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The role of glutathione S-transferase Pi (GSTPi) in asthma

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By

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ABSTRACT

In the United States, over 30 million Americans suffer from asthma. More than 9 million of these asthmatics are children. Asthma is a complex genetic disease involving a multifaceted relationship between genetic background and environmental exposures. Among those genes consistently linked to asthma are the glutathione S-transferase (GST) family of genes, a group of phase II detoxification enzymes and redox regulators responsible for host defense against many environmental toxins and reactive oxygen species (ROS) including those found associated with diesel exhaust particles (DEP), environmental tobacco smoke (ETS), and mold. GSTPi is the predominant GST enzyme expressed in the lung. Many epidemiological studies have implicated a single nucleotide polymorphism in the GSTPi gene, GSTP1 Ile105Val (rs1695), as a predictor for asthma. While evidence supports a strong role for GSTPi in allergic airway disease, very little is known about the regulation of GSTPi in asthma and its contribution to oxidative stress in asthma. The studies in this dissertation focus on determining GST activity regulation during allergic inflammation and how it contributes to the development of asthma. We demonstrate that children in the Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS) birth cohort (N=570) carrying the GSTP1 Val105 allele, an allele associated with decreased GST enzyme activity, are significantly likely to wheeze when exposed to DEP, ETS, or mold. Exposure to multiple allergens increased the risk for wheezing independently of the GSTP1 genotype, suggesting that multiple environmental exposures can overwhelm the genetic effect. In order to address the regulation of GSTPi expression and total GST activity, we utilized mouse models of asthma. We demonstrate that allergen or interleukin-13 (IL-13) treatment attenuates GSTPi expression and total GST activity in the lung, although IL-13 is not required for this down-regulation. In addition, these studies demonstrate that IL-13 induced and not allergen
induced attenuation of GSTPi expression and total GST activity is dependent on signal transducer and activator of transcription 6 (STAT6), suggesting that down-regulation of GSTPi expression and total GST activity can occur by STAT6 dependent and independent pathways. In addition, data suggests that down-regulation of GSTPi activity following allergen treatment may result in a decreased ability to neutralize oxidative stress intermediates and consequently, contribute to the asthma phenotype. The studies presented in this dissertation provide evidence that GSTPi expression and GST activity are regulated in asthma and warrant further study as a potential therapeutic target for asthma, especially asthma exacerbated by oxidative stress.
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AHR</td>
<td>Airway hyperresponsiveness</td>
</tr>
<tr>
<td>APTI</td>
<td>Airway pressure-time index</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
</tr>
<tr>
<td>Asp</td>
<td><em>Aspergillus fumigatus</em></td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>CCAAPS</td>
<td>Cincinnati Allergy and Air Pollution Study</td>
</tr>
<tr>
<td>CDNB</td>
<td>1-chloro-2,4-dinitrobenzene</td>
</tr>
<tr>
<td>DEP</td>
<td>Diesel exhaust particles</td>
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<tr>
<td>ETS</td>
<td>Environmental tobacco smoke</td>
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<td>GSH</td>
<td>Glutathione</td>
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<td>HBEC</td>
<td>Human bronchial epithelial cell</td>
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<td>House dust mite</td>
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<td>IL-4</td>
<td>Interleukin-4</td>
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<td>Interleukin-13</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
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<tr>
<td>Term</td>
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<td>------------------------------------------------</td>
</tr>
<tr>
<td>IT</td>
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<tr>
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<td>Janus tyrosine kinase</td>
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<td>Kelch-like ECH-associated protein-1</td>
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<tr>
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<td>Nuclear factor-erythroid 2 related factor 2</td>
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<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>P-Value</td>
<td>Probability that the difference between two groups occurs by chance</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>STAT6</td>
<td>Signal transducer and activator of transcription 6</td>
</tr>
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</table>
CHAPTER 1

INTRODUCTION
Overview

Asthma, a chronic disease characterized by variable airflow limitation, airway inflammation, and airway hyperresponsiveness (AHR), is a major public health concern, especially in children (Elias et al., 2003; Hewer, 1998). Asthma prevalence in children increased by 75% between 1980 and 2004, and it is now the most common chronic disease of childhood, affecting more than 9 million children (13%) in the U.S. (Bloom et al., 2006). Although there is a strong genetic predisposition for asthma, these shifts in prevalence point toward environmental causations. The glutathione S-transferases (GSTs) represent a major group of detoxification enzymes and redox regulators important in host defense to numerous environmental toxins and reactive oxygen species (ROS) including those found in cigarette smoke (Conklin et al., 2009) and air pollution (Diaz-Sanchez and Riedl, 2005). GSTs play important roles in modifying peroxidase and isomerase activities and facilitating responses to oxidative stress reactions. They are also involved in the pathways that detoxify polycyclic aromatic hydrocarbons such as benzo[a]pyrene (Sheehan et al., 2001). GSTs neutralize the electrophilic sites of compounds by conjugating them to the tripeptide thiol, glutathione (GSH), which has an electron-donating capacity. The resulting product is more water-soluble allowing for successful detoxification and lung protection from potential damage caused by electrophilic compounds and products of oxidative stress (Hayes and Pulford, 1995). Evidence suggests that the level of expression of GSTs is a crucial factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals (Hayes and Pulford, 1995). The most abundant mammalian GSTs are the class alpha, mu, and pi enzymes. GSTP1, the predominant cytosolic GST expressed in lung epithelium (Fryer et al., 1986), is encoded by a 2.8-kb gene located on chromosome 11q13, a known “hot spot” for asthma-related genes (Doull et al., 1996; Kano et al., 1987). A single nucleotide polymorphism
at position 313 in \textit{GSTPI} converts an adenine to a guanine (A→G) (rs1695) (Ahmad et al., 1990). The resulting isoleucine to valine substitution in codon 105 of exon 5 (Ile\textsuperscript{105}→Val\textsuperscript{105}) significantly lowers GST enzyme activity (Watson et al., 1998). This \textit{GSTPI} variant has been associated with asthma in multiple studies (Aynacioglu et al., 2004; Carroll et al., 2005a; Carroll et al., 2005b; Fryer et al., 2000; Gilliland et al., 2002; Lee et al., 2005; Nickel et al., 2005; Tamer et al., 2004). Many of these studies implicate the \textit{GSTPI} Ile105Val polymorphism as a predictor for asthma. In one study, subjects with the \textit{GSTPI} homozygous Val\textsuperscript{105}/Val\textsuperscript{105} genotype have been reported to have a 3.55-fold increased risk of having atopic asthma compared to nonatopic asthma (Tamer et al., 2004). Furthermore, a recent report demonstrated that GSH homeostasis is altered in children with severe asthma leading to increased oxidative stress (Fitzpatrick et al., 2009). Despite this and other evidence supporting a strong role for GST in asthma, very little is known about the regulation of GSTs in asthma. The \textbf{central hypothesis} for this dissertation is \textbf{GST activity is regulated during allergic inflammation and decreased GST activity contributes to the development of asthma.}

\textbf{Asthma}

Approximately 300 million people worldwide suffer from asthma (World Health Organization, 2007). Asthma is a chronic inflammatory disorder of the airways in which constriction and inflammation causes narrowing of the airways, making it difficult to breathe. Asthma symptoms can include wheezing, coughing, shortness of breath, and tightening of the chest. Asthma is commonly divided into two categories: allergic (extrinsic) asthma and non-allergic (intrinsic) asthma. Those experiencing allergic asthma are typically genetically susceptible and have inappropriate immune responses to a variety of otherwise innocuous
antigens. Some of these allergens include house dust mites and their droppings, pollens, molds, and pet dander. People with non-allergic asthma often experience the same symptoms as those with allergic asthma, but their asthma is not triggered by allergens. Instead, irritants in the air such as environmental tobacco smoke, perfumes, paint fumes, and air pollution can trigger non-allergic asthma. Respiratory infections, dramatic changes in the weather, and exercise can also be triggers for non-allergic asthma.

Asthma is a complex genetic disease. In 2006, a study found that 118 genes were associated with asthma or atopy in 492 scientific articles. Only 10 of these genes were replicated in more than 10 independent populations (Ober and Hoffjan, 2006). This highlights the difficulties of genetic studies in which the phenotypic impact of these genes is modest and replication in different populations is limited (von Mutius, 2009). In addition, the prevalence rates of asthma differ in developed vs. less developed countries and urban vs. rural areas (Pope, 2000). Asthma prevalence rates have also differed in populations with the same ethnic background (Wong and Chow, 2008). There is a strong genetic predisposition for asthma, but these shifts in prevalence point toward environmental causations. This multifaceted interaction between genetic and environmental factors contributes to asthma being considered a ‘complex disease.’

**Importance of Interleukin-13 (IL-13) In Allergic Inflammation**

In 1989, IL-13 was first identified as P600, a protein produced by activated $T_{h2}$ cells (Brown et al., 1989). A member of the class of Type I cytokines, IL-13 shares the Type I tertiary structure consisting of a four $\alpha$-helical hydrophobic bundle core (Leonard and Lin, 2000). At the amino acid level, IL-13 shares a 25% homology with IL-4. Among the conserved regions is a
25-amino-acid hydrophobic structural core with only minimal hydrophobic substitutions (Chomarat and Banchereau, 1998). However, IL-4 is mouse or human-specific, whereas IL-13 is not. Human IL-4 acts only on human cells and mouse IL-4 acts only on mouse cells (Park et al., 1987). In contrast, mouse IL-13 acts equally on human or mouse cells. Nonetheless, IL-13 does seem to be species-selective since human IL-13 activity is greater on human cells as opposed to mouse cells (de Vries, 1996; Minty et al., 1993). With four exons and three introns, the gene encoding IL-13 is found 12 kb upstream of the gene for IL-4 in the same orientation on human chromosome 5q31, a region linked with asthma. The 5q31 chromosome is also the location for the genes encoding IL-3, IL-5, IL-9, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Daniels et al., 1996; Marsh et al., 1994; Meyers et al., 1994; Postma et al., 1995; Smirnov et al., 1995).

IL-13 plays a pivotal role in the pathogenesis of asthma (Grunig et al., 1998; Wills-Karp et al., 1998). Similar to IL-4, human IL-13 promotes human B-cell proliferation and induces class switching to IgG4 and IgE with CD40-CD40 ligand co-stimulation (Oettgen and Geha, 2001). Both IL-4 and IL-13 can induce surface antigen expression that includes the low-affinity IgE receptor CD23 (FceRII) and major histocompatibility complex (MHC) Class II (Chomarat and Banchereau, 1998). In monocytes and macrophages, IL-13 can increase expression of integrin family members critical in adhesion: CD11b, CD11c, CD18, and CD29 (Zurawski and de Vries, 1994) as well as Class II MHC and CD23 (de Vries, 1998). IL-13 action on monocytes and macrophages also inhibits the production of pro-inflammatory mediators including prostaglandins (Endo et al., 1996), reactive oxygen and nitrogen intermediates (Doherty et al., 1993; Sozzani et al., 1995), IL-1, IL-6, IL-8, TNF-α, and IL-12 (Lentsch et al., 1997). In eosinophils, IL-13 has been shown to promote survival, activation, and recruitment (Horie et al.,
IL-13 activates mast cells and contributes to IgE priming of mast cells (de Vries, 1998). IL-13 also induces endothelial cell expression of vascular cell adhesion molecule-1 (VCAM-1), a molecule important to eosinophil recruitment (Bochner et al., 1995). In smooth muscle cells, IL-13 induces proliferation and methacholine-induced contractions in vitro (Wills-Karp, 2001) as well as an upregulation of type I collagen in human skin fibroblasts (Oriente et al., 2000). Further, IL-13 activity in epithelial cells induces chemokine expression (Li et al., 1999), alters mucociliary differentiation and ciliary beating (Laoukili et al., 2001), and results in goblet cell metaplasia (Grunig et al., 1998; Wills-Karp et al., 1998; Zhu et al., 1999).

Unlike IL-4, IL-13 seems to be crucial to the effector phase of allergic inflammation since IL-13 is not important in the incipient CD4 T cell differentiation into Th2 cells. In other words, IL-13 is important to sustaining Th2-mediated inflammation rather than initiating the Th2 response. Transgenic mice overexpressing IL-13 display airway inflammation and mucus hypersecretion (Zhu et al., 1999). On the contrary, blocking IL-13 eliminates allergic inflammation in an autonomous manner from IL-4 (Grunig et al., 1998; Wills-Karp et al., 1998). The mechanism by which IL-13 contributes to AHR remains unclear. Allergen challenged IL-13-deficient mice fail to show AHR or mucus hypersecretion despite the presence of IL-4 and IL-5 (Grunig et al., 1998; Walter et al., 2001; Wills-Karp et al., 1998), and the addition of recombinant IL-13 (rIL-13) restores these features (Walter et al., 2001; Webb et al., 2004). However, in a different mouse model of asthma, allergen challenged IL-13-deficient mice were shown to have attenuated mucus hypersecretion despite the persistence of AHR (Webb et al., 2000). Furthermore, IL-13 overexpression in the adult mouse lung was found to cause emphysema-like symptoms and mucus metaplasia. In addition, they discovered that IL-13 was an
effective stimulator of matrix metalloproteinases (MMPs) and cathepsin proteases (Zheng et al., 2000). IL-13 overexpression is also found to be associated with conspicuous fibrosis, whereas an IL-4 overexpression fails to exhibit fibrosis (Rankin et al., 1996; Zhu et al., 1999).

IL-13 signaling utilizes the Janus tyrosine kinase (JAK)/JAK-signal transducer and activator of transcription 6 (STAT6) pathway (Kaplan et al., 1996; Takeda et al., 1996a). JAKs are tyrosine kinases in which there are four mammalian members: JAK1, JAK2, JAK3, and Tyk2 (Ihle, 2001). While JAK3 is expressed in hematopoietic cells, JAK1, JAK2, and Tyk2 are more universally expressed (Leonard and Lin, 2000). Upon IL-13 binding to its receptor, JAK1 and Tyk2 are activated (Keegan et al., 1995; Roy and Cathcart, 1998; Welham et al., 1995). This activation then leads to phosphorylation of the IL-13 receptor, which directs STAT6 recruitment to the receptor, ensued by phosphorylation and activation of STAT6 (Mikita et al., 1996). The phosphorylated STAT6 monomers then dimerize and translocate into the nucleus where they can initiate transcription of downstream genes (Mikita et al., 1998). The activation of STAT6 can be sustained in the presence of either IL-4 or IL-13 (Andrews et al., 2002; Wang et al., 1999). Maintenance of STAT6 activation requires JAK activity as well as an incessant cycle of activation, deactivation, nuclear export, and reactivation (Andrews et al., 2002).

STAT6 also plays a crucial role in the pathogenesis of allergic asthma. Studies using STAT6-deficient mice have found them to have impaired macrophage function in response to IL-13 (Takeda et al., 1996a) and diminished IL-4 mediated functions such as T_{H}2 differentiation, cell surface marker expression, and Ig class switching to IgE (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996b). Furthermore, the importance of STAT6 has been implicated for many components of allergic asthma. STAT6-deficient mice develop attenuation
of AHR, eosinophilic inflammation, and mucus production in response to ovalbumin (Akimoto et al., 1998).

**Contribution of Environmental Factors (DEP, ETS, and Mold) to Asthma**

About 85% of children who develop asthma have an allergic response to aeroallergens (Peden, 2000). In fact, early-life allergic sensitization to aeroallergens was found to be significantly associated with the later development of asthma symptoms (Sherrill et al., 1999). This suggests that early-life exposure to aeroallergens may play an important role in the developmental stages of the immature immune system. Of particular interest are exposures to diesel exhaust particles (DEP), environmental tobacco smoke (ETS), and mold. Each of these environmental exposures has been implicated in the exacerbation of asthma and asthma symptoms in infants and children (Biagini et al., 2006; Brunekreef et al., 1989; Cho et al., 2006b; Jaakkola et al., 1993; Jaakkola et al., 2001; Larsson et al., 2001; Peden, 2000; Ronmark et al., 1999; Ryan et al., 2007; Strachan and Sanders, 1989; von Mutius, 2002; Waegemaekers et al., 1989).

Most DEP are fine (0.1-2.5 μm) or ultrafine (<0.1 μm) in size, permitting them to be respirable and capable of reaching the peripheral airways (Riedl and Diaz-Sanchez, 2005). Studies have shown that DEP can promote alterations in T_{h}2 immunity and IgE production (Pandya et al., 2002; Peden, 2000). DEP are composed of elemental carbon cores with large surface areas capable of binding chemicals, transition metals, and airborne allergens. In mice, intratracheal DEP administration causes increased nitric oxide production, cellular infiltration, mucus production, and AHR (Lim et al., 1998). DEP interactions with allergens such as grass
pollen, dog, cat, and house dust mite (HDM) has been shown to augment allergen-induced responses (Diaz-Sanchez et al., 1997). Moreover, DEP exposure has been shown to exacerbate ovalbumin (OVA) and HDM-induced allergic asthma in mice (Ichinose et al., 2004).

Approximately 43% of children between the ages of two and eleven years old are exposed to ETS in the home (Pirkle et al., 1996). ETS, also known as secondhand smoke, is the mixture of sidestream smoke, the smoke given off by the burning end of a tobacco product, and mainstream smoke, the smoke exhaled by a smoker. Comprised of more than 4,000 chemicals, ETS consists of a vapor phase containing volatile agents (e.g., benzene, vinyl chloride, and acrolein) and a particulate phase (tar) containing semi-volatile and non-volatile agents (e.g., nicotine, aromatic amines, and polycyclic aromatic hydrocarbons (PAHs)) (Hecht, 2002; Jaakkola and Jaakkola, 1997). 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).

Mold exposure has also been shown to induce asthma and allergic rhinitis via IgE-mediated mechanisms (Edmondson et al., 2005). The optimum growing conditions for molds are warm, damp, and humid conditions. The risk for wheezing was found to be increased by two-fold in infants in homes that had visible mold or water damage and up to six-fold in aeroallergen-sensitized infants (Cho et al., 2006b). Some of the more common species of molds studied include Cladosporium, Penicillium, Alternaria, and Aspergillus, although there are estimated to be hundreds of thousands of different fungal species. Aspergillus fumigatus is a commonly used allergen in mouse models of asthma. Exposure to Aspergillus fumigatus induces strong airway hyperreactivity, high IgE levels, elevated peripheral blood and lung eosinophils, and pulmonary inflammation (Kurup and Grunig, 2002).
Gene-Environment Interactions

A gene-environment interaction has been defined as “a different effect of an environmental exposure on disease risk in persons with different genotypes” or “a different effect of a genotype on disease risk in persons with different environmental exposures” (Ottman, 1996). Genotyping allows for identification of susceptible individuals within populations and better estimation of the true magnitude of effect of an environmental exposure compared to population-based studies. At the statistical level, an interaction consists of a genetic effect between two groups: exposed and non-exposed individuals or the disparity of environmental effect between two genetically different groups (Guo, 2000). Gene-environment effects on asthma development have, thus far, concentrated mainly on gene interactions with air pollutants, tobacco smoke, and microbial exposures (von Mutius, 2009). In the previously mentioned review of nearly 500 disease association studies, 25 genes associated with asthma or atopy phenotypes were identified in six or more populations (Ober and Hoffjan, 2006). Among the genes consistently associated with asthma is the glutathione S-transferase (GST) gene family. Consequently, Chapter 2 of this dissertation analyzes the gene-environment interactions between DEP, ETS, and mold and a polymorphism (GSTP1 Ile105Val, rs1695) in a GST family member, glutathione S-transferase Pi (GSTPi) in a Cincinnati birth cohort.

Glutathione S-transferases (GSTs) In the Lung

The environment contains numerous xenobiotics, chemical compounds that are foreign to the body, which all organisms are exposed to on a continual basis. These xenobiotics provide neither energy nor any biologic necessity, but can gain entry into the body through the foods we eat, through our skin, or of particular interest, via inhalation. If left to accumulate in the body,
some xenobiotics can damage and harm the body. Similarly, an abundance of free radicals or reactive oxygen species (ROS) in the body can cause damage to the body via oxidative stress. Air pollution, tobacco smoke, herbicides, and pesticides are examples of free radical sources found in the environment. Fortunately, the body is able to combat this xenobiotic assault and oxidative stress with cellular antioxidant defense systems. Included in the protective system are GSTs, a family of phase II detoxification enzymes and redox regulators important in host defense to a broad spectrum of environmental toxins and ROS, including those found associated with allergens, mold, cigarette smoke, and air pollution (Conklin et al., 2009; Diaz-Sanchez and Riedl, 2005; Hayes and Pulford, 1995; Kumar et al., 2009). All eukaryotic species possess multiple GST isoenzymes (Hayes and Pulford, 1995). GSTs play important roles in modifying peroxidase and isomerase activities and facilitating responses to oxidative stress reactions. They are also involved in detoxifying pathways of polycyclic aromatic hydrocarbons such as benzo[a]pyrene (Sheehan et al., 2001). GSTs neutralize the electrophilic sites of ROS by conjugating them to the tripeptide thiol, glutathione (GSH), which has an electron-donating capacity. The resulting product is more water-soluble and easier to eliminate from the body, allowing for successful detoxification and lung protection from potential damage caused by electrophilic compounds and products of oxidative stress (Hayes and Pulford, 1995).

In humans, GSTs are divided into eight families: Alpha, Kappa, Mu, Omega, Pi, Sigma, Theta, and Zeta (Mannervik et al., 1992; Pemble and Taylor, 1992). The majority of mammalian GSTs are cytosolic (Alpha, Mu, Omega, Pi, Sigma, Theta, and Zeta), whereas the Kappa family contains the mitochondrial GSTs. The cytosolic GSTs are dimeric and contain subunits that vary from 199 to 244 amino acids. Mouse and human cytosolic GSTs within a class share more than 40% identity while GSTs share less than 25% identity between classes. The Kappa GSTs are also
dimeric with subunits the length of 226 amino acids. Furthermore, mice, rats, and humans are known to have only a single Kappa GST (Hayes et al., 2005; Hayes and Pulford, 1995). In humans, the most abundant cytosolic GSTs are GST Alpha1, Alpha2, Mu1, Pi1, Theta1, and Theta2 (Hoensch et al., 2002). This dissertation focuses on the Pi class of GSTs.

**Glutathione S-transferase Pi (GSTPi)**

A single gene in the Pi subfamily, *GSTP1*, is the predominant cytosolic GST expressed in lung epithelium (Fryer et al., 1986). GSTP1 is a 2.8 kb gene located on chromosome 11q13, a known “hot spot” for asthma-related genes (Doull et al., 1996; Kano et al., 1987). A single nucleotide polymorphism at position 313 in *GSTP1* results from conversion of an adenine to a guanine (A→G) (rs#1695) (Ahmad et al., 1990). The resulting isoleucine to valine substitution in codon 105 of exon 5 (Ile105→Val105) significantly lowers GST enzyme activity (Watson et al., 1998). This *GSTP1* variant has been associated with asthma in multiple studies (Aynacioglu et al., 2004; Carroll et al., 2005a; Carroll et al., 2005b; Fryer et al., 2000; Gilliland et al., 2002; Lee et al., 2005; Nickel et al., 2005; Tamer et al., 2004). Many of these studies implicate the *GSTP1* Ile105Val polymorphism as a predictor for asthma. In one study, the *GSTP1* homozygous Val105/Val105 genotype was more frequent among asthmatics compared to control subjects, and those subjects with the Val105/Val105 genotype were reported to have a 3.68-fold increased risk of having asthma (Tamer et al., 2004).

The *GSTP1* Ile105Val polymorphism has been analyzed in relation to different environmental exposures. As previously discussed, DEP, ETS, and mold are a few of several environmental factors and components of air pollution that have been examined for their involvement in exacerbating asthma and/or asthma symptoms (Biagini et al., 2006; Cho et al.,
Chemicals bound to the surface of DEP have been shown to induce ROS (Hiura et al., 1999; Riedl and Diaz-Sanchez, 2005). Moreover, cigarette smoke has also been shown to contain a high concentration of ROS (Huang et al., 2005). In fact, an association between the \textit{GSTP1} polymorphism, ETS, and lung cancer has been observed (Gilliland et al., 2006; Miller et al., 2003; Wenzlaff et al., 2005). Similarly, mold exposure has also been associated with increased intracellular levels of ROS (Huttunen et al., 2000; Jussila et al., 2001). Though the mechanism by which DEPs, ETS, and mold manipulate the development of asthma and asthma symptoms is unknown, there continues to be convincing evidence that implicates oxidative stress as a contributor to inflammatory responses in the airway (Diaz-Sanchez et al., 1997; Howard et al., 1998; Leem et al., 2005; Nel et al., 2001).

Unlike humans, mice carry two homologous \textit{GSTPi} genes that lie in tandem: \textit{GSTP1} and \textit{GSTP2}. They are separated by about 2.5 kb of DNA and are both actively transcribed. Their cDNA are 97\% identical and differ in only six amino acids. Furthermore, transcription of \textit{GSTP1} is more abundant than \textit{GSTP2} (Bammler et al., 1994). Mice lacking both \textit{GSTPi} genes were initially generated to study the effects of skin tumorigenesis induced by a polycyclic aromatic hydrocarbon (7,12-dimethylbenz anthracene) and a tumor-promoting agent (12-\textit{O}-tetradecanoylphorbol-13-acetate). GSTPi null mice appeared healthy, had no organ abnormalities, and reproduced normally. However, GSTPi-deficient mice were found to have significantly more skin papillomas than GSTPi-sufficient mice (Henderson et al., 1998). A subsequent study reported that mice lacking both \textit{GSTPi} genes developed more lung tumors than their wild-type counterparts when exposed to three different chemicals that are constituents of tobacco smoke (Ritchie et al., 2007). Interestingly, a recent study, also utilizing GSTPi-deficient mice, found that their exposure to OVA resulted in an increase in eosinophilia, goblet cell
hyperplasia, airway remodeling, airway resistance, and IL-5 compared to OVA-exposed wild-type mice (Zhou et al., 2008a).

Evidence suggests that the expression level of GST is a vital factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals (Hayes and Pulford, 1995). Consequently, GST constitutive expression may play a role in a tissue’s ability to manage particular types of chemical stress (Knight et al., 2007). GST activity was found to correlate significantly with GSTP1 mRNA expression level ($R^2=0.77$, $P<0.001$) (Bauer et al., 2006). Interestingly, in the aforementioned study using GSTPi-deficient mice, GSTP1 expression in the lungs of wild-type mice was significantly attenuated after OVA treatment (Zhou et al., 2008b). However, GST activity was not reported. Despite this and other evidence supporting a strong role for GST in asthma, very little is known about the regulation of GST in asthma. Thus, Chapter 3 of this dissertation uses mouse models of asthma to determine the regulation of GSTPi expression and total GST activity as well as the localization of GSTPi in the lung.

**Nuclear Factor-erythroid 2 Related Factor 2 (Nrf2)**

Nrf2 is a cap’n’collar (CNC)-basic region/leucine zipper (bZIP) transcription factor involved in the transcriptional regulation of many antioxidant genes, including those involved in the GST pathway. It is richly expressed in the intestine, kidney, and lung: organs that need to continually use detoxification reactions (Itoh et al., 1997). To induce antioxidants and detoxifying enzymes, Nrf2 binds to the cis-acting antioxidant response element (ARE). However, before Nrf2 can bind to the ARE, it must be dissociated from Kelch-like ECH-associated protein-1 (Keap1), a cytoplasmic, cysteine-rich, actin-bound protein (Itoh et al., 1999; Kobayashi et al., 2002). Under basal conditions, Keap1 retains the inactive form of Nrf2 in
the cytoplasm, preventing Nrf2 from functioning as a transcription factor. Several protein kinase pathways can activate phosphorylation of Nrf2, which leads to Keap1-Nrf2 dissociation, nuclear Nrf2 translocation, and ARE binding (Cho and Kleeberger, 2009). By activating ARE-bearing genes, such as GSTs, Nrf2 initiates cellular response pathways against oxidative injury, inflammation/immunity, apoptosis, and carcinogenesis (Cho et al., 2006a). In fact, Nrf2 was found to directly bind and activate the mouse GSTPi gene, indicating that Nrf2 acts upstream of GSTPi (Ikeda et al., 2002).

Mice with a disrupted Nrf2 gene (Nrf2−/−) were found to have increased pulmonary hyper-permeability, macrophage inflammation, and epithelial injury, compared to wild-type mice, in situations of hyperoxia (Hayes and McLellan, 1999). Nrf2-deficient mice also had a significant reduction in mRNA expression of glutathione peroxidase 2 (GPX2), the main cigarette smoke-inducible isoform of a group of antioxidant enzymes that scavenge hydrogen peroxide and organic hydroperoxides (Singh et al., 2006). Interestingly, the lungs of Nrf2-deficient mice were found to have decreased GSTPi transcript levels compared to wild-type mice following OVA treatment. In this same study, using an OVA model of asthma, Nrf2-deficient mice were reported to have increased susceptibility to airway inflammation and AHR. In addition, Nrf2-deficient mice treated with OVA had increased oxidative stress, measured by a decrease in reduced glutathione/oxidized glutathione (GSH/GSSG) ratio, in lung tissue compared to their wild-type counterparts (Rangasamy et al., 2005).
**Experimental Rationale**

Asthma is a complex disease that entails an intricate relationship between genetic and environmental factors. In asthmatics, oxidative stress can occur from exposure to environmental toxins found in air pollution. Diesel exhaust particles (DEP), environmental tobacco smoke (ETS), and mold are three environmental agents that have been implicated in the development of asthma. They have also been shown to contain compounds that contribute to oxidative stress, including reactive oxygen species (ROS) and polycyclic aromatic hydrocarbons (PAHs). IL-13, a key mediator in the pathogenesis of asthma, induces airway hyperresponsiveness (AHR), goblet cell metaplasia, and mucus hypersecretion. IL-13 levels are elevated in response to environmental exposures that can cause oxidative stress. GSTPi is one of the predominant detoxification enzymes in the lung that can help neutralize compounds contributing to oxidative stress. Numerous epidemiological studies have demonstrated an association between the \( GSTP1 \) Ile105Val polymorphism with asthma. However, very little is known about the regulation of GSTPi expression and GST activity in asthma. Consequently, the aims of this dissertation intend to answer the following questions:

1) Are infants carrying the \( GSTP1 \) Val\textsuperscript{105} allele more susceptible to wheezing in the context of multiple exposures including diesel exhaust particles (DEP), environmental tobacco smoke (ETS), and mold?

2) How are GSTPi expression and total GST activity regulated in mouse models of asthma?
To answer the first question, the studies in Chapter 2 of this dissertation aim to determine the gene-environment interactions in the Cincinnati Allergy and Air Pollution Study (CCAAPS) birth cohort between the \textit{GSTP1} Ile105Val polymorphism and environmental exposures (DEP, ETS, and mold exposure) in the development of wheezing. Those infants carrying the \textit{GSTP1} Val^{105} allele were significantly more likely to wheeze when exposed to DEP, ETS, or mold. In Chapter 3 of this dissertation, we determine if GSTPi expression and total GST activity are regulated during allergic inflammation using mouse models of asthma. Studies completed in this chapter suggest that GSTPi expression levels and total GST activity are down-regulated in asthma by STAT6 dependent and independent pathways.
References


CHAPTER 2

Associations between multiple environmental exposures and glutathione S-transferase P1 on persistent wheezing in a birth cohort.*

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Abstract

Objective: To determine the impact of environmental exposures (diesel exhaust particle (DEP), environmental tobacco smoke (ETS), and mold) that may contribute to oxidative stress on persistent wheezing in the Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS) birth cohort and to determine how the impact of these exposures is modified by the GSTP1 Ile105Val polymorphism.

Methods: A land-use regression model was used to derive an estimate of each child’s DEP exposure. ETS exposure was determined by questionnaire data. Each child’s home was evaluated for visible mold by a trained professional. Children in the CCAAPS cohort were genotyped for the GSTP1 polymorphism (N=570). Persistent wheezing was defined as wheezing at both 12 and 24 months.

Results: High DEP exposure conferred increased risk for wheezing phenotypes but only among the Val105 allele carriers. Infants with multiple exposures were significantly more likely to persistently wheeze despite their genotype.

Conclusion: There is evidence for an environmental effect of DEP among carriers of the GSTP1 Val105 allele in the development of persistent wheezing in children. The protective effect of the GSTP1 Ile105 genotype may be overwhelmed by multiple environmental exposures that converge on oxidative stress pathways.
Introduction

The increasingly common occurrence of childhood wheeze and asthma, particularly in affluent westernized society, is well-documented (1998). Environmental factors associated with wheezing in early life include traffic exhaust exposure through diesel exhaust particles (DEP) (Ryan et al., 2007; WHO, 2004), environmental tobacco smoke exposure (ETS) (Biagini et al., 2006; Huang et al., 2005), and mold exposure (Iossifova et al., 2007; Riedl and Diaz-Sanchez, 2005; WHO, 2004). The relationship between the glutathione S-transferase P1 (GSTP1) Ile105Val polymorphism and asthma has been reported in several populations, but these studies have not examined the interplay of the combined genetic and environmental factors on longitudinal wheezing status during early childhood (Fryer et al., 2000; Gilliland et al., 2002a).

In humans, the glutathione S-transferase (GST) class of multifunctional enzymes are divided into eight families: Alpha, Kappa, Mu, Omega, Pi, Sigma, Theta, and Zeta (Mannervik et al., 1992; Pemble and Taylor, 1992). A single gene in the Pi subfamily, GSTP1, is the predominant cytosolic GST expressed in lung epithelium (Fryer et al., 1986). GSTP1 is a 2.8 kb gene located on chromosome 11q13, a known “hot spot” for asthma-related genes (Doull et al., 1996; Kano et al., 1987). A single nucleotide polymorphism at position 313 in GSTP1 converts an adenine to a guanine (A→G) (Ahmad et al., 1990). The resulting isoleucine to valine substitution in codon 105 of exon 5 (Ile105→Val105) significantly lowers GST enzyme activity (Watson et al., 1998).

Delineating the factors that are contributory or protective to persistent wheezing in early childhood is critical to advance our understanding of asthma. There is limited information about how genetic and environmental factors interact to influence longitudinal asthmatic/wheezing status over time. DEP, ETS, and mold exposures are common and each has been shown to
aggravate respiratory symptoms. The gene-environment effect related to these individual and/or combined exposures has not been evaluated with regard to longitudinal wheezing status. The purpose of this study was to investigate whether exposure to DEP, ETS, and/or mold uniquely modifies wheezing and persistent wheezing in young children, especially among those with the \textit{GSTP1} I105V polymorphism. Our study evaluates the modified effect of this polymorphism upon exposure to not only ETS and mold, but distinctively DEP exposure associated with traffic and their combined exposures utilizing the well-characterized Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS) birth cohort.

\textbf{Methods}

\textit{Study Participants}

The CCAAPS study is a longitudinal birth cohort of high-risk children having at least one atopic parent. A complete description of the study’s recruitment, methods, and objectives has been published (LeMasters et al., 2006). Briefly, infants with at least one atopic parent (based on allergy skin prick testing) were enrolled between 2001 and 2003 in a seven county area of Cincinnati, Ohio. Families were recruited based on the proximity of their home residence to truck and bus traffic by geocoding residential addresses located on birth records (Figure 1; online). All infants recruited for the CCAAPS study were carried to term (>35 weeks), and no premature infants were eligible. Parental asthma diagnosis history and shortness of breath symptoms were collected at the time of the parent skin prick test (SPT). Infant subjects were evaluated by skin prick testing with a panel of 15 aeroallergens and two foods (egg white and milk) at both 12 and 24 months of age. Annual questionnaires administered to parents with regards to infant respiratory symptoms were adapted from the International Study of Asthma and
Allergies in Children (ISAAC) (Asher et al., 1995). At the time of recruitment, administered questionnaires also collected information on household smoking habits and demographics. This study was approved by the Institutional Review Board.

**DNA Collection and GSTP1 Gene Polymorphism Genotyping**

Buccal cells were collected using a nylon bristle cytology brush. Genomic DNA was isolated using the Zymo Research Genomic DNA II Kit™ (Orange, CA). Genotyping was accomplished using the LightTyper platform (Roche Diagnostics, GmbH, Mannheim, Germany). The PCR primers (GSTP1 Forward: 5’-TGGACATGGTGAGATGACGGCG-3’ and GSTP1 Reverse: 5’-GGTCAGCCCAAGCCACCT-3’) and hybridization probes (5’-LCR640-AGGGAGACGTATTTGCAGCGGAGG-3’ and 5’-ACCCTGGTGCAGATGCTCACATAGTTGGTGAGFL-3’) were designed using the LightCycler Probe Design Software 2.0 (Roche Diagnostics, GmbH, Mannheim, Germany). Genotypes were confirmed by randomly re-genotyping 10% of the population. Genotypes were dichotomized to carriers and non-carriers of the Val^{105} allele.

**Outcome Definitions**

Parents were asked the following ISAAC adapted question: “In the past 12 months, have you ever noticed your child wheezing?” Infant wheezing at ages 12 and 24 months was defined as parental report of the child wheezing at the respective study visit one or more times in the past 12 months. Persistent wheezing was defined as parental report of the child wheezing at both the 12- and 24-month visits.
Environmental Exposure Definitions

The environmental exposures evaluated were DEP, ETS, and visible mold. Average daily levels of DEP at each infant’s home were calculated using a land-use regression (LUR) model of exposure as previously described (Ryan et al., 2007). Briefly, ambient levels of fine particulate matter with aerodynamic diameter <2.5 µm (PM$_{2.5}$) were measured at 24 sampling sites located throughout the greater Cincinnati, Ohio metropolitan area. The PM$_{2.5}$ chemical composition has been previously described (Martuzevicius et al., 2004). Elemental carbon was measured at these different monitoring sites and estimated source signatures in the airshed were determined to determine how much elemental carbon was attributable to traffic alone. This estimate was used to estimate truck and bus DEP exposure as previously reported (Hu et al., 2005). Geographic, traffic, and land-use data within 400 meters (m) of each sampling site was collected in a geographic information system (GIS). From these data a LUR model with a coefficient of determination ($R^2$) of 0.75 was developed that included elevation, number of trucks within 400 m of the sampling site, and the length of bus routes within 100 m of the sampling site. The estimated model parameters were subsequently applied to the same geographic variables determined for each infant’s home residence at the time of study enrollment when they were approximately seven months of age. This estimate was used to obtain unique estimates of their early life exposure to DEP. The median exposure to DEP was estimated to be 0.34 µg/m$^3$ (range=0.23-0.88). The level of 0.5µg/m$^3$ was chosen to determine high vs. low exposure based upon the distribution of estimated DEP and prior results indicating an approximate 2-fold increased risk for wheezing at 12 months at this exposure level, and this level represented the top quintile (Ryan et al., 2007). The LUR model was further evaluated deriving a LUR model with 6 sampling sites removed. The estimated DEP was subsequently compared to the sampled DEP.
and was generally found to slightly underpredict the sampled values (manuscript currently in review).

Infants were defined as exposed to household ETS if the parent reported at least one smoker (person that smoked one or more cigarettes per day) living in the infant’s home. Infants were defined as exposed to mold if an in-home trained professional observed any visible mold, water damage, or moldy odor at the time of the home evaluation, generally before the infant’s first birthday (Cho et al., 2006). Infants in homes that did not meet any of these criteria were considered unexposed to visible mold. Multivariate models were adjusted for race (Caucasian vs non-Caucasian) and gender. In analyses evaluating wheezing at 12 and 24 months, daycare attendance was also defined at 12 and 24 months.

**Statistical Analysis**

Racial differences for demographics, environmental exposures, health outcomes, and genotype and allele frequencies were compared using the chi-squared statistic. Unadjusted odds ratios (OR) and 95% Confidence Intervals (95% CI) were calculated using logistic regression to evaluate the univariate associations between outcomes, environmental exposures, and genotype. Three-way contingency tables were used to assess associations of environmental exposures, genotype, and health outcomes stratified by both genotype and exposure. Due to the significant difference in allele frequency between racial groups, a race-stratified analysis was also performed for Caucasians and non-Caucasians. The associations of DEP, ETS, and visible mold exposures and genotype with each outcome were evaluated using conditional logistic regression adjusting for daycare attendance, race, and gender. Since parent education, income, and health insurance were all highly correlated with race, we chose to adjust only for race. Race, however,
was not found to be a significant covariate in adjusted models evaluating the independent associations of DEP (p=0.46), ETS (p=0.46), and mold (p=0.44) with wheezing at 12 months of age. Figure 2, therefore, present associations for racial groups combined between environmental exposures and GSTP1 genotype. All possible gene-environment interactions to evaluate the effect modification of DEP, ETS, and mold exposure by genotype were evaluated in each model. An interaction was removed from the model if the p-value was greater than 0.20. All analysis was performed using SAS software (version 8.2 for Windows, SAS Institute, Cary, NC, USA).

Results

Subjects, exposures, and health outcomes

Of the 570 study participants, 464 (81.4%) infants were Caucasian and 106 (18.6%) were non-Caucasian (Table I). Of the non-Caucasian infants, 86.8% were African Americans defined as both parents being African American. Non-Caucasian infants were significantly more likely than Caucasians to have a household income less than $40,000 (69.5% versus 26.7%; p<0.001), have higher exposure to DEP ≥0.50 μg/m³ (p<0.001), and have visible mold in their homes (p=0.01). ETS exposure did not significantly differ between the racial groups. Wheezing at 24 months of age (34.6% versus 17.2%) and persistent wheezing (24.6% versus 10.9%) were significantly increased in non-Caucasian versus Caucasian infants.

Effect of exposure on wheezing phenotypes

Exposure to DEP ≥0.50 μg/m³ increased the risk of wheezing at 24 months (OR=2.15, 95% CI=1.24-3.55) and persistent wheezing (OR=2.41, 95% CI=2.29-4.51) (data not shown). Similarly, ETS exposure was associated with wheezing at 12 months (OR=1.73, 95%
CI=1.15-2.62), 24 months (OR=2.15, 95% CI=1.34-3.44), and persistent wheezing (OR=1.80, 95% CI=1.02-3.20). Mold exposure was significantly associated with infant wheezing at 24 months (OR=1.76, 95% CI=1.09-2.85) and persistent wheezing (OR=2.00, 95% CI=1.07-3.71). A comparison of the wheezing percentage among all infants versus those stratified by race revealed similar trends.

**GSTP1 alleles in study subjects**

The GSTP1 allele frequencies were significantly different (p=0.002) among Caucasians (Ile=67.2%, Val=32.8%) and non-Caucasians (Ile=56.1%, Val=43.9%). Similar allelic differences were noted in other studies (Watson et al., 1998). Overall, 81.4% of the children were homozygous for the GSTP1 Ile<sup>105</sup> allele, and 18.6% were carriers of the Val<sup>105</sup> allele. When children carrying at least one Val<sup>105</sup> allele (Ile/Val or Val/Val) were combined and compared to those homozygous for the Ile<sup>105</sup> allele, the non-Caucasian children were significantly more likely than Caucasians to be carriers of the Val<sup>105</sup> allele (21.9% versus 14.7%; p=0.02). GSTP1 genotype data were in Hardy-Weinberg equilibrium when stratified by race and gender.

**GSTP1 and environmental exposures**

After stratifying by genotype, high DEP exposure (≥0.50 µg/m³) was associated with an increased risk for wheezing among carriers of the Val<sup>105</sup> allele (Figure 2A). Again, racial groups were combined since race was not found to be a significant covariate in evaluating the independent associations of each environmental exposure with wheezing at 12 months of age. The Ile/Ile genotype conferred a protective effect against wheezing when infants were exposed to high amounts of DEP. This finding was consistent at 12 months (p=0.01), 24 months (p=0.002),
and with persistent wheezing (p=0.003) (Figure 2A). Similarly, carriers of the Val\textsuperscript{105} allele were significantly more likely to wheeze at 12 and 24 months if they were exposed to ETS (p=0.05 and p=0.01, respectively) compared to those infants with the protective Ile/Ile genotype (Figure 2B). Carriers of the Val\textsuperscript{105} allele exposed to visible mold were also significantly more likely to wheeze at 24 months of age (p=0.04) (Figure 2C).

The effect of each exposure (DEP, ETS, and visible mold) was independently evaluated with respect to wheezing at 12 months, 24 months, and persistent wheezing after adjusting for genotype, daycare attendance, race, and gender (Table II). High DEP exposure (≥0.50 μg/m\textsuperscript{3}) conferred a significant risk for persistent wheezing (OR=2.13, 95% CI 1.11-4.07). There was a trend noted between high DEP exposure (≥0.50 μg/m\textsuperscript{3}) and \textit{GSTP1} genotype on wheezing (DEP-\textit{GSTP1} interaction, p=0.08; Table IIA). Similar interactions between ETS and visible mold exposures with the \textit{GSTP1} Ile/Val or Val/Val genotypes were not observed and were subsequently removed from the models (Table IIB and C). ETS exposure was significantly associated with wheezing at both 12 (OR=1.78, 95% CI=1.17-2.71) and 24 months of age (OR=2.06, 95% CI=1.27-3.35), and an elevated risk was observed for persistent wheezing (Table IIB). Visible mold exposure significantly increased the risk of wheeze at 24 months (OR=2.18, 95% CI=1.30-3.63) and persistent wheezing (OR=2.57, 95% CI=1.33-4.96) (Table IIC). Thus, all three exposures were significant in a univariate model.

\textit{Multiple exposures associated with wheezing}

We next adjusted for all three exposures simultaneously as well as genotype, daycare attendance, race, and gender (Table III). In this model, high DEP exposure (≥0.50 μg/m\textsuperscript{3}) was associated with wheezing at 24 months (OR=1.93, 95% CI=1.06-3.53) and with persistent
wheezing (OR=2.13, 95% CI=1.03-4.41). Exposure to ETS was significantly associated with wheezing at 12 (OR=1.73, 95% CI=1.11-2.70) and 24 months (OR=1.90, 95% CI=1.13-3.18). Similarly, visible mold exposure was associated with wheezing at 24 months (OR=2.12, 95% CI=1.25-3.60) and persistent wheezing (OR=2.47, 95% CI=1.27-4.80) (Table III).

Environmental exposure load overwhelms genotype effect

In order to evaluate the relationship of total environmental “load” with wheezing, the additive or synergistic effect of having none, one, or two or more environmental exposures (DEP, ETS, or mold) was investigated. One-third (32.2%) of CCAAPS infants were not exposed to any of the three environmental exposures whereas 43.2% were exposed to one pollutant and 25.6% were exposed to two or more. Carriers of the Val\textsuperscript{105} allele were found to be at risk for wheezing if they were exposed to two or more pollutants compared to unexposed infants at 12 months (p=0.02), 24 months (p<0.01), and with persistent wheezing (p<0.01). In addition, infants homozygous for the Ile\textsuperscript{105} allele exposed to two or more air pollutants were at significantly increased risk for wheezing compared to those who were not exposed at 24 months (p=0.03) and with persistent wheezing (p=0.01) (Figure 2D). The protective effect of the Ile/Ile genotype against wheezing previously observed with each of the individual exposures alone disappears when infants have multiple exposures. This trend is most evident with DEP exposure alone, but this same trend can be seen when infants are exposed to any one exposure (Figure 2). Environmental load was not significantly different between races (Table I). Clearly, long-term exposure to more pollutants places the infant at greater risk (almost 4 fold) of persistent wheezing irrespective GSTP1 genotype (Table IID).
Discussion

To our knowledge, this study is the first to investigate the impact of complex environmental exposures (DEP, ETS, and mold) along with genetics, specifically GSTP1, on persistent wheezing in children. Our data supports that DEP, ETS, and/or mold exposure are risk factors for wheezing by 24 months of age. Furthermore, the presence of the Val\textsuperscript{105} allele, which has been shown to significantly lower GST enzyme activity (Watson et al., 1998), confers susceptibility to these environmental exposures compared to the Ile\textsuperscript{105} allele. The Ile/Ile GSTP1 genotype conferred protection against wheezing among the DEP exposed group, however, infants exposed to multiple environmental exposures were significantly more likely to persistently wheeze irrespective of genotype. Thus, the Ile\textsuperscript{105} GSTP1 genotype may confer protection from persistent wheezing, but strong environmental exposure converging on a similar pathway may overwhelm the genetic effect.

Other investigations have reported associations between asthma and the GSTP1 polymorphism (Gilliland et al., 2002a; Gilliland et al., 2006; Gilliland et al., 2002b). The Val\textsuperscript{105} allele was shown to have a protective effect in children age 8 to 11 years against respiratory illness (Gilliland et al., 2002b), and in adults age 20 to 34 years exposed to DEP and secondhand smoke against increased nasal allergic responses (Gilliland et al., 2006). Contrary to these studies, our study examined infancy and early childhood, a period of time in which the lung undergoes critical development and asthma symptoms are just beginning to develop. Early childhood wheezing and early persistent wheezing may be a precursor to asthma in these young children. One limitation of this study is that it is difficult to definitively determine the cause of wheezing in this young age group. The use of the well-studied ISAAC-adapted questions was used to characterize wheezing as a precursor to asthma.
One mechanism by which environmental exposures may lead to lung injury is by inducing inflammatory cells to generate reactive oxygen species (ROS) leading to oxidative injury (Owen et al., 1991; Riedl and Diaz-Sanchez, 2005; Vachier et al., 1992). DEP, ETS, and mold are three common environmental exposures that lead to increased generation of ROS and have been shown to cause respiratory symptoms (Biagini et al., 2006; Cho et al., 2006; Ryan et al., 2007). DEP are respirable, with over 90% in the fine (0.1-2.5 μm) or ultrafine (<0.1 μm) size range. The DEP are composed of elemental carbon cores with large surface areas capable of binding organic polycyclic aromatic hydrocarbons and transition metals, which have the potential to induce ROS (Riedl and Diaz-Sanchez, 2005). Cigarette smoke has also been shown to contain a high concentration of ROS (Huang et al., 2005). Studies have demonstrated an association between ETS exposure during early childhood with the subsequent development of asthma (von Mutius, 2002). Mold exposure has also been associated with increased intracellular levels of ROS (Jussila et al., 2001) and respiratory illness in children (Jaakkola et al., 1993). Though the mechanism by which DEP, ETS, and mold may contribute to the development of asthma and asthma symptoms is unknown, there is mounting evidence implicating oxidative stress as a contributor to the airway inflammatory response (Leem et al., 2005; Nel et al., 2001). GSTs can neutralize the electrophilic sites of reactive oxygen species (ROS) by conjugation to the tripeptide thiol, glutathione (GSH), which has an electron-donating capacity. The resulting product is more water-soluble promoting ROS detoxification and thereby protecting the lung from oxidative damage (Hayes and Pulford, 1995). This may be one mechanism for the observed genetic effect of GSTP1 in this study. We cannot rule out that the observed association between GSTP1 and wheezing is due to a linked polymorphism in the same gene and/or another gene. Another limitation of the study is the relatively small sample size of non-Caucasian children in
the cohort (N=106). Race was not found to be a significant covariate in multivariate models evaluating independent associations of the three environmental exposures with wheezing at 12 months resulting in the combining of the racial groups. Although ideally it would be beneficial to stratify by race, the power of the study would have been severely compromised, particularly for African-Americans.

An important strength of this study is the longitudinal birth cohort design. In particular, DEP estimate exposure using multiple monitoring sites and a LUR model is unique to the CCAAPS cohort. Given the importance of early-life exposures, DEP, ETS, and mold exposure were determined through age two. Although we did collect data regarding treatment in the wheezing infants, we did not have complete data on all the children. In-utero smoke exposure was an independent risk factor for wheezing at 12 and 24 months but not persistent wheezing in only the Caucasian infants. There was no association among the non-Caucasian infants. We recognize that one limitation of the exposure assessment is that infants are not only exposed in the home. In order to address this, we adjusted for daycare attendance (also used as a proxy for exposure to respiratory infections). Future analyses will consider cumulative exposures and effect on asthma development as children age.

Overall, the relationship between DEP and wheezing was found to be stronger at 24 months of age than 12 months suggesting that longer exposure results in an elevated risk of wheezing. Interestingly, ETS exposure at 12 months of age was associated with wheezing at both 12 and 24 months of age suggesting that a shorter exposure time is needed to see an effect in infants. Since infants’ lungs are still developing, household ETS exposure is generally much closer to the child’s personal living space than is exposure to traffic exhaust and therefore highly likely to have a stronger impact during this sensitive stage of development. This reflects the
findings of a recent study that reported exposure to parental smoking during the first year of life is associated with persistent wheezing (De Sario et al., 2006).

In conclusion, these data suggest that the \textit{GSTP1} genotype should be considered when evaluating asthma/wheezing in young children exposed to high DEP levels, ETS, and/or visible mold. These data provide evidence that carriers of the Val\textsuperscript{105} allele may be more susceptible to high DEP exposure with regard to the development of persistent wheezing. High environmental load converging on an oxidative stress pathway may overwhelm the genetic effect.
References


variations of PM2.5 concentration and composition throughout an urban area with high freewy density-the Greater Cincinnati study. Atmospheric Environment. 38, 1091-1105.


| Table I. Demographics, Exposures, and Health Outcomes of Infants. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Total           | Caucasian       | Non-Caucasian   | p-value         |
| **Gender**      |                 |                 |                 |                 |
| Male            | 298 (52.3)      | 244 (52.6)      | 54 (50.1)       | 0.76            |
| Female          | 272 (47.7)      | 220 (47.4)      | 52 (49.1)       |                 |
| **Family Income** |             |                 |                 |                 |
| < $40,000       | 196 (34.7)      | 123 (26.7)      | 73 (69.2)       | <0.001          |
| ≥ $40,000       | 369 (65.3)      | 337 (73.3)      | 32 (30.5)       |                 |
| **Daycare Attendance at 12 months** |         |                 |                 |                 |
| Yes             | 43 (7.6)        | 35 (7.6)        | 8 (7.7)         | 0.96            |
| No              | 524 (92.4)      | 428 (92.4)      | 96 (92.3)       |                 |
| **Daycare Attendance at 24 months** |         |                 |                 |                 |
| Yes             | 70 (12.4)       | 54 (11.7)       | 16 (15.4)       | 0.30            |
| No              | 497 (87.6)      | 409 (88.3)      | 88 (84.6)       |                 |
| **DEP Estimate (binary)** |             |                 |                 |                 |
| < 0.50 µg/m³   | 469 (82.3)      | 396 (85.3)      | 73 (68.9)       | <0.001          |
| ≥ 0.50 µg/m³   | 101 (17.7)      | 68 (14.7)       | 33 (31.1)       |                 |
| **ETS Exposure** |             |                 |                 |                 |
| Yes             | 158 (28.5)      | 121 (27.0)      | 37 (34.9)       | 0.11            |
| No              | 396 (71.5)      | 327 (73.0)      | 69 (65.1)       |                 |
| **Mold Exposure** |             |                 |                 |                 |
| Yes             | 297 (56.5)      | 258 (59.2)      | 39 (43.3)       | 0.01            |
| No              | 229 (43.5)      | 178 (40.8)      | 51 (56.7)       |                 |
| **Environmental Load*** |             |                 |                 |                 |
| None            | 178 (32.2)      | 144 (31.0)      | 34 (32.1)       | 0.09            |
| One             | 246 (43.2)      | 209 (45.1)      | 37 (34.9)       |                 |
| Two or more     | 146 (25.6)      | 111 (23.9)      | 35 (33.0)       |                 |
| **Wheezing at 12 months** |         |                 |                 |                 |
| Yes             | 145 (25.7)      | 116 (25.6)      | 29 (31.5)       | 0.34            |
| No              | 383 (72.5)      | 320 (73.4)      | 63 (68.5)       |                 |
| **Wheezing at 24 months** |         |                 |                 |                 |
| Yes             | 99 (19.9)       | 72 (17.2)       | 27 (34.6)       | <0.001          |
| No              | 399 (80.2)      | 348 (82.8)      | 51 (65.4)       |                 |
| **Persistent Wheezing** |        |                 |                 | <0.001          |
| Yes             | 60 (12.9)       | 43 (10.9)       | 17 (24.6)       |                 |
| No              | 404 (87.1)      | 352 (89.1)      | 52 (75.4)       |                 |
| **Total n**     | 570            | 464            | 106             |                 |

*DEP estimate ≥ 0.50 µg/m³, ETS exposure, and/or mold exposure

**The total n is different from the n for each individual demographic because not all data was available for each subject.**
<table>
<thead>
<tr>
<th>Table II. Adjusted Odds Ratios (OR) for Infant Wheezing, Environmental Exposures, and GST-P1 Genotype.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. DEP Estimate Exposure</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Wheezing at 12 Months</td>
</tr>
<tr>
<td>DEP Estimate ≥ 0.59 µg/mL²</td>
</tr>
<tr>
<td>Il/Val or Val/Val Genotype</td>
</tr>
<tr>
<td>Daycare Attendance*</td>
</tr>
<tr>
<td>Non-Caucasian Race</td>
</tr>
<tr>
<td>Male Gender</td>
</tr>
<tr>
<td>DEP*Il/Val or Val/Val Genotype</td>
</tr>
<tr>
<td><strong>B. ETS Exposure</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Wheezing at 12 Months</td>
</tr>
<tr>
<td>ETS Exposure</td>
</tr>
<tr>
<td>Il/Val or Val/Val Genotype</td>
</tr>
<tr>
<td>Daycare Attendance*</td>
</tr>
<tr>
<td>Non-Caucasian Race</td>
</tr>
<tr>
<td>Male Gender</td>
</tr>
<tr>
<td>ETS*Il/Val or Val/Val Genotype</td>
</tr>
<tr>
<td><strong>C. Mold Exposure</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Wheezing at 12 Months</td>
</tr>
<tr>
<td>Mold Exposure</td>
</tr>
<tr>
<td>Il/Val or Val/Val Genotype</td>
</tr>
<tr>
<td>Daycare Attendance*</td>
</tr>
<tr>
<td>Non-Caucasian Race</td>
</tr>
<tr>
<td>Male Gender</td>
</tr>
<tr>
<td>Mold*Il/Val or Val/Val Genotype</td>
</tr>
<tr>
<td><strong>D. Environmental Lead</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Wheezing at 12 Months</td>
</tr>
<tr>
<td>Environmental Lead*</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>One</td>
</tr>
<tr>
<td>Two or more</td>
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<tr>
<td>Il/Val or Val/Val Genotype</td>
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<tr>
<td>Daycare Attendance*</td>
</tr>
<tr>
<td>Non-Caucasian Race</td>
</tr>
<tr>
<td>Male Gender</td>
</tr>
<tr>
<td>Total Lead*Il/Val or Val/Val Genotype</td>
</tr>
</tbody>
</table>

* Daycare attendance at 2 months for 12-month wheezing; attendance at 24 months for 24 month and persistent wheezing.

ns: not significant at the 0.05 level and therefore removed from model (interaction only).

ETS exposure, DEP estimate ≥ 0.59 µg/mL² and/or mold exposure.

ref: reference category.
Table III: Adjusted Associations Estimated as Odds Ratios (OR) of Infant Wheezing with Demographic and Environmental Factors.

<table>
<thead>
<tr>
<th></th>
<th>Wheezing at 12 Months</th>
<th></th>
<th>Wheezing at 24 Months</th>
<th></th>
<th>Persistent Wheezing</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>p-value</td>
<td>OR</td>
<td>95% CI</td>
<td>p-value</td>
</tr>
<tr>
<td>Male Gender</td>
<td>1.47</td>
<td>0.97-2.23</td>
<td>0.07</td>
<td>1.92</td>
<td>1.16-3.17</td>
<td>0.01</td>
</tr>
<tr>
<td>Non-Caucasian Race</td>
<td>1.20</td>
<td>0.69-2.07</td>
<td>0.52</td>
<td>2.54</td>
<td>1.36-4.75</td>
<td>0.00</td>
</tr>
<tr>
<td>Daycare Attendance*</td>
<td>1.88</td>
<td>0.91-3.91</td>
<td>0.09</td>
<td>1.81</td>
<td>0.95-3.47</td>
<td>0.07</td>
</tr>
<tr>
<td>DEP Estimate Exposure ≥ 0.50µg/m³</td>
<td>0.92</td>
<td>0.40-2.14</td>
<td>0.85</td>
<td>1.93</td>
<td>1.06-3.53</td>
<td>0.03</td>
</tr>
<tr>
<td>ETS Exposure</td>
<td>1.73</td>
<td>1.11-2.70</td>
<td>0.02</td>
<td>1.90</td>
<td>1.13-3.18</td>
<td>0.02</td>
</tr>
<tr>
<td>Mold Exposure</td>
<td>1.22</td>
<td>0.79-1.86</td>
<td>0.37</td>
<td>2.12</td>
<td>1.25-3.60</td>
<td>0.01</td>
</tr>
<tr>
<td>Hl/Val or Val/Val GST-P1 Genotype</td>
<td>0.94</td>
<td>0.59-1.47</td>
<td>0.77</td>
<td>1.25</td>
<td>0.76-2.07</td>
<td>0.38</td>
</tr>
<tr>
<td>Hl/Val or Val/Val GST-P1*DEP Estimate ≥ 0.50µg/m³</td>
<td>2.09</td>
<td>0.69-6.30</td>
<td>0.19</td>
<td>ns</td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>

* Defined as daycare attendance during the first year of life for wheezing at age 12 months, attendance during the second year of life for wheezing at 24 months of age and lifetime attendance for persistent wheezing.

ns: not significant at the 0.20 level and therefore removed from model (interaction only).

ETS, mold and/or high DEP

ref: referent category
Figure 1. Geographic location of CCAAPS infants’ homes. Infants lived within a 7-county area of Cincinnati, Ohio. Families were recruited on the basis of the proximity of their home residence to truck and bus traffic by geocoding residential addresses located on infant birth records.
Figure 2. Individual environmental exposures, \textit{GSTPI} genotype, and infant wheezing. \textbf{A}, High DEP estimate exposure levels were significantly associated with wheezing at 12 months, 24 months, and with persistent wheezing only in infants carrying the Val$^{105}$ allele. \textbf{B}, ETS exposure was significantly associated with wheezing at 12 and 24 months only in infants carrying the Val$^{105}$ allele. 
\textbf{C}, Mold exposure was significantly associated with wheezing at 24 months in infants carrying the Val$^{105}$ allele. 
\textbf{D}, Environmental load, \textit{GSTPI} genotype, and infant wheezing. 
At 12 months, only infants carrying the Val$^{105}$ allele and who were exposed to 2 or more exposures were significantly likely to wheeze. At 24 months and with persistent wheezing, all infants despite their genotype were significantly likely to wheeze when exposed to 2 or more exposures. *P value < 0.05.
CHAPTER 3

Down-regulation of Glutathione S-transferase Pi in Asthma Contributes to Enhanced Oxidative Stress*

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* Manuscript in preparation
Abstract

GSTPi is one of the predominant detoxification enzymes in the lung. While evidence implicates an important role for GSTPi in asthma, the regulation of GSTPi expression and its contribution to oxidative stress in asthma has not been delineated. Both GSTPi expression and total GST activity were significantly attenuated in the lungs of mice following allergen and IL-13 exposure. IL-13 was sufficient by not necessary for the observed down-regulation of GSTPi expression and total GST activity. Nrf2, a transcription factor upstream of GSTPi, was found to display the same expression pattern as GSTPi in these models. GSTPi expression and GST activity were down-regulated in these asthma models via STAT6 dependent and independent mechanisms. This finding extended to human asthma whereby GSTPi expression was attenuated in asthmatic children compared to non-asthmatic children. GSTPi-deficient mice exhibited increased oxidative stress (increase in %CySS) compared with wild-type mice following allergen challenge. Collectively, these data suggest that down-regulation of GSTPi activity following allergen treatment may result in a decreased ability to neutralize oxidative stress intermediates and consequently, contribute to the asthma phenotype. These data further support that GSTPi may be an important therapeutic target for the oxidative stress observed in children with asthma, especially severe asthma.
Introduction

Asthma, a chronic inflammatory disorder of the airways, is a major public health concern, especially in children (Hewer, 1998). Asthma prevalence in children increased by 75% between 1980 and 2004, and it is now the most common chronic disease of childhood, affecting more than 9 million children (13%) in the U.S. (Bloom et al., 2006). A recent review of nearly 500 asthma gene association studies identified 25 genes associated with asthma phenotypes in six or more populations (Ober and Hoffjan, 2006). Among those consistently associated with asthma is the glutathione S-transferase (GST) family of genes, a major group of detoxification enzymes and antioxidants important in host defense against numerous environmental toxins and neutralization of reactive oxygen species (ROS) including those found associated with polluted air (Diaz-Sanchez and Riedl, 2005), cigarette smoke (Conklin et al., 2009), and mold (Huttunen et al., 2000; Jussila et al., 2001). Each of these has been implicated in the development of asthma (Larsson et al., 2001; Peden, 2000; Waegemaekers et al., 1989).

GSTs are a family of phase II enzymes. GSTs neutralize the electrophilic sites of compounds such as ROS by conjugating them to the tripeptide thiol, glutathione (GSH), which has electron-donating capacity. The resulting product is more water-soluble, allowing for successful detoxification and lung protection from potential damage caused by electrophilic compounds and products of oxidative stress (Hayes and Pulford, 1995). Consequently, GSTs play important roles in modifying peroxidase and isomerase activities that can generate these compounds and contribute to oxidative stress. For example, chemicals bound to the surface of DEP can induce ROS, some of which can be metabolized by GSTs (Hiura et al., 1999; Riedl and Diaz-Sanchez, 2005). In addition, exposure to allergens such as mold has been associated with increased intracellular levels of ROS (Huttunen et al., 2000; Jussila et al., 2001). GSTs are also
involved in detoxifying pathways of polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (Sheehan et al., 2001). Cigarette smoke has been shown to contain a high concentration of PAHs (Ding et al., 2006), and an association between a glutathione S-transferase Pi (GSTPi) polymorphism (rs1695), ETS, and lung cancer has been observed (Gilliland et al., 2006; Miller et al., 2003; Wenzlaff et al., 2005).

The main cytosolic GST expressed in lung epithelium, GSTP1 (GSTP1), is encoded by a 2.8-kb gene which maps to a region linked to the development of asthma: chromosome 11q13 (Doull et al., 1996; Fryer et al., 1986; Kano et al., 1987). GSTPi has been shown to play a unique, non-redundant role in total pulmonary GST activity (Conklin et al., 2009; Henderson et al., 1998). Many epidemiologic studies have implicated the GSTP1 Ile105Val polymorphism (rs1695) as a predictor for asthma (Aynacioglu et al., 2004; Carroll et al., 2005a; Carroll et al., 2005b; Fryer et al., 2000; Gilliland et al., 2002; Lee et al., 2005; Nickel et al., 2005; Schroer et al., 2008; Tamer et al., 2004). Although the contribution of the Val105 allele is not fully understood, the GSTP1 Val105 allele has been reported to have significantly lower GST enzyme activity (Watson et al., 1998). In addition, we have previously shown that exposure to DEP, environmental tobacco smoke (ETS), and mold each conferred an increased risk for wheezing in children that were carriers of the Val105 allele (Schroer et al., 2008). A recent study using mice deficient in both mouse GSTPi genes (GSTP1/P2-deficient mice (Henderson et al., 1998)) reported an increase in lung resistance compared to wild-type mice in response to ovalbumin treatment, further supporting a role for GSTPi in allergic airway disease (Zhou et al., 2008). Despite this and other evidence supporting a strong role for GSTPi in asthma, very little is known about the regulation of GSTPi in asthma.
GST expression has been shown to be an important factor in determining the sensitivity of cells to a wide range of toxic chemicals (Hayes and Pulford, 1995). The levels of GSTPi mRNA expression correlates to GST activity ($R^2=0.77$, $P<0.001$) (Bauer et al., 2006). The aim of this investigation was to determine how GSTPi expression and total GST activity are regulated in a mouse model of asthma and to identify the localization of GSTPi in the lung utilizing mouse models of asthma. Our data reveal that GSTPi expression and total GST activity are significantly down-regulated in the lung in a mouse model of asthma following either allergen or IL-13 treatment.

**Methods**

**Animals and care.** Animals were maintained in a pathogen-free vivarium and handled under institutional animal care using committee–approved procedures. Wild-type C57BL/6 and Balb/c mice were purchased from Jackson Laboratories (Bar Harbor, ME).

**Allergen treatment of mice.** An extract of house dust mite (HDM) (*Dermatophagoides pteronyssinus*) was purchased from Greer Laboratories (Lenoir, NC). Wild-type C57Bl/6 and Balb/c mice from The Jackson Laboratory (Bar Harbor, ME) along with IL-13$^{-/-}$ (Balb/c background) originally obtained from Andrew McKenzie (McKenzie et al., 1998) (Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom, STAT6$^{-/-}$ (Balb/c background) from The Jackson Laboratory (Bar Harbor, ME), and GSTPi$^{-/-}$ mice generated on a 129xF1 background (Henderson et al., 1998) and backcrossed for at least six generations with C57Bl/6 mice (obtained from Colin J. Henderson, PhD., University of Dundee) were immunized as previously described (Tabata et al., 2006). For the kinetic experiments, each
sequential intratracheal (IT) challenge was performed at one-week increments. One day after the last challenge, the AHR in response to acetylcholine (50 μg/kg) was measured as the airway pressure-time index (APTI), as previously described (Walters et al., 2002). After APTI measurement, blood, bronchoalveolar lavage fluid (BALF), and lung tissues were harvested.

Wild-type Balb/c mice were IT challenged with 100 μg of *Aspergillus fumigatus* (*Asp*) crude protein extract from Greer Laboratories (Lenoir, NC) on days 0, 2, 4, 7, 9, 11, 14, 16, and 18 (three times a week for 3 weeks). On day 19, APTI was used to measure AHR and blood, BALF, and lung tissues were harvested.

Wild-type Balb/c mice were intraperitoneally (IP) sensitized on days 0 and 14 with 50 μg ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO) with Imject Alum Adjuvant (Thermo Scientific, Rockford, IL) according to the manufacturer’s instructions. The mice were then IT challenged with 50 μg OVA on days 24 and 27. On day 29, APTI was used to measure AHR and blood, BALF, and lung tissues were harvested.

**IL-13 treatment of mice.** Wild-type C57Bl/6 and Balb/c mice, STAT6^-/-^ mice, and GSTPi^-/-^ mice and were IT challenged with 5 μg of hIL-13 (Peprotech, Rocky Hill, NJ) on days 0, 3, and 6. On day 7, APTI was used to measure AHR and blood, BALF, and lung tissues were harvested.

**Quantitative RT-PCR.** Total RNA from mouse lungs was isolated using TRIzol reagent (Invitrogen Corp, Carlsbad, CA), digested with RNase-free DNase, and purified with the RNeasy kit from Qiagen, Inc (Valencia, CA). cDNA was generated using the SuperScript® First-strand Synthesis System for RT-PCR (Invitrogen). Primers for mouse GSTPi (forward: 5’-ATCTTGAGACACCTTGGC-3’ and reverse: 5’-CCTTCACGTAGTCATTTTACC-3’)

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were designed using the LightCycler Probe Design Software 2.0 (Roche Diagnostics, GmbH, Mannheim, Germany). Primers for mouse GSTK1, GSTM1, GSTT1, Nrf2, and human GSTPi were previously published (Li et al., 2004; Rasmi et al., 2006; Ruiz-Laguna et al., 2006; Watanabe et al., 2009). The PCR products were quantified using the LightCycler 480 system with SYBR Green I Master Mix according to the manufacturer’s instructions (Roche Diagnostics, GmbH, Mannheim, Germany). Values were normalized to either GAPDH or 18S rRNA (Schmittgen and Zakrjasek, 2000).

**Total GST activity in mouse lung.** Enzymatic activity of GST was quantified using the GST assay kit according to the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO). Lung lysate was processed in 0.1 M sodium phosphate buffer, pH=6.5, containing 2 mM EDTA. The amount of protein in each lung lysate was analyzed using Coomassie Plus™ Protein Assay Reagent (Thermo Scientific, Rockford, IL). 10 µg of protein was used per sample to assess GST activity after conjugation to 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). GST activity was analyzed at 340 nm over a time period of 6 minutes. The linear range of detection for the kit was 0 to 0.25 mg/ml GST. Experimental OD$_{340}$ values fell within range of the GST control provided by the kit’s manufacturer.

**Sample collection and analysis of cysteine (Cys) and cystine (CySS).** Lung tissue was extracted and frozen immediately following the sacrifice of each mouse. Each sample was weighed and the lung tissue was crushed in 250 µl of glutathione (GSH) preservation solution containing perchloric acid (5% final), iodoacetic acid (13.4 mM final), boric acid (0.1 M final), and an internal standard γ-glutamyl-glutamate (γ-Glu-Glu) (5 nM final). Samples were vortexed
and centrifuged at 4°C for 1 minute at 13,000Xg. 300 μl of the supernatant were extracted and transferred to a fresh tube, and the pH of the sample was adjusted to 9.0 ± 0.2 with 3M KOH. After incubating at room temperature for 20 minutes, the samples were dansylated and stored in the dark for 24 hours at room temperature. The reactions were terminated by the addition of 300 μl of chloroform and 25 μl of the sample was used for high-performance liquid chromatography (HPLC) analysis (Yeh et al., 2008).

**GSTPi Immunohistochemistry.** Slide-mounted paraffin sections were deparaffinized, rehydrated, and antigen retrieval was performed in a steamer using high-pH target retrieval according to the manufacturer’s instructions (Dako, Denmark). After treating with diluted hydrogen peroxide to inactivate endogenous peroxidase activity and blocking using diluted donkey serum, slides were incubated at 4°C for 18 hours with a 1:150 dilution in PBS of rabbit anti-GSTPi antibodies (gift from Colin J. Henderson, PhD., University of Dundee). Sections were then washed with PBS and incubated with biotinylated secondary antibody directed against the primary antibody type. After washing with PBS, slides were developed using a peroxidase-labeled avidin detection system (Vector Labs, Burlingame, CA), coverslipped, and photographed using an RT Slider digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

**Statistical analysis.** All statistical analysis was performed using PRISM software (GraphPad Software Inc., La Jolla, CA). The statistical significance between two groups in figures 1, 2, 4A-B, 6A-C, and 9, was determined by a two-tailed t-test in which a P ≤ 0.05 was considered statistically significant. To determine the statistical significance between multiple groups in
 GSTPi expression and total GST activity are decreased in allergen (HDM, Asp, and OVA) challenged mice.

We examined the level of expression of GSTPi and total GST activity following allergen sensitization and challenge with HDM, Asp, or OVA in mice. In all models, mice displayed a significant increase in AHR measured by APTI following allergen treatments compared to PBS treatment as expected (data not shown). We observed a marked decrease in the level of GSTPi mRNA expression in the lungs of mice following HDM, Asp, or OVA challenge compared to control treated mice (Figure 1A, C, data not shown). We next examined total GST activity in the lungs and observed a decrease in total GST activity in the lungs of HDM treated mice (Figure 1B). This observation was not allergen-specific since a decrease in total lung GST activity was found following Asp treatment (Figure 1D). Direct application of PBS or up to 100μg HDM to this assay had no effect on GST activity (data not shown).

Expression of GST family members (GSTK1, GSTM1, and GSTT1) is decreased in allergen (HDM, Asp, and OVA) challenged mice.

Although it has been reported that GSTPi accounts for over 90% of the GST activity toward CDNB in the lung (Fryer et al., 1986), other family members may also contribute to the total activity. Thus, we defined the expression of the other GST family members also found to be expressed in the lung (GSTK1, GSTM1, and GSTT1). The mu and theta classes (GSTM1 and
GSTT1), like the pi class (GSTPi), are cytosolic GSTs whereas the kappa GSTs (GSTK1) are mitochondrial (Knight et al., 2007). Similar to GSTPi, lung transcript levels of these GST family members were also significantly attenuated following HDM, Asp, and OVA treatment when normalized to 18SrRNA (Figure 2A-C; data not shown).

**Kinetics of down-regulation of GSTPi expression and total GST activity following allergen challenge.**

In order to determine the kinetics of the observed decrease in GSTPi expression and total GST activity following acute and chronic allergen treatment, we analyzed the lungs of mice exposed to HDM after sensitization alone and after sensitization followed by weekly IT challenges for up to 11 weeks. There were no significant differences in either GSTPi expression or total GST activity levels following IP sensitizations. However, following the first IT challenge, there was a significant decrease in the GSTPi expression fold change (HDM treatment normalized to PBS treatment) (Figure 3A). The total GST activity fold change was significantly decreased by the second IT challenge (Figure 3B). Following only a single IT challenge (without prior sensitization), there was no significant difference in GSTPi expression between HDM and PBS treated mice (data not shown). After sensitization and weekly IT challenges for up to 7 weeks, GSTPi expression and total GST activity remained depressed. After 7 IT challenges, the fold changes between HDM and PBS treatments in both GSTPi expression and total GST activity ceased to be significantly different, and after 9 or 11 weekly challenges, there were no observed depression in GSTPi expression or total GST activity.
IL-13 is sufficient to suppress GSTPi mRNA expression but is not required for HDM suppression of GSTPi expression and total GST activity down-regulation.

IL-13 is a key mediator in the pathogenesis of allergic asthma (Wills-Karp et al., 1998). In order to elucidate the role IL-13 plays in regulating GSTPi transcript levels and total GST activity, we treated mice with IL-13. As expected, mice treated with IL-13 in this model displayed significantly increased AHR measured by APTI compared to PBS treated mice (data not shown). Similar to what we observed following allergen treatment, IL-13 treatment attenuated GSTPi transcript levels and total GST activity (Figure 4A, B). Like HDM, the IL-13 down-regulation of total GST activity was time-dependent. Measurement of total GST activity following each IL-13 treatment (1 IT, 2ITs, or 3ITs), the total GST activity fold change was significantly decreased following the third IL-13 treatment (Figure 4E).

To determine whether IL-13 was necessary for the observed decrease in GSTPi expression and total GST activity, IL-13-deficient mice were treated with HDM. HDM treatment of these mice resulted in decreased GSTPi expression and total GST activity (Figure 4C, D).

HDM suppression of GSTPi mRNA expression and total GST activity down-regulation is signal transducer and activator of transcription 6 (STAT6) independent.

IL-13 signaling mediates its effect through the Janus tyrosine kinase (JAK)/JAK-signal transducer and activator of transcription 6 (STAT6) pathway (Kaplan et al., 1996; Takeda et al., 1996). To elucidate whether HDM down-regulation of GSTPi was dependent on STAT6, STAT6-deficient mice were treated with HDM. As expected, HDM exposed wild-type mice display increased AHR measured by APTI, whereas STAT6-deficient mice treated with HDM do not display an increase in AHR (Kuperman et al., 1998). In response to allergen exposure,
STAT6-deficient mice have also been shown to have markedly attenuated migration of inflammatory cells into the airways including eosinophilic inflammation and attenuated mucus production (Akimoto et al., 1998). GSTPi transcript levels and total GST activity in the lungs of STAT6-deficient mice were significantly decreased after HDM treatment (Figure 5A, B). Thus, STAT6 was not required for the observed decrease in GSTPi expression and total GST activity after HDM treatment.

**IL-13 induced GSTPi expression and total GST activity down-regulation is STAT6 dependent.**

To specifically examine whether IL-13 down-regulation of GSTPi was dependent on STAT6, STAT6-deficient mice were treated with IL-13. IL-13 treatment of STAT6-deficient mice does not result in an increase in AHR, as previously reported (Kuperman et al., 2002). Interestingly, neither GSTPi expression nor total GST activity levels revealed any significant change following IL-13 treatment in STAT6-deficient mice (Figure 5C, D). This demonstrates that IL-13 induced down-regulation of GSTPi expression and total GST activity require STAT6 in contrast to HDM induced down-regulation.

**Nuclear factor-erythroid 2 related factor 2 (Nrf2) expression is decreased in allergen (HDM, Asp, and OVA) and IL-13 challenged mice.**

Nrf2 is a transcription factor involved in the transcriptional regulation of many antioxidant genes, including those involved in the GST pathway. Nrf2 was found to directly bind and activate the mouse GSTPi gene, indicating that Nrf2 acts upstream of GSTPi (Ikeda et al., 2002). In Nrf2-deficient mice, GSTPi expression is reduced, further supporting the contribution
Nrf2 makes to GSTPi expression (Rangasamy et al., 2005). Since Nrf2 is upstream of GSTPi and may be a potential mechanism for GSTPi down-regulation, we wanted to determine if Nrf2 was also down-regulated in mouse models of asthma. HDM treatment in mice resulted in decreased Nrf2 transcript levels compared to their control counterparts (Figure 6A). Similar to GSTPi expression, the observed decreased in Nrf2 transcript levels was not allergen specific but was also detected following Asp and OVA treatment in wild-type mice (Figure 6B, data not shown). In order to elucidate the contribution of IL-13 to Nrf2 down-regulation, mice were treated with IL-13. IL-13 treatment was sufficient to significantly decrease Nrf2 expression levels (Figure 6C). Furthermore, Nrf2 expression levels were significantly depleted in both IL-13- and STAT6-deficient mice treated with HDM (Figure 6D, E).

**Lungs of GSTPi-deficient mice treated with HDM have increased oxidative stress.**

Our data thus far supported that GSTPi expression and total GST activity was significantly down-regulated in our mouse model of asthma. In order to determine if the observed decrease was biologically relevant, we determined levels of oxidative stress in wild-type and GSTPi-deficient mice. The substrate that GSTs rely on to catalyze detoxification reactions, GSH, is produced from cysteine (Cys), glycine, and glutamate. Cys availability is often a limiting factor for the rate of GSH synthesis (Cotgreave and Gerdes, 1998). Cys and its oxidized disulfide form, cystine (CySS), represent the major extracellular thiol/disulfide redox control system in mammals (Jones, 2006). To establish the contribution of decreased total GST activity to Cys oxidation, the %CySS (CySS/(Cys+CySS)) was analyzed in the lungs of wild-type and GSTPi-deficient mice treated with HDM. An increase in %CySS is indicative of oxidative stress. The lungs of GSTPi-deficient but not wild-type mice treated with HDM had
approximately 25% more CySS compared to control treated mice (P<0.05), indicating increased oxidative stress following HDM treatment in GSTPi-deficient mice (Figure 7).

**Localization of GSTPi in the lung**

To determine the localization of GSTPi expression in the lung and the relevant cell type(s) in which it is regulated, GSTPi immunohistochemistry was performed on mouse lungs treated with PBS, HDM, and IL-13. GSTPi expression was expressed predominantly in epithelial cells, although there was also some expression in type II pneumocytes (Figure 8A). Interestingly, GSTPi expression appeared to be absent in goblet cells induced by either HDM or IL-13 in wild-type mice (Figure 8B, C). No staining was detected in GSTPi-deficient mice (Figure 8D-F). In contrast to wild-type mice, STAT6-deficient mice treated with HDM or IL-13 do not develop goblet cell hyperplasia. In these mice, GSTPi was mainly expressed in epithelial cells similar to wild-type mice treated with PBS (Figure 8G-I).

**GSTPi expression is decreased in nasal epithelial cells (NECs) of asthmatic children.**

Our data support a decrease in GSTPi expression in mouse models. To evaluate whether GSTPi was similarly down-regulated in children with asthma, we quantified GSTPi expression in RNA from NECs of control (non-atopic, non-asthmatic children) versus asthmatic children. Similar to the observed down-regulation of GSTPi expression in mouse models of asthma, GSTPi expression was also attenuated in NECs of asthmatic children compared to non-atopic, non-asthmatic controls (Figure 9).
Discussion

Previous studies support the relevance of GSTPi in asthma, however this is the first study to analyze the regulation and detection of GSTPi expression in asthma. Our findings demonstrate that GSTPi expression and total GST activity are down-regulated in mouse models of asthma. Similarly, our findings extend to human asthma whereby GSTPi expression was attenuated in asthmatic children. The down-regulation occurs via both STAT6 dependent and independent mechanisms. Specifically, IL-13, but not HDM induced GSTPi down-regulation was STAT6 dependent. This suggests that HDM can induce down-regulation of GSTPi expression and total GST activity by IL-13 and STAT6 autonomous pathways. This study also uncovers a functional role for GSTPi in regulating Cys oxidation, revealing that GSTPi plays an important role in neutralizing oxidative stress in asthma.

Since GSTPi is an enzyme that plays a critical role in cellular detoxification of endogenous and xenobiotic substrates and protection against oxidative stress, it is logical that evidence exists supporting a substantial role for GSTPi in allergic asthma, a disease caused by numerous electrophilic substrate-containing allergens. We had predicted that following allergen exposure there would be an increased necessity for GSTPi-driven detoxification in the lung, marked by an increase in GSTPi expression and total GST activity. Therefore, it was surprising to observe a decrease in GSTPi expression and total GST activity following allergen exposure. One possibility is that the mechanism by which GSTPi neutralizes allergen-induced oxidative stress is transient. Our kinetic experiments reveal that the down-regulation of GSTPi expression and total GST activity is time-dependent. A significant HDM-induced drop in GSTPi expression occurs after sensitization and a single IT challenge and remains low from 2IT challenges to 5IT challenges. However, this observation appears to be resolving by week 7 after which GSTPi
expression increases slightly with each consecutive IT. The fold change in total GST activity follows a similar trend. Taken together, our data reveal that the levels of GSTPi expression and total GST activity are dependent on the chronicity of allergen exposure.

The mechanism by which down-regulation of GSTPi expression and total GST activity occur likely involves Nrf2. HDM and IL-13 treated mice displayed decreased Nrf2 expression. IL-13-deficient and STAT6-deficient mice also display decreased Nrf2 expression following HDM exposure, which indicates that IL-13 contributes but is not necessary for HDM induced Nrf2 down-regulation and that this down-regulation is STAT6 independent. These data indicate that the mechanism of the down-regulation of GSTPi expression and total GST activity observed during asthma likely involves Nrf2.

Results from our studies also support previous data establishing a significant correlation between GST activity and the level of GSTPi mRNA expression (Bauer et al., 2006). We observed a decrease in GSTPi gene expression following sensitization and one IT challenge whereas the total GST activity did not decrease until after two IT challenges. This suggests that the observed decrease in GSTPi gene expression precedes the decrease in total GST activity. Surprisingly, we did not observe a change in GSTPi protein expression analyzed by western blot (data not shown). The assay used for measuring GSTPi protein expression in the lung may not be sensitive enough to detect modest changes in protein levels. Furthermore, all of the GSTPi protein may not be functionally active in total lung lysate, and the functional GSTPi may be altered following allergen exposure without changing the total amount of protein present. Hence, an assay specifically measuring functionally active GSTPi may be useful for future studies. It remains possible that HDM directly affects GST activity, however, this is unlikely since we see similar results with IL-13 as well.
The observed decrease in GSTPi expression and total GST activity is not likely due to the dilutional effect of migratory cells infiltrating the lung in response to allergen exposure because STAT6-deficient mice demonstrated similar decreases. STAT6-deficient mice have marked attenuation of lung inflammation, AHR, and mucus production (Akimoto et al., 1998; Malaviya and Uckun, 2002). Since STAT6-deficient mice have diminished airway inflammation, a decrease in GSTPi expression and total GST activity in STAT6-deficient mice treated with HDM indicates that this decrease is likely due to resident cells and not migratory cells.

Our data demonstrate a functional role for GSTPi in regulating Cys oxidation. In the presence of GSTPi, oxidative stress caused by HDM treatment was neutralized. As a result, there is no detectable change in oxidative stress marked by a difference in %CySS. However, in the absence of GSTPi, this balance was disrupted causing increased oxidative stress marked by an increase in %CySS following HDM treatment. A complete lack of GSTPi may cause regulation of Cys oxidation to be volatile, making it more difficult for the lungs to neutralize oxidative stress caused by HDM exposure. Since GSH is composed of Cys and GSTPi uses GSH as a cofactor for cellular detoxification, the increase in %CySS following HDM challenge in the absence of GSTPi suggests that GSTPi is important for GSH homeostasis. A recent study reported that severe asthmatic children were shown to have altered GSH homeostasis leading to oxidative stress (Fitzpatrick et al., 2009). Thus, down-regulation of GSTPi in wild-type mice caused by allergen exposure may lead to a disruption of GSH homeostasis causing enhanced oxidative stress.

Our study has revealed that GSTPi expression and total GST activity are dysregulated in mouse models of asthma. Furthermore, GSTPi plays an important role in neutralizing oxidative stress. Oxidative stress has been implicated as a key mechanism in the pathogenesis of asthma.
(Andreadis et al., 2003). The antioxidant and detoxification capacity of GSTPi and its potential role in GSH homeostasis suggest that the GSTPi pathway could be a critical therapeutic target for asthma and its symptoms in response to oxidative stressors found in the ambient air we breathe. In fact, GSH has already been shown to alleviate IL-13 induced asthma in mice (Lowry et al., 2008).

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Figure 1. Allergen exposure attenuates GSTPi expression and total GST activity in mouse lungs. (A, B) Wild-type mice were IP sensitized twice and IT challenged twice with HDM. (C, D) Mice were IT challenged nine times with Asp. (A, C) Total lung RNA was isolated to determine expression levels of GSTPi normalized to 18SrRNA by real-time RT-PCR. (B, D) Total lung protein was isolated to determine total GST activity using CDNB as a substrate. * indicate statistically significant differences (P<0.05), and values are the mean ± standard deviation. These data are representative of at least 3 experiments.
Figure 2. HDM exposure attenuates mouse lung expression of GSTK1, GSTM1, and GSTT1. (A-C) Mice were IP sensitized twice and IT challenged twice with HDM. Total lung RNA was isolated to determine expression levels of: (A) GSTK1, (B) GSTM1, and (C) GSTT1 normalized to 18SrRNA by real-time RT-PCR. * indicate statistically significant differences (P<0.05), and values are the mean ± standard deviation. These data are representative of at least 3 experiments.
**Figure 3. Kinetics of GSTPi regulation.** Mice were IP sensitized once, twice, or IP sensitized twice and IT challenged 1, 2, 3, 5, 7, 9, or 11 times with HDM. Data is represented as fold changes in (A) GSTPi expression or (B) total GST activity in HDM treated mice relative to PBS treated mice. (A) Total lung RNA was isolated to determine expression levels of GSTPi normalized to 18S rRNA by real-time RT-PCR. (B) Total lung protein was isolated from mouse lung to determine total GST activity using CDNB as a substrate. * indicates a significant difference between HDM and PBS treated mice (P<0.05). Under the most stringent conditions, adjusting for multiple comparisons using the Bonferroni correction (P<0.004), there are significant differences between HDM and PBS treated mice in both GSTPi expression and total GST activity after 2 and 3 IT challenges. Values are the mean ± standard deviation. N=3-5 mice per a treatment group.
A. GSTPI Expression Fold Change

IP Sensitization: 1 + + + + + + + +
# IT Challenges: 0 0 1 2 3 5 7 9 11

B. GST Specific Activity Fold Change

IP Sensitization: 1 + + + + + + + +
# IT Challenges: 0 0 1 2 3 5 7 9 11
Figure 4. IL-13 is sufficient but not necessary for HDM-induced suppression of GSTPi expression and total GST activity down-regulation. (A-B) Wild-type mice were IT challenged 3 times with IL-13. (A) Total lung RNA was isolated to determine expression levels of GSTPi normalized to 18SrRNA by real-time RT-PCR. (B) Total lung protein was isolated from mouse lung to determine total GST activity using CDNB as a substrate. (C-D) IL-13+/+ and IL-13−/− mice were IT challenged 3 times with HDM and analyzed for: (C) GSTPi expression (D) total GST activity. (E) Wild-type mice were IT challenged 1, 2, or 3 times with IL-13. Data are represented as fold changes in total GST activity in IL-13 treated mice relative to PBS treated mice. * indicate statistically significant differences (P<0.05). # indicate a significant difference between IL-13 and PBS treated mice (P<0.05). Values are the mean ± standard deviation. These data are representative of 2 to 3 experiments.
Figure 5. HDM-induced GSTPi expression and total GST activity down-regulation is STAT6 independent, but IL-13-induced down-regulation is STAT6 dependent. (A-B) STAT6+/- and STAT6-/- mice were IP sensitized twice and IT challenged twice with HDM. (A) Total lung RNA was isolated to determine expression levels of GSTPi normalized to 18S rRNA by real-time RT-PCR. (B) Total lung protein was isolated from mouse lung to determine total GST activity using CDNB as a substrate. (C-D) STAT6+/- and STAT6-/- mice were IT challenged 3 times with IL-13 and analyzed for (C) GSTPi expression and (D) total GST activity. * indicate statistically significant differences (P<0.05), and values are the mean ± standard deviation.
Figure 6. Expression of Nrf2 in Mouse Models of Asthma. Total lung RNA was isolated to determine expression levels of Nrf2 normalized to 18SrRNA by real-time RT-PCR. (A) Wild-type mice were IP sensitized twice and IT challenged twice with HDM. (B) Wild-type mice were IT challenged 9 times with Asp. (C) Wild-type mice were IT challenged 3 times with IL-13. (D) IL-13+/+ and IL-13−/− mice were IP sensitized twice and IT challenged twice with HDM. (E) STAT6+/+ and STAT6−/− mice were IP sensitized twice and IT challenged twice with HDM. * indicate statistically significant differences (P<0.05), and values are the mean ± standard deviation. These data are representative of 1 to 3 experiments.
Figure 7. Oxidative stress is increased in GSTPi−/− but not wild-type mice in an allergen induced asthma model. Wild-type and GSTPi−/− mice were IP sensitized twice and IT challenged twice with HDM. Lung tissue was collected for high-performance liquid chromatography (HPLC) analysis of Cys and CySS. * indicate statistically significant differences (P<0.05), and values are the mean ± standard deviation. Cys=cysteine, CySS=cystine
**Figure 8. Localization of GSTPi in the lung.** Wild-type, GSTPi<sup>−/−</sup>, and STAT6<sup>−/−</sup> mice were treated with PBS, HDM, or IL-13 and analyzed for GSTPi expression in the lung by immunohistochemistry. (A) Wild-type mice treated with PBS displayed positive GSTPi expression predominantly in epithelial cells and some GSTPi expression in Type II pneumocytes. (B, C) GSTPi expression was not detected in goblet cells of wild-type mice treated with HDM or IL-13. (D-F) No staining was detected in GSTPi<sup>−/−</sup> mice treated with either PBS, HDM, or IL-13. (G-I) GSTPi expression in STAT6<sup>−/−</sup> mice treated with PBS, HDM, or IL-13 was mainly found in epithelial cells and some expression in Type II pneumocytes. All images were captured at 100X and inset images were captured at 400X.
Figure 9. GSTPi expression in NECs. RNA was isolated primarily from human nasal epithelial cells as previously published (Guajardo et al., 2005). RNA was isolated to determine expression levels of GSTPi normalized to GAPDH by real-time RT-PCR. Children were divided into 2 groups: Control (non-atopic, non-asthmatic children) and Asthmatics (stable asthmatic children and acute asthmatic children experiencing an acute exacerbation of asthma and presenting to the Emergency Department). Values are the mean ± standard deviation. *P<0.05
CHAPTER 4

SUMMARY, DISCUSSION, AND UNPUBLISHED DATA
Asthma is the most common chronic disease among children, and evidence suggests that early-life exposure to aeroallergens can lead to the development of asthma symptoms later in life. However, since asthma is a complex genetic disease, identifying genes that demonstrate notable functional relevance has important gene-environment and therapeutic implications. The studies in this dissertation concentrate on the contribution of decreased GST activity to the development of asthma and the regulation of GSTPi expression and total GST activity in allergic inflammation. In this section, the results of the experiments presented in this dissertation will be summarized and associated questions will be discussed along with future studies and ongoing work.

**Gene-environment Interactions Between GSTP1 Ile105Val and Environmental Exposures**

The first aim of this study was to determine the gene-environment interactions in the Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS) birth cohort between the GSTP1 Ile105Val polymorphism and environmental exposures (DEP, ETS, and mold) in the development of wheezing. This GSTP1 polymorphism has been analyzed in relation to many different individual environmental exposures. However, this is the first study to analyze multiple, complex environmental exposures along with genetics on persistent wheezing in children. When we analyzed the environmental effect within a genotype, we found that children exposed to ETS, mold and particularly DEP exposure associated with traffic were significantly more likely to wheeze when they carried the GSTP1 Val105 allele. However, infants exposed to multiple environmental exposures were significantly more likely to persistently wheeze irrespective of genotype, indicating that strong environmental exposure converging on a similar pathway may overwhelm the genetic effect.
How do the functions of the \textit{GSTPI} Ile\textsuperscript{105} and Val\textsuperscript{105} alleles differ?

A comparison of the \textit{GSTPI} Ile\textsuperscript{105} and Val\textsuperscript{105} alleles using \textit{Escherichia coli} expression found GST enzyme activity to be reduced by approximately 30\% for the Val\textsuperscript{105} allele (Ali-Osman et al., 1997; Zimniak et al., 1994). Moreover, individuals carrying the \textit{GSTPI} Val\textsuperscript{105} allele have been shown to have significantly lower GST enzyme activity (Watson et al., 1998). While promising groundwork has been laid out by these studies, it would be advantageous to analyze GST activity specifically in the CCAAPS children to help further link a functional gene-environment interaction and possibly track their GST enzyme activity over time. In addition, utilizing transgenic mice that express the human versions of each of these alleles may give us more insight into their functional relevance in the development of asthma. A similar technique has recently been published examining a SNP in human metalloproteinase-1 (MMP-1) (Coon et al., 2009).

How do the gene-environment interactions between \textit{GSTPI} Ile105Val and environmental exposures change as the infants get older and become more exposed?

Contrary to our studies, the Val\textsuperscript{105} allele was shown to have a protective effect in children age 8 to 11 years against respiratory illness and in adults age 20 to 34 years exposed to DEP and secondhand smoke against increased nasal allergic responses (Gilliland et al., 2002; Gilliland et al., 2006). One potential reason for this contradiction is that our study examined infancy and early childhood, as opposed to late childhood and adulthood. Asthma is difficult to diagnose at a young age, but early childhood wheezing and early persistent wheezing may be a precursor to asthma in these young children. Furthermore, older children and adults have longer environmental exposure than infants. Our data revealed that multiple environmental exposures
can overwhelm the genetic effect since infants were significantly likely to persistently wheeze when exposed to two or more environmental exposures, regardless of their GSTP1 genotype. Appropriately, it is seems logical that despite the fact that the aforementioned studies examined specific environmental exposures, these older children and adults are very likely to be exposed to many environmental stimuli, which could contribute to the disparate findings. Indeed, when we analyzed the parents of the CCAAPS infants (N=743), we found that contrary to CCAAPS infant analysis, the Val\textsuperscript{105} allele conferred a protective effect against asthma when exposed to DEP, ETS, or mold (Figure 1A-C). It is possible that by being older, adults carrying the Val\textsuperscript{105} allele have had the opportunity to adapt to their GST enzyme deficiency by having other detoxification systems compensate for this loss over time. Interestingly, those parents carrying the Val\textsuperscript{105} allele still showed increased likelihood of asthma upon exposure to high DEP, ETS, or mold, although this trend was not significant. As expected, parents were significantly more likely to have asthma when exposed to two or more environmental exposures regardless of their genotype (Figure 1D). Accordingly, using a mouse model of asthma, our data support that with increased allergen exposure, there is a decrease in total GST activity.

An alternative explanation is that the GSTP1 genotype could play a different role in response to different environmental stimuli during the developing years of life compared to older children and adults. Infancy and early childhood is a period of time when the lungs are still undergoing critical development. It is possible that childhood asthma and adult asthma may be phenotypically different diseases (Martinez, 2007). A similar difference in age effect was found looking at the association of a defensin-β1 (DEFB1) gene polymorphisms and asthma (Levy et al., 2005). This reinforces the strength of the CCAAPS and its longitudinal birth cohort design. It
will be particularly interesting to follow these infants and their response to environmental exposures throughout their childhood.

**Regulation of GSTPi Expression and Total GST Activity In Mouse Models of Asthma**

Along with this study and the considerable amount of epidemiologic evidence that supports a strong role for GSTPi in asthma, a recent study using GSTPi-deficient mice showed an increase in lung resistance compared to wild-type mice, further supporting the link between GSTPi and asthma (Zhou et al., 2008). Despite this, very little is known about the regulation of GSTPi in asthma. Thus, in Chapter 3 the aim was to determine the regulation of GSTPi expression and total GST specific activity in mouse models of asthma. The study showed that GSTPi expression and total GST specific activity were attenuated in the lungs of mice following allergen treatment. IL-13 treatment in mice and use of IL-13-deficient mice indicate that IL-13 alone is sufficient to induce a decrease in GSTPi expression and total GST activity but IL-13 is not necessary. Interestingly, studies utilizing STAT6-deficient mice treated with allergen or IL-13 reveal that IL-13 mediated down-regulation of GSTPi expression and total GST activity are STAT6 dependent. Nrf2, a transcription factor that regulates GSTPi, was found to display the same expression pattern as GSTPi in these models. Therefore, our data suggest that down-regulation of GSTPi expression and total GST activity can occur by STAT6 dependent and independent mechanisms that likely involve Nrf2. The results of these studies raise several questions:
Why do GSTPi expression and total GST activity decrease in response to allergen exposure?

Since GSTPi is an important enzyme in the lung involved in detoxification and protection against oxidative stress, we had predicted that GSTPi expression and total GST activity would increase following allergen exposure. Thus, we were surprised to see that GSTPi expression and total GST activity decrease in response to allergen exposure. GSTP1 expression in the lung was shown to decrease following OVA treatment in another study as well (Zhou et al., 2008). One potential explanation for this is that the mechanism by which GSTPi neutralizes allergen-induced oxidative stress is transient as suggested by our kinetic experiments examining the timing of GSTPi expression and total GST activity down-regulation (Chapter 3 Discussion). The observed decrease in the level of GSTPi mRNA could be attributed to two possibilities: 1) transcription of the gene is decreased 2) the half-life of the GSTPi mRNA is decreased due to increased degradation of the transcript. To assess the rate of GSTPi transcription, a nuclear run-on assay could be used to compare control and allergen treated cells. A transient decrease in GSTPi expression and total GST activity may be an important signal to allow for repair prior to detoxification. An initial repair process may be critical to cells in response to damage occurring from allergen exposure that may hinder successful detoxification. Another possibility for GSTPi down-regulation is that GSTPi acts as an upstream modulator that may signal and activate other detoxification pathways. Thus, a decrease in GSTPi expression may indicate the initiation of downstream detoxification pathways. The surprising decrease in GSTPi expression and total GST activity could also indicate that GSTPi is maladaptive in response to allergen exposure.

Evidence from our data indicates that down-regulation of GSTPi expression and total GST activity can occur by at least two mechanisms. This study shows that one of these
mechanisms involving IL-13 is STAT6 dependent. As such, it would be interesting to evaluate genes that are differentially expressed in lungs of GSTPi-deficient mice that are IL-13 treated versus allergen treated using mRNA expression profiling by microarray analysis. This may help identify additional genes and pathways that interact with GSTPi. Interestingly, microarray analysis of lung tissue derived from GSTPi-deficient mice compared to wild-type mice indicated that many of the most up-regulated genes were found to be associated with asthma (Ritchie et al., 2007).

**Is Nrf2 activation required for GSTPi expression and total GST activity?**

Nrf2 was found to directly bind and activate the transcription of the mouse GSTPi gene, indicating that Nrf2 acts upstream of GSTPi (Ikeda et al., 2002). While the studies in this dissertation demonstrate that Nrf2 expression parallels GSTPi expression in mouse models of asthma, additional studies will be necessary to determine whether or not Nrf2 activation of GSTPi is required for GSTPi expression and total GST activity. Although it has been reported that Nrf2-deficient mice were found to have decreased GSTPi transcript levels compared to wild-type mice following OVA treatment (Rangasamy et al., 2005), this study shows that down-regulation of GSTPi expression and total GST activity can occur by at least two different mechanisms: one that is IL-13-induced and STAT6 dependent and one that is not. Therefore, it would be interesting to look at the levels of GSTPi expression and total GST activity in not only Nrf2-deficient mice treated with IL-13 but also in Nrf2- and STAT6-deficient mice.
Why is there a decrease in GSTPi mRNA expression and total GST activity but no decrease in GSTPi protein expression?

Since a decrease in GSTPi expression and total GST activity in mouse lung following allergen and IL-13 exposures was observed, a decrease in the amount of GSTPi protein was expected. However, as observed by the Western blot shown in Figure 2, no difference in GSTPi expression following HDM treatment was observed. As mentioned in the discussion section of Chapter 3, one reason is the fact that the Western blot is not sufficiently sensitive. This leads to another question: Is all the GSTPi protein detected functionally active? Since the GST activity assay measures total GST activity and not specifically GSTPi activity, it is difficult to interpret how much activity is actually due to GSTPi. In the future, a specific assay distinctively measuring GSTPi activity would be beneficial.

Another explanation is that GSTPi undergoes post-translational modification. *In vitro* evidence exists for different post-translational modifications of GSTPi such as phosphorylation at Thr109, Ser28, Ser154, and Ser184; *O*-glycosylation at Thr5; methylation at unknown sites; *N*-glycosylation at unknown sites (Johnson et al., 1992; Kuzmich et al., 1991; Tew, 2007). Although the biological relevance of these post-translational modifications has yet to be determined, there is initial data that imply these modifications have an effect on GST function. In particular, methylation of a GST subunit was found to inhibit the conjugating activity toward CDNB (Johnson et al., 1992). Furthermore, GST methyltransferase activity was detected in the lungs of rats (Johnson et al., 1992). Taken together, it is reasonable to hypothesize that post-translational modifications will influence GSTPi activity.

Since there was no difference in GSTPi protein expression in control versus HDM treated lungs, the decrease in total GST activity following HDM treatment could also be due to the
presence of an enzyme inhibitor. To address this, total GST activity could be measured in a sample that contained equal parts of lung lysate from HDM treated and PBS treated mice. In this experiment, if an inhibitor contributes to a decrease in total GST activity, we would expect the total GST activity of the mixed sample to be similar to that of HDM treated mice and lower than that of PBS treated mice. However, if a GST enzyme inhibitor is not present, we would expect the level of total GST activity of the mixed mouse sample to be approximately halfway in between the activity levels of PBS treated mice alone and HDM treated mice alone.

**What is the contribution of decreased GST activity to glutathione homeostasis?**

A recent report demonstrated that GSH homeostasis was altered in children with severe asthma leading to increased oxidative stress (Fitzpatrick et al., 2009). To evaluate the impact of the observed decrease in GSTPi expression and total GST activity to GSH homeostasis, the reduced glutathione to oxidized glutathione ratio (GSH/GSSG) in bronchoalveolar lavage fluid (BALF) of GSTPi-deficient and wild-type mice treated with HDM is currently being analyzed by high performance liquid chromatography (HPLC) (collaboration with Dr. Lou Ann Brown and Dr. Anne Fitzpatrick, Emory University). Increased oxidative stress characterized by a decreased GSH/GSSG ratio (low GSH or high GSSG) was recently observed in mice treated with OVA (Park et al., 2009). Consequently, it is predicted that mice treated with HDM will have a significantly lower GSH/GSSG ratio compared to mice treated with PBS. Furthermore, GSTPi-deficient mice are predicted to have a significantly lower GSH/GSSG ratio compared to wild-type mice following HDM exposure. These data would indicate a functional role for GSTPi in regulating GSH homeostasis. GSTPi uses GSH as a cofactor for cellular detoxification. Once GSH has donated its electron (reduced GSH), it is reactive and can be oxidized by binding to
another reactive GSH to become oxidized (GSSG). A build-up of GSSG can alter airway GSH homeostasis, leading to increased vulnerability to lung injury (Koike et al., 2007). Bronchial endothelial lining fluid has been shown to contain large amounts of GSH, 96% of which is in the reduced form (Cantin et al., 1987). Taken together, it is predicted that without GSTPi to bind the abundant reduced GSH, GSH homeostasis is altered, and this can lead to an increase in oxidative stress.

Localization of Pulmonary GSTPi Expression In Mouse Models of Asthma.

Consistent with previously published results, GSTPi IHC indicates that GSTPi is expressed predominantly in epithelial cells, including some expression in Type II pneumocytes (Conklin et al., 2009). Interestingly, based on analysis of GSTPi expression in mouse lungs using IHC, it also appears that goblet cells resulting from HDM or IL-13 treatment do not express GSTPi.

Is GSTPi expression and total GST activity down-regulated in human epithelial cells?

As discussed in Chapter 3, GSTPi expression was attenuated in human nasal epithelial cells (NECs) collected from Asthmatic versus Control children. This analysis was also evaluated with these children were separated into three different groups: 1) Control (non-atopic, non-asthmatic children), 2) Stable Asthmatics, and 3) Acute Asthmatics (children experiencing an acute exacerbation of asthma and presenting to the Emergency Department) (Guajardo et al., 2005) (Figure 3B). Although, while separating the Asthmatic group into Stable and Acute Asthmatics yields results that are not significant, a decrease in GSTPi expression in both groups of asthmatic children (Stable and Acute Asthmatics) compared to Control children is still
observed. Current work with Dr. Kelly Metz consists of actively recruiting more patients and increasing the sample size for each group in hopes of seeing a significant decrease in GSTPi expression among the asthmatic children. These results would further support the observed down-regulation of GSTPi expression in mouse models of asthma. To further examine GSTPi expression and total GST activity in epithelial cells in response to allergen exposure, human bronchial epithelial cells (HBECs) were treated with HDM (Figure 3A). Following HDM exposure, HBECs displayed attenuated GSTPi expression compared to PBS treated HBECs. This observation consistently reflects the GSTPi expression down-regulation seen in the lungs of mice using our mouse models of asthma. Given that multiple studies have observed a correlation between GSTPi expression and GST activity, it is predicted that total GST activity will also be significantly attenuated following HDM treatment. Furthermore, the use of HBECs helps place the observed down-regulation of GSTPi expression and total GST activity in mouse models of asthma into a human context.

**Is GSTPi expression and total GST activity down-regulation due to the dilutional effect of goblet cell hyperplasia following HDM treatment?**

The decrease in GSTPi expression and total GST activity is unlikely due to the dilutional effect of goblet cell hyperplasia following allergen treatment. It has been demonstrated that OVA-induced increases in mucus-containing cells are STAT6-dependent (Kuperman et al., 1998). Furthermore, STAT6-deficient mice develop attenuation of AHR, eosinophilic inflammation, and mucus production in response to ovalbumin (Akimoto et al., 1998). Consistent with these findings, STAT6-deficient mice treated with HDM in our mouse model of asthma do not acquire goblet cells. However, following HDM treatment, the lungs of STAT6-deficient mice
still display decreased GSTPi expression and total GST activity as shown in Chapter 3 despite the absence of goblet cells. This suggests that GSTPi expression and total GST activity down-regulation is not likely due to the presence of goblet cells following HDM treatment.

Do goblet cells express GSTPi?

Based on GSTPi IHC results in mouse lung, GSTPi expression appeared to be absent in goblet cells induced by either HDM or IL-13. To further solidify the observed absence of GSTPi expression in goblet cells, it would be important to analyze isolated goblet cells to determine if they have attenuated GSTPi expression and total GST activity. A mouse Clara cell line, mtCC1-2, has been shown to induce Mucin5ac (MUC5AC) promoter activation upon stimulation with IL-13 (Evans et al., 2004; Magdaleno et al., 1997). Clara cells are non-ciliated secretory epithelial cells that have been identified as goblet cell progenitors induced by pulmonary allergen exposure in vivo (Chen et al., 2009). MUC5AC is the major MUC gene expressed in goblet cells (Fahy, 2002). It would be interesting to analyze the levels of GSTPi transcript and protein expression as well as total GST activity in these cells upon stimulation. Based on GSTPi IHC results, it is expected that upon stimulation, mtCC1-2 cells will have no or very low GSTPi expression and total GST activity.

An alternative approach would involve the use of mice with conditional expression of SAM-pointed domain–containing Ets-like factor (SPDEF) (Park et al., 2007). SPDEF has recently been shown to regulate a transcriptional network that induces mouse goblet cell differentiation and mucus production (Chen et al., 2009). It is predicted that lungs from mice over-expressing SPDEF will have attenuated GSTPi expression and total GST activity compared to wild-type mice.
Is GSTPi expression and total GST activity down-regulation due to the dilutional effect from migratory cells present after HDM treatment?

As indicated in the discussion of Chapter 3, it is unlikely that the observed decrease in GSTPi expression and total GST activity is due to the dilutional effect of migratory cells infiltrating the lung in response to allergen exposure. As previously mentioned, in response to allergen exposure, STAT6-deficient mice have diminished AHR, markedly attenuated eosinophilic inflammation and migration of inflammatory cells into the airways, and attenuated mucus production (Akimoto et al., 1998). To further confirm that down-regulation of GSTPi expression and total GST activity is unlikely due to the dilutional effect of migratory cells, it would be beneficial to analyze total GST activity in whole lung lysates of wild-type and STAT6-deficient mice treated with PBS or HDM. Using whole lung lysate from these mice, total lung weight and protein concentration can be taken into account when calculating the total GST activity. Since STAT6-deficient mice have markedly attenuated migration of inflammatory cells into the airways, we can assess the involvement of migratory cells to total GST activity. We predict that migratory cells do not contribute the observed decrease in total GST activity following HDM exposure. As such, we would still expect a decrease in total GST activity in HDM treated wild-type and STAT6-deficient mice even after adjusting for total lung weight and protein.

Lessons From Human Genotypes and Mouse Models of Asthma

Investigating genotypes associated with disease outcome can be helpful in developing hypotheses regarding critical molecular mechanisms and signals that are impaired in disease. Epidemiologic studies analyzing the GSTPI Ile105Val polymorphism, including the one
presented in this dissertation, have identified a functional mutation in multiple human populations that is strongly associated with asthma. However, research involving human subjects has its limitations. Ethics and difficulties recruiting enough willing participants prove to be substantial hurdles when it comes to research involving human subjects. Fortunately, mouse models provide a relevant mammalian platform in which genetic manipulations can be performed, oftentimes mimicking human tendencies. The analysis of the GSTP1 Ile105Val polymorphism in the CCAAPS birth cohort led us to the hypothesis that GSTPi was regulated in allergic inflammation. To test this hypothesis, mouse models of asthma were utilized to perform these investigations in a more controlled manner. Collectively, the results indicate GSTPi expression and total GST activity are down-regulated in asthma. GSTPi expression was also found to be down-regulated in children with asthma, supporting that our observations in mouse models of asthma are applicable to human asthma. The mechanism by which GSTPi down-regulation contributes to asthma likely involves enhanced oxidative stress. Our results demonstrate that upon allergen exposure, GSTPi deficiency in mice results in increased oxidative stress, a key mechanism implicated in the pathogenesis of asthma (Andreadis et al., 2003). Increased oxidative stress has also been associated with severely asthmatic children shown to have altered GSH homeostasis (Fitzpatrick et al., 2009). Collectively, these data suggest that the decrease in GSTPi activity following allergen treatment results in a decreased ability to neutralize oxidative stress intermediates. These data also indicate that GSTPi may be an important therapeutic target for asthma and its symptoms in response to oxidative stressors. Interestingly, GSH has already been shown to alleviate IL-13 induced asthma in mice (Lowry et al., 2008). The results of this dissertation not only reinforce the importance of future research involving GSTPi and its role in asthma but also highlight the complementary nature of using
mouse models to understand disease etiology with human research to ultimately influence clinical care and disease treatment.
References


Figure 1. Individual environmental exposures, GSTP1 genotype, and parental asthma in the CCAAPS birth cohort.

DEP, ETS, mold, and environmental load exposure definitions are the same as previously published (Schroer et al., 2008). Parental asthma was defined as a history of asthma and current asthma symptom. The environmental effect within a genotype is analyzed and compared in each graph. **A**, High DEP estimate exposure levels were significantly associated with asthma in parents who were only carrying the Ile\textsuperscript{105} allele. **B**, ETS exposure was significantly associated with asthma in parents only carrying the Ile\textsuperscript{105} allele. **C**, Mold exposure was significantly associated with asthma in parents only carrying the Ile\textsuperscript{105} allele. **D**, Environmental load, GSTP1 genotype, and parental asthma. All parents despite their genotype were significantly likely to have asthma when exposed to 2 or more exposures. N=743 *P value < 0.05.
A. DEP exposure, GSTP1 genotype, and parental asthma.

B. ETS exposure, GSTP1 genotype, and parental asthma.

C. Mold exposure, GSTP1 genotype, and parental asthma.

D. Environmental Load, GSTP1 genotype, and parental asthma.
**Figure 2. Lung GSTPi protein expression does not change following HDM treatment.**

Total lung lysates were prepared in RIPA buffer and protein concentration was analyzed using Coomassie Plus™ Protein Assay Reagent (Thermo Scientific, Rockford, IL). 30 μg/lane total protein was separated by SDS-polyacrylamide gel electrophoresis. GSTPi was detected using a 1:1,000 dilution of an antibody raised to mouse GSTPi (gift from Colin J. Henderson, PhD., University of Dundee). Monoclonal anti-β-Actin antibody produced in mouse was used at a 1:10,000 dilution (Sigma-Aldrich, St. Louis, MO). Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies: anti-rabbit IgG-HRP for GSTPi and goat anti-mouse IgG-HRP for β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA). Bands were detected using Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK). GSTPi^{+/+} mice were IP sensitized twice and IT challenged twice with HDM. Lung protein from a GSTPi^{-/-} mouse was used as a negative control. Data is represented as GSTPi Arbitrary Units (AU) normalized to B-Actin AU. This data is representative of at least 3 experiments.
Figure 3. GSTPi expression in HBECs and NECs.

A, HBECs were continuously treated for 1 week with PBS or 250 μg/ml HDM. RNA was isolated to determine expression levels of GSTPi normalized to GAPDH by real-time RT-PCR. GSTPi expression in HDM treated HBECs was significantly less than PBS treated HBECs. These data are representative of 3 independent experiments. B, NECs were collected as previously published (Guajardo et al., 2005). Children were divided into 3 groups: Control (non-atopic, non-asthmatic children), Stable Asthmatics, and Acute Asthmatics (Children experiencing an acute exacerbation of asthma and presenting to the Emergency Department). Values are the mean ± standard deviation. *P<0.05