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Modulation of Respiratory Tract Responses by Acetaminophen

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Modulation of Respiratory Tract Responses by Acetaminophen

Gregory James Smith, Ph.D.

University of Connecticut, 2015

The prevalence of asthma is increasing, and the cause(s) of this increase are unknown. The etiology of asthma is complex and includes both genetic, developmental, and environmental components. Epidemiological evidence suggests that acetaminophen (APAP, N-acetyl-*p*-aminophenol) may play a role in the pathogenesis of asthma. The goal of this thesis was to test the plausibility of the “acetaminophen hypothesis” in asthma, which states that therapeutic use of acetaminophen is linked to the increase in asthma prevalence through pro-oxidant effects of the drug.

The airways of the respiratory system represent the largest surface area of the body exposed to the atmosphere. Several physiological adaptations of the respiratory tract aid in maintaining homeostasis under variable atmospheric conditions. These include oxidant responses, sensory irritation-reflex responses, and inflammatory responses. Respiratory tract responses can be activated by environmental exposures and disease states. In some situations they can be compromised. For example, asthmatic airways mount exaggerated immune responses to normally innocuous atmospheric variables, and may have altered oxidant responses. The measurement of respiratory responses can provide direct data on airway status.

Described herein are studies examining the effects of acetaminophen on acute airway oxidant responses, acute sensory irritation-reflex responses, and longer-term inflammatory responses in a murine model of allergic airway disease (asthma). The first study assessed murine acute respiratory

oxidant responses after APAP treatment alone, or in combination with environmental tobacco smoke (ETS). Near-therapeutic doses of APAP induced airway oxidant responses similarly to ETS. Notably, APAP potentiated both ETS induced oxidant responses and ETS induced sensory irritant-reflex irritant responses. In a second study, the effects of acetaminophen on airway inflammatory responses to house dust mite extract (HDM) were examined. Unexpectedly, the effects of HDM exposure were substantially diminished by APAP. These studies suggest that APAP has acute pro-oxidant effects in the airways at near-therapeutic doses, and thus may have detrimental effects in individuals predisposed to oxidant injury, or exposed to oxidant air pollutants such as ETS. Despite documenting pro-oxidant effects of APAP in the airways, these studies call into question the overall biological plausibility of the “APAP hypothesis” in asthma.

Modulation of Respiratory Tract Responses by Acetaminophen

Gregory James Smith

B.S., University of Vermont, 2011

A Dissertation

Submitted in Partial Fulfillment of the

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at the

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Gregory James Smith

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APPROVAL PAGE

Doctor of Philosophy Dissertation

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2015

DEDICATION

To Mom and Dad:

Thank you for always fostering my curiosity and ensuring that I
will never forget twelve is a dozen.

&

To Grandma Lila, and Grandpa and Grandma Harris

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Many friends, family members, colleagues, and mentors have contributed to my personal and professional development throughout the duration of this work. I am forever grateful to them. I would especially like to thank my graduate advisor Dr. John Morris. The importance of his role in shaping my career as a scientist cannot be overstated. Without Dr. Morris' guidance, expertise, and good judgement I could not have made it to this point. I also owe a great many thanks to the other members of my doctoral dissertation committee: Drs. José Manautou, Michelle Cloutier, and Roger Thrall. The example provided by my committee members, both individually and as a team, is truly one I will always seek to emulate.

“If I have seen a little further it is by standing on the shoulders of giants.”

~Isaac Newton

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LIST OF ABBREVIATIONS

| | |
|--------|--|
| 5-PP | 5-Phenyl-1-Pentyne |
| ANOVA | Analysis of variance |
| APAP | N-acetyl- <i>p</i> -aminophenol |
| ARE | Antioxidant response element |
| BAL | Bronchoalveolar lavage |
| CBR | Cannabinoid receptor |
| CO | Carbon monoxide |
| COX | Cyclooxygenase |
| CYP | Cytochrome P450 |
| DC | Dendritic cell |
| DEM | Diethylmaleate |
| DEP | Diesel exhaust particles |
| EC | Epithelial cell |
| ELF | Endothelial lining fluid |
| ETS | Environmental tobacco smoke |
| GCL/C | Glutamylcysteine ligase catalytic subunit |
| GM-CSF | Granulocyte macrophage-colony stimulating factor |
| GSH | Glutathione (reduced) |
| GSSG | Gluthione disulfide (oxidized) |
| GST | Glutathione S-Transferase |
| HDM | House dust mite |

| | |
|----------|--|
| IL | Interleukin |
| Keap1 | Kelch-like ECH-associated protein |
| LPS | Lipopolysaccharide |
| NAPQI | N-acetyl- <i>p</i> -benzoquinone-imine |
| NPSH | Non-protein sulfhydryl |
| Nqo1 | NAD(P)H dehydrogenase, quinone 1 |
| Nrf2 | Nuclear factor erythroid 2-related factor 2 |
| OVA | Ovalbumin |
| PAH | Polycyclic aromatic hydrocarbon |
| PG | Prostaglandin |
| ROS | Reactive oxygen species |
| RTM | Respiratory transitional mucosa |
| SD | Standard Deviation |
| SE | Standard Error |
| -SH | Sulfhydryl |
| TBM | Tracheobronchial mucosa |
| TLR | Toll-like receptor |
| TRPV1/A1 | Transient receptor potential vanilloid 1/ankyrin 1 |
| UDP | Uridine diphosphate |

Chapter 1: Review of the Literature

Asthma

1.1 The Epidemic

Currently, it is estimated that 300 million people suffer from asthma worldwide, including more than 25 million in the United States (Braman, 2006, Moorman *et al.*, 2012). Asthma is the most common chronic disease among children, and the leading cause of missed school days. The disease is a burden on society, causing approximately 2 million emergency room visits, and more than 400,000 hospitalizations annually in the United States. The total cost to society of increased absenteeism and medical care is an estimated \$56 billion (Barnett and Nurmagambetov, 2011). The past three decades have witnessed a worldwide epidemic-scale increase in asthma prevalence (Eder *et al.*, 2006). In the United States, the population prevalence of asthma increased from 3.1 to 8.6 percent in the years spanning 1980 to 2010 (Moorman *et al.*, 2007, Moorman *et al.*, 2012). Unfortunately, the cause (s) of the increase are unknown.

It is unlikely that changes in human genetics could account for the upward trend in asthma prevalence in such a short period of time, suggesting that environmental factors are responsible (Varner *et al.*, 1998). Changes in environmental risk factors for asthma such as air pollutants, pathogens, diet and lifestyle, and acetaminophen use may provide mechanistic clues. With regard to environmental factors, several hypotheses have been proposed to explain the increase in asthma prevalence including the “hygiene hypothesis,” the “vitamin D hypothesis,” and the “acetaminophen (APAP, *n*-acetyl-*para*-aminophenol) hypothesis”. None of these hypotheses are mutually exclusive, or have been proven.

The hygiene hypothesis suggests that early life exposures to certain microbes influence the development of an appropriately regulated immune system (von Mutius, 2007). Specifically, decreased early-life microbial exposure may shift the balance of immune responses from Th1 to Th2. Th1 responses are associated with the inflammatory response to infections whereas Th2 responses promote IgE production and the maturation of mast cells and eosinophils. Th2-type responses generate cytokines (e.g. Il-4, Il-5, Il-13) which are generally associated with the development of allergy and asthma. The hygiene hypothesis is supported by epidemiological studies showing that children who grew up in farming households have lower rates of asthma than those in urban areas (Elliott *et al.*, 2004). The basic premise of the “vitamin D” hypothesis is that vitamin D is essential for the development and regulation of the immune system, and deficiency of the vitamin is associated with higher rates of asthma and allergy (Bozzetto *et al.*, 2012). Low vitamin D increases the severity of certain early-life viral infections which are associated with asthma and the development of Th2 weighted responses (Jackson *et al.*, 2008, Jartti *et al.*, 2010).

Mitigating the rising prevalence of asthma requires sound knowledge of the factors which cause it to increase. Therefore, this work investigates one of the recently identified risk factors for asthma, acetaminophen use. Specifically, the biological plausibility of a causal relationship between acetaminophen use and asthma pathogenesis is the focus of this dissertation. The APAP hypothesis forms the basis of this work, and thus its formulation will be discussed in more detail.

1.2 The APAP Hypothesis

In the early 1980's, there was a paradigm shift from aspirin to acetaminophen use in the treatment of pain and fever (Varner *et al.*, 1998). This shift was driven by the association of aspirin with Reyes syndrome, a condition of unknown cause which leads to fatal liver and brain swelling. The onset of increasing acetaminophen use coincided with the rise in asthma prevalence which led to the early hypothesis by Varner *et al.* (1998) that the loss of a protective effect of aspirin was responsible for the increase in asthma. This is logical, as aspirin inhibits the formation of prostaglandin E₂ (PGE₂) by cyclooxygenase-2 (COX-2). PGE₂ can inhibit Th1 responses and enhance Th2 responses which are associated with asthma (Kalinski, 2012). Although now considered a misconception, at the time of the Varner report it was not thought that APAP inhibits COX-2 (Graham *et al.*, 2013). Shaheen *et al.* published the first epidemiological study to show that frequent APAP use was associated with asthma (Shaheen *et al.*, 2000). This study also describes a potential mechanism for the association. Specifically, that APAP may influence the pathogenesis of asthma by compromising airway oxidant defenses, and enhancing oxidant burden. Soon after this report, the same group published a larger ecological analysis showing that national acetaminophen sales predicted asthma prevalence (Newson *et al.*, 2000). Since then, many others have found a strong association between APAP use and asthma prevalence (Etminan *et al.*, 2009). The largest study to date, the International Study of Asthma and Allergies in Childhood, included >200,000 children across multiple asthma centers world-wide and found a dose-dependent increase in asthma risk with APAP use (Beasley *et al.*, 2011). A large prospective study, The Nurse's Health Study also found a dose-dependent increase in the incidence of asthma with APAP use in adult women (Barr *et al.*, 2004). Additionally, prenatal acetaminophen use has been

associated with childhood asthma (Shaheen *et al.*, 2010). That the association is present in multiple ages, sexes, and in different regions of the world adds to its potential importance.

Despite the multitude of positive associations between APAP and asthma, limitations of epidemiological data preclude confirmation of a causal relationship between them. That the association could be explained by confounding factors has been argued in great detail (Heintze and Petersen, 2013). The most often cited limitation is that most studies did not collect data on the reasons why APAP was being administered. Therefore, different types of indications (ear infection, respiratory tract infection, general pain, etc.) could not be adjusted for in their analysis. This may have led to a positive association through confounding by indication. For example, it is not known how frequently APAP was indicated for fever caused by infections compared to other causes of fever or pain. Certain respiratory tract infections which cause fever are independent risk factors for asthma. In those cases APAP could be an innocent bystander; simply a predictor of infections. Supporting this explanation are several studies which found that adjusting for concurrent respiratory infections diminished the association (Lowe *et al.*, 2010, Sordillo *et al.*, 2015). Another study found an association between APAP use and asthma symptoms in early life, but the association was no longer present during a follow-up with subjects at 7 years of age, suggesting that the association was real but temporary (Kreiner-Moller *et al.*, 2012). The current data leave the question of whether or not APAP influences the pathogenesis of asthma controversial and unanswered. Thus there is a need for more direct evidence to determine whether increased APAP use has caused the rise in asthma prevalence. Well controlled animal studies can provide causal data while eliminating the influence of confounding factors that are present in epidemiological studies. However, few animal studies investigating the plausibility of the APAP hypothesis have been performed. Such studies are provided herein.

Proposed pathophysiological effects of APAP which may provide lines of inquiry into the association include: 1) APAP's ability to cause antioxidant glutathione (GSH) loss and oxidative stress, 2) a lack of cyclooxygenase (COX) pathway inhibition with the drug causing enhanced Th2-mediated allergic responses, and 3) an antigenic activity of APAP which causes increased IgE (Eneli *et al.*, 2005, Shaheen *et al.*, 2000). More recent data suggest that APAP does inhibit COX enzymes, therefore, lack of COX inhibition seems less likely (Graham *et al.*, 2013). As far as a possible antigenic activity of APAP is concerned, the only evidence comes from a single low-powered epidemiological study, and several case-reports of APAP induced anaphylaxis (Eneli *et al.*, 2005).

The most developed of these proposed mechanisms is that concerning oxidative stress. Oxidative stress is a hallmark of asthma and is thought to be important in asthma pathogenesis. Markers of oxidant levels are higher in people with asthma and polymorphisms in antioxidant genes such as glutathione transferase are risk factors for asthma (Riedl and Nel, 2008, Holguin, 2013). However, asthma is an inflammatory disease and oxidative stress may be a result as well as a cause of active airway inflammation confounding full definition of its role (Andreadis *et al.*, 2003, Bhalla *et al.*, 2009, Henricks and Nijkamp, 2001). Animal studies confirm the importance of oxidative stress in asthma, e.g. Nrf2 knockout mice demonstrate enhanced susceptibility to allergic airway disease (Rangasamy *et al.*, 2005, Williams *et al.*, 2008). At sufficient doses, APAP causes severe liver toxicity primarily through oxidative stress caused by its reactive metabolite, N-acetyl-P-benzoquinone-imine (NAPQI) (McGill and Jaeschke, 2013). Hepatotoxic doses of APAP have been associated with extrahepatic GSH loss and tissue injury in the nose, and lung (Gu *et al.*, 2005, Hart *et al.*, 1995).

1.3 Asthma Pathogenesis

Asthma is a chronic inflammatory disorder of the airways characterized by episodic airway obstruction caused by inflammation and airway smooth muscle hyperresponsiveness (NHLBI, 2007). Symptoms of asthma include wheezing, coughing, shortness of breath, and sensations of tightening of the chest which may occur in the absence of inflammation. Typically asthma symptoms are reversible, but in some cases persistent inflammation can lead to permanent structural changes and irreversible airflow obstruction.

The airways are exposed to variable environmental conditions which include exposure to fluctuating weather conditions, microbes, pollutants, and allergens. The ability of the airways to respond appropriately to environmental exposure is critical to maintaining airway homeostasis, and ultimately gas exchange. Healthy airways adapt to changes in the airway environment through a variety of physiological mechanisms. These include the efficient mucociliary clearance and phagocytosis of foreign materials, production of airway lining fluid, and the formation of a physical and dynamic barrier to the environment (Pinkerton *et al.*, 2015). In asthma, an inappropriate over-reaction to certain environmental exposures occurs. The mechanism (s) for this inappropriate response are not completely understood, but may be inflammatory in nature, and depend on both an individual's genetic composition and the exposure at hand. A diversity of genotypes and environmental exposures influence the development of asthma, producing what are known as asthma phenotypes (Wenzel, 2012). For example, while they both have asthma, the pathophysiology of one individual's disease may differ greatly from another in terms of genetic susceptibility, environmental triggers, age of onset, clinical and physiological features, and response to therapy. Therefore, different "asthma phenotypes" arose as a means to more adequately describe the spectrum of asthma.

Asthma is currently separated into several phenotypes. Historically, there were thought to be two primary phenotypes among asthmatic individuals, specifically allergic vs. non-allergic. This is now considered to be an oversimplification (Lambrecht and Hammad, 2015, Wenzel, 2012). Differing phenotypic classifications have now been proposed. A useful classification identifies five separate phenotypes: early onset allergic, late onset eosinophilic, exercise induced, obesity related, and neutrophilic. Allergic asthma, including early onset and late onset eosinophilic asthma, is the most common asthma phenotype. Non-allergic asthma, typically neutrophilic asthma, often develops later in life. Individuals with allergic (eosinophilic) asthma mount inappropriate immune responses to normally innocuous inhaled and ingested materials. These include-but are not limited to-pollen, pet dander, certain foods such as peanuts, as well as house dust mites (HDM) and their waste products. Those with non-allergic (neutrophilic) asthma often experience similar symptoms to those with allergic asthma. However, individuals with non-allergic (neutrophilic) asthma do not have adaptive immune system reactivity to allergens. Non-allergic asthma triggers include irritants such as environmental tobacco smoke (ETS), respiratory tract infections, certain medications, cold weather, and exercise. Many of the aforementioned non-allergic triggers are risk factors for allergic asthma, and can cause exacerbations in individuals with allergic asthma as well. The focus of the following discussion will be on the allergic type as it is the most common and is modeled in the experiments to follow (See Chapter 3). These experiments will rely on a murine model of HDM induced allergic asthma. It is difficult to relate this model directly to asthma phenotypes in humans. The airway response in this model consists of both eosinophilic and neutrophilic inflammation. This model is also characterized by airway hyperresponsiveness (Bracken *et al.*, 2015), another key clinical feature of asthma in humans.

The clinical features of asthma result from an integrated and multicellular response. The airway epithelium, airway smooth muscle, neuronal tissue, and immune system are all involved (Burleson *et al.*, 2015). In individuals with allergic asthma, disease progresses through three stages: sensitization, acute inflammation, and chronic disease (Maes *et al.*, 2010). Allergen sensitization in the airways begins with initial allergen contact at the airway epithelial barrier (Lambrecht and Hammad, 2012). The airway epithelial barrier is continuous and composed primarily of a layer of epithelial lining fluid (ELF), and underlying airway epithelial cells (EC) (Reynolds *et al.*, 2015). The airway ECs are held together by intracellular junctions formed by a variety of specialized transmembrane proteins. Intracellular junctions include the tight junctions, adherens junctions, and desmosomes. The intracellular junctions regulate the paracellular flow of materials across the epithelium, and also have cell signaling functions. The airway epithelial lining fluid consists (from the air interface inward) of a thick mucus layer, and a thin periciliary fluid layer. The movement of EC cilia propel the mucus layer towards the proximal end of the trachea. This action carries out foreign material trapped in the thick mucus layer on top. The thick mucus consists primarily of gel forming mucins, MUC5AC and MUC5B, produced by secretory ECs (goblet cells, and others). This “mucociliary apparatus” is a highly effective clearance mechanism. However, some materials, especially those that are water soluble (eg. vapors, small molecules, and proteins), can diffuse across the epithelial lining fluid to interact with the epithelium. Inhaled HDM proteins, for example, can diffuse across the lining fluid to activate EC pattern recognition receptors such as Toll Like Receptor-4 (TLR-4) (Calderon *et al.*, 2014). HDM also has intrinsic protease activity which can disrupt epithelial cell-cell contacts, or cleave protease activated receptors (Post *et al.*, 2012). ECs respond to the presence of HDM and other allergens in their microenvironment by

releasing cytokines, and chemokines. This cytokine and chemokine release coordinates and modulates the activity of the immune system.

One immune cell in particular, the dendritic cell (DC), is key to linking the HDM exposure response of ECs with the adaptive immune system (Cook and Nakano, 2015, Lambrecht and Hammad, 2009). The epithelium contains a network of immature DCs, and additional immature DCs in the circulation may be recruited. Related to monocytes/macrophages, DCs normally sample the airway epithelial microenvironment taking up various particles and proteins via receptor mediated endocytosis and phagocytosis. When HDM proteins are taken up by DCs they are processed into smaller antigens which are displayed on the DC surface. Concurrent EC cytokine and chemokine release then facilitates the activation of the antigen presenting DCs (Lambrecht and Hammad, 2009).

Stimulation of the epithelium, resulting in cytokine and chemokine release, is a requirement for airway DC activation (Lambrecht and Hammad, 2012). This stimulation can result from extrinsic factors (microbial infections, air-pollutant exposure, protease allergens, etc.) and may be modulated by intrinsic factors such as intracellular junction integrity. Airway oxidative stress is a common feature of asthma, can be induced by air-pollutants, and can also stimulate the epithelium (Riedl and Nel, 2008, Holguin, 2013). Oxidative stress as well as oxidant air-pollutant exposure will be discussed in more detail below. Individuals with asthma often have impaired epithelial barrier function due to epithelial junction protein defects. For example, decreased expression of the tight junction protein E-cadherin has been found to be higher in some asthmatic populations (Nawijn *et al.*, 2011). Genome wide association studies have identified several EC and tight-junction related asthma susceptibility genes (Heijink *et al.*, 2014). Whether these abnormalities in cell-junction proteins cause asthma or are an effect of another underlying process in asthma is not

fully understood (Heijink *et al.*, 2014). Disruption of EC junctions may influence the pathogenesis of asthma by increasing basal inflammatory signaling and therefore lowering the threshold for EC mediated activation of DCs (Lambrecht and Hammad, 2012). An impaired epithelial barrier may also enhance the flux of allergen across it, thus increasing the probability of initial sensitization, and adaptive immune responses. The fact that most people with asthma are allergic, but most with allergies do not develop asthma suggests that epithelial barrier dysfunction or other epithelial specific pathologies may be key to producing the respiratory effect of allergen exposure seen in asthma.

Activated DCs carrying antigen proteins migrate through afferent lymphatic ducts to nearby lymph nodes and present the antigens for recognition by T-lymphocytes (Holgate, 2012). In the lymph node the activated DC induces naïve T-helper type lymphocyte differentiation to either memory or effector T-helper 2 type (Th2) lymphocytes that express HDM antigen specific T-cell receptors. The release of cytokines by activated Th2 cells, which include interleukins (IL)-4, 5, and 13, initiates an inflammatory cascade leading to the final steps in allergen sensitization and the development of immunological memory (Paul and Zhu, 2010). Specifically, Th2 cytokines promote mast-cell differentiation and maturation, and eosinophil maturation and survival. HDM specific Th2 lymphocytes along with their cytokines also cause naïve B-lymphocytes to undergo clonal expansion/B-cell class-switching to HDM specific immunoglobulin E and G (IgE, and IgG1). This is in contrast to T-helper 1 type (Th1) cytokines which are released in response to microbial infections, and are not associated with IgE production. Rather, Th1 responses tend to counteract Th2 responses in a regulatory fashion. Upon repeat exposure to HDM, a more robust response driven by HDM specific memory-Th2 lymphocytes and -B-lymphocytes occurs in allergic individuals. The features of the response include Th2 cytokine release followed by

chemotaxis of eosinophils to the airway, IgE mediated mast cell degranulation, increased vascular and epithelial permeability, bronchial smooth muscle hyperreactivity, and mucus metaplasia (Burleson *et al.*, 2015).

This multifaceted response to repeat allergen exposure results in the clinical features of allergic asthma primarily by affecting the conducting regions of the lower airways. It may also affect the upper airways (eg. nasal passages) to cause allergic rhinitis (Grossman, 1997). The primary clinical features of asthma are wheezing, coughing, and shortness of breath which result from several physiological changes in the airways (Fish and Peters, 1998). Wheezing is caused by high-velocity, turbulent airflow in the airways due to a decrease in airway caliber. Decreased airway caliber is primarily caused by bronchoconstriction associated with bronchial hyperreactivity (an exaggerated response to various inhaled stimuli). Airway inflammation is thought to be the cause of bronchial hyperreactivity. Coughing, which is initiated by the mechanical stimulus of excess mucus on airway sensory nerves, is intended to clear mucus from the airways. Decreased airway caliber and mucus production result in a loss of efficient gas exchange and increased effort of breathing. Together these processes contribute to the sensation of being short of breath. Additionally, frequent and severe inflammatory activity in the airways is associated with airway remodeling processes such as thickening of the basement membrane, and bronchial smooth muscle hypertrophy. These changes can lead to irreversible airway obstruction.

1.4 Mouse Models of Asthma

Several different mouse models of asthma have been developed (Maes *et al.*, 2010). The current models do not recapitulate all of the aspects of the human disease, therefore, they are more commonly referred to as “allergic airway disease” models. Despite their inherent differences,

mouse models of allergic airway disease have been very helpful in understanding the pathogenic mechanisms of asthma, particularly because of their ease of manipulation, and the availability of gene knockout mice. For example, the role of granulocyte macrophage colony stimulating factor (GM-CSF), and TLR-4 in the allergic response were defined in mouse models of HDM induced asthma (Hammad *et al.*, 2009, Cates *et al.*, 2004). The most widely used model is based on sensitizing mice to the protein ovalbumin (OVA) (Maes *et al.*, 2010). As OVA is not a typical human allergen, its relevancy has been debated. OVA is typically administered by inhalation aerosol, and requires the use of an adjuvant to enhance the immune response. Another recently developed model, considered to be more relevant than OVA, is the HDM model. Typical HDM models do not require an adjuvant. HDM contains a mixture of several different protein allergens, as well as endotoxin/lipopolysaccharide (LPS). Several of the proteins contained in HDM are proteases which can enhance HDM's allergenicity. Additionally, LPS in HDM can modulate the immune system and cause inflammation.

HDM allergies are one of the most highly prevalent in humans (Calderon *et al.*, 2014). High levels of HDM antigen in the home are associated with a high risk of developing HDM allergy, and are also a risk factor for asthma (IOM, Institute of Medicine (US) Committee on the Assessment of Asthma and Indoor Air, 2000). HDM administration in mice produces robust respiratory inflammation, asthma associated Th2 cytokine responses, and bronchial hyperresponsiveness (Cates *et al.*, 2004). Like in human asthma, HDM specific IgE is present in the serum of mice treated with HDM (Bracken *et al.*, 2015). Importantly, the effects of other environmental or pharmacological exposures can be examined by combining these exposures with HDM (Mitchell *et al.*, 2012). In chapter 3, the effects of APAP on allergic responses in a HDM model of allergic airway disease are examined.

APAP

1.5 Indication and Mechanisms of Action

APAP is a low molecular weight phenolic compound that has analgesic and antipyretic properties (Graham *et al.*, 2013). It is one of the most commonly used non-prescription medications worldwide. APAP was first introduced in 1893, but was not widely used until the 1960's, and then only by adults. In the early 1980's, use of APAP in children became more common (Varner *et al.*, 1998). More recently it was estimated that greater than 97% of children are exposed to APAP before age two (Kogan *et al.*, 1994). APAP has similar pharmacological actions to those of the non-steroidal anti-inflammatory drugs (NSAIDs) such as Ibuprofen, and is indicated for pain relief (analgesic) and fever reduction (antipyretic). Unlike the NSAIDs, APAP has only a weak anti-inflammatory effect (Graham *et al.*, 2013).

It is believed that APAP exerts its analgesic and antipyretic effects primarily by inhibiting the COX enzymes, and consequently their conversion of arachidonic acid to prostaglandins (PG). In humans APAP has been shown to selectively inhibit COX-2 (Hinz *et al.*, 2008). Other mechanisms have been proposed, some involving COX inhibition as the initiating step and others are unrelated to COX inhibition. For example, there is evidence to suggest that APAP may augment the endocannabinoid system. The effects of APAP are inhibited by cannabinoid receptor (CBR) antagonists, and diminished in CBR knockout mice (Ottani *et al.*, 2006, Mallet *et al.*, 2008). Inhibition of COX could lead to the availability of more arachidonic acid which is used in the synthesis of endocannabinoids, therefore, the effects seen in these studies may require COX inhibition first. On the other hand, a recently discovered fatty acid amide hydrolase metabolite of APAP, AM404, may cause analgesia directly by acting on transient receptor potential vanilloid 1

(TRPV1) expressed in the central nervous system (Mallet *et al.*, 2010). AM404 may also block the re-uptake of endocannabinoids, thus increasing the inhibitory activity of these compounds on the transduction of pain signals.

1.6 Metabolism and Toxicity

APAP is extensively metabolized, leaving very little of the parent APAP compound to be eliminated. The majority of the drug is eliminated via renal excretion as glucuronide and sulphate conjugates in the urine, catalyzed by UDP-glucuronosyl-transferases and sulfotransferases (McGill and Jaeschke, 2013). A smaller fraction of the parent APAP undergoes cytochrome P450 (CYP450) metabolism to form the highly reactive intermediate, N-acetyl-P-benzoquinone-imine (NAPQI). Several different CYP isoforms, including 2E1, 3A4, 1A2, and 2D6, catalyze the biotransformation of APAP to NAPQI (Hinson *et al.*, 2010).

APAP is considered to be a safe drug at the recommended therapeutic dose (15 to 20 mg/kg) (McGill and Jaeschke, 2013). At therapeutic doses, NAPQI is efficiently detoxified by conjugation with GSH. At supratherapeutic doses, glucuronidation and sulfation pathways are overwhelmed leading to a greater fraction of the parent APAP being bioactivated to NAPQI by CYP450s. At sufficient doses of APAP (>300 mg/kg), high levels of NAPQI eventually result in severe hepatotoxicity and liver failure. It is well known that excess NAPQI causes the depletion of cellular GSH (Jaeschke *et al.*, 2012). The preferred antidote for APAP overdose is N-acetylcysteine, a compound which can conjugate with NAPQI similarly to GSH, and may also help replenish cellular GSH stores (Lauterburg *et al.*, 1983). There is some debate over the exact mechanism by which APAP causes hepatotoxicity. However, it is generally accepted that severe GSH loss causes

oxidative stress and the initiation of multiple processes. These processes include disruption of mitochondrial function, NAPQI-protein adduct formation, and inflammation that ultimately lead to cellular necrosis (Hinson *et al.*, 2010).

In human cases of APAP overdose, respiratory tract effects are typically not observed or perhaps they are overlooked. A literature search reveals only one case-study in which a human presented with diffuse alveolar pulmonary damage following an overdose of APAP (Price *et al.*, 1991). On the other hand, respiratory tract effects of APAP are commonly identified in animal studies. APAP-induced respiratory tract lesions were first identified by Placke *et al.* (1987), who found that high doses of APAP (600 mg/kg, orally) caused necrosis of the bronchial epithelium of mice. Doses below 600 mg/kg did not produce lesions in tissues other than the liver. Additional studies, found nasal epithelial necrosis in mice after high doses of APAP (Hart *et al.*, 1995, Jeffery and Haschek, 1988). It is likely that local generation of NAPQI plays a role in these respiratory tract effects (Placke *et al.*, 1987). The airways are a site of high xenobiotic metabolizing activity, and the CYP450 isoforms necessary for the biotransformation of APAP to NAPQI are expressed throughout the respiratory tract (Ding and Kaminsky, 2003). However, the relative contribution to respiratory tract toxicity of respiratory APAP metabolism versus hepatic metabolism is not clear. Liver specific knockout mice with 95% less CYP450 activity than wild-type exhibit decreased lung toxicity with APAP, but no decrease in nasal tissue toxicity (Gu *et al.*, 2005). This suggests both hepatic metabolism and respiratory tract metabolism of APAP may contribute to different degrees depending on the respiratory tract region. Additionally, GSH loss in the liver may affect lung GSH levels, and impair respiratory tract detoxification of APAP/NAPQI. High levels of GSH are found in the epithelium and ELF, throughout the respiratory tract (Gould and Day, 2011).

These stores of GSH can be replenished by de novo synthesis in the respiratory tract, but also via uptake from circulating GSH produced by the liver.

Oxidants and the Airways

1.7 Oxidant Defenses

Oxygen is an inherently reactive element. As the airways make up the largest surface area of the body exposed to the atmosphere, they are exposed to greater levels of oxygen than other organs. One of the byproducts of exposure to a high oxygen environment are reactive oxygen species (ROS) (van der Vliet, 2015). Normal cellular metabolism can also lead to the production of ROS. ROS, such as superoxide and hydrogen peroxide, are the result of the addition of extra electrons to oxygen. High levels of reactive oxygen species can disrupt the balance between the oxidative and reductive chemical reactions (redox balance) in airway cells. A redox imbalance favoring oxidative reactions causes oxidative stress.

The airways have evolved effective oxidant defenses that aid in the maintenance of redox homeostasis in a high oxygen environment (van der Vliet, 2015). The collective term for these cellular defenses is antioxidants. They include soluble compounds such as GSH and vitamins (eg. C and E), and enzyme systems such as the peroxidases, thioredoxin, and superoxide dismutase. Cellular stores of antioxidants can be depleted by high concentrations of oxidants. Cellular antioxidant signaling mechanisms such as the nuclear factor erythroid 2-related factor 2 (Nrf2) have evolved in aerobic organisms (Bataille and Manautou, 2012). These signaling mechanisms can counteract the effects of antioxidant depletion.

The airways are exposed to many oxidants in addition to oxygen. Systemically delivered compounds such as APAP may have oxidant effects in the airways (Placke *et al.*, 1987, Gu *et al.*, 2005). Oxidant air pollutants may also be present in the airway environment. Many air pollutants are reactive oxidants or contain oxidant components which can cause oxidative stress. These include ozone, diesel exhaust, and ETS (Gilmour *et al.*, 2006). In addition to affording protection against high oxygen environments, airway antioxidants can mitigate the effects of oxidant air pollutants such as ETS. They also are involved in the detoxification of reactive compounds such as NAPQI (McGill and Jaeschke, 2013). Oxidants can activate the Nrf2 pathway, a pathway regulated by the transcription factor Nrf2 (Goldring *et al.*, 2004, Tirumalai *et al.*, 2002). The Nrf2 pathway can upregulate enzymes involved in the synthesis of antioxidants, as well as specific enzymes for the detoxification of oxidant xenobiotics. The focus of this section will be on the antioxidant GSH, and the Nrf2 pathway as they are key cellular factors in the detoxification of NAPQI, and are involved in the detoxification of ETS.

GSH is the reduced form of a tripeptide composed of one amino acid each of γ -glutamate, cysteine, and glycine (Meister and Anderson, 1983). GSH is the most abundant low molecular weight thiol in eukaryotic cells. It is present in the lower airways from 100 to 400 μ M, and in the upper airways from 1.5 to 2mM (Gould and Day, 2011, Potter *et al.*, 1995, Cantin *et al.*, 1987). This is in contrast to the plasma where only $<5\mu$ M GSH is present (Cantin *et al.*, 1987). GSH biosynthesis occurs in a two-step process (Meister and Anderson, 1983). First, glutamyl-cysteine ligase (Gcl) catalyzes the formation of γ -glutamylcysteine from glutamate and cysteine, then glycine is added by glutathione synthetase.

The antioxidant functions of GSH depend on its central cysteine moiety, specifically the chemical reactivity of this cysteine's free thiol group (-SH). The reaction of oxidants with GSH can occur

spontaneously, or can be catalyzed by glutathione S-transferases (GSTs). The conjugation of NAPQI and GSH occurs primarily through direct reactivity of NAPQI with GSH, but NAPQI can also be a good substrate for GST pi (Hinson *et al.*, 2010). Acrolein, a primary oxidant component of ETS, is similarly detoxified through direct or enzymatic conjugation with GSH (Parent *et al.*, 1998, Berhane *et al.*, 1994). In the process of conjugation with certain oxidants, such as NAPQI, GSH can be eliminated from the body as a mercapturic acid, and thus is lost (Mitchell *et al.*, 1973a). The thiol group may also donate its hydrogen in reactions with oxidants, forming oxidized glutathione (GS⁻). Two molecules of GS⁻ then bind to form glutathione disulfide (GSSG) which may be exported and/or recycled by GSH disulfide reductase (Meister and Anderson, 1983).

With sufficient oxidant burden, GSH loss can lead to an inability to detoxify both exogenous and endogenous oxidant chemicals. For example, GSH depletion by diethylmaleate (DEM) enhances hyperbaric oxygen toxicity (Deneke *et al.*, 1985). Similarly, DEM greatly enhances the nasal toxicity of naphthalene (Phimister *et al.*, 2004). As alluded to earlier, a dose-dependent loss of hepatic GSH occurs after APAP administration: little to no toxicity is observed up to 70% GSH loss when significant NAPQI adducts begin to form, and at approximately 90% loss, hepatocellular necrosis occurs (Mitchell *et al.*, 1973b, Hinson *et al.*, 2010). In the following experimental chapters, the degree of GSH loss is used as a biomarker of airway tissue oxidant burden following APAP.

Nrf2 is a key regulator of the cellular response to oxidative stress. Nrf2 is highly conserved among vertebrates and is found in multiple tissues and cell types (Kobayashi *et al.*, 2002). Nrf2 is a member of the Cap'n'collar-type basic leucine zipper transcription factor proteins. Under basal conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) through hydrogen bonding (Kobayashi and Yamamoto, 2005). Keap1 facilitates the targeting of

Nrf2 for proteasomal degradation by Cullin 3 ubiquitin ligase. Under conditions of oxidative stress, or in the presence of oxidants, Nrf2 is released from Keap1 and translocates into the nucleus. It is thought that oxidants interact with cysteine residues on Keap1 which cause a conformational change, relaxing the Keap 1-Nrf2 bond and allowing it to translocate (Dinkova-Kostova *et al.*, 2002). Once in the nucleus, Nrf2 binds as part of a complex with other transcription factors to antioxidant response elements (ARE) located in the upstream promoter regions of several cytoprotective genes (Bataille and Manautou, 2012). Emphasizing the importance of Nrf2 in cytoprotection, mice that do not express Nrf2 are more susceptible to acetaminophen hepatotoxicity (Enomoto *et al.*, 2001), and the airway toxicity of high oxygen (Cho *et al.*, 2002). Nrf2-deficient mice were reported to have increased susceptibility to airway inflammation following treatment with ovalbumin (OVA). Notably, these mice had increased oxidative stress, as measured by lower airway GSH levels compared to wild-type controls (Rangasamy *et al.*, 2005). Additionally, Nrf2-deficiency enhances susceptibility to cigarette smoke induced emphysema (Rangasamy *et al.*, 2004).

Nrf2 activation can induce the expression of genes involved in multiple detoxification pathways. For example, the gene encoding Gcl, the enzyme which catalyzes the rate-limiting step in GSH synthesis, contains an ARE and can be upregulated by Nrf2 in response to low GSH (Chan and Kwong, 2000). NAD(P)H dehydrogenase, quinone 1 (Nqo1) is also induced by Nrf2 (Aleksunes *et al.*, 2006). Nqo1 is involved in the detoxification of reactive quinones, such as NAPQI. The upregulation of these genes in airway tissues has been successfully used as a biomarker of oxidative stress (Cichocki *et al.*, 2014a, Klaassen and Reisman, 2010). In the studies detailed in Chapter 2 of this dissertation, Nrf2 dependent genes are used to document oxidative stress in the airways following APAP, and ETS exposure.

1.8 Respiratory Sensory Irritation Reflexes

Sensation of temperature, chemicals, osmolarity, and mechanical stresses from breathing or airway secretions, is extremely important for proper function, and protection of the airways. These functions are primarily accomplished by airway sensory neurons (Alarie, 1973). The airways are innervated by the sensory afferents of cranial nerves 5 and 10, the trigeminal and vagus nerves, respectively. Nerve endings from the trigeminal and vagus nerves project into the respiratory tissue to just below the epithelium near the basement membrane, but generally do not project past the epithelial tight junctions. Excitation of these neurons by a number of different factors (chemicals, epithelial damage, immune cell infiltration, and cytokines) can cause sensations of pain and burning which lead to respiratory reflexes such as coughing and sneezing, plasma extravasation, and mucus secretion. Activation of sensory nerves can also lead to neuropeptide release which promotes inflammation, vasodilation, and bronchoconstriction.

The transient receptor potential (TRP) family of non-selective cation channel receptors plays an important role in the sensation of many different stimuli (Clapham, 2003). Two important receptors of this family expressed in airway sensory neurons are the TRP ankyrin 1 (TRPA1) and vanilloid 1 (TRPV1) receptors (Bessac and Jordt, 2008). These receptors respond to both exogenous and endogenous stimuli, such as inflammatory mediators (e.g. prostaglandins), capsaicin from hot peppers, and acrolein (a byproduct of the combustion of organic materials, such as tobacco). Both TRPA1 and TRPV1 are involved in sensing different ranges of environmental temperatures, and their activation elicits pain or burning sensations. TRPV1 is activated at greater than 42°C, while TRPA1 is activated by extreme cold less than 0°C. It is thought that capsaicin and other TRPV1 agonists shift the threshold of thermal activation of TRPV1 to a lower temperature, resulting in indirect activation. TRPV1 agonists contain a vanilloid ring structure

which binds to one of the TRPV1 ion channel-gating helices to promote activation. With TRPA1 ligand binding, a conformational change in the TRPA1 ion channel leads to the stabilization of its open state directly. TRPA1, is considered to be a cellular sensor of oxidants, and oxidative stress. Most of the agonists for TRPA1 tend to be oxidants, and it has been found that TRPA1 agonists covalently bind to cysteine residues on the N-Terminal tail. This activation mechanism is analogous to the oxidant binding mechanism of the Keap1-Nrf2 pathway, and GSH.

The sensory irritation reflex provides an important means of measuring the effects of air-pollutants on the airways. Many of the chemicals contained in ETS are sensory irritants (Willis *et al.*, 2011). In chapter 2, the effects of APAP on the sensory irritation reflex response induced by ETS, or two of its constituent chemicals acrolein and cyclohexanone, are measured. In mice, inhalation exposure to sensory irritant chemicals elicits a characteristic reflexive epiglottal closure at the end of inspiration, termed braking (Willis *et al.*, 2011). This respiratory sensory irritation reflex response can be measured with plethysmography. During plethysmography, mice are held in a chamber which has a pressure transducer attached, in order to measure changes in pressure induced by the mouse's breathing. Other changes in the airways or in breathing patterns can be observed following exposure to irritants. These include increased or decreased respiratory rate, coughing, tissue edema, and vasodilation. Sensory irritation in other animals can be measured, but the readily quantified braking response is unique to mice. Of the many pathways initiated by sensory nerve stimulation in the mouse, the braking response is the most sensitive (Morris *et al.*, 2003).

1.9 Environmental Tobacco Smoke

Exposure to airborne oxidants such as ozone, diesel exhaust particles (DEP), and ETS influence the pathogenesis and prevalence of asthma (Gilmour *et al.*, 2006). Each of these environmental oxidant exposures has been defined as a risk factor for asthma and exacerbations of asthma symptoms. ETS was chosen as the model oxidant air pollutant for the following studies because of the world-wide commonality of exposure to ETS in non-smokers, especially children. Additionally, the airway effects of tobacco smoke, and tobacco smoke irritants have been the subject of research in our lab for a number of years.

ETS, is a mixture of side stream smoke, from the burning end of a tobacco product, and mainstream smoke which has been exhaled by an active smoker (Jaakkola and Jaakkola, 1997). ETS is comprised of over 4,500 different compounds, many of which are oxidants. ETS consists of a vapor phase and an aerosol-particulate phase. The vapor phase contains volatile agents (*e.g.*, acrolein, cyclohexanone) and the particulate phase contains both semi-volatile and non-volatile agents (*e.g.*, nicotine, aromatic amines, and polycyclic aromatic hydrocarbons (PAHs)) (Jaakkola and Jaakkola, 1997). The temperature at which the tobacco is burned can greatly influence the toxicity of the smoke. Lower temperatures are present during the generation of side stream smoke leading to a more incomplete combustion of the tobacco, and thus more constituents such as carbon monoxide, and acrolein. Once in the environment, tobacco smoke can undergo further physicochemical changes which may modulate its toxicity (Schick and Glantz, 2006). For example, the equilibrium of certain constituents may shift between the particle and vapor phases (CDC, 2010). ETS contains constituents that are known to cause cancer and other adverse health effects.

Approximately 50% of children between the ages of 2 and 19 years old are exposed to ETS in the home (Quinto *et al.*, 2013, Pirkle *et al.*, 1996). High levels of ETS exposure also occur while children are riding in automobiles with active smokers (King *et al.*, 2012). ETS causes increased airway epithelial permeability, inflammation, and oxidative stress in humans (Morrison *et al.*, 1999). Several studies have found associations between parental smoking and asthma development in children (Strachan and Cook, 1997, Jaakkola and Jaakkola, 2002a). Maternal smoking has the strongest effect, and the number of smokers in the household increases the strength of the association. ETS has also been associated with asthma in adults (Jaakkola and Jaakkola, 2002b). In mice, ETS exposure was found to increase bronchial hyperreactivity, eosinophilia, and promote a Th2-cytokine response following allergen exposure (Seymour *et al.*, 1997, Seymour *et al.*, 2003). Several other studies in animals have observed that ETS enhances allergic sensitization, and worsens disease (Lanckacker *et al.*, 2013, Mitchell *et al.*, 2012, Van Hove *et al.*, 2008).

The effects of tobacco smoke in allergic airway disease models are known to vary depending on the type of exposure (ETS vs. main stream) (Van Hove *et al.*, 2008, Maes *et al.*, 2010). Most studies have used chronic exposure paradigms, and high smoke concentrations to mimic the effects of active smoking. Few studies have investigated the effects of low concentration ETS on the airways. Importantly, one study has shown that ETS enhances Th-2 driven airway inflammation in a murine HDM model using low ETS exposure levels of greater relevance to non-smokers (Mitchell *et al.*, 2012).

Experimental Section

1.10 Central Theme and Hypotheses

The cause (s) of the rise in asthma prevalence are unknown. Epidemiological studies suggest that increased APAP use may be linked to asthma, leading to the formulation of the “APAP hypothesis”. For the APAP hypothesis to be plausible, it follows that there should be demonstrable effects of APAP in the airways at non-hepatotoxic doses. A full understanding of the relationship between APAP dose and airway effects is needed to establish the role of APAP in asthma pathogenesis. However, few studies have addressed the biological plausibility of the “APAP hypothesis,” or the airway effects of APAP at non-hepatotoxic doses. Therefore, the overarching goal of this work was to address this knowledge gap. From this goal a central theme of this dissertation emerges: *investigating the effects of non-hepatotoxic doses of APAP on the airways.*

Highlighted in the “APAP hypothesis” is the assertion that APAP plays a role in asthma pathogenesis through exertion of pro-oxidant effects on the airways. The pro-oxidant basis of the acetaminophen hypothesis stems from the well-characterized toxic effect of APAP in the liver as well as the evidence of extrahepatic toxicity described previously. This pro-oxidant effect of APAP may influence the airway effects of other oxidant air-pollutants which overlap in pathways of detoxification, such as ETS. Additionally, oxidative stress is implicated in the pathogenesis of asthma, and many environmental risk factors, such as ETS, are oxidants. An interaction between APAP and ETS is likely. Taken together, this evidence prompted the hypothesis tested in chapter 2 of this dissertation: *that APAP acts as a pro-oxidant in the airways, and potentiates the acute effects of ETS.* This hypothesis is tested in mice through the measurement of several biomarkers of airway oxidative stress (GSH loss, and Nrf2 gene expression) following APAP treatment alone,

and in combination with ETS. The effects of APAP on the airways function *in vivo*, is also examined through measurements of the sensory irritation reflex response to APAP alone and in combination with ETS. Results indicated that APAP acted as a pro-oxidant in the airways of the mouse and potentiated the acute airway response to ETS. These results, by providing biological plausibility, supported the “APAP hypothesis.

Asthma is a chronic disease, therefore, isolating individual acute effects of APAP on respiratory responses as described above is not entirely sufficient to test the biological plausibility of the APAP hypothesis. Mouse models of allergic airway disease, such as the HDM model, provide an opportunity to examine the effects of APAP in a complex pathophysiological system. Additionally, these models allow us to examine cause and effect relationships as they are employed in a properly-controlled setting. In chapter 3, it is hypothesized that *APAP will enhance HDM induced inflammation in a mouse model of allergic airway disease*. This hypothesis is tested in mice by measuring the allergic-inflammatory responses to HDM following APAP treatment. Bronchoalveolar lavage, lavage protein measurement, lung tissue cytokine levels, histology, and serum immunoglobulin, are used to characterize the HDM allergic-inflammatory response. A positive response to HDM includes increased lung immune cellularity, increased lavage protein, and increased cytokine signaling. Surprisingly our initial results indicated that rather than enhancing disease development, APAP blocked the development of HDM-induced allergic airway inflammation. The subsequent studies were designed to fully characterize this effect.

Chapter 2: Acetaminophen is an airway oxidant and potentiates respiratory tract oxidant and irritant responses to environmental tobacco smoke in mice

This work has been recommended for publication in the peer-reviewed journal

Environmental Health Perspectives.

2.1 Abstract

Background: Acetaminophen can cause oxidative injury in the liver; it is not known if acetaminophen causes oxidative stress in the respiratory tract. If so, this widely used analgesic may potentiate the adverse effects of oxidant air pollutants.

Objectives: The goals of this study were to determine if acetaminophen induces respiratory tract oxidative stress and/or potentiates the oxidative stress and irritant responses to an inhaled oxidant: environmental tobacco smoke (ETS).

Methods: Female C57Bl/6J mice were administered acetaminophen (100 mg/kg ip) and/or side stream tobacco smoke (as a surrogate for ETS, 5 mg/m³ for 10 minutes) with airway oxidative stress being assessed by depletion of tissue antioxidants (as estimated by non-protein sulfhydryl, NPSH levels) and/or induction of oxidant stress response genes. In addition, the effects of acetaminophen on airway irritation reflex responses to ETS were examined by plethysmography.

Results: Acetaminophen depleted NPSH in nasal, thoracic extrapulmonary, and lung tissues, and also induced the oxidant stress response genes, glutathione cysteine ligase, catalytic subunit, and NAD(P)H dehydrogenase, quinone 1, in these sites. ETS produced a similar response. The response to acetaminophen plus ETS was equal to or greater than the sum of the responses to either agent alone. Although without effect by itself, acetaminophen greatly increased the reflex irritant response to ETS.

Conclusions: Acetaminophen, at supratherapeutic levels, induces oxidative stress throughout the respiratory tract and potentiates the oxidative responses to environmentally relevant ETS exposure. These results highlight the potential for this widely used drug to modulate the responsiveness to oxidant air pollutants.

2.2 Introduction

Oxidative stress results from an imbalance between antioxidants and pro-oxidants within a cell. Oxidative stress is a common mechanism for respiratory tract injury by inhaled and also systemically delivered toxic agents. For example, oxidative stress contributes to the airway injury produced by inhaled diesel exhaust, ozone, and environmental tobacco smoke (ETS) (Holguin, 2013). Systemically delivered toxicants such as styrene and naphthalene can be bioactivated throughout the respiratory tract, and induce oxidative stress as well (Cruzan *et al.*, 2012, Spiess *et al.*, 2010). Oxidative stress contributes to the development and/or exacerbation of respiratory diseases, including asthma. For example, biomarkers of oxidative stress are elevated in asthma and individuals with low antioxidant levels are susceptible to the development of this disease (Larkin *et al.*, 2015, Riedl and Nel, 2008). Additionally, oxidant air pollutants, including the ubiquitous indoor air pollutant ETS, are associated with increased prevalence and/or severity of asthma (Gilmour *et al.*, 2006, IOM, Institute of Medicine (US) Committee on the Assessment of Asthma and Indoor Air, 2000, Kanchongkittiphon *et al.*, 2015). This report focuses on the potential for the over-the-counter analgesic, acetaminophen, to induce airway oxidative stress and potentiate the airway response to the inhaled oxidant stressor, ETS.

Acetaminophen, (APAP, *n*-acetyl-*para*-aminophenol), is a commonly used medicine for relieving pain and reducing fever in adults and children. APAP is a known hepatotoxicant. The majority of APAP is metabolized in the liver by glucuronidation and sulfation pathways and is safely excreted; however, a fraction of APAP is metabolically activated in the liver to the pro-oxidant metabolite N-acetyl-P-benzoquinone-imine (NAPQI) (McGill and Jaeschke, 2013). NAPQI is highly reactive, causes cellular oxidative stress, and covalently binds to cellular macromolecules (Jaeschke *et al.*, 2012). Detoxification of NAPQI consumes the important antioxidant glutathione

(GSH). NAPQI induces the nuclear factor-erythroid 2-related factor-2 (NRF2) dependent oxidative stress gene response pathway (Bataille and Manautou, 2012, Klaassen and Reisman, 2010) causing induction of genes involved in multiple detoxification pathways. This includes the enzyme which catalyzes the rate-limiting step of GSH synthesis, glutamyl-cysteine ligase, catalytic subunit (GCLC), as well as NAD(P)H dehydrogenase, quinone 1 (NQO1), which is involved in the detoxification of reactive quinones (Aleksunes *et al.*, 2006, Chan and Kwong, 2000). Induction of NRF2 pathway genes can be used as a sensitive biomarker for cellular oxidative stress (Cichocki *et al.*, 2014a, Cichocki *et al.*, 2014b, Klaassen and Reisman, 2010) .

The metabolic activation of APAP to NAPQI is catalyzed by a variety of cytochrome P450 isoforms including CYP2E1, CYP3A4, and CYP1A2 (Hinson *et al.*, 2010). These CYPs are expressed in the respiratory tract, suggesting that similar metabolic activation may occur in this site as well (Ding and Kaminsky, 2003). When administered directly to the lungs, NAPQI induces a neurogenic inflammatory response (Nassini *et al.*, 2010) and hepatotoxic doses of acetaminophen are known to deplete GSH and cause injury in the nose and lungs (Hart *et al.*, 1995, Gu *et al.*, 2005). It is not known if the bioactivation capacity of respiratory tissues is sufficient to induce oxidative stress at non-hepatotoxic, supratherapeutic doses of APAP. Were APAP to induce oxidative stress in respiratory tissues, it could enhance the response to other oxidant stressors. The current study is focused on determining if APAP induces oxidative stress in respiratory tissues, and if so, if APAP enhances the oxidative stress and respiratory tract irritant responses to environmentally relevant ETS exposure. ETS was selected because it is a common air pollutant and both acetaminophen and ETS have been associated with increased prevalence of asthma (Etminan *et al.*, 2009, Gilmour *et al.*, 2006).

The hypothesis that APAP acts as a pro-oxidant in the airways and enhances the response to ETS was tested in a mouse model by determining if APAP and/or APAP+ETS: 1) causes a loss of GSH, and 2) activates the oxidant stress response pathway utilizing two NRF2-dependent genes as biomarkers: *Gclc* and *Nqo1*. The effects of APAP on the response to ETS were further characterized by examining the ETS-induced irritation reflex response. This response is caused by ETS stimulation of nasal trigeminal chemosensory nerves through the oxidant sensitive transient receptor potential ankyrin 1 (TRPA1) channel (Andre *et al.*, 2008). Results indicated that APAP induces oxidative stress in the entire respiratory tract and potentiates the oxidant response to environmentally relevant ETS exposure.

2.3 Materials and Methods

Experimental Approaches. The first studies were aimed at determining if APAP induced oxidative stress in respiratory tissues. Towards this end, animals were euthanized 0-3 hrs after APAP administration (ip), and nasal respiratory/transitional mucosa (RTM), intrathoracic airways (tracheal/mainstream bronchial mucosa, TBM), and lung (left lobe) samples were collected. Oxidative stress was assessed by determination of tissue non-protein sulfhydryl levels (NPSH, as a surrogate for GSH), and expression levels of two NRF2-dependent oxidant stress response genes: *Gclc* and *Nqo1* (Cichocki *et al.*, 2014b). The response of NRF2 *-/-* mice to APAP was also investigated to confirm a role for NRF2 in any gene expression changes. Plasma levels of acetaminophen were determined in animals euthanized 15 min after dosing (the time of peak plasma concentration, (Gu *et al.*, 2005, Lin *et al.*, 1996), to assess the therapeutic relevancy of the APAP dosing regimen.

The interaction between APAP and ETS was assessed by examining the nasal RTM response to these agents alone and in combination, using the measures described above for APAP. Side-stream smoke was used as a surrogate for ETS. Mice were exposed for 10 min to a nominal exposure concentration of 5 mg/m³ to approximate the ETS concentrations achieved in a closed automobile containing a smoker, or clean filtered air in the same apparatus (Sendzik *et al.*, 2009). The 10 min duration corresponded to the burn time of a single cigarette. In addition, the effect of APAP on the reflex irritation response of mice to ETS was examined. Stimulation of trigeminal chemosensory nerves causes a brainstem-mediated characteristic change in breathing pattern that can be assessed non-invasively during exposure (Alarie, 1973, Vijayaraghavan *et al.*, 1993, Willis *et al.*, 2011). Stimulation of these nerves is also pro-inflammatory (Andre *et al.*, 2008, Nassini *et al.*, 2010, Caceres *et al.*, 2009). The rationale for the focus on the nose for the APAP-ETS study was multifold. The nose is the first airway exposed to ETS in the mouse, and is a common site of inhaled toxicant-induced injury in the rodent (Morris, 2012). Nasal injury in nose-breathing rodents is a sentinel for lower airway injury in mouth breathing humans, and the ETS-induced irritant reflex response is mediated via the trigeminal nerve and, therefore, originates in the nose (Cichocki *et al.*, 2014b, Morris, 2012, Gloede *et al.*, 2011).

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 that any effects of APAP were oxidant specific rather than generalized in nature. Cyclohexanone activates chemosensory nerves by the transient receptor potential vanillin 1 (TRPV1) receptor, while acrolein acts through TRPA1 (Bautista *et al.*, 2006, Saunders *et al.*, 2013). Second, the effect of APAP was examined in animals

pretreated with 5-phenyl-1-pentyne (5-PP) to inhibit CYP metabolism (Morris, 2013, Roberts *et al.*, 1998) and bioactivation of APAP. Third, the GSH depleting agent, diethyl maleate (DEM), was administered to determine if modulation of nasal GSH status could replicate the effects of APAP. This agent is conjugated with GSH via glutathione-S-transferases, resulting in depletion of tissue GSH levels (Boyland and Chasseaud, 1967, Phimister *et al.*, 2004).

Animal procedures. Female C57Bl/6J mice (9 to 11 weeks of age) were used for all experiments. Female mice were used because there is a rich database on respiratory reflex responses to irritants, including ETS, in female mice (Willis *et al.*, 2011, Ha *et al.*, 2015) and female mice are more sensitive than male mice to the acute respiratory tract effects of metabolically activated toxicants (Van Winkle *et al.*, 2002). Mice were obtained from Jackson Laboratories. Age-matched Nrf2-null and wild-type (C57BL/6J background) mice were kindly provided by Dr. Jose Manautou. Initial Nrf2 ^{-/-} breeding pairs were obtained from Dr. Angela Slitt at the University of Rhode Island. Mice were 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). Food (Lab Diet; PMI Nutrition International) and tap water were provided *ad libitum*. All animals were treated humanely and with regard for alleviation of suffering. Animal procedures were approved by the University of Connecticut Institutional Animal Care and Use Committee.

Unless otherwise indicated all chemicals were obtained from Sigma Aldrich (St. Louis, Missouri). APAP, dissolved in 37° C saline (10 mg/ml), was administered via ip injection. When administered, the cytochrome P450 inhibitor 5-PP (GFS chemicals) was given ip at a dose of 100

mg/kg (10 mg/ml in olive oil) 1hr prior to APAP treatment (Morris, 2013). DEM was administered at a dose of 250 mg/kg (0.33 M solution in olive oil, ip, (Phimister *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. Nasal RTM tissues were removed from the ventral portions of the nasal cavity by microdissection (Cichocki *et al.*, 2014a) (Olfactory mucosa was not collected as this is neural, non-respiratory, tissue.) The intrathoracic TBM airways and the left lobe of the lungs were removed. For NPSH determination tissue samples were homogenized in 5% trichloroacetic acid-3 mM EDTA and were stored at -80 °C until analysis. For qRT-PCR, tissues were immediately placed in an aqueous RNA stabilization buffer containing saturating ammonium sulfate, 20 mM EDTA, and 25 mM Sodium Citrate, at pH 5.2 and stored at -80 °C until analysis.

Breathing Pattern Analysis. Mice were held in a double plethysmograph (Buxco Inc.) connected to a directed airflow nose-only inhalation chamber (CH Technologies) for irritant exposure to allow monitoring of breathing parameters during the exposure. Animals were placed in the plethysmograph for a 15-min acclimatization, a 5-min baseline, and then a 10 minute exposure to irritant. The sensory irritation reflex response, characterized by a pause (termed braking) at the onset of each expiration due to glottal closure, was quantitated by measuring the duration of braking (Willis *et al.*, 2011). Breathing patterns were monitored using Emka Technologies 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. Control mice were exposed to clean filtered air in the same exposure chamber. 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 2.5 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). To achieve constant concentration smoke exposures, side stream cigarette smoke was continuously generated by passing filtered and humidified air over a lit cigarette centered in one port of a 2L four neck boiling flask (Kimble-Chase). High concentration smoke from the flask was drawn into a second identical flask using a peristaltic pump and fed using positive pressure into the inhalation chamber. Smoke was generated from Kentucky 2R4F reference cigarettes that had been stored for at least 24 hr at 55% relative humidity. For smoke exposures, particulate levels were measured by a Casella Microdust Pro Analyzer. Nominal suspended particle concentration over the duration of smoke exposures was 5 mg/m³. Airborne carbon monoxide (CO) levels were monitored continuously during exposure with a Digital CO Detector (DCO1001, General Tools). CO levels averaged 25 to 35 ppm throughout the exposures.

Analytical Techniques. NPSH levels were used as a surrogate for GSH. NPSH was determined by the method of Sedlak and Lindsay (1968) and normalized to protein content using the Lowry

method (Lowry *et al.*, 1951). Data are expressed as percent of control. Control levels averaged approximately 18, 8, and 12 nmol/mg protein in RTM, TBM and lung, respectively. For determination of plasma APAP levels, blood was collected by cardiac puncture with heparinized syringes. Plasma, prepared by centrifugation, was diluted 1:10 in 5% trichloroacetic acid-3 mM EDTA and then analyzed for APAP content by HPLC based on the method of Lin et al. (1996). APAP eluted with a retention time of 11 minutes using a mobile phase of 7% acetonitrile-0.1% trichloroacetic acid, a flow rate of 1 mL/min, and an Agilent Eclipse-Pro Plus LC-18 (5 μ m, 4.6 x 250 mm) column, and was detected by UV absorbance at a wavelength of 254 nm. Levels were determined in plasma from 4 APAP-injected and 4-vehicle injected mice (to confirm the absence of contaminating peaks).

For qRT-PCR of selected oxidant stress response genes, total RNA was isolated from respiratory tissue homogenates using an RNeasy kit from Qiagen. 1 μ g of total RNA was used for first strand cDNA synthesis by an iScript cDNA synthesis kit (Bio-rad). RT-PCR was performed using SYBR Green as an indicator with an ABI 7500 Real-Time PCR system on the Fast setting. PCR reactions contained 10 ng of cDNA (4 μ l), 500 nM of each primer (1 μ l total), and 5 μ l of 2x SYBR Green PCR Master mix for a 10 μ l total volume. The PCR was carried out according to the manufacturer's recommended thermal cycling protocol. Data were normalized to β -Actin as the internal reference control mRNA. Results are represented as the fold change in expression of target genes over control calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primers were designed with Life Technologies OligoPerfect™ designer and obtained from Invitrogen (Life Technologies).

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). Individual data values were excluded *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 ANOVA followed, as appropriate, by Newman-Keuls test. When appropriate data were log transformed to correct for heteroscedasticity. Sensory irritation time course data were analyzed by repeated-measures ANOVA followed by Newman-Keuls test. A p-value less than 0.05 was required for statistical significance.

2.4 Results

APAP causes oxidative stress in airways. To examine the time course of the response to APAP mice were euthanized 1, 2 or 3 hrs after 100 mg/kg APAP treatment and tissues collected from RTM, TBM and lung. In all tissues, NPSH levels were approximately 80% of control 1 hr after APAP ($p < 0.05$) and returned to control levels by 2-3 hrs (Figure 2.1a). At a dose of 60 mg/kg RTM NPSH was not depleted by APAP, averaging 96 ± 7.7 % of control. Liver NPSH levels averaged $60 \pm 3.9\%$, $83 \pm 2.7\%$, and $106 \pm 3.5\%$, at 1, 2 and 3 hr, respectively after a 100 mg/kg dose of APAP. Both *Gclc* and *Nqo1* genes were significantly induced in all respiratory tissues at a dose of 100 mg/kg, albeit with somewhat differing magnitudes and time courses (Figure 2.1b, c). Dose response relationships for RTM gene induction are shown in Supplemental Material, Figure S1.1. While *Gclc* was somewhat induced at a dose of 60 mg/kg APAP, *Nqo1* was not. In NRF2 $-/-$ mice, basal RTM expression of *Gclc* and *Nqo1* were approximately 7- and 70-fold lower than wild-type control, respectively; APAP caused a less than 2-fold or less induction of either gene and the levels of induction were not statistically significant (Supplemental Material, Figure S2.2). Serum APAP levels were determined in mice euthanized 15 min after a 100 mg/kg dose.

No APAP (or contaminating peak) was detected in vehicle-injected controls (n=4), APAP levels averaged 35 ± 6 $\mu\text{g/mL}$ (n=4) in treated mice.

APAP-ETS interaction. Initial studies focused on the time course of NRF2-dependent gene induction, if any, following ETS (Figure 2.2). ETS exposure levels averaged 6.3 ± 0.6 mg/m^3 (mean \pm SD). *Gclc* was slightly induced, with *Gclc* levels averaging 1.1- and 1.2-fold of control at 1 and 2 hr respectively ($p < 0.05$). *Nqo1* was only increased 2 hrs after exposure to 2-fold of control ($p = 0.02$, t-test).

To examine the potential for an APAP-ETS interaction, mice were exposed to ETS 1 hr after 100 mg/kg APAP administration because this is a time when there is depletion of NPSH but no induction of antioxidant genes (see Figure 2.1). The dose of 100 mg/kg was selected because at this dose APAP modulated all measures of oxidative stress. Mice were euthanized immediately after ETS exposure for determination of NPSH and 2 hours after ETS (3 hours after APAP) for assessment of gene expression because both *Gclc* and *Nqo1* were induced at that time by ETS (Figure 1.2).

ETS alone produced a 21% loss of NPSH, similar to the 20 % loss caused by APAP; APAP followed by ETS exposure caused a loss of 40%, ($p = 0.04$, compared to control, Figure 2.3a). NPSH was significantly depleted from control in only the APAP+ETS groups. Although the NPSH levels in APAP treated mice were of similar magnitude as in the initial study (Figure 2.1a), in this case the group did not differ from control. ETS exposure levels averaged 5.2 ± 0.4 mg/m^3 (mean \pm SD).

Gclc was significantly increased over control levels in the APAP and the APAP+ETS groups (Figure 2.3b). In the ETS group, *Gclc* expression levels averaged 1.2-fold of control, similar to

that in the previous experiment (Figure 2.2a), but in this case a significant difference from control was not observed. In the APAP and APAP-ETS groups, *Gclc* expression averaged 2.8 and 3.7-fold of control suggesting an additive or greater interaction. The difference in *Gclc* levels in the APAP and APAP+ETS groups approached statistical significance ($p=0.07$).

Nqo1 was significantly increased over control in only the APAP+ETS group (Figure 2.3c) and the levels in the group were significantly higher than in either the APAP or ETS groups. In the ETS group, *Nqo1* levels averaged 1.3-fold of control levels, a response somewhat less than in the previous experiment (Figure 2.3b); direct comparison of the response level in this and the preceding experiment did not reveal a significant difference. *Nqo1* was not induced in the APAP group, consistent with the lack of *Nqo1* expression changes in RTM 3 hr after APAP in the earlier experiment (see Figure 2.1c). ETS exposure levels averaged $4.7 \pm 0.5 \text{ mg/m}^3$ (mean \pm SD).

Irritation Reflex. ETS induces the sensory irritation reflex response as indicated by induction of braking at the onset of each expiration; no such response was observed after APAP, but an enhanced response was seen in APAP+ETS mice (Figure 2.4). This response was quantitated by calculating the 1 min average duration of braking in each animal (Figure 2.4e). While APAP alone did not elicit this response, ETS ($4.5 \pm 0.6 \text{ mg/m}^3$, mean \pm SD) produced a moderate response which was significantly increased in APAP-pretreated mice, indicating that APAP potentiated the irritation response.

To examine the oxidant basis for this interaction, the effects of APAP on the irritation response to the TRPA1 specific oxidant vapor, acrolein, and the non-oxidant, TRPV1 agonist vapor, cyclohexanone were examined (Figures 2.5a,b). APAP was without effect on the response to cyclohexanone, but potentiated the response to acrolein. To establish a dose-response, two

additional dose groups (60, and 200 mg/kg) were included in the acrolein sensory irritation experiment. The response to acrolein was slightly but not significantly ($p=0.6$) elevated by a 60 mg/kg dose of APAP (Figure 2.5a) and significantly ($p=0.001$) elevated at a dose of 100 mg/kg. At 200 mg/kg APAP produced braking during the baseline (data not shown). Additional studies were done to further characterize the role of oxidative stress in the APAP potentiation of the irritant response (Figure 2.5c). These studies focused on the APAP potentiation of the acrolein irritant response, rather than ETS, because acrolein is a single agent known to act through TRPA1. As observed previously, the irritation response to acrolein was potentiated by APAP. The potentiation was absent in 5-PP-treated mice. Pretreatment with DEM 1 hr prior to acrolein exposure potentiated the acrolein response. Nasal RTM NPSH levels in the DEM treated mice averaged $75 \pm 5.4\%$ percent of control (compared to $100 \pm 6.6\%$ in controls, $p=0.04$, $n=4$ in each group). This level is similar to that caused by APAP (Figure 2.1a). 5-PP was without effect on the irritant response to acrolein (in mice not given APAP), and the DEM vehicle was without effect on the response to acrolein. Therefore, the 5-PP alone group, the DEM vehicle treated group, and data from non-treated mice were pooled to form the composite control group for this study.

2.5 Discussion

The present study demonstrates that APAP, at supratherapeutic doses, modulates airway oxidative stress- and respiratory irritant-responses to ETS. That adverse respiratory responses to ETS can be enhanced by the commonly used analgesic APAP, is a novel concept. Greater than 97% of children use acetaminophen prior to age two (Kogan *et al.*, 1994). Historically, ETS exposure in non-smoking populations has been high. During the period from 1988-1991, greater than 80% of non-smoking adults in the U.S. were passively exposed to ETS (Pirkle *et al.*, 1996). More recently, ETS exposure in non-smokers has ranged from 52.5% in 1999 to 25.3% in 2012 (Homa *et al.*,

2015). Approximately 30% of children are exposed to ETS in an automobile (King *et al.*, 2012). The ubiquitous use of APAP, coupled with the high frequency of exposure to ETS, highlights the widespread potential for adverse health effects were a toxicologically significant interaction between APAP and ETS to occur.

Our initial studies indicated that a 60 mg/kg dose of APAP did not cause significant changes in all measures of oxidative stress: nasal GSH was not significantly decreased, *Gclc* expression was significantly higher than control, but *Nqo1* expression was not. Additionally, 60 mg/kg caused a slight increase in the acrolein response but the increase was not statistically significant. APAP doses of 100 mg/kg were used for subsequent studies because this was the lowest dose which produced significant changes in all parameters. Future studies could more thoroughly define the effects of APAP at lower doses. Interestingly, at 200 mg/kg, APAP produces a sensory irritation response suggesting there may be sufficient electrophile produced at this dose to interact with airway sensory nerves. This finding is consistent with the observation that NAPQI can directly activate the TRPA1 channel (Nassini *et al.*, 2010). The human recommended dose of APAP is 15 mg/kg. Therapeutic levels are 5-20 µg/ml and hepatotoxicity is associated with blood levels exceeding 150 µg/ml (Rumack and Matthew, 1975). Although the dose level used in this study is higher than the recommended dosage, peak blood levels were 35 µg/ml. This is similar to that reported by Gu *et al.* (2005), who also report that blood APAP decreases to 10 µg/ml within 1 hour after a 100 mg/kg dose. Thus, this dosing regimen resulted in near therapeutic APAP levels that were well below the threshold for overt liver toxicity. Female mice were used for these studies. Female mice are less susceptible to APAP hepatotoxicity than males (Sheng *et al.*, 2013), but more sensitive to the pulmonary toxicity of metabolically activated chemicals such as naphthalene (Van Winkle *et al.*, 2002). Future studies would be needed to determine if male mice are more sensitive

to the pulmonary effects of APAP and ETS, however if they are more sensitive, then a more dramatic interaction between APAP and ETS than what is reported herein would be anticipated.

At the 100 mg/kg dose APAP clearly induced oxidative stress in the airways as indicated by depletion of NPSH and induction of oxidant stress response genes. It has previously been shown that APAP depletes nasal NPSH (Gu *et al.*, 2005), but at much higher, hepatotoxic doses. That Nrf2 *-/-* mice demonstrated reduced responsiveness to APAP provides evidence that the oxidant stress gene response was mediated, at least in part, through this transcription factor. The toxic response to APAP is due to metabolic activation via CYP to the strong electrophile NAPQI and CYP is expressed throughout the respiratory tract of the mouse (Ding and Kaminsky, 2003, Hinson *et al.*, 2010). Perhaps local activation of APAP is involved in the responses observed herein. The current results do not, however, rule out the possibility of hepatic events (escape of activated APAP, depletion of blood GSH) as a contributing factor to the oxidative stress response observed in this study (Gu *et al.*, 2005, Phimister *et al.*, 2004). Since, 5-PP inhibits CYP metabolism systemically these data do not provide insights into the exact role of hepatic versus local nasal activation of APAP.

ETS contains thousands of chemicals, many of which are oxidants. In the current study, 10 min exposure to 5 mg/m³ ETS caused a modest oxidative stress response as indicated by slight induction of an oxidative stress gene response. While responses of similar magnitude were observed in the first ETS study and the ETS-APAP study, these changes were not universally detected as statistically significant. This suggests the ETS exposure represents a threshold response level. If so, this may represent a response of concern because the exposure level is similar to that obtained in a car containing an active smoker and the exposure was only of 10 min duration.

Future studies would be required to determine if slightly higher concentrations or longer duration ETS exposure results in a repeatedly observed oxidative stress response.

The interaction between APAP and ETS is likely due to the oxidant properties of both agents. The NPSH depletion response of both agents appeared to be additive, although the statistical basis for this was not clear-cut. The induction of *Gclc* also appeared to be additive, although the differences between APAP and APAP + ETS did not quite attain statistical significance. The *Nqo1* response clearly indicated a synergistic interaction between APAP and ETS, with neither agent alone producing a response, but the combination causing a clear induction. A synergistic interaction was also observed with respect to the reflex irritation response to ETS. APAP alone did not cause reflex irritation, but it markedly increased the irritation response to ETS.

The finding that APAP greatly enhances the irritation response to ETS in these studies suggests that APAP can alter complex integrated airway responses. ETS stimulates chemosensory nerves via the oxidant sensitive TRPA1 receptor (Andre *et al.*, 2008). The current studies confirm an oxidant basis for the APAP-ETS interaction. APAP potentiated the irritant response to the oxidant acrolein, which is a known TRPA1 agonist. That APAP was without effect on the non-oxidant TRPV1 agonist, cyclohexanone, suggests the effects of APAP are oxidant specific. Treatment with 5-PP blocked the modulation of acrolein irritation indicating that oxidant induced sensory irritation is due to the generation of a metabolite, likely NAPQI, rather than a pharmacological effect of the parent APAP. APAP is known to be activated by nasal CYP and the nasal toxicity of APAP is independent of liver activation (Gu *et al.*, 2005). DEM at a dose which produced a similar level of GSH loss to APAP, mimicked the effects of APAP on acrolein irritation suggesting that GSH depletion may contribute to the interaction. DEM has been shown to potentiate the nasal toxicity of another metabolically activated toxicant, naphthalene (Phimister *et al.*, 2004). Perhaps nasal

GSH acts to detoxify acrolein and depletion of GSH enhances acrolein penetration to chemosensory nerve endings which innervate the nasal mucosa. Overall these findings provide strong evidence of an oxidant basis for the effect of APAP on respiratory responses, and also suggest that tissue antioxidant levels may be a determinant of sensitivity to inhaled irritant chemicals.

The public health impact of ETS is well studied. ETS exposure is associated with increased asthma incidence and severity (Coogan *et al.*, 2015, IOM, Institute of Medicine (US) Committee on the Assessment of Asthma and Indoor Air, 2000, Kanchongkittiphon *et al.*, 2015). Multiple epidemiological studies, in both adults and children, reveal an association between the increased APAP use since 1980 and the increased asthma prevalence since that time (Barr *et al.*, 2004, Beasley *et al.*, 2011, Etminan *et al.*, 2009). This has led to the “APAP hypothesis”, specifically that acetaminophen may contribute to asthma causation, perhaps through its oxidant properties (McBride, 2011). This hypothesis, however is controversial due to the potential for confounding in epidemiological studies and lack of experimental evidence (Heintze and Petersen, 2013, Holgate, 2011). The current studies lend credence to the APAP hypothesis, by supporting a novel and biologically plausible mechanism whereby APAP may contribute to the development of asthma, specifically by enhancing the effects of other asthma risk factors such as ETS. However, the current studies only provide information on single short-term exposure to APAP and ETS. Future studies would be required to address the effects of repeated exposures to these agents and on potential differences in outcome if ETS exposure proceeded APAP administration. Nonetheless oxidative stress is thought to play a role in asthma pathogenesis (Holguin, 2013, Riedl and Nel, 2008) and the current studies indicate an oxidative stress-based interaction between environmentally relevant levels ETS and near therapeutic levels of APAP.

Conclusions

Multiple lines of evidence indicate that in the mouse, APAP, at supratherapeutic levels, acts as an airway oxidant and potentiates acute airway responses to environmentally relevant levels of another airway oxidant, ETS. These results suggest that APAP may exert adverse effects on the respiratory tract and, perhaps, should be used with caution in individuals exposed to cigarette smoke. APAP avoidance may be prudent, particularly in individuals susceptible to the development of asthma who are exposed to airborne oxidants such as ETS.

2.6 Tables and Figures

Table 2.1. Mouse primer sequences for qRT-PCR (listed 5'-3'):

| Gene | Forward | Reverse |
|-------------|----------------------|----------------------|
| <i>ActB</i> | GCAACGAGCGGTTCG | CCCAAGAAGGAAGGCTGGA |
| <i>Nqo1</i> | TTTAGGGTCGTCTTGGCAAC | GTCTTCTCTGAATGGGCCAG |
| <i>Gclc</i> | TTCATGATCGAAGGACACCA | CTGCACATCTACCACGCAGT |

Figure 2.1.

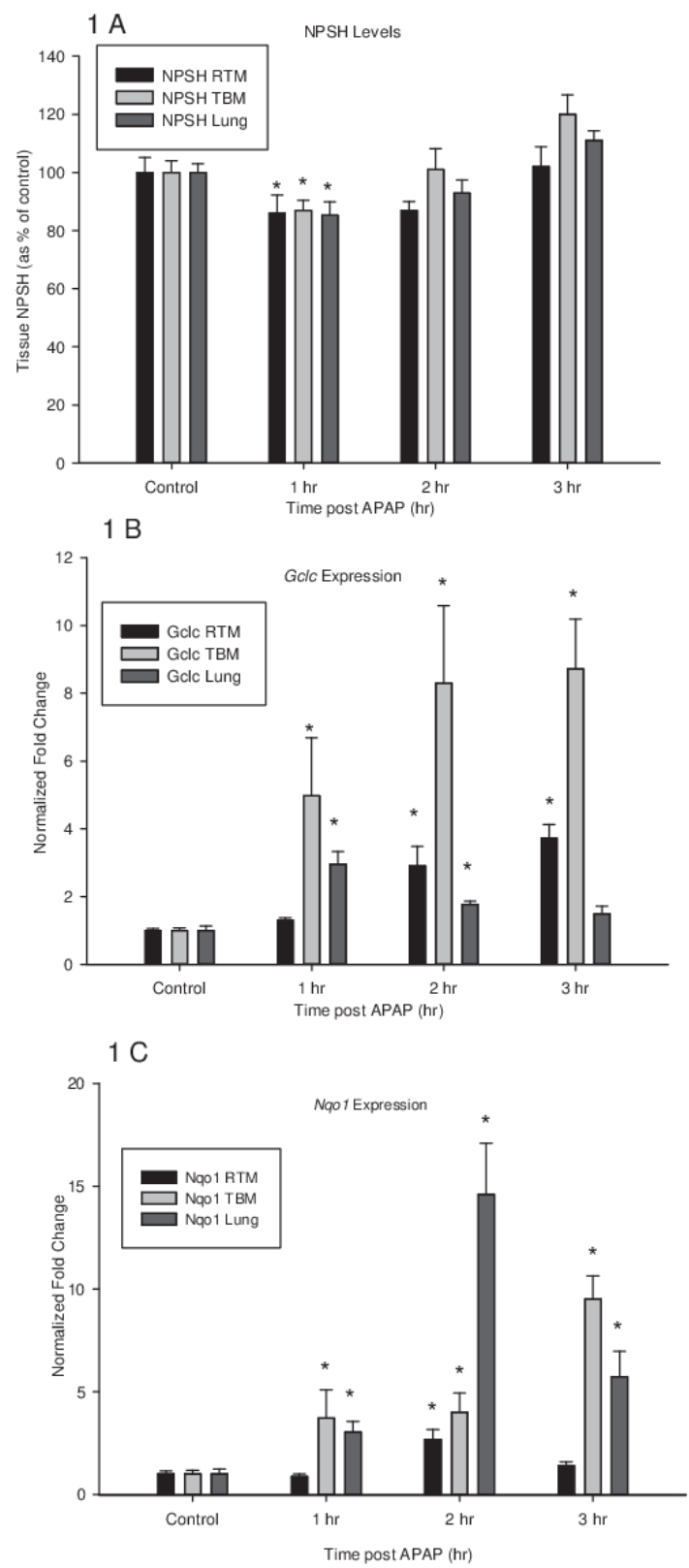


Figure 2.1. NPSH levels and oxidant stress response gene expression in the respiratory tract following APAP administration. (A) Time course of NPSH levels in nasal respiratory/transitional mucosa (RTM), thoracic extra pulmonary airway mucosa (trachea/mainstem bronchi- TBM), and lung parenchyma (Lung) 1, 2, or 3 hours after APAP administration (100 mg/kg, ip). Data are presented as mean \pm SE and are normalized to total protein (*= p < 0.05, compared to respective control). Groups contained 4-6 mice. (B, C) Time courses of (B) *Gclc* and (C) *Nqo1* expression, in nasal RTM, TBM, and Lung 1, 2, or 3 hours after APAP administration (100 mg/kg, i.p). Data are presented as mean fold change \pm SE, and are normalized to control values (* = p<0.05, compared to respective control). Groups contained 4-6 mice.

Figure 2.2.

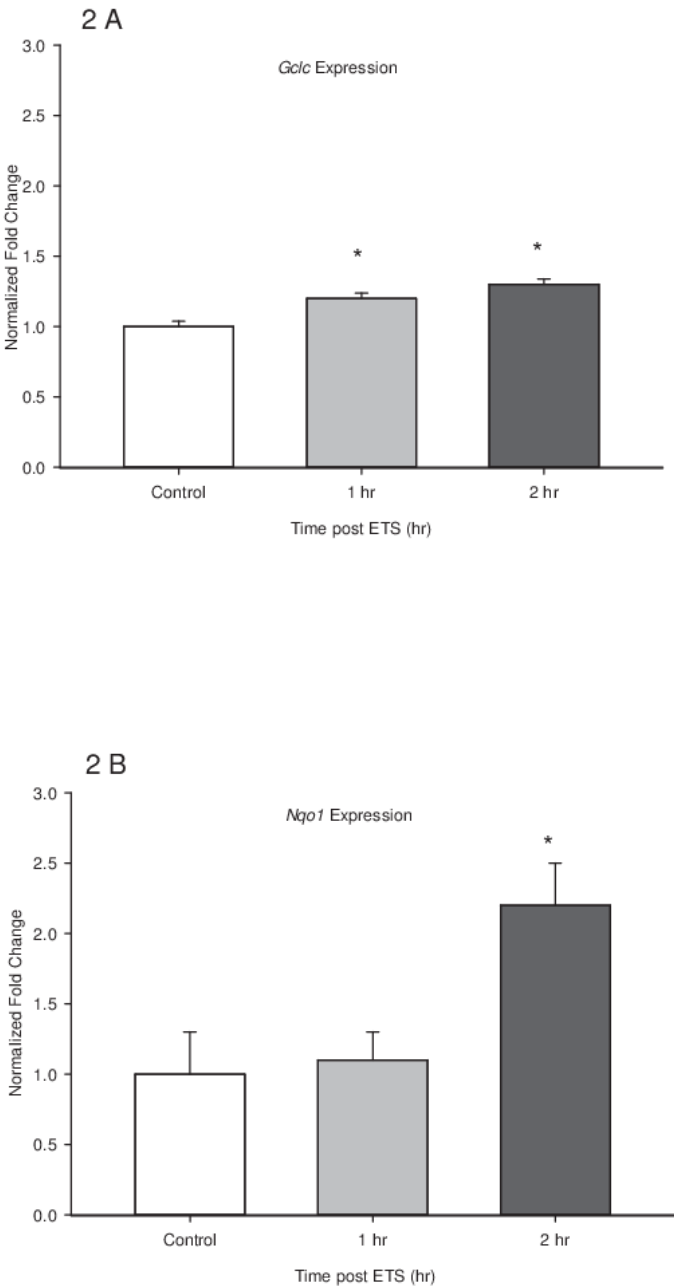


Figure 2.2. Time course of oxidant stress response gene expression in nasal respiratory/transitional mucosa following 10 minute exposure to 6 mg/m³ ETS. (A) *Gclc*, and (B) *Nqo1* expression data are presented as mean fold change \pm SE, and are normalized to control values (* indicates $p < 0.05$ compared to respective control, $n=6$ in all groups).

Figure 2.3.

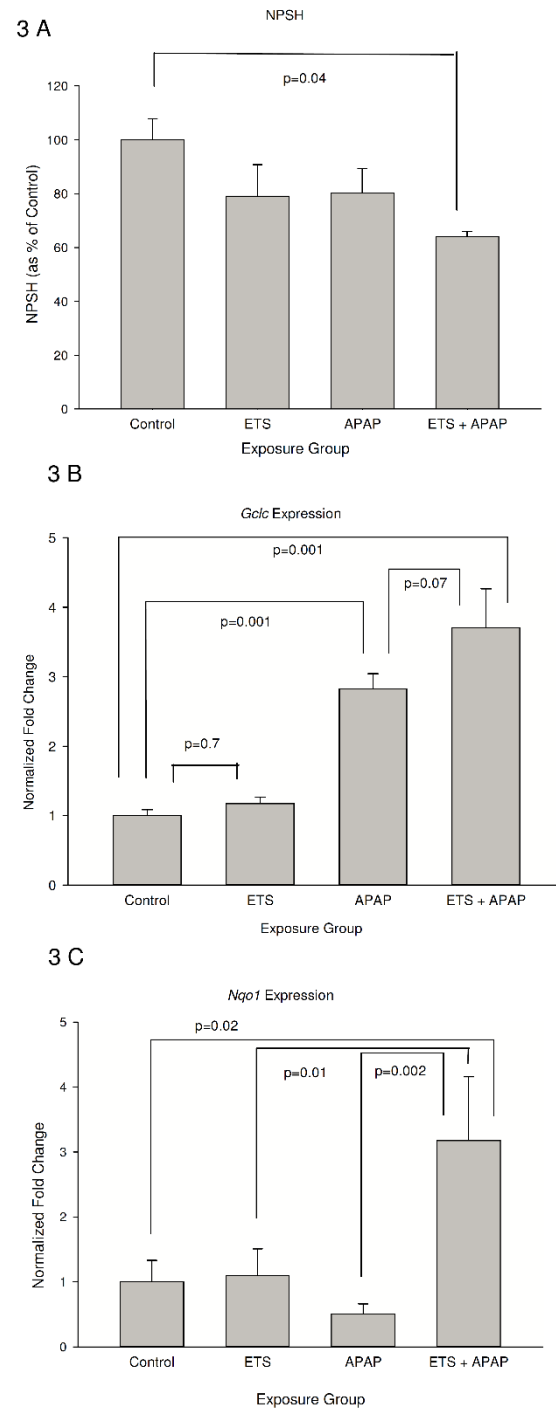


Figure 2.3. Effect of ETS (5 mg/m³), APAP, and the combination of ETS+APAP on NPSH depletion and oxidant stress response gene expression. (A) NPSH levels in nasal respiratory/transitional mucosa in controls and after APAP, ETS, or APAP+ETS. Data are presented as mean \pm SE and are normalized to total protein (*= p < 0.05, compared to respective control). Groups contained 5-7 mice. (B) *Gclc* and (C) *Nqo1* expression in nasal respiratory/transitional mucosa in controls and after APAP, ETS, or APAP+ETS. APAP was administered at a dose of 100 mg/kg ip; ETS exposures were of 10 minute duration at a total particulate concentration of 5 mg/m³. *Gclc* expression in the APAP group was significantly higher than control (p=0.001) *Nqo1* expression in the APAP groups did not differ from control levels (p=0.26). Data are presented as mean fold change \pm SE, and are normalized to control values (* = p<0.05, compared to respective control). Groups contained 4-6 mice.

Figure 2.4.

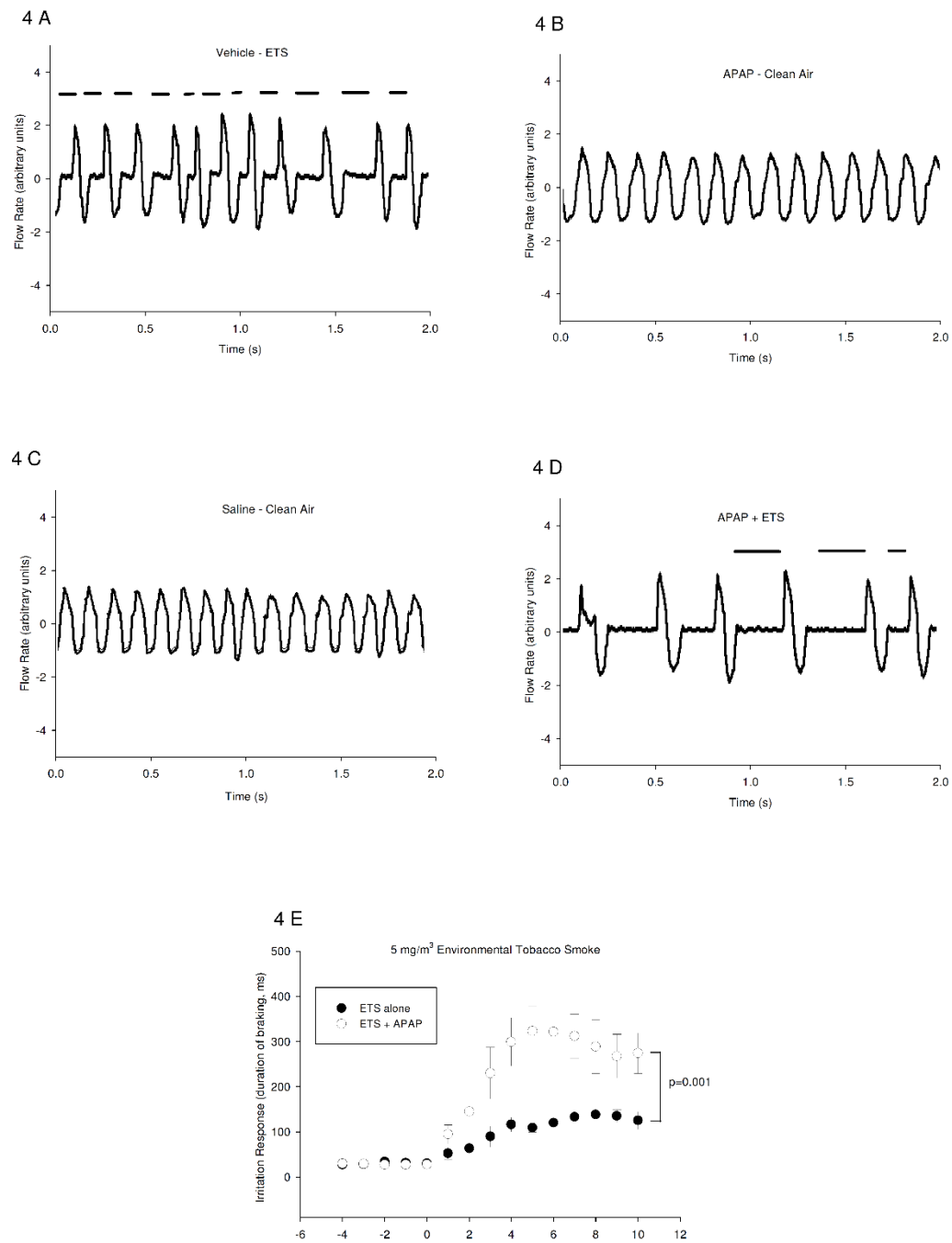


Figure 2.4. Representative breathing patterns of mice exposed to (A) APAP vehicle (saline) followed by 5 mg/m³ ETS, (B) 100 mg/kg APAP followed by clean air, (C) APAP vehicle (saline) followed by clean air, (C) 100 mg/kg APAP followed by ETS. The clean air or ETS exposure occurred 1 hr following vehicle or APAP injection. Plotted are representative respiratory air flow rate patterns (arbitrary units, expiration is up) during these exposures. No braking is observed in control exposures (C) or with APAP alone (B). Marked braking, indicated by the periods of zero flow (and depicted with the heavy bars) was observed in ETS and ETS+APAP exposed mice. (E) Time course of modulation of ETS-induced reflex irritation response by APAP. Data are presented as mean duration of braking (ms) \pm SE. Groups contained 5-10 mice (p values shown in figure)

Figure 2.5.

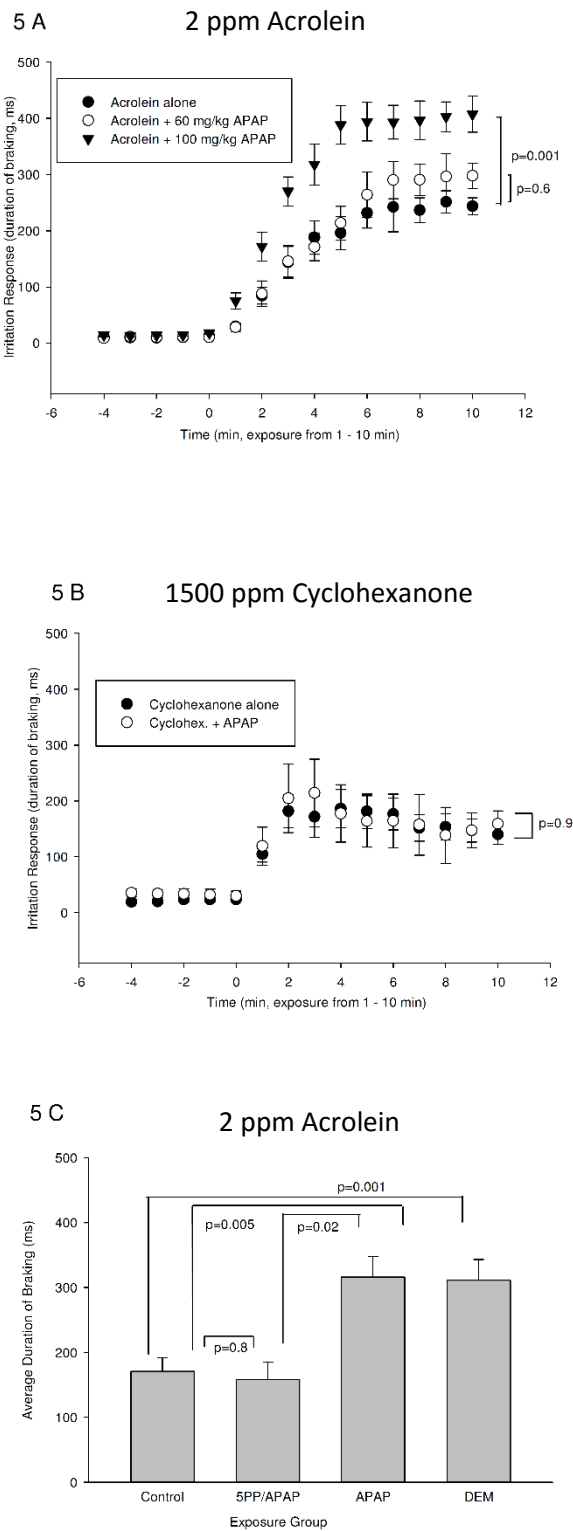
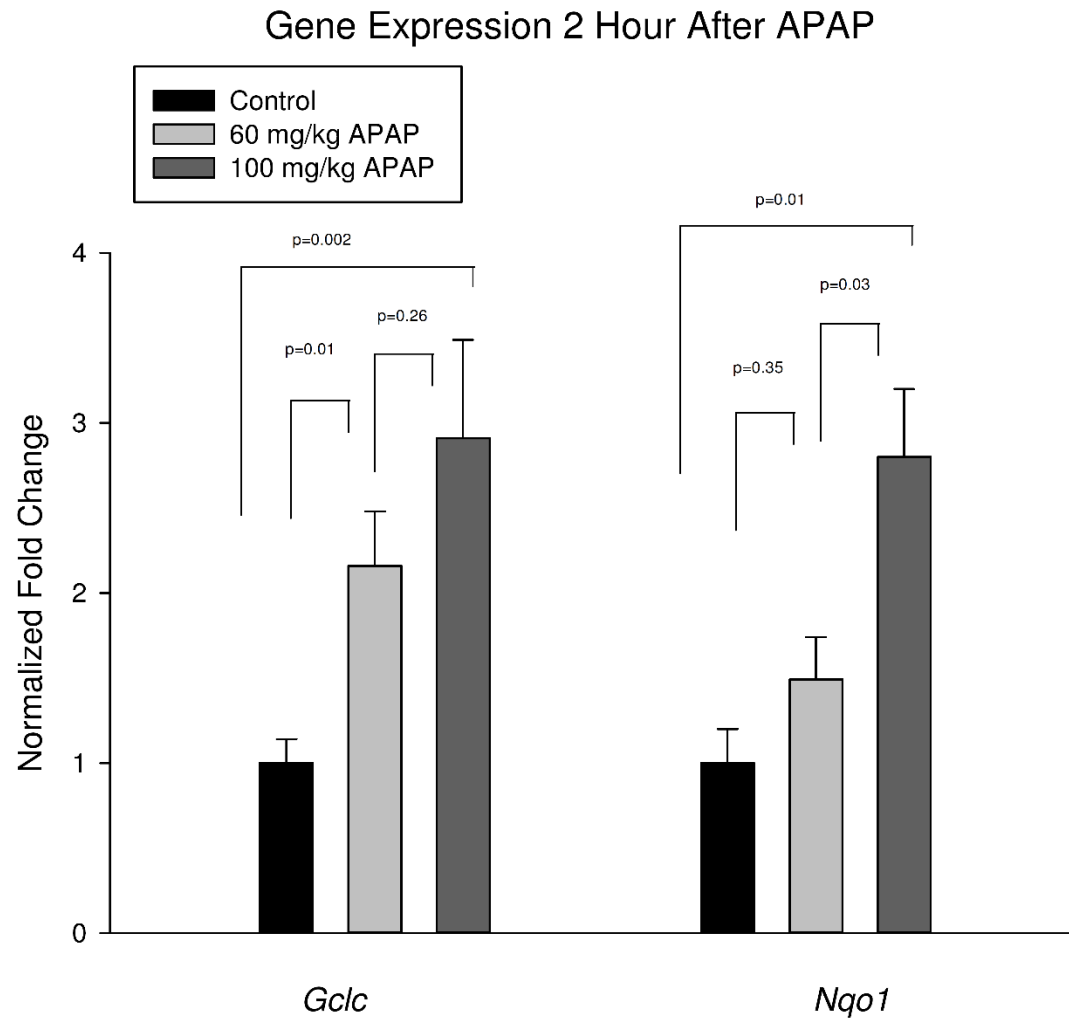


Figure 2.5. Nasal trigeminal chemosensory nerve reflex irritation responses to (A) 2.3 ± 0.4 ppm acrolein (mean \pm SD) following vehicle, 60 mg/kg, or 100 mg/kg APAP, and (B) 1590 ± 130 ppm cyclohexanone following vehicle or 100 mg/kg APAP administration. (C) Modulation of the nasal trigeminal chemosensory nerve irritant reflex response to 2.9 ± 0.15 ppm acrolein (control) (mean \pm SD) by: APAP, 5-PP pretreatment followed by APAP, or DEM. (A, B) Time courses include a 5 min clean air baseline (-5 to 0 min) followed by irritant exposure starting at time 0 (p values are shown in figures). Data are presented as mean duration of braking (ms) \pm SE. Groups contained 5-10 mice. (C) Data are presented as mean duration of braking (ms) \pm SE (p values are shown in figures). Groups contained 4-8 mice.

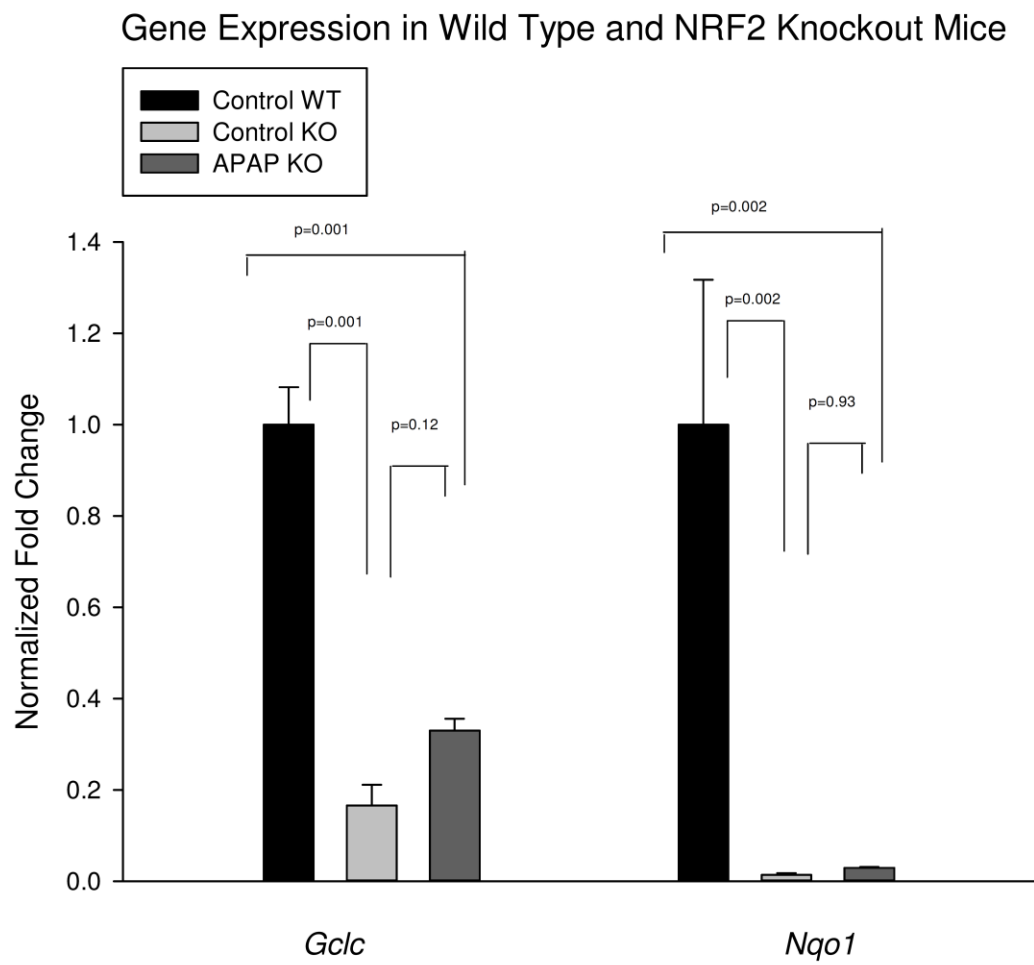
2.7 Supplemental Material

Supplemental Material, Figure S2.1.



Supplemental Material, Figure S2.1.: Dose response data for induction of *Gclc* and *Nqo1* by APAP. While both genes demonstrated apparent dose response relationships, *Gclc* was significantly induced at a dose of 60 mg/kg while *Nqo1* was not. Both genes were significantly induced at a dose of 100 mg/kg. Data are presented as mean fold change \pm SE, and are normalized to control values (p values are indicated in the figure). Groups contained 4-6 mice.

Supplemental Material, Figure S2.2.



Supplemental Material, Figure S2.2: Effect of APAP on *Gclc* and *Nqo1* expression in NRF2 wild type (WT) and knockout (KO) mice. Expression of both genes was significantly lower in knockout than wild type mice. Neither gene was significantly induced by APAP in the knockout strain. Data are presented as mean fold change \pm SE, and are normalized to control values (p values are indicated in the figure). Groups contained 4-6 mice.

Chapter 3: Acetaminophen attenuates house dust mite induced allergic airway disease in mice

3.1 Abstract

Epidemiological evidence suggests that APAP may play a role in the pathogenesis of asthma, likely through pro-oxidant mechanisms. However, no studies have investigated the direct effects of APAP on the development of allergic inflammation. In order to determine the likelihood of a causal relationship between APAP and asthma pathogenesis, we explored the effects of APAP on inflammatory responses in a murine HDM model of allergic airway disease. The HDM model consisted of once daily intranasal instillations for up to two weeks. APAP was administered 1 hour prior to HDM during either week 1 or 2. The effects of HDM exposure were substantially diminished in APAP treated groups compared to controls in this model as indicated by markedly reduced airway inflammation (as assessed by bronchoalvolar lavage), lower cytokine expression in lung tissue, and diminished histological evidence of perivascular and peribronchiolar immune cell infiltration. The anti-inflammatory effect of APAP was not abrogated by an inhibitor of cytochrome P450 metabolism, suggesting that the effect was due to the parent compound or a non-CYP450 generated metabolite. Taken together, our studies do not support the biological plausibility of the “APAP hypothesis” that APAP use may contribute to the causation of asthma.

3.2 Introduction

Asthma is a chronic respiratory tract disease characterized by airway inflammation, episodic airway obstruction, and airway hyperresponsiveness affecting more than 25 million people in the United States (Moorman *et al.*, 2012). The past three decades have witnessed a worldwide epidemic-scale increase in asthma prevalence (Eder *et al.*, 2006), the cause(s) of which are unknown. The increase in asthma prevalence occurred concomitantly with the expansion in acetaminophen (APAP, *n*-acetyl-*para*-aminophenol) use following identification, in the early 1980s, of the Reyes Syndrome risk from aspirin (Varner *et al.*, 1998). The association between APAP use and asthma has prompted the “APAP hypothesis,” which states that APAP contributes to the increase in asthma, likely through its pro-oxidant effects (McBride, 2011, Holgate, 2011).

Multiple epidemiological studies have found a strong association between APAP use and asthma prevalence in children and adults, several of which are included in a metaanalysis (Etminan *et al.*, 2009). The largest study featured, the International Study of Asthma and Allergies in Childhood, included >200,000 children across multiple asthma centers world-wide and found a dose-dependent increase in asthma risk with APAP use (Beasley *et al.*, 2011). A large prospective study, The Nurse’s Health Study also found a dose-dependent increase in the incidence of asthma with APAP use in adult women (Barr *et al.*, 2004). Additionally, prenatal acetaminophen use has been associated with childhood asthma (Shaheen *et al.*, 2010). However, due to inherent limitations of epidemiological data, a causal relationship has not been established. One such limitation is the possibility of confounding by indication; for example, APAP is often used to treat fever caused by infections, and certain respiratory tract infections are independent risk factors for asthma (Heintze and Petersen, 2013). Supporting this explanation are several studies that found that adjusting for concurrent respiratory infections diminished the association (Lowe *et al.*, 2010, Sordillo *et al.*,

2015). Another study found an association between APAP use and asthma symptoms in early life, but during a follow-up with subjects at 7 years of age the association was no longer present, suggesting that the association was real but temporary (Kreiner-Moller *et al.*, 2012).

Despite evidence supporting confounding biases which could explain the APAP-asthma association, uncertainty and the near ubiquitous use of APAP, make it worthy of further investigation. The most developed of several proposed mechanisms for the association is that oxidative stress caused by APAP plays a role. Oxidative stress is a hallmark of asthma and is thought to be important in asthma pathogenesis. Markers of oxidant levels are higher in people with asthma and polymorphisms in antioxidant genes such as glutathione transferase are asthma risk factors (Riedl and Nel, 2008, Holguin, 2013). However, asthma is an inflammatory disease and oxidative stress may be a result as well as a cause of active airway inflammation making difficult full definition of its role (Andreadis *et al.*, 2003, Bhalla *et al.*, 2009, Henricks and Nijkamp, 2001). Animal studies confirm the importance of oxidative stress in asthma, e.g. mice with reduced antioxidant defenses (nuclear factor erythroid 2-related factor 2 (Nrf2) knockout mice) demonstrate enhanced susceptibility to allergic airway disease (Rangasamy *et al.*, 2005, Williams *et al.*, 2008). At sufficient doses APAP causes severe liver toxicity primarily through oxidative stress caused by its reactive metabolite, N-acetyl-P-benzoquinone-imine (NAPQI) (McGill and Jaeschke, 2013). Hepatotoxic doses of APAP have been associated with extrahepatic GSH loss and tissue injury in the nose, and lung (Hart *et al.*, 1995, Gu *et al.*, 2005).

The effects of APAP at high doses in the liver and airways suggest that an oxidative stress based mechanism is plausible. However, limited information is available on the respiratory tract effects of APAP at non-hepatotoxic doses. Recent work in our laboratory focused on the potential for non-hepatotoxic doses of APAP to cause oxidative stress in the airways. At near-therapeutic doses,

APAP causes airway GSH loss, airway cellular oxidative stress response pathway induction, and potentiates acute airway responses to Environmental Tobacco Smoke (ETS) (Smith *et al.*, 2015). That APAP acts as a pro-oxidant in the respiratory tract suggests that it could promote the development of asthma similarly to other oxidant asthma risk factors such as ETS, ozone, and diesel exhaust (Gilmour *et al.*, 2006). Our previous studies focused on the acute pro-oxidant effects of APAP on the airways, and while they support a proposed mechanism for the association between APAP and asthma which may involve oxidative stress and the potentiation of the effects of ETS, they do not provide any direct evidence linking APAP treatment with the development of future allergic disease. Thus the overarching goal of these studies was to investigate the effects of APAP on allergic responses of the airways in a murine HDM model of allergic airway disease. We hypothesized that APAP administration would enhance the development of allergic airway disease caused by HDM, similar to the enhancement of HDM induced inflammation observed with other pro-oxidants such as ETS.

Interestingly, our initial studies indicated that APAP did not enhance the response to HDM antigen, rather the opposite effect was observed. APAP effectively blocked the response to HDM. The approach of the experiments featured in this work then became to extensively characterize and document the unexpected attenuation of the HDM response by APAP through measurements of airway inflammatory cell influx, cytokine mRNA profiles, HDM specific serum immunoglobulin, and airway histological presentation.

3.3 Results

HDM Dose-Response. Initial studies were aimed at defining the dose response relationships for HDM in order to delineate both a minimally and maximally effective dose. Based on previous studies 5 dose levels ranging from 0.625 μ g to 10 μ g were used (Bracken et al., 2015). Mice were administered HDM intranasally once daily for two weeks with a 2 day rest period in between weeks 1 and 2. Mice were euthanized on day 12, 24 hrs following the last dose of HDM (Figure 3.1A). HDM produced a dose-dependent increase in the number of airway inflammatory cells as measured by the presence of increased eosinophils, neutrophils, and lymphocytes in the BAL fluid (Figures 3.1 B-D). (The amounts of these cells in control mice groups were negligible, accounting for <0.5% of the cells present). Lung histopathology is described below. Macrophage numbers were significantly higher than control at HDM doses of 5 μ g or higher (Figure 3.1E). No consistent increase in BAL cellularity was observed with the 0.625 μ g dose. The 1.25 μ g dose was the lowest dose that caused a consistent, marked inflammatory response. The response to 10 μ g was no higher than that to 5 μ g suggesting these were maximally effective doses relative to inflammatory cell influx in this model.

APAP Attenuates the HDM-Inflammatory Response. To examine the effect of APAP on airway inflammation induced by minimally (1.25 μ g) and maximally (10 μ g) effective doses of HDM, mice were treated daily with APAP, or vehicle (control) one hour prior to HDM. APAP was administered at a non-hepatotoxic dose of 100 mg/kg based on our previous studies (Smith *et al.*, 2015) which indicated that at this dose and time (1 hr) APAP causes depletion of the antioxidant GSH throughout the respiratory tract and potentiates the effects of another oxidant, ETS. APAP was administered during either week 1 or week 2 of the HDM model and mice were euthanized on day 12 (Figure 3.2A). APAP was administered for no more than 5 days as this is the maximum

recommended duration of treatment for children (Tylenol® Package Insert 2015). As observed previously (see Figure 3.1), both the 1.25 and 10 µg doses of HDM resulted in significant influx of inflammatory cells in the BAL. The inflammatory cell response to the 1.25µg dose of HDM was virtually absent in animals treated with APAP (Figure 3.2B). The attenuation of the HDM response was less pronounced at the 10µg dose level, and was less consistent across all cell types, however, significantly fewer eosinophils were observed in the APAP treated animals at this HDM dose (Figure 3.2C). For both HDM dose groups, APAP was more effective at attenuating the inflammatory response when given in week 2 than week 1. No significant differences were observed between the control and APAP groups with respect to macrophage numbers (Figure 3.1D) HDM at both doses significantly elevated BAL supernatant protein levels, a response blocked by APAP treatment during week 2 (Figure 3.2D).

Time Course of APAP's Effect on HDM response. To determine the time course of the effects of APAP on the HDM response, we performed an experiment in which groups of mice treated with HDM, or APAP and HDM were euthanized on each day of the second week of the HDM model. The lungs of euthanized mice were lavaged and lung tissue samples were collected for determination of mRNA levels of selected genes by qRT-PCR. The genes selected for qRT-PCR analysis and the functional basis for their selection are provided in Table 3.1. The genes included the TH2 cytokines *IL-4*, *IL-5* and *IL-13*, the pro-inflammatory genes *Mip2* and *INos*, genes associated with allergic airway disease, *CaSR* and *Muc5AC*, and genes associated with APAP bioactivation and oxidative stress response, *Cyp2E1* and *Nqo1*.

HDM induced a progressive increase in lavage inflammatory cell numbers during week 2 of this model (Figure 3.3). The eosinophil, neutrophil, and lymphocyte cellular influx caused by HDM was absent in mice treated with APAP (Figure 3.3A-C). The macrophage influx was also attenuated by APAP (Figure 3.3D). An upward trend in BAL fluid protein concentration was observed during week 2 of HDM treatment that was absent in the APAP treated group (Figure 3.3E). The cell counts and BAL supernatant protein levels on day 12 repeat the results of our previous experiments (See Figure 3.2B-D). With respect to the time course of the inflammatory response, the attenuating effects of APAP were fully apparent by day 10 of the model, the first day in which increased inflammatory cells were observed. (Figures 3.3A, and C).

Based on the inflammatory cell influx response data, lung tissues from animals euthanized on days 8, 10 and 12 were analyzed for gene expression. These represent days prior to an inflammatory cell influx (day 8), the first day in which a consistent increased in inflammatory cells occurs (day 10) and the last day of the model (day 12). Several, but not all of the examined genes were significantly induced by HDM. Specifically, HDM resulted in 2-fold or more, statistically significant, induction of *Il-4*, *Il-5*, *Muc5AC*, and *Mip2* (Figure 3.4A-D). The effects of APAP on gene expression were apparent on day 12 of the models. Specifically, the expression of *Il-4* and *Mip2* was significantly lower in groups treated with APAP. The *Il-5* and *Muc5AC* responses were also attenuated by APAP but the effect did not attain statistical significance ($p=0.08$ and $p=0.053$, respectively). Notably, APAP was without effect on expression of any of these genes on day 10. The genes *Il-13*, *CaSR*, *INos*, *Nqo1* (Supplemental Figure S3.1), and *Cyp2E1* (Figure 3.4E) were not significantly elevated over control levels by HDM and the degree of change was less than 1.5-fold in all cases. APAP did not alter the expression of these genes (Supplemental Figure S3.1).

Interestingly, the expression of *Cyp2E1* was significantly lower than control in mice treated with HDM on day 12 of the model, a response prevented by APAP (Figure 3.4E).

Effect of CYP450 inhibition. To determine whether the attenuation of the HDM induced inflammatory response was due to the parent APAP molecule or a CYP450 metabolite, we performed a separate experiment in which mice were treated with the CYP450 inhibitor, 5-phenyl-1-pentyne (5PP). Were a CYP450 metabolite involved it would be anticipated that the attenuating effects of APAP would be absent in the 5PP treated mice. 5PP was administered 1 hr prior to APAP for a maximum of two days (on day 8, and 9) based on our previous studies (Morris, 2013, Smith *et al.*, 2015) in which it was shown that 5PP at this dosage blocked the airway oxidant response to APAP. In the current study, mice were euthanized on day 10 as this is the first day of the model in which the attenuating effects of APAP on inflammation were observed (See Figure 3.3A-C). The effect of APAP is apparent in the 5PP treated groups with respect to decreased eosinophils and neutrophils. For neither cell type was the response different between the APAP and 5-PP APAP treated groups. 5PP alone may have caused a slight decrease in inflammation (Figure 3.5A, and B). This decrease was not statistically significant. In both the APAP and 5PP-APAP treated groups there appears to be decreased lymphocytes, although no statistically significant differences were found (Figure 3.5C). Fewer macrophages were observed in the APAP treated group, a response which was not different from the 5PP-APAP group (Figure 3.5D).

Histological Effects. To confirm the results of previous endpoints, a qualitative histological assessment of lungs from HDM exposed mice with or without APAP treatment, and controls was performed. No inflammation was apparent in vehicle- or APAP-treated control mice. A focal and moderate degree of lung inflammation was present in HDM exposed mice (Figure 3.6, Panel A). Macrophages, and polymorphonuclear cells were observed in perivascular and peribronchiolar

clusters distributed throughout the majority of the lung lobes. Fewer clusters of inflammatory cells were observed in APAP treated groups, and some lobes did not have any indication of inflammation. Both HDM, and APAP+HDM groups had increased small airway PAS staining indicative of enhanced mucus production (Figure 3.6, Panel B). The degree of staining was similar between the two groups although appeared to be somewhat reduced in the APAP+ HDM groups. No evidence of increased PAS staining was observed in the control groups.

Effect of APAP on HDM Specific Serum Immunoglobulin. HDM-specific IgE and IgG1 was not detectable in sera of mice on day 12 of the model in any treatment group (control, APAP, HDM or HDM+APAP) (Data not shown).

3.4 Discussion

The epidemiological evidence suggesting that APAP use may influence the pathogenesis of asthma is controversial; however, the effects of APAP on the development of asthma have not been rigorously examined in an animal model to assess the biological plausibility of the “APAP hypothesis.” Therefore, the objective of this study was to evaluate the effects of APAP treatment on inflammatory responses in a murine HDM model of allergic airway disease. A HDM model of allergic airway disease was chosen for its environmental and human relevance as allergies to HDM are highly prevalent in humans, especially among those with asthma (Boulet *et al.*, 1997, Calderon *et al.*, 2014) . The response to HDM in mice exhibits several of the principle features of allergic disease in humans, and importantly HDM produces a respiratory allergic response in mice that has been extensively characterized (Cates *et al.*, 2004, Cates *et al.*, 2007, Johnson *et al.*, 2004). It is important to note however, that the HDM model is a model of acute allergic airway disease and

not chronic asthma. Immunological tolerance in HDM models has been shown to occur after long-term exposure (Bracken *et al.*, 2015). Therefore, our studies were limited to 2 weeks in duration and designed to examine changes in the early response to both minimal and maximal doses of HDM. Our HDM model produced a robust and dose dependent increase in inflammation as measured by increased airway eosinophilia, and neutrophilia similar to that observed by other investigators using HDM models (Bracken *et al.*, 2015, Cates *et al.*, 2004). Previous studies observed a maximal response at a 25 ug dose (Cates *et al.*, 2004). The response to HDM in our model peaked at a 10 µg dose, likely influenced by a difference in the day on which BAL was performed (day 12 in our model, compared to day 15 in the Cates *et al.*, 2004 study), and perhaps differences in HDM extract composition (LPS, and protein levels).

Mouse models of asthma have been defined as having two main phases, an early allergic sensitization phase, followed by a challenge phase (Maes *et al.*, 2010). The timing of co-exposures to other pro-oxidants such as ETS (e.g. during sensitization vs. challenge) has been shown to produce pleiotropic disease outcomes in asthma models (Botelho *et al.*, 2011, Lanckacker *et al.*, 2013, Maes *et al.*, 2010). Therefore, in our experiments investigating the effects of APAP on the response to HDM, we gave APAP during either the first or the second week of the HDM model exposure to account for the possibility of differential effects during earlier vs later phases of the model. The 100 mg/kg dose used in this study causes significant GSH loss throughout the respiratory tract at 1 hr, but elicits peak blood APAP levels of 35 µg/ml, which are only slightly higher than therapeutic levels (5-20 µg/ml) (Smith *et al.*, 2015).

We hypothesized that APAP would enhance the response to HDM based on our previous studies that indicated that APAP was an airway pro-oxidant and potentiated the effects of another airway oxidant, ETS, following acute exposure (Smith *et al.*, 2015). Unexpectedly, APAP caused a

dramatic attenuation of HDM induced allergic airway disease. APAP was more effective at the 1.25µg HDM dose level, than the 10µg HDM dose level suggesting that the attenuation might be overwhelmed at higher antigen burdens. APAP was more effective during week 2 of the HDM model despite the fact that mice had been previously exposed to antigen for a week. The inflammatory endpoints we measured, airway eosinophilia and neutrophilia in particular, are the result of differentially regulated processes (Lambrecht and Hammad, 2015). Therefore, the lack of an increase in any of the cell types, or in BAL protein, after APAP treatment is highly suggestive of a general anti-inflammatory effect. This is unexpected because APAP is generally considered to be a weak anti-inflammatory drug (Graham *et al.*, 2013). For example, APAP is not effective in treating severe inflammation such as with rheumatoid arthritis, but can be effective at blocking lower grade inflammation such as that caused by dental procedures. Furthermore, APAP blocked the attenuating effect of HDM on *Cyp2E1* expression, a gene that is not directly related to the HDM inflammatory response.

Lungs from mice exposed to HDM had mild to moderate peribronchiolar and perivascular inflammation upon histological examination. That clusters of inflammatory cells were absent or less apparent in APAP treated mice, confirms the decreased inflammatory cell presence in the BAL fluid of this group. As expected, the degree of inflammation observed in this study was less than that observed in our previous studies which used a higher dose of HDM (Bracken *et al.*, 2015). However, a similar perivascular and peribronchiolar clustering pattern was observed in the previous studies. Neither HDM-specific IgG1 nor IgE was detected in sera of HDM treated mice. Bracken reports low levels of these antibodies on day 15 of their model that used 25 ug intranasal instillation of HDM (5 days per week). The lack of IgG1 and IgE in our model is, perhaps, not

surprising considering the shorter time period (12 vs 15 days) and lower HDM dosage (10 vs 25 ug).

We measured a battery of cytokines chosen to encompass several different allergic, inflammatory, and metabolic processes. Initially, this gene expression analysis was intended to determine specific gene expression patterns caused by both HDM and the combination of HDM and APAP treatment. *Il-4*, *Il-5*, and *Il-13* were chosen as they are primary effector cytokines of the Th2-type allergic response (Lambrecht and Hammad, 2015). Associated with both allergic and non-allergic inflammatory responses; *Muc5AC*, *MIP2*, and *INOS* are effectors of increased mucus glycoprotein production, neutrophil influx, and airway and vascular smooth muscle tone, respectively (Coleman, 2002, Evans *et al.*, 2015, Hamid *et al.*, 1993, Wolpe *et al.*, 1989). *Cyp2E1* is one of the primary cytochrome P450 enzymes responsible for the biotransformation of APAP into NAPQI, while *Nqo1* is involved in the detoxification of NAPQI (Hinson *et al.*, 2010, Moffit *et al.*, 2007). Importantly, APAP appears to have attenuated the increase in all genes that were increased by HDM. This was indicated by statistically significant decreases in *Il-4*, and *MIP2*, and slight decreases in *Il-5* (p=0.08) and *Muc5AC* (p=0.053) on day 12. The slight decrease in *Muc5AC* gene expression did not translate to a marked difference in apparent mucus production in the qualitative histological assessment. This could be due to the timing of *Muc5AC* induction relative to tissue collection or the influence of mucin proteins such as *Muc5b* which were not examined. No other clear changes were observed except a decrease in *Cyp2E1* with HDM, which was reversed by APAP on day 12.

A recent study has shown that *CaSR* may be a gatekeeper of airway hyperresponsiveness and inflammation in allergic asthma (Yarova *et al.*, 2015). Yarova *et al.* observed that *CaSR* mRNA and protein expression were elevated in asthmatic humans, and mice. Additionally, treatment with

CaSR antagonists effectively blocked the characteristic features of asthma in their mixed allergen (combination of OVA, and extracts of HDM, *Alternaria*, and *Aspergillus*) model. We did not observe any changes in *CaSR* expression with HDM exposure in our model despite a marked inflammatory response by other measures. Additionally APAP markedly attenuated the response to HDM without producing any alteration in *CaSR* expression. These results suggest that CaSR may not be the key to inflammation in all models of asthma, and may not be critical to HDM allergen sensitization.

The mechanism of APAP's effect on the response to HDM is unknown. That 5PP did not block the effect of APAP suggests that the effect is not due to a CYP metabolite, but more likely the parent compound or a metabolite not generated by CYPs. This explains the apparent discordance between the results of the current study and our previous studies in which the acute pro-oxidant effects of APAP was blocked by 5PP (Smith *et al.*, 2015). In this study, the acute pro-oxidant effects did not translate to an increase in inflammation by HDM over a longer period of time. It is possible that repeat dosing has an anti-oxidant effect in the airways through upregulation of NRF2 (O'Connor *et al.*, 2014). However, this is not likely the cause of the decreased inflammation seen here, as the NRF2 mediated gene *Nqo1* was not induced throughout the time course. Highlighted by our previous study demonstrating an acute pro-oxidant interaction between APAP and ETS, is that rather than promoting asthma pathogenesis directly, APAP may predispose individuals to other pro-oxidant asthma causative factors such as ETS (Smith *et al.*, 2015). The potential for APAP to potentiate the effects of other airborne oxidants such as ETS was not investigated here, and therefore remains an important consideration. Perhaps in concert with these agents, the effects that we have observed in the current study are overwhelmed.

That there were no differences in cytokine expression, or BAL protein levels between the HDM and APAP+HDM groups on day 10, a time at which significant changes in BAL cellularity were observed indicating the changes in gene expression lagged the changes in cellularity. This suggests that the cytokine expression following APAP treatment is most likely an effect of the decreased inflammation by APAP rather than a cause of the decreased inflammation. A possible mechanism, consistent with the lack of an effect on day 10, is that APAP could be influencing the trafficking of immune cells. Decreased immune cell migration could be caused by activation of the cannabinoid receptor system by APAP; either by diverting metabolism of arachidonic acid to endocannabinoids through inhibition of PG synthesis, or through a direct effect of its recently discovered metabolite and anandamide analogue, AM404 (Graham *et al.*, 2013). Cannabinoid receptor activity has been shown to decrease the recruitment of immune cells (Lunn *et al.*, 2006, Rieder *et al.*, 2010). Activation of the cannabinoid system also enhances immune cell apoptosis in mice (Lunn *et al.*, 2006, Rieder *et al.*, 2010). This effect may also be involved in the attenuation of the HDM model, however inflammatory cell numbers were never increased in the model suggesting that an effect on cell trafficking into the lungs is more likely than an enhanced apoptosis of cells within the lungs. Further studies are necessary to examine these possibilities.

In conclusion, we provide direct biological data, which do not support the APAP hypothesis in asthma. In agreement with recent epidemiological studies that suggest the association between APAP use and asthma can be explained by confounding factors, we show that APAP does not potentiate allergic airway disease in our model. In fact, the response to HDM was virtually absent in APAP treated mice. More studies will be needed to confirm these results in other models of airway inflammation and asthma, and to determine the mechanism of the observed effects. The

mechanistic insights provided by the modulation of allergic airway disease by APAP may provide novel information on the pathogenesis of asthma, and potential novel therapeutic targets.

3.5 Methods

Mice and dosing. All animal protocols received prior approval by the University of Connecticut Institutional Animal Care and Use Committee (A12-013). Experiments were performed on 9 to 12 week old female C57Bl/6J mice obtained from The Jackson Laboratory. Mice were 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). Food (Lab Diet; PMI Nutrition International) and tap water were provided *ad libitum*.

All chemicals were obtained from Sigma Aldrich, unless otherwise indicated, and were of USP grade, or better. APAP dissolved in 37°C saline (10 mg/ml) was administered via i.p. injection. The cytochrome P450 inhibitor 5-phenyl-1-pentyne (5PP) (GFS chemicals) was given ip at a dose of 100 mg/kg (10 mg/ml in olive oil) 1 hr prior to APAP treatment (Morris, 2013). For euthanasia, mice were anesthetized by urethane injection (1.3 g/kg i.p.) followed by exsanguination through the abdominal aorta or cardiac puncture.

Murine Model of HDM Antigen-induced Allergic Airway Disease. Based on the exposure protocol of Bracken et al. (2015), equal parts *Dermatophagoides pteronyssinus* (*Der. p.*) and *Dermatophagoides farinae* (*Der. f.*) lyophilized HDM extracts (Greer Laboratories) were resuspended in phosphate-buffered saline (PBS) at concentrations from 12.5 to 200 ng dry weight/μL. The lyophilized HDM extract mixture contained 3,131 endotoxin units/mg of HDM,

and 3.32 % *Der. p./Der. f.*, and the same lot number was used throughout the experiments. The HDM suspension was delivered in the morning between 9:00 and 12:00AM by intranasal instillation in a single 50 μ L volume while the mice were lightly anesthetized with isoflurane (2.5%).

Bronchoalveolar Lavage and Tissue Collection. Following euthanasia, lungs were lavaged *in situ* with PBS (3 x 1 mL lavages). BAL fluid cells were pelleted at 2000 x g for 5 minutes, and lavage supernatant was collected and frozen at -20°C for total protein determination. Total protein was measured by the Lowry method (Lowry *et al.*, 1951). Cell pellets were resuspended in PBS containing 2% bovine serum albumin (BSA). Total cell counts were obtained with a hemocytometer and Turk's solution. Slides of BAL cells were prepared by cytocentrifugation and stained with the Protocol Hema 3 system (Fisher Healthcare). Differential white blood cell counts were performed on a minimum of 200 cells. No eosinophils, neutrophils, or lymphocytes were observed in the vehicle control and APAP groups that received HDM vehicle instillations. Lungs collected for qRT-PCR analysis were inflated with and stored in aqueous RNA stabilization buffer containing saturating ammonium sulfate, 20 mM EDTA, 25mM sodium citrate, at pH 5.2. When collected, blood was obtained via cardiac puncture from anesthetized mice and spun at 1000 x g for 10 minutes to obtain serum.

Quantitative Real Time-PCR. Total RNA was isolated from mouse lung tissue homogenates using an RNeasy kit from Qiagen. 1 μ g of total RNA was used for first strand cDNA synthesis by an iScript cDNA synthesis kit (Bio-rad). RT-PCR was performed using SYBR Green as an indicator with an Applied Biosystems 7500 Fast Real-Time PCR System. PCR reactions contained 10 ng of cDNA (4 μ l), 500 nM of each primer (1 μ l total), and 5 μ l of 2x SYBR Green PCR Master mix (10 μ l total volume). The PCR was carried out according to the manufacturer's recommended

thermal cycling protocol. β -Actin was used as the internal reference control mRNA. Results are represented as the fold change in expression of target genes over control calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primer sequences (Table 3.2.) were designed with the Life Technologies OligoPerfect™ designer, or obtained from Harvard Primer Bank (Harvard Medical School), and synthesized by Invitrogen (Life Technologies).

Serum Immunoglobulin Measurement. Detection of serum HDM specific IgE and IgG was performed as described previously (Bracken *et al.*, 2015). Briefly, Nunc MaxiSorp, flat-bottom plates (Thermo Scientific) were coated with 10, or 2 μ g/ml of HDM extract in sodium bicarbonate buffer (pH 9.5), for IgE or IgG respectively. Blocking was performed with BD OptEIA assay diluent (BD Biosciences). Serum samples were added for the IgE assay in 2-fold serial dilutions (1/20-1/2,560) and for the IgG assay in 10-fold serial dilutions (1/20-1/200,000,000). For antibodies, biotin-SP-conjugated goat anti-mouse IgE (Southern Biotech) and biotin-SP-conjugated goat anti-mouse IgG Fc γ subclass 1-specific (Jackson ImmunoResearch) were used followed by streptavidin-HRP (BD Biosciences). Development was performed with a BD OptEIA TMB substrate reagent set (BD Biosciences), and reaction were quenched with 1M phosphoric acid. Absorbance measurements at 450 and 570 nm were made with a Tecan Safire 2 microplate reader.

Histology. Following euthanasia, lungs not subject to lavage were removed, inflated and stored in 4% buffered formalin. Formalin fixed lungs were then processed by the Connecticut Veterinary Medical Diagnostic Center using standard techniques. Briefly, paraffin embedded lung sections were stained with hematoxylin and eosin (H&E) and periodic acid-schiff (PAS). Lung sections were examined qualitatively via light microscopy and representative images of each treatment group were taken.

Statistical Analysis. Data are reported as mean \pm SE unless otherwise indicated in the text. Data were analyzed with Graphpad Prism software (Graphpad Software, Inc.). Statistically outlying data points were excluded *a priori* if they were greater than 3x the SD of the group. Data were compared by one-way ANOVA followed by the Newman-Keuls post-hoc test for multiple comparisons. When appropriate data were log transformed to correct for heteroscedasticity. A p-value less than 0.05 was considered statistically significant.

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3.7 Tables and Figures

Table 3.1. Mouse gene names and functions for qRT-PCR analysis.

| <i>Gene</i> | <i>Name</i> | <i>Function</i> | <i>Reference</i> |
|----------------------|--|--|-------------------------------|
| <i>Il-4</i> | Interleukin 4 | Th2 type cytokine response | (Lambrecht and Hammad, 2015) |
| <i>Il-5</i> | Interleukin 5 | Th2 type cytokine response | (Lambrecht and Hammad, 2015) |
| <i>Il-13</i> | Interleukin 13 | Th2 type cytokine response | (Lambrecht and Hammad, 2015) |
| <i>CaSR</i> | Calcium sensing receptor | Airway inflammation, hyperresponsiveness | (Yarova <i>et al.</i> , 2015) |
| <i>Muc5AC</i> | Mucin 5AC | Airway mucus gel formation | (Evans <i>et al.</i> , 2015) |
| <i>Mip2</i> | Macrophage inflammatory protein 2 | Neutrophil chemotactic factor | (Wolpe <i>et al.</i> , 1989) |
| <i>INos</i> | Inducible nitric oxide synthase | Mast cell activation, smooth muscle tone | (Coleman, 2002) |
| <i>Cyp2E1</i> | Cytochrome P450, family 2, subfamily E | APAP metabolism to NAPQI | (Hinson <i>et al.</i> , 2010) |
| <i>Nqo1</i> | NAD(P)H dehydrogenase, quinone 1 | Detoxification of NAPQI | (Moffit <i>et al.</i> , 2007) |

Table 3.2. Mouse primer sequences for qRT-PCR (listed 5'-3')

| <i>Gene</i> | <i>Forward</i> | <i>Reverse</i> |
|----------------|-------------------------|------------------------|
| <i>β-Actin</i> | GCAACGAGCGGTTCG | CCCAAGAAGGAAGGCTGGA |
| <i>Il-4</i> | CGAGCTCACTCTCTGTGGTG | TGAACGAGGTCACAGGAGAA |
| <i>Il-5</i> | CTCTGTTGACAAGCAATGAGACG | TCTTCAGTATGTCTAGCCCCTG |
| <i>Il-13</i> | CCTGGCTCTTGCTTGCCTT | GGTCTTGTGTGATGTTGCTCA |
| <i>CaSR</i> | AGCAGGTGACCTTCGATGAGT | ACTTCCTTGAACACAATGGAGC |
| <i>Muc5AC</i> | CTGTGACATTATCCCATAAGCCC | AAGGGGTATAGCTGGCCTGA |
| <i>Mip2</i> | CCAACCACCAGGCTACAGG | GCGTCACACTCAAGCTCTG |
| <i>INos</i> | TGAAGAAAACCCCTTGTGCT | TTCTGTGCTGTCCCAGTGAG |
| <i>Cyp2E1</i> | GGGACATTCCTGTGTTCCAG | CTTAGGGAAAACCTCCGCAC |
| <i>Nqo1</i> | TTTAGGGTCGTCTTGGCAAC | GTCTTCTCTGAATGGGCCAG |

Figure 3.1: HDM Dose Response

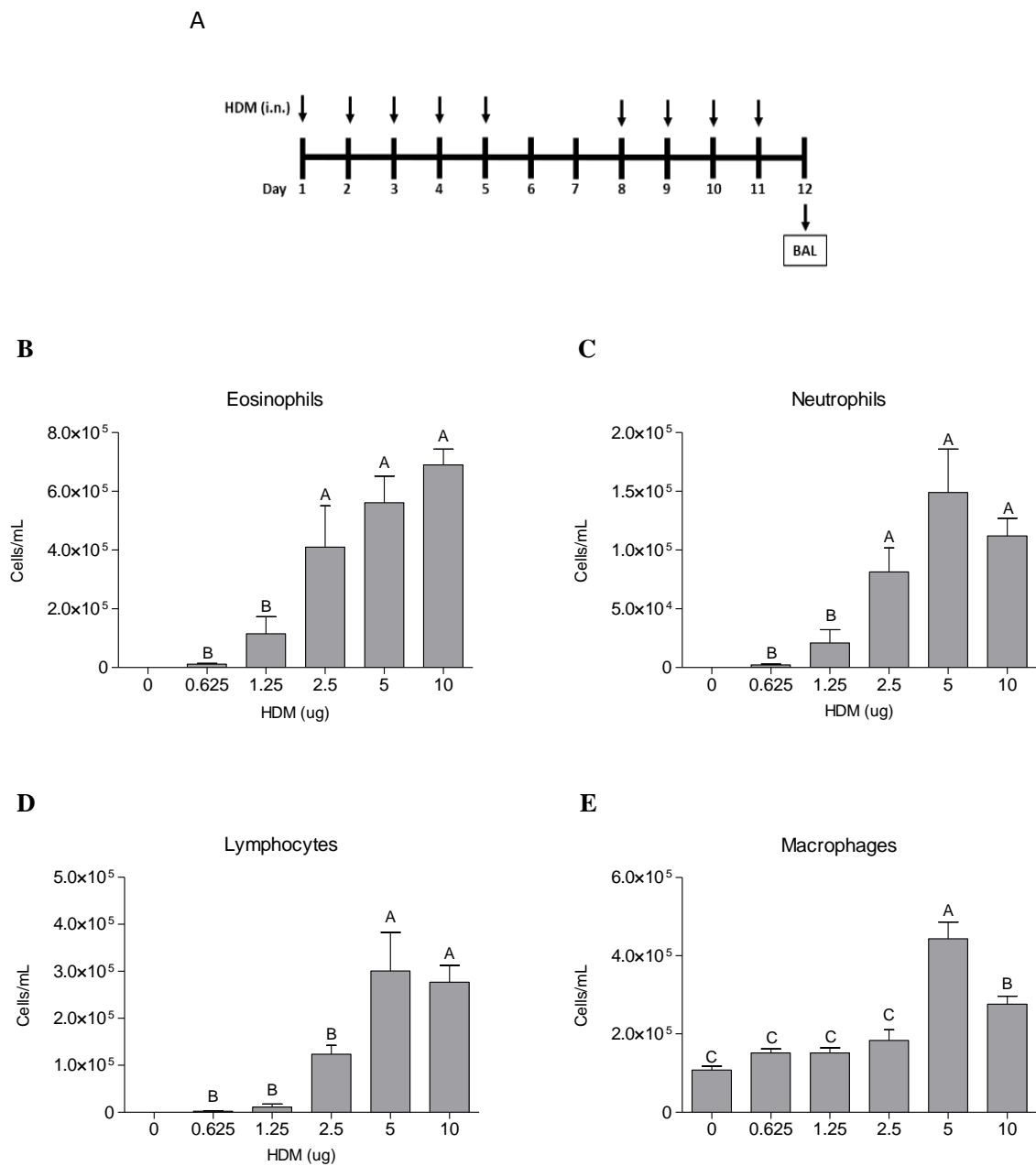


Figure 3.1. HDM produces a dose-dependent increase in BAL cellularity. (A) HDM model timeline. For the two-week HDM exposure model C57Bl/6J mice were anesthetized and administered from 0 to 10µg HDM extract in 50µl PBS i.n. for up to two weeks on days indicated with black arrows above the timeline. Mice were euthanized and BAL was performed on day 12. (B) Eosinophils, (C) neutrophils, (D) lymphocytes, and (E) macrophages. BAL cell data are presented as mean cells/ml \pm SE (n=6 mice/group). Data were analyzed by ANOVA followed by Newman-Keuls test; bars with differing superscripts differ from each other at the $p<0.05$ level.

Figure 3.2: Effect of APAP on HDM Response

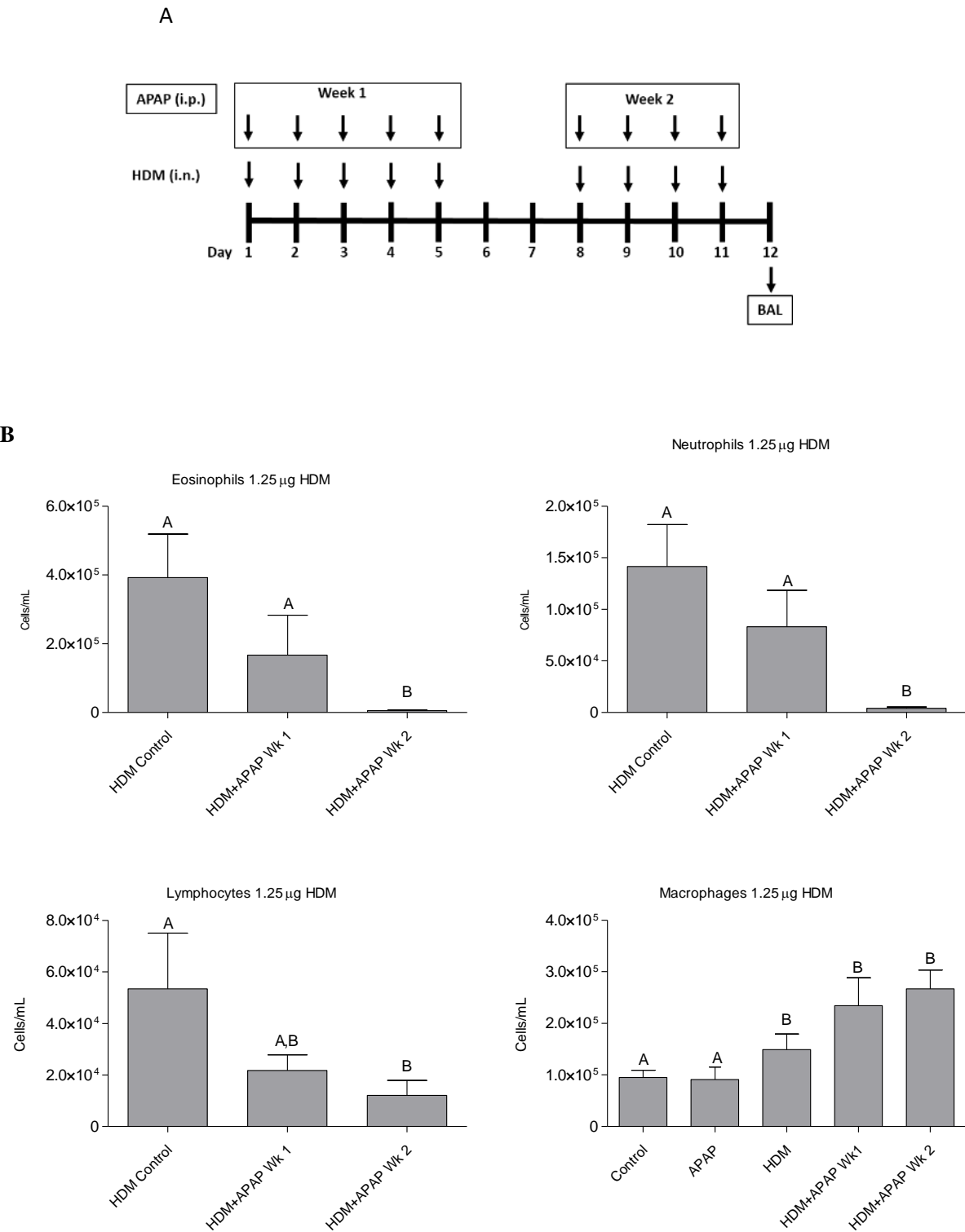
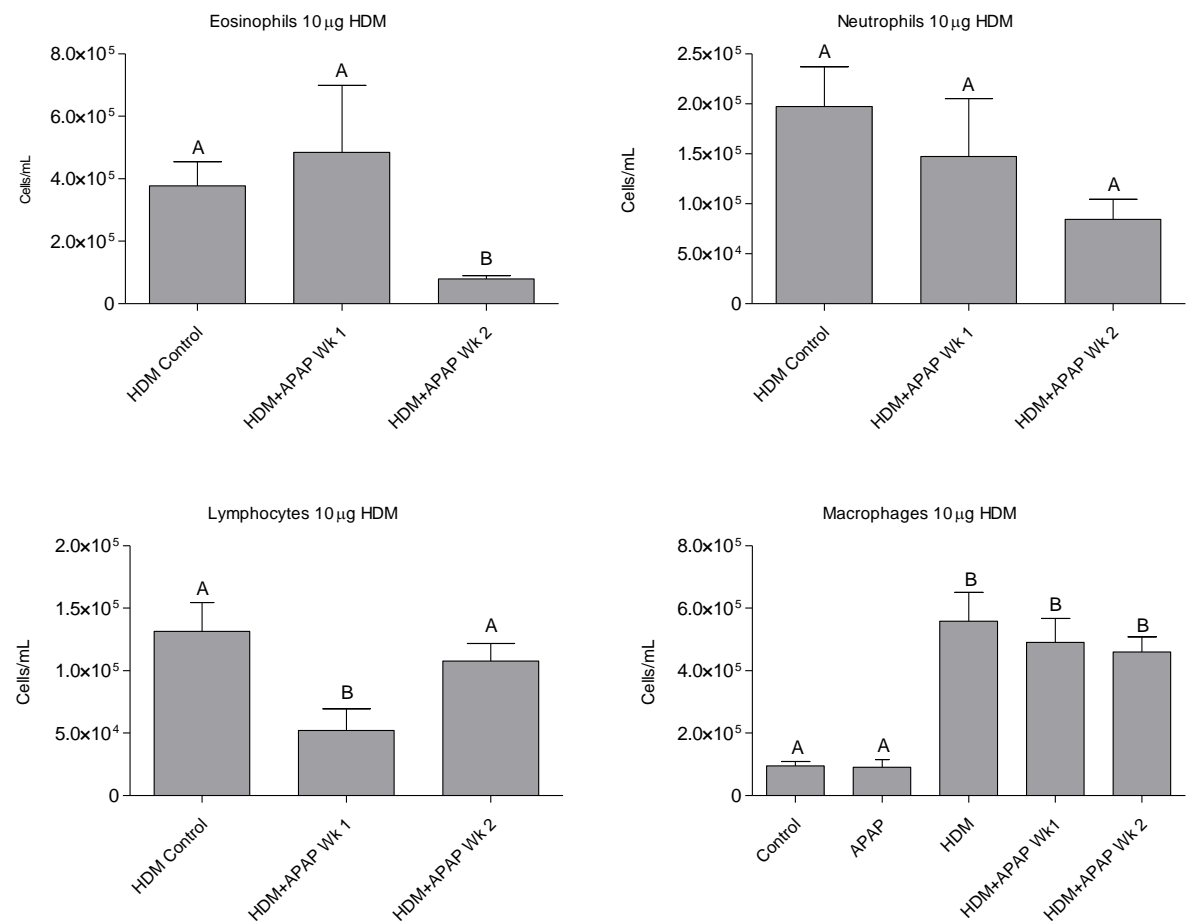


Figure 3.2 continued: Effect of APAP on HDM Response

C



D

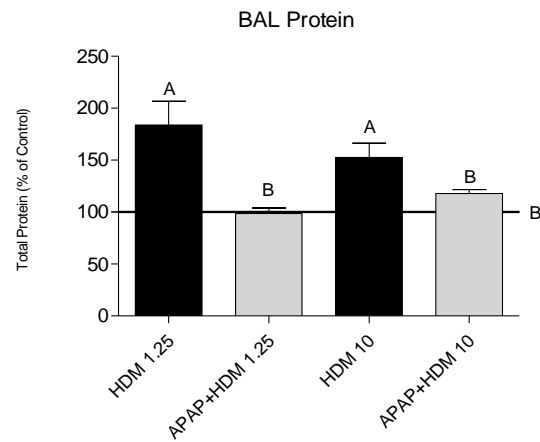


Figure 3.2. APAP attenuates the inflammatory response to HDM. (A) HDM model timeline with APAP administration either for 5 days during week 1, or 4 days during week 2. Mice were administered either a 1.25 or 10 μ g dose of HDM in either of the weeks of the 2 week protocol. Mice were euthanized and BAL was performed on day 12. Black arrows above the timeline represent days mice received doses of HDM and or APAP. BAL cells of mice administered either vehicle control (HDM Control), APAP during week 1 (HDM+APAP Wk1), or APAP during week 2 (HDM+APAP Wk2), and a (B) 1.25 μ g or (C) 10 μ g dose of HDM (i.n.). Differential cell count data are presented as mean cells/ml \pm SE (n=5 mice/group). (D) Protein levels represented as mean percent of control \pm SE in BAL fluid supernatants (n=5 mice/group). The black line at y=100% represents the mean of the pooled control values (n=10 mice/group). Data were analyzed by ANOVA followed by Newman-Keuls test; line and bars with differing superscripts differ from each other at the p<0.05 level. All data are representative of two independent experiments.

Figure 3.3: Bronchoalveolar Lavage Time Course

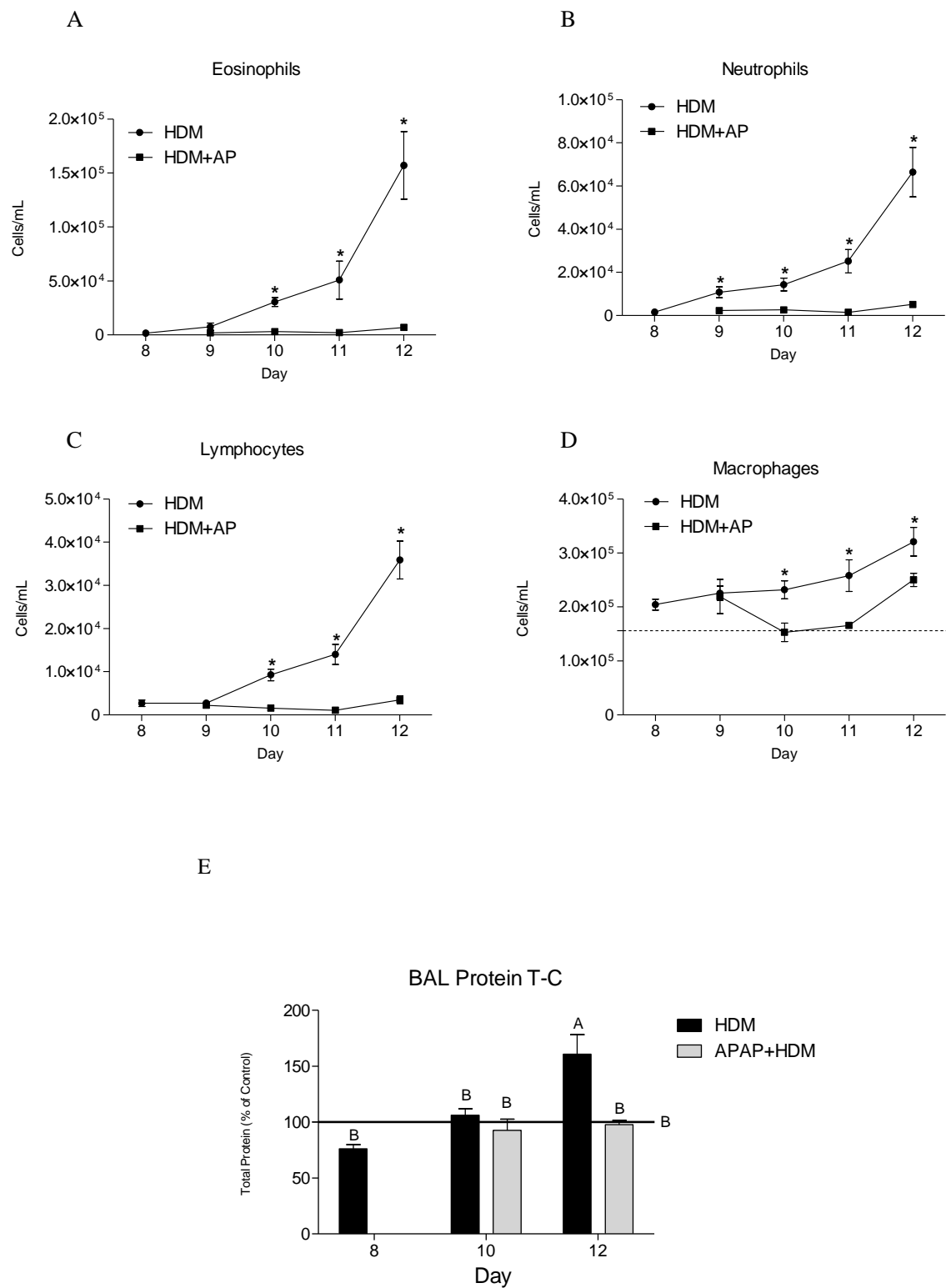


Figure 3.3. Time course of the effect of APAP on the HDM induced inflammatory response. Mice were administered APAP during week 2 (days 8-11), along with a 1.25 μ g dose of HDM following the same dosing regimen as in Figure 2A. BAL fluid was collected from separate groups of mice during each day of week 2. (A-D) BAL cell data are presented as mean cells/ml \pm SE (n=4-6 mice/group). (* $p < 0.05$ compared to HDM; ANOVA with Newman-Keuls test). No inflammatory cell types were observed in control groups. (E) Protein levels in BAL fluid supernatants represented as mean percent of control \pm SE (n=5 mice/group). The vehicle control and APAP alone groups were not significantly different, therefore they were pooled to form the control group (black line at y=100%, n=10 mice/group). Data were analyzed by ANOVA followed by Newman-Keuls test; line and bars with differing superscripts differ from each other at the $p < 0.05$ level. All data are representative of two independent experiments.

Figure 3.4: Gene Expression Time Course

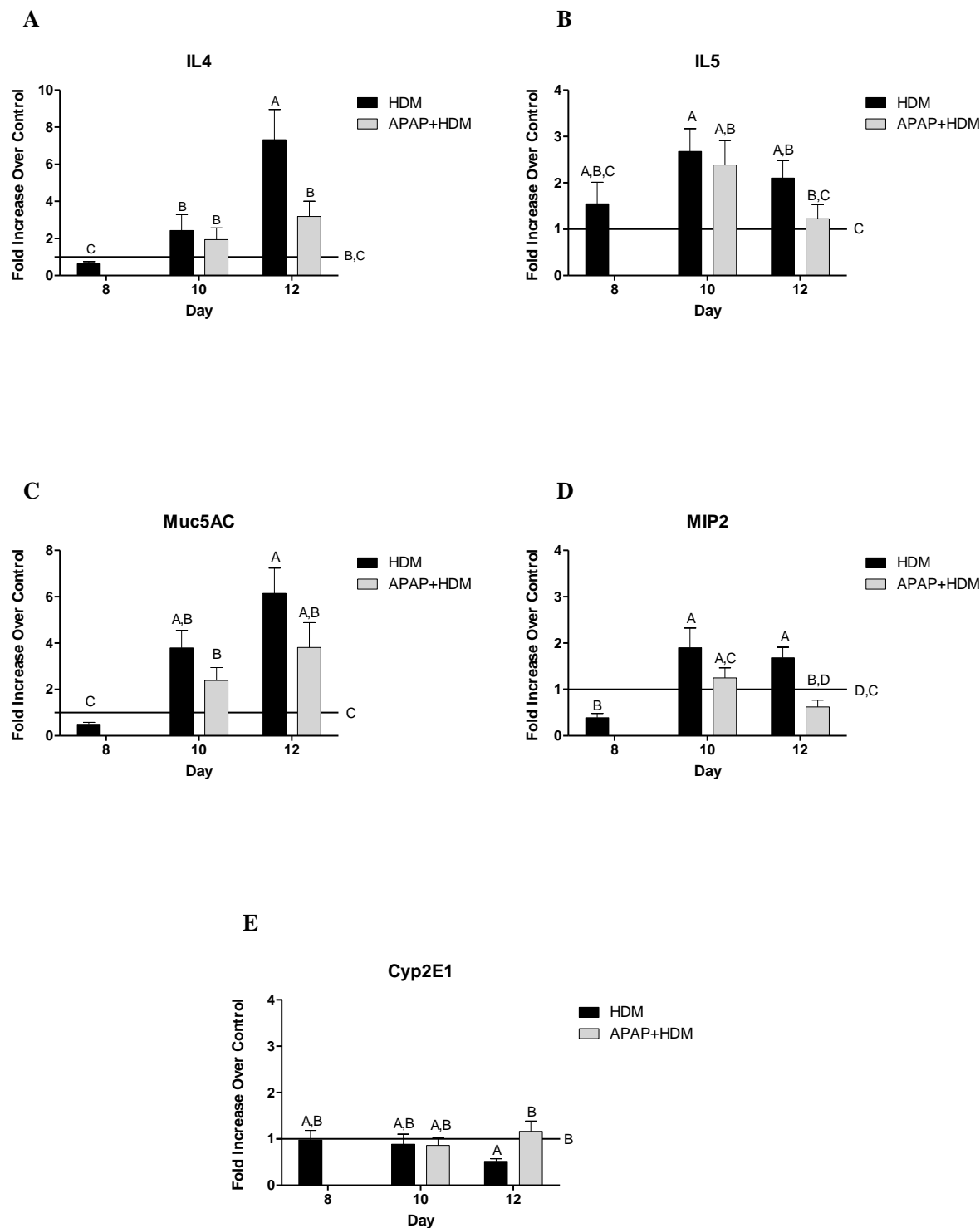


Figure 3.4. Time courses of gene induction in the lung for selected genes modulated by HDM and APAP. (A) *Il-4*, (B) *Il-5*, (C) *Muc5AC*, (D) *Mip2* (E) *Cyp2E1*. Lung tissue was collected from groups of mice on days 8, 10, and 12 of the time course experiment. For all genes analyzed, the vehicle control and APAP alone groups did not differ significantly, therefore they were combined to form pooled control group for statistical analysis. Individual gene names are indicated at the top of each figure, and data are presented as mean fold increase \pm SE, over pooled control (black line at $y=1$). Line and bars with differing superscripts differ at the $p<0.05$ level (ANOVA with Newman-keuls test). Data are representative of two independent experiments ($n=4-6$ mice/group).

Figure 3.5: Effect of CYP450 Inhibition

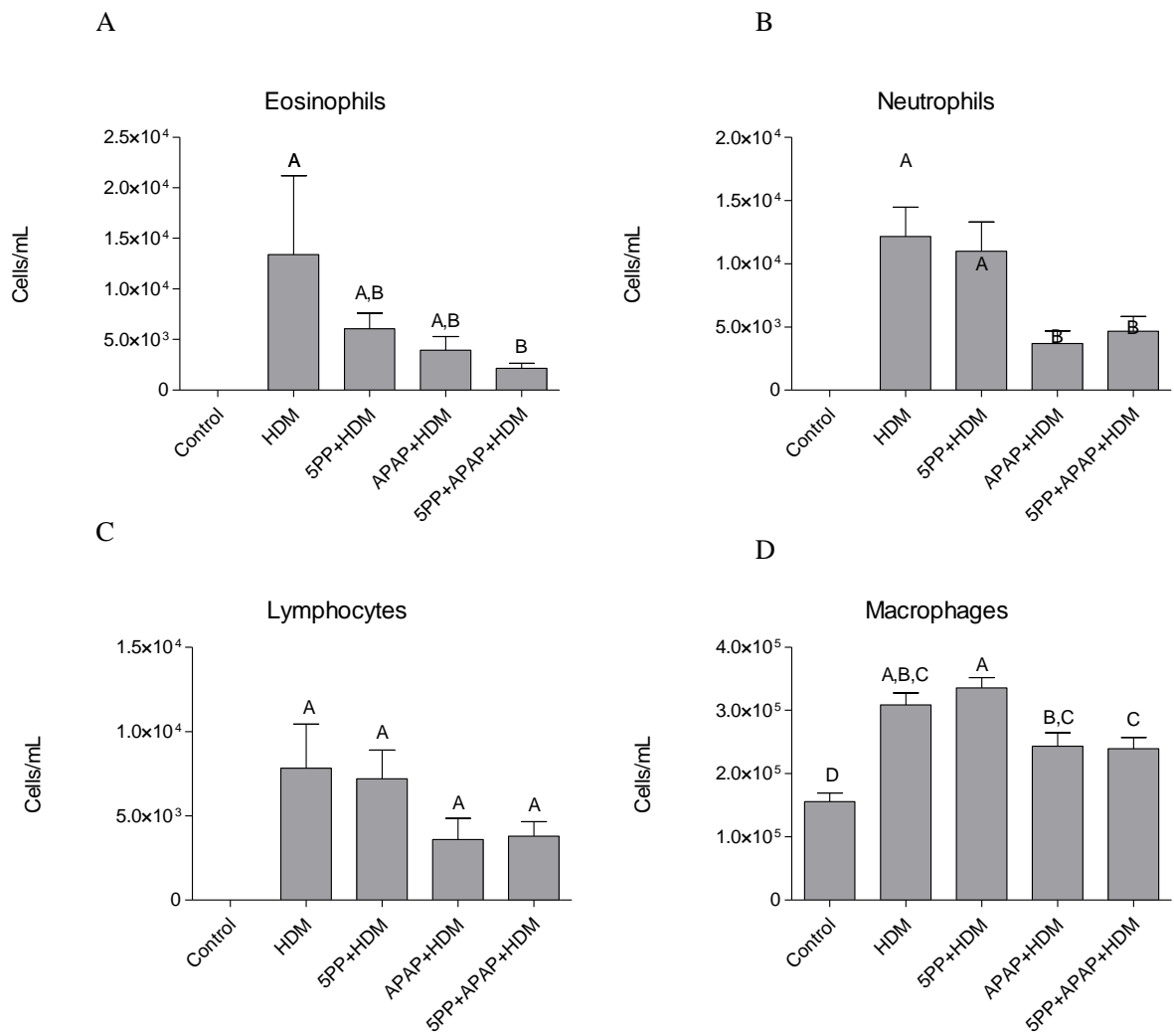


Figure 3.5. Effect of CYP450 inhibition on attenuation of HDM response by APAP. Treatment of animals with 5-PP one hour prior to APAP treatment began once per day on day 8 and ended on day 9. BAL was performed on day 10. A 1.25 µg dose of HDM was used. (A-D) Differential cell count data are presented as mean cells/ml \pm SE (n=4-6 mice/group). Bars with differing superscripts are differ at the p<0.05 level (ANOVA with Newman-Keuls test). No inflammatory cell types were observed in control groups and data are representative of two independent experiments.

Figure 3.6: Histological Assessment

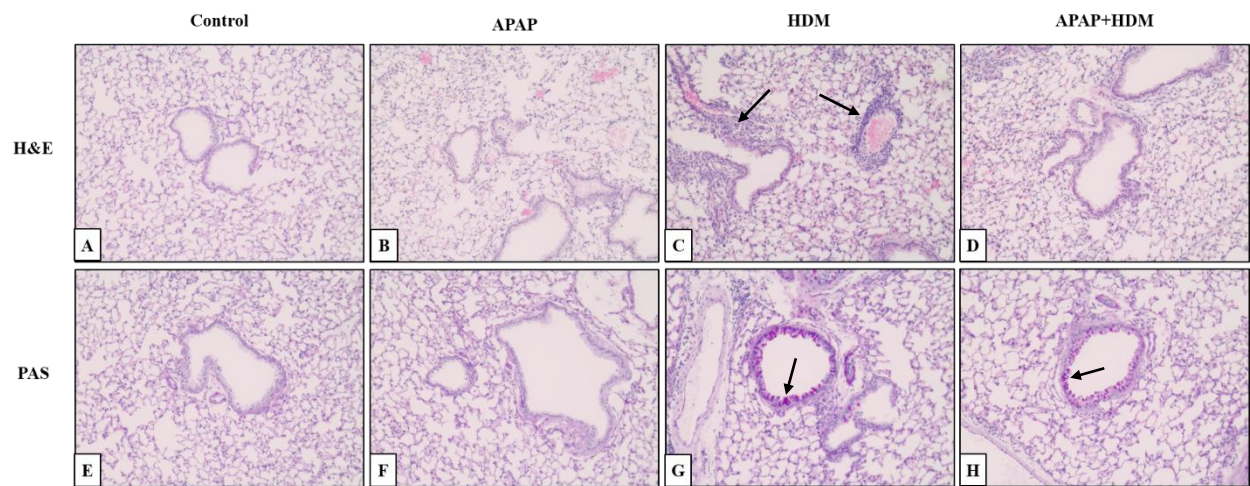
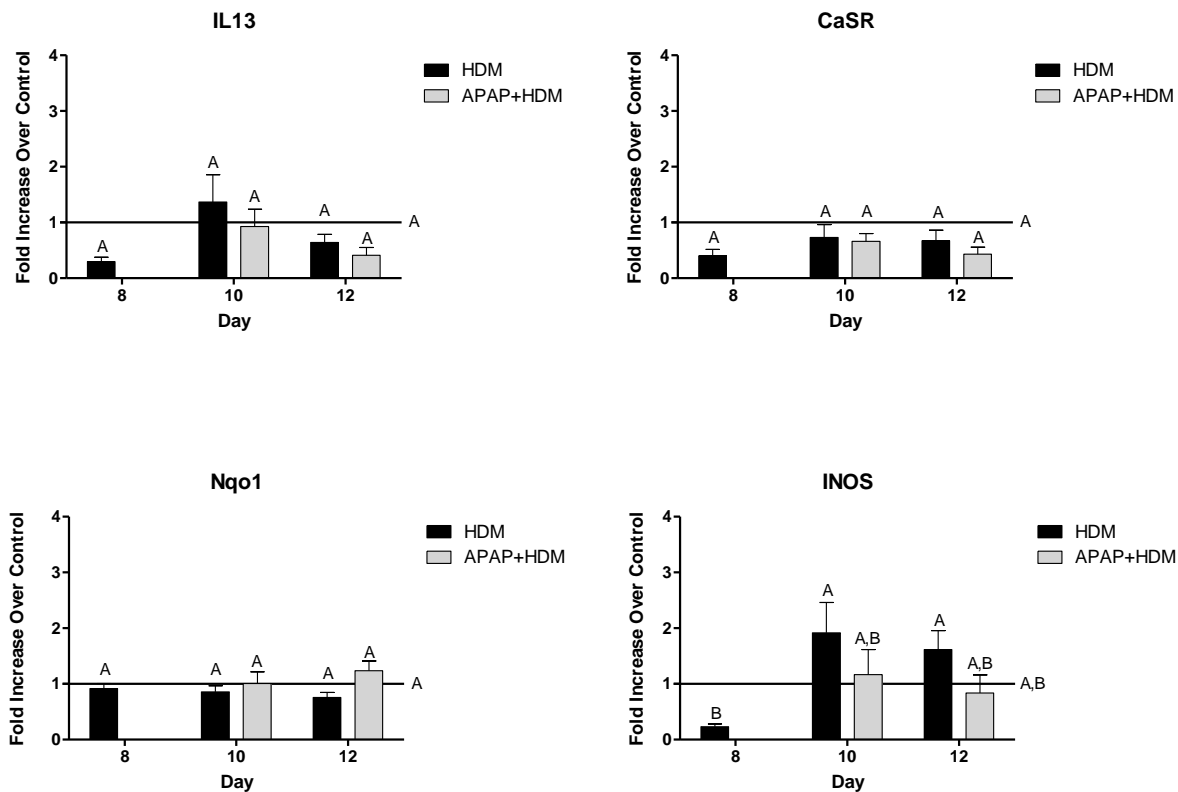


Figure 3.6. HDM-exposed mice demonstrate moderate perivascular/peribronchiolar inflammation which is largely absent in APAP treated mice. Lungs were fixed in formalin, sectioned, and stained with H&E or PAS. (A-D) Arrows indicate examples of perivascular/peribronchiolar inflammation. H&E. x200. (E-H) Arrows indicate examples of mucus production by airway goblet cells. PAS x200. Images are representative of lungs from each group indicated above each image column, and two independent experiments (n=3 mice/group).

Supplemental Figure S3.1: Gene Expression Time Course



Supplemental Figure S3.1. Time courses of gene induction in the lung for selected genes modulated by HDM and APAP. (A) *Il-13*, (B) *CaSR*, (C) *Nqo1*, (D) *INos*. Lung tissue was collected from groups of mice on days 8, 10, and 12 of the time course experiment. For all genes analyzed, the vehicle control and APAP alone groups did not differ significantly, therefore they were combined to form pooled control group for statistical analysis. Individual gene names are indicated at the top of each figure, and data are presented as mean fold increase \pm SE, over pooled control (black line at y=1). Line and bars with differing superscripts differ at the p<0.05 level (ANOVA with Newman-keuls test). Data are representative of two independent experiments (n=4-6 mice/group).

Chapter 4: Conclusions

The goal of these studies was to investigate the effects of non-hepatotoxic doses of APAP on acute and inflammatory responses of the airways. Two original hypotheses, prompted by the “APAP hypothesis” in asthma, were tested: 1) *APAP acts as a pro-oxidant in the airways and enhances the response to ETS* and 2) *APAP administration would enhance the development of allergic airway disease caused by HDM*. The studies described in Chapter 2 support the first hypothesis by demonstrating that APAP causes airway oxidative stress and enhances respiratory irritant-responses to ETS. In Chapter 3 an attenuating effect of APAP on the HDM induced airway inflammatory response was described, providing evidence against the second hypothesis.

In our investigation of the acute airway effects of APAP, described in Chapter 2, several observations were made that support the first hypothesis. First, APAP caused significant GSH loss throughout the respiratory tract, and induction of Nrf2 mediated gene expression. Nrf2 oxidant defense pathway responses represent the first step in the hierarchical oxidative stress model that describes stepwise cellular responses to progressively increasing oxidative stress and GSH loss (Riedl and Nel, 2008). Low doses of APAP were not expected to produce the overt signs of toxicity, such as pro-inflammatory cytokine release and histopathological changes, which are further up in the hierarchical oxidative stress model. Therefore, we examined airway Nrf2 pathway induction as this is considered to be a biomarker of the early response to oxidative stress alongside GSH levels. Both cellular GSH and Nrf2 pathway genes are principle components of the detoxification pathways of APAP and other oxidants including ETS.

The next experiments in Chapter 2 were aimed at determining whether APAP could potentiate the effects of other oxidants and whether airway GSH status modulates airway oxidant sensitivity. It

was observed that APAP treatment enhanced the ETS induced oxidative stress response, and the combination of APAP+ETS caused greater GSH loss than either APAP or ETS alone. Loss of airway GSH caused by the specific GSH depleting agent DEM was associated with an enhanced sensory irritation response to acrolein.

In healthy airways, the sensory nerves which initiate the sensory irritation-reflex response are separated from the atmosphere by the airway epithelial barrier. GSH and other antioxidants in the epithelial barrier protect airway function from damage by airborne oxidants. The results of the DEM experiment, suggest that the airway epithelial antioxidant barrier is impaired by APAP. It is likely that GSH loss following APAP and DEM increased the amount of acrolein penetrating to the airway sensory nerves. These findings suggest that tissue antioxidant levels may be a determinant of sensitivity to inhaled irritant chemicals, a novel observation that has not been previously reported. Antioxidant levels may also influence the sensitivity of the airways to other types of airborne oxidant chemicals, such as ozone, which were not examined in these studies.

At 200 mg/kg, APAP produces a sensory irritation response suggesting there may be sufficient oxidant produced at this dose to interact with airway sensory nerves. Other possibilities include: APAP induced oxidative stress causes the release of paracrine signals which can activate sensory nerve responses, or perhaps there is sufficient metabolism of APAP in the sensory nerve endings to generate NAPQI. Others have found that NAPQI can activate the TRPA1 channel in expression systems and isolated nerves, and cause plasma extravasation after instillation of NAPQI via the trachea *in vivo* (Nassini et al. 2010). Conceptually, it seems less likely that the effects observed in Chapter 2 of this thesis are due to escape of free NAPQI from the nasal epithelium due to the high

reactivity of NAPQI. In all of the models in the studies performed by Nassini et al., NAPQI was administered directly. In their expression systems and isolated nerves, concentrations of 100 μ M or greater were required to elicit significant TRPA1 responses, and in the trachea 1mM NAPQI was instilled. In our studies, a blood APAP concentration of 35 ug/mL equates to approximately 230 μ M. Therefore, considering that only about 5% of a therapeutic dose of APAP is converted to NAPQI, the relevance of direct administration of 100 μ M NAPQI is questionable. The high relevance of the present studies is emphasized by the observation that APAP has pro-oxidant effects after systemic administration of near-therapeutic doses *in vivo*.

The methods used in this thesis to examine the effects of APAP on respiratory responses in Chapter 2 (eg. use of whole lung tissue homogenates) may actually underestimate the oxidant effects of APAP on specific airway regions or cell-types. For example, the toxicity of APAP is dependent on CYPs and CYP expression is known to vary throughout the airways (e.g. Clara/Club cells and nasal olfactory epithelial cells have high CYP expression) (Ding and Kaminsky, 2003). Regions with high rates of biotransformation of APAP to NAPQI may be subject to greater effects, which could not be measured in the present studies. The sensory irritation-reflex response in mice is mediated by nerves of the upper airways, although sensory nerves also innervate the lower airways (Vijayaraghavan *et al.*, 1993). As water soluble vapors (e.g. acrolein) are almost completely scrubbed from the air by the rodent nose, less sensory irritation from these chemicals occurs in the lower airways of mice (Morris *et al.*, 2003, Morris, 2012). Unlike rodents, humans primarily breathe through their mouths. Additionally, the human nose is less efficient at scrubbing vapors. Taken together this evidence suggests that the findings in Chapter 2 may be predictive of greater effects in the lower airways of humans, and that the effects may not be uniform throughout the airways.

The findings described in Chapter 2 provide strong evidence of an oxidant basis for the effect of APAP on respiratory responses. The Keap1-Nrf2 system, TRPA1 receptor, and GSH all have thiol residues which are oxidant labile, and were influenced by APAP treatment in our studies. APAP potentiated ETS induced respiratory responses: including GSH loss, Nrf2 pathway gene induction, and oxidant sensitive TRPA1 receptor mediated stimulation of chemosensory nerves (Andre et al. 2008). That APAP potentiated the acrolein response but not cyclohexanone confirm an oxidant basis for the APAP-ETS interaction. Overall, that APAP acts as a pro-oxidant adds indirect support for the APAP hypothesis in that it provides biological plausibility to the concept that APAP may be a causative factor in asthma. These studies highlight another possibility. ETS is a known causative factor for asthma. Therefore, it is possible that APAP may facilitate the development of asthma in individuals who are also exposed to cigarette smoke through an oxidant based interaction. Epidemiological studies providing information on both APAP use and estimated levels of cigarette smoke exposure exist. However, an interaction between APAP and ETS in existing epidemiological data has not been investigated.

The experiments featured in Chapter 3 of this thesis represent a characterization of the unexpected effect of APAP on HDM induced inflammation. In essence, the second hypothesis of this thesis was that APAP would enhance the response to HDM. Previous studies demonstrated that APAP had pro-oxidant effects at near-therapeutic doses, so it was postulated that APAP might enhance HDM inflammation similarly to ETS and other oxidants (Smith *et al.*, 2015). The data described in Chapter 3 did not support the second hypothesis. APAP caused a marked attenuation of most inflammatory endpoints that were measured. In APAP treated groups, fewer inflammatory cells were observed in the lung, and all genes increased by HDM alone were decreased.

In these studies, a HDM model of allergic airway disease was chosen over more frequently used models such as OVA because of its environmental and human relevance. Additionally, HDM causes a respiratory allergic response in mice without the use of additional immune modulators called adjuvants (Cates *et al.*, 2004). That HDM does not require adjuvant to produce an allergic response suggests it is highly potent. It is likely that a combination of intrinsic protease activity, and high LPS levels contribute to this (Calderón *et al.*, 2015). Despite the potency of HDM compared to other allergens, to produce inflammation in experimental models of asthma on a reasonable time-scale it is necessary to use relatively high amounts of concentrated HDM.

The high dose of allergen needed to generate mouse models of asthma is an obvious drawback of these models not often discussed in the literature. For perspective, HDM at concentrations of greater than 10µg/g of dust in the home are associated with a high likelihood of developing allergy and asthma (Sporik *et al.*, 1990). Using a body surface area extrapolation, it can be estimated that a 60kg adult would need to inhale about 600 grams of dust to get an equivalent dose to the 25µg dose used in many HDM models. Furthermore, this 25µg dose is administered on multiple days. Many differences between these models and asthma in humans can be expected, as asthma in humans is a chronic disease that develops over longer periods of time. Nevertheless, mouse models of asthma have proven to be useful in delineating many of the pathogenic processes in asthma. In the present investigation, mouse models revealed a novel and potentially useful effect of a widely used therapeutic agent.

Pilot studies suggested that APAP did not potentiate HDM responses, thus further investigations were primarily designed to characterize this effect. Though several important mechanistic insights were provided by these investigations. The acute pro-oxidant effects of APAP, but not the attenuation of HDM inflammatory responses by APAP, were blocked by an inhibitor of CYP

metabolism (Smith *et al.*, 2015). This observation may be helpful in understanding the apparent dichotomy observed between the potentiation of acute oxidant responses and the attenuation of inflammatory responses in chapters 2 and 3, respectively. This suggests that the acute effects of APAP and its effect on HDM responses are mediated by two different compounds. It is likely, that a toxicological effect of NAPQI is responsible for the acute effects, while it is possible that a heretofore unappreciated pharmacological effect of APAP can attenuate longer-term HDM induced inflammation. The decrease in HDM induced gene expression in APAP treated mice occurred after the onset of decreased cellular influx. This suggests that decreased cytokine expression following APAP treatment is not the primary cause of decreased cellular influx. A possible mechanism for this, detailed in Chapter 3, is that APAP could be influencing the trafficking of immune cells. Further studies will be necessary to determine the mechanism by which APAP decreases HDM induced inflammation.

At a superficial level, the dose of APAP required to modulate respiratory responses in both Chapters 2 and 3 (100 mg/kg) appears to be substantially higher than a therapeutic dose (15-20mg/kg) (Rumack and Matthew, 1975). However, interspecies extrapolation of dose levels from the mouse to human based solely on body weight, is misleading. Approximating the dose based on surface area is not a perfect method, but provides a more accurate representation of equivalent doses between mouse and human (Reagan-Shaw *et al.*, 2008). In the case of APAP, using a body surface area extrapolation from a 100 mg/kg dose in mice gives an approximate 8 mg/kg in a 60kg adult human. For a 20kg child, a 100 mg/kg dose in mice is equivalent to 12 mg/kg. Based on this conversion metric alone, it would appear that a 100 mg/kg dose in mice is particularly relevant to human therapeutic use. However, a number of factors lead to the difficulty in extrapolating drug doses from mouse to human by any means: for example, mice tend to eliminate drugs faster, and

have greater CYP expression per gram of tissue than humans (Martignoni *et al.*, 2006). This could mean that in humans the effects of APAP we observed may be distributed over a greater period of time and thus could be less relevant. As the disposition of acetaminophen is different between mice and humans, and animal models are never perfect, a better way to determine potential toxicity of a compound is to estimate the tissue burden empirically. Therefore, peak APAP levels in the blood were measured by HPLC. This facilitated a direct comparison between the APAP blood levels in mice after a 100mg/kg dose and those produced by a therapeutic dose in humans. Notably, the pro-oxidant effects observed in these studies were associated with near therapeutic blood levels (Rumack and Matthew, 1975). Measurements of blood APAP levels in these studies provided additional confidence that a 100mg/kg dose in mice may cause similar airway oxidant burden, and anti-inflammatory effects during therapeutic use of APAP by humans.

That the acute effects of APAP, are not predictive of longer-term responses in an asthma models is not entirely surprising. Asthma is a complex chronic disease; the acute effects observed in Chapter 2 are arguably mediated by much simpler mechanisms. Certain populations are more susceptible to developing asthma and mouse models cannot encompass all of the nuances of human disease. For example, there are certain individuals in the human population that may be more susceptible to the effects of airway pro-oxidants, such as those with polymorphisms in GSH conjugating enzymes or those who are exposed to high levels of oxidant air pollutants (Strachan and Cook, 1997, Liang *et al.*, 2013). The existence of these individuals bolsters the relevance of the data presented in Chapter 2, in spite of the findings in Chapter 3. It is possible that APAP may be acting in concert with ETS and other oxidants in susceptible populations, to produce a “perfect storm,” for the development of asthma. Further studies are needed to examine this possibility.

In summary, this body of work demonstrates that the ubiquitous analgesic and antipyretic agent, APAP, has a multitude of effects in the respiratory tract at near-therapeutic doses. Importantly, these results clearly indicate APAP is an airway pro-oxidant and also potentiates the effect of the known pro-oxidant asthma causative factor ETS. That APAP can act to potentiate the airway effects of known asthma causative factors is a highly novel concept that may be of great clinical importance. It certainly suggests that APAP should be avoided in individuals sensitive to oxidants or those exposed to cigarette smoke. The effects of APAP in the HDM model are clearly contradictory to the APAP hypothesis relative to the potential role of APAP in the increasing prevalence of asthma. It is recognized that the murine HDM model does not perfectly mimic the human disease, nevertheless, these results provide strong evidence that APAP does not likely contribute to the pathogenesis of asthma, unless it does so by potentiating the effects of other causative factors. This possibility was not investigated in the current project, but is highly worthy of pursuit. As is the case with most scientific inquiry, this thesis undoubtedly asks more questions than could be answered in its scope. Although not providing definitive answers these results highlight the strong potential for unanticipated adverse pulmonary side-effects to occur following APAP use.

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