to ETS and could lead to an inflammatory response. That said, the therapeutic use of APAP may be detrimental to those regularly exposed to environmental toxins and lead to the onset of asthma, as supported by the APAP hypothesis.
Works Cited


