The Effect of Bisphenol A Exposure on Mast Cell Function
and Pulmonary Inflammation Associated with Asthma

by

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TABLE OF CONTENTS

Acknowledgements ii
List of Figures vi
List of Abbreviations viii
Abstract xiv
Chapter 1: Introduction 1
   Asthma 1
   Mast Cells 2
   Bisphenol A 6
   BPA Endocrine Disruption 11
   BPA Epigenetic Modification 14
   BPA and Inflammation Associated with Asthma 16
   Summary 19
   References 25
Chapter 2: Bisphenol A at Environmentally Relevant Concentrations Enhances Histamine and Cysteiny l Leukotriene Release from Bone Marrow-derived Mast Cells 37
   Abstract 37
   Introduction 38
   Materials and Methods 41
   Results 43
   Discussion 46
LIST OF FIGURES

Figure 1.1: BPA monomer is formed through a condensation reaction between phenol and acetone in a 2-to-1 ratio 22

Figure 1.2: The polymerization reaction to form polycarbonate plastic involves a reaction catalyzed by HCl in the presence of a 1-to-1 ratio of BPA monomer and phosgene, while polymerization to form epoxide resin involves a reaction catalyzed by NaOH in the presence of BPA monomer and epichlorohydrin, usually in a 1-to-1 ratio 23

Figure 1.3: Comparison of the molecular structures of BPA and E2 24

Figure 2.1: Percent increase in histamine release from BMMCs following 30-minute treatment with E2 or BPA, and CysLT release following 30-minute treatment with BPA 51

Figure 2.2: Percent increase in histamine release from BMMCs pretreated with ICI for 1 hour before treatment with 10 nM E2 or 10 nM BPA for 30 minutes, and CysLT release from BMMCs pretreated with or without 1 μM ICI before treatment with 10 nM BPA 52

Figure 2.3: Percent increase in histamine release and CysLT release from BMMCs pretreated with 10 μM U0126 or 3 mM EGTA for 1 hour before treatment with 10 nM BPA for 30 minutes 53

Figure 3.1: Levels of CysLTs and PGD$_2$ secreted after IgE crosslinking from BMMCs of animals exposed perinatally to BPA 75

Figure 3.2: Levels of TNF-α, IL-13, IL-4, IL-5, and IL-6 secreted after IgE crosslinking from BMMCs of animals exposed perinatally to BPA 76

Figure 3.3: Percentage increase in histamine release after IgE crosslinking from BMMCs of animals exposed perinatally to BPA, as compared to IgE-free control 77

Figure 3.4: Decreased levels of global DNA methylation in BMMCs from adult animals with perinatal BPA exposures 78

Figure 4.1: Anti-OVA IgE measured in sera of female and male offspring with developmental exposure to BPA and subsequent OVA challenge 107

Figure 4.2: Production of cytokines IL-13 and IFN-γ from stimulated splenocytes of male and female offspring with developmental exposure to BPA and subsequent OVA challenge 108
Figure 4.3: Airway influx of total leukocytes as measured in BALF of female and male offspring, airway influx of macrophages; PMNs; lymphocytes; and eosinophils as measured in BALF of female and male offspring, and percent composition of macrophages; PMNs; lymphocytes; and eosinophils among total leukocytes in BALF from female and male offspring all with developmental BPA exposure and subsequent OVA challenge

Figure 4.4: Airway production of cytokines IL-4, IL-13, TNF-α, and IL-17 measured in BALF of female and male offspring, airway production of CysLTs measured in BALF of female and male offspring, and lung concentration of RANTES measured in tissue homogenates from female offspring all with perinatal exposure to BPA and subsequent OVA challenge

Figure 4.5: Representative lung sections from OVA-challenged mice stained with H&E depicting no inflammation, thin inflammatory infiltrate around the bronchiole and in the interbronchial-interarteriolar space, and dense peribronchiolar inflammation

Figure 5.1: Conceptual summary of research aims

Figure 5.2: A proposed model for BPA-induced mast cell dysregulation and the broader effect of perinatal BPA exposure on pulmonary inflammation after allergen challenge in adulthood

Figure 5.3: Comparison of the molecular structures of BPA, BPB, BPF, BPAF, and BPS
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-LO</td>
<td>5-Lipoxygenase</td>
</tr>
<tr>
<td>5-mC</td>
<td>5-Methylcytosine</td>
</tr>
<tr>
<td>a/a</td>
<td>Viable Yellow Agouti Homozygous Recessive</td>
</tr>
<tr>
<td>Al(OH)₃</td>
<td>Aluminum Hydroxide</td>
</tr>
<tr>
<td>AMA</td>
<td>American Medical Association</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator Protein-1</td>
</tr>
<tr>
<td>Aᵥ/y</td>
<td>Viable Yellow Agouti</td>
</tr>
<tr>
<td>Aᵥ/y/a</td>
<td>Viable Yellow Agouti Heterozygous Dominant</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar Lavage</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Mouse Strain with a Th2-dominant Immune Response</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar Lavage Fluid</td>
</tr>
<tr>
<td>β-hex</td>
<td>β-Hexosaminidase</td>
</tr>
<tr>
<td>BMMC</td>
<td>Bone Marrow-derived Mast Cell</td>
</tr>
<tr>
<td>BPA</td>
<td>Bisphenol A</td>
</tr>
<tr>
<td>BPA/kg</td>
<td>Bisphenol A Per Kilogram</td>
</tr>
<tr>
<td>BPAF</td>
<td>Bisphenol AF</td>
</tr>
<tr>
<td>BPB</td>
<td>Bisphenol B</td>
</tr>
<tr>
<td>BPF</td>
<td>Bisphenol F</td>
</tr>
<tr>
<td>BPS</td>
<td>Bisphenol S</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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</table>
BW/day  Body Weight Per Day
C57BL/6  Mouse Strain with a Th1-dominant Immune Response
Ca\(^{2+}\)  Calcium Ion
CO\(_2\)  Carbon Dioxide
COX-1  Cyclooxygenase-1
COX-2  Cyclooxygenase-2
CpG  Cytosine Phosphate Guanine
CTMC  Connective Tissue Mast Cell
CysLT  Cysteinyl Leukotriene
DDE  Dichlorodiphenyldichloroethylene
DNA  Deoxyribonucleic Acid
DNP  2,4-Dinitrophenol
E2  17\(\beta\)-Estradiol
EDC  Endocrine Disrupting Chemical
EGTA  Ethylene Glycol Tetraacetic Acid
EIA  Enzyme Immunoassay
EPA  Environmental Protection Agency
ER  Estrogen Receptor
ER\(_\alpha\)  Estrogen Receptor Alpha
ER\(_\beta\)  Estrogen Receptor Beta
ERK  Extracellular Signal-regulated Kinase
ERR-\(\gamma\)  Estrogen-Related Receptor-Gamma
FceRI  High-affinity Immunoglobulin E Receptor
GAB2  Growth-factor-receptor-bound Protein 2-associated Binding Protein 2
<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>GADS</td>
<td>Growth-factor-receptor-bound Protein-2-releated adaptor protein</td>
</tr>
<tr>
<td>GD</td>
<td>Gestational Day</td>
</tr>
<tr>
<td>GPER</td>
<td>G-protein Coupled Estrogen Receptor 1</td>
</tr>
<tr>
<td>GPR30</td>
<td>G-protein Coupled Estrogen Receptor 1</td>
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<td>GRB2</td>
<td>Growth-factor-receptor-bound Protein 2</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
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<td>HMC-1</td>
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</tr>
<tr>
<td>ICI</td>
<td>ICI 182,780</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-Gamma</td>
</tr>
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<td>IgE</td>
<td>Immunoglobulin E</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
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<tr>
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<td>Interferon-gamma-induced Protein-10</td>
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<tr>
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<td>Janus Kinase</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LAT</td>
<td>Linker for Activation of T Cells</td>
</tr>
<tr>
<td>LOAEL</td>
<td>Low Observed Adverse Effect Level</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated Protein Kinase</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Michigan Cancer Foundation-7 (a human breast cancer cell line)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemotactic Protein-1</td>
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<tr>
<td>MEK</td>
<td>Extracellular Signal-regulated Kinase Kinase</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>--------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mg/kg</td>
<td>Milligrams Per Kilogram</td>
</tr>
<tr>
<td>mg/mL</td>
<td>Milligrams Per Milliliter</td>
</tr>
<tr>
<td>MIP-3</td>
<td>Macrophage Inflammatory Protein-3</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro Ribonucleic Acid</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mL/min</td>
<td>Milliliter Per Minute</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MMC</td>
<td>Mucosal Mast Cell</td>
</tr>
<tr>
<td>n</td>
<td>Number of Study Subjects</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>ng/kg</td>
<td>Nanograms Per Kilogram</td>
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<tr>
<td>ng/mL</td>
<td>Nanograms Per Milliliter</td>
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<tr>
<td>NHANES</td>
<td>National Health And Nutrition Examination Survey</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NOAEL</td>
<td>No Observed Adverse Effect Level</td>
</tr>
<tr>
<td>NTAL</td>
<td>Non-T-cell Activation Linker</td>
</tr>
<tr>
<td>NTP</td>
<td>National Toxicology Program</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>pg/mL</td>
<td>Picograms Per Milliliter</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
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<td>------------</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinisitol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PLCγ1</td>
<td>Phospholipase Cγ1</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear Neutrophil</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal Day</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Peroxisome Proliferator-activated Receptor-Gamma</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated And Normal T Cell Expressed and Secreted</td>
</tr>
<tr>
<td>RBL-2H3</td>
<td>Rat Basophilic Leukemia Cell Line-2H3</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SHC</td>
<td>Src Homology 2-domain-containing Transforming Protein C</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Inhibitory Ribonucleic Acid</td>
</tr>
<tr>
<td>SLP76</td>
<td>Src Homology 2-domain-containing Leukocyte Protein of 65 kDa</td>
</tr>
<tr>
<td>SOCS-1</td>
<td>Suppressor Of Cytokine Signaling-1</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcription 3</td>
</tr>
<tr>
<td>SYK</td>
<td>Spleen Tyrosine Kinase</td>
</tr>
<tr>
<td>Th</td>
<td>T Helper Cell</td>
</tr>
<tr>
<td>Th1</td>
<td>T Helper Cell Type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T Helper Cell Type 2</td>
</tr>
<tr>
<td>Th17</td>
<td>T Helper Cell Type 17</td>
</tr>
<tr>
<td>TR</td>
<td>Thyroid Hormone Receptor</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
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<tr>
<td>Symbol</td>
<td>Definition</td>
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<td>µg/kg</td>
<td>Micrograms Per Kilogram</td>
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<td>µL</td>
<td>Microliter</td>
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<td>Micromolar</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-Alpha</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
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ABSTRACT

Bisphenol A (BPA) is a widely used monomer of polycarbonate plastics and epoxide resin implicated in asthma pathogenesis when exposure occurs to the developing fetus. Widespread exposure to BPA is evident by detectable levels present in 93% of the United States population. This project tested the hypothesis that exposure to environmentally relevant levels of BPA enhances pro-inflammatory mediator release from mast cells, a key cell type involved in the development of atopic asthma, and leads to worsened adulthood pulmonary inflammation after perinatal exposure in an allergen-induced rodent model of asthma.

Pro-inflammatory mediator release was examined using bone marrow-derived mast cells (BMMCs) following in vitro or in vivo BPA exposure. Exposure to environmentally relevant levels of BPA (1-1000 nM) in vitro increased BMMC histamine (p≤0.030) and cysteinyl leukotriene (CysLT) (p≤0.029) release – a response that was not inhibited by estrogen receptor antagonism, but was inhibited by blocking extracellular signal-regulated kinase signaling (p≤0.003) or by chelating extracellular calcium ions (p≤0.037). Perinatal BPA exposure through maternal diet in mice with mixed C57BL/6 and C3H/HeJ backgrounds increased the release of CysLTs (p≤0.036), TNF-α (p≤0.019), prostaglandin D₂ (p=0.009), and IL-13 (p=0.001) and decreased global DNA methylation.
The influence of BPA exposure on pulmonary inflammation and allergic sensitivity was tested using an ovalbumin sensitization and airway challenge model in BALB/c mice exposed to BPA during *in utero* and early postnatal development through the maternal diet. At 12-weeks-old, BPA-exposed offspring displayed increased sera anti-OVA IgE levels ($p \leq 0.038$) and production of IL-13 ($p \leq 0.028$) and IFN-$\gamma$ ($p < 0.0001$) from OVA-stimulated splenocytes, indicating enhanced allergen sensitization. However, pulmonary inflammation, as assessed by total and differential leukocyte counts, cytokines, and histopathological scoring, was either not different or reduced in mice exposed to BPA.

In this work, exposure to environmentally relevant levels of BPA *in vitro* and *in vivo* resulted in upregulated release of pro-inflammatory mediators from mast cells and enhanced allergen sensitization. However, BPA exposure did not worsen pulmonary inflammation following allergen challenge. Based on these results, minimizing BPA exposures during the perinatal period may be an important means of reducing the risk of asthma and other allergic diseases later in life.
CHAPTER 1

INTRODUCTION

Asthma

Asthma is a chronic inflammatory disease of the airways characterized by reversible airway obstruction, bronchospasm, airway hyperreactivity, airway remodeling, and recurrence of clinical symptoms such as wheezing, coughing, shortness of breath, and chest congestion (Barrios et al., 2006). Asthma is categorized into two classifications: atopic asthma and non-atopic asthma. These classifications can also be designated as extrinsic and intrinsic asthma or allergic and non-allergic asthma.

Development of allergic asthma is typically seen early in life, and asthmatic episodes are initiated in response to a particular allergen such as pollen or dander. Non-allergic asthma, on the other hand, usually develops later in life and its cause is unclear. Non-allergic asthmatic episodes have been linked to non-allergen triggers such as stress, exercise, air pollution, cigarette smoke, or repeated exposure to occupational hazards.

The worldwide prevalence of asthma has steadily risen since the 1970s, and current reports estimate that the number of individuals diagnosed with asthma is about 300 million globally (WHO, 2007; Anandan et al., 2010), with 23 million of those cases in the United States alone (CDC, 2012). The rapid growth in asthma prevalence has put a significant economic strain on healthcare systems. In the United States, the direct cost of
treated asthma is estimated to be $50.1 billion a year (Barnett and Nurmagambetov, 2011). Factoring in another $5.9 billion for indirect costs, such as work absenteeism, raises the approximate economic burden of asthma to $56 billion (Barnett and Nurmagambetov, 2011). Despite successful advances in asthma treatment, asthma mortality remains high, especially in underdeveloped countries with limited resources to diagnose and treat asthma. Global asthma mortality rates reach nearly 300,000 yearly, compared to the US alone where mortality reaches approximately 3,000 yearly (CDC, 2012). Interestingly, epidemiologic studies have repeatedly indicated that the prevalence of asthma is highest in industrialized areas, suggesting that environmental exposure to certain products may play a role in asthma pathogenesis (Anandan et al., 2010).

**Mast Cells**

Mast cells are resident leukocytes of myeloid origin commonly found in many vascularized tissues, especially those in contact with the external environment such as skin, lungs, and intestines. Mast cells exhibit different phenotypes dependent upon the tissue to which they are localized (Bienenstock et al., 1983; Damas and Lecomte, 1983; Schulman et al., 1983). In the lung and certain other tissues, mast cells exhibit two distinct phenotypes: mucosal mast cells (MMCs) and connective tissue mast cells (CTMCs) (Irani et al., 1986). MMCs and CTMCs in the lung are distinguishable by their unique profiles of proteases, which are induced by T cells in MMCs and constitutively expressed in CTMCs (Xing et al., 2011). In experimental settings, primary mast cells are routinely derived from collected bone marrow after stimulation with growth factors to
induce differentiation; however, the phenotype of these mast cells differ from both MMC and CTMC phenotypes (Hunter et al., 2012).

The main characteristic of mast cells is cytoplasmic granules containing preformed pro-inflammatory mediators, which include histamine, cytokines, lipid-derived mediators, proteases, and proteoglycans. Mast cells play an important role in the induction of allergic asthma since they can become activated in response to a particular antigen. While dormant, the mast cell exists with half a million IgE molecules bound to the cell surface by transmembrane high-affinity Fcε receptors (FcεRIs) (Amin, 2012). In the presence of an antigen, several IgE molecules will bind to a single antigen in a process called crosslinking, which initiates a complex intracellular signaling cascade to induce mast cell release of pro-inflammatory mediators by both degranulation and de novo synthesis (Walls et al., 2001). Mast cells may also be activated by other stimuli such as complement proteins, cell injury, and chemical and biologic substances, although, these processes are less understood.

IgE crosslinking on the mast cell surface directly results in FcεRI aggregation. Subsequently the lipid-raft protein Lyn, a Src-family kinase, phosphorylates tyrosine residues on the FcεRI β-chain and γ-chains to serve as Lyn, SYK (spleen tyrosine kinase), and Fyn docking sites (Okayama et al., 2012). Independently or together, Lyn and SYK phosphorylate membrane-bound scaffold protein LAT (linker for activation of T cells), while Lyn, SYK, and Fyn phosphorylate NTAL (non-T-cell activation linker). Activation of LAT versus activation of NTAL thereby establishes a divergence in the FcεRI-initiated signaling cascade for mediator release. LAT phosphorylation results in
direct recruitment of GRB2 (growth-factor-receptor-bound protein 2), GADS (GRB2-related adaptor protein), and PLCγ₁ (phospholipase Cγ₁) and indirect recruitment of SHC (Src homology 2 (SH2)-domain-containing transforming protein C), SLP76 (SH2-domain-containing leukocyte protein of 65 kDa), SOS (son of sevenless), and Vav. Once formed, the GRB2-SOS-SHC complex can induce the mitogen-activated protein kinase (MAPK) pathway to synthesize lipid-derived mediators or induce cytokine transcription, while PLCγ₁ in the PLCγ₁-GADS-SPL76-VAV complex induces degranulation through PKC (protein kinase C) signaling and calcium ion (Ca^{2+}) influx. In a similar fashion, NTAL, also referred to as LAT2, forms a GRB2-SOS complex that activates the MAPK pathway to induce cytokine transcription, and also forms a complex with VAV, GRB2, GAB2 (GRB2-associated binding protein 2), and PI3K (phosphatidylinositol 3-kinase) to induce cytokine transcription or Ca^{2+} influx for degranulation. The redundancy in LAT-induced and NTAL-induced mast cell activation has been studied using bone marrow-derived mast cells (BMMCs) that are LAT and/or NTAL deficient. LAT knockout (KO) BMMCs displayed similarly diminished degranulation and synthesis of mediators as LAT-NTAL double KOs, yet, NTAL KO BMMCs displayed a higher capacity for degranulation and mediator synthesis (Gilfillan and Tkaczyk, 2006). Thus, LAT-mediated mast cell activation is viewed as the primary signaling cascade, and NTAL-mediated activation is viewed as a secondary cascade.

The end result of either LAT- or NTAL-mediated signaling in mast cells is the release of pro-inflammatory mediators. The most well characterized mediator released by mast cells is histamine, a biologic amine. Mast cells are capable of releasing large
quantities of histamine, approximately 3 picograms per cell (Shahid et al., 2009). In allergic responses, histamine acts as a vasodilator allowing other leukocytes to infiltrate the inflamed tissues, but also can act to stimulate release of mediators from macrophages, basophils, eosinophils, fibroblasts, lymphocytes, neutrophils, epithelial cells, and endothelial cells.

Mast cells have been demonstrated to release over 60 different cytokines, chemokines, and growth factors (Galli et al., 2005). Most notable in relation to asthma and allergic disease is mast cells’ release of Th2 cytokines. Specific inflammatory responses mediated by T helper type 2 (Th2) cells, a subclass of T helper (Th) lymphocytes, play a large role in the mechanism leading to airway obstruction. Th2 cells release a highly characteristic profile of cytokines that includes IL-4, IL-5, IL-9, and IL-13. These cytokines are capable of initiating immediate hypersensitivity responses mediated by IgE. IL-4, IL-9, and IL-13 stimulate B cells to produce IgE, while IL-5 and IL-13 act on airway smooth muscle and epithelium to elicit airway hyperreactivity and glycoprotein production (Chung and Barnes, 1999). IL-4, IL-5, and IL-13 also play a role in eosinophil maturation and recruitment to the lungs, while IL-9 stimulates mast cell proliferation and differentiation (Chung and Barnes, 1999).

The lipid mediators released in greatest quantity by mast cells are prostaglandin (PG) D$_2$ and leukotriene (LT) C$_4$. PGD$_2$ is produced through the cyclooxygenase-1 and -2 pathway, while LTC$_4$, which can be further metabolized into LTD$_4$ and LTE$_4$ (collectively referred to as the cysteinyL LTs (CysLTs)), is produced through the 5-lipoxygenase (5-LO) pathway. PGD$_2$ is generally thought of as a vasodilator and airway
constrictor, however, a study of allergen-induced pulmonary inflammation using PGD$_2$ receptor KO mice indicated that PGD$_2$ signaling plays a role in lymphocyte recruitment, eosinophil recruitment, Th2 cytokine production, and development of airway hyperresponsiveness (Matsuoka et al., 2000). Similarly, the primary activity of the CysLTs in asthma is believed to be airway smooth muscle constriction. However, an allergen-induced pulmonary inflammation study in mice lacking LTC$_4$ synthase, the final enzyme in the 5-LO pathway needed for LTC$_4$ synthesis, indicated a role for the CysLTs in eosinophil recruitment, mast cell recruitment and activation, mucus secretion, goblet cell hyperplasia, IgE and IgG1 production, Th2 cytokine production, and airway hyperresponsiveness (Kim et al., 2006).

Due to the complexity involved in regulating inflammatory responses, it is difficult to define the precise role of each cell type or signal involved in asthma pathophysiology. While mast cells are important for the induction of allergic asthma and regulating acute responses, their role becomes diminished in comparison to other leukocytes as chronic asthma is established. Cells including eosinophils, neutrophils, macrophages, and lymphocytes take on more prominent roles as they are and continue to be recruited to the lungs. Many of the pro-inflammatory mediators released by mast cells continue to be released by other cell types as well. Thus, completely understanding how an environmental exposure influences inflammation associated with asthma, especially from a mechanistic standpoint, is very difficult.

**Bisphenol A**
Bisphenol A (BPA, also (CH₃)₂C(C₆H₄OH)₂, 2,2-bis(p-hydroxyphenyl)-propane, or p,p'-isopropylidenebisphenol) is a monomer of polycarbonate plastics and epoxide resin. First synthesized in 1891, BPA monomer is produced by a condensation reaction with two parts phenol and one part acetone (Figure 1.1). Polymerization to generate polycarbonate plastics occurs through a 1-to-1 addition of BPA monomer and phosgene catalyzed by HCl, while polymerization of BPA-containing epoxide resin is most commonly formed by a 1-to-1 addition of BPA monomer and epichlorohydrin catalyzed by NaOH (Figure 1.2). In 2009, the yearly production of BPA was estimated to be 6 billion pounds, with an additional 100 tons released directly into the atmosphere (Vandenberg et al., 2009). BPA is a regular component of food-related consumer products such as baby bottles, food storage containers, plastic wrap, water bottles, and the linings of metal cans; however, BPA is also found in non-food-related items such as medical tubing, dental sealants, thermal receipts, and paper towels (Vandenberg et al., 2007).

In 1993, Krishnan et al. was the first to report that BPA monomers are released from polycarbonate containers when several failed experiments led to the discovery that water autoclaved in polycarbonate flasks contained dissociated BPA molecules (Krishnan et al., 1993). BPA monomers readily dissociate from polymers through hydrolysis of the ester bond. While BPA depolymerization occurs at normal conditions such as room temperature and neutral pH, high temperatures, low or high pH, age, repeated washing, and general use increase the amount of BPA monomers leaching from a polycarbonate or epoxide products (Kang et al., 2006; Richter et al., 2007). Due to the widespread use of
BPA in food-related items, the main route of human exposure is through ingestion of tainted food and drink. However, minor routes of exposure through inhalation and dermal absorption exist due to the presence of BPA in indoor air, outdoor air, household dust, thermal receipts, and other paper goods (Vandenberg et al., 2007).

Many studies from Europe, Japan, and the United States have reported regular and widespread exposure to BPA in adults and children as evidenced by measurable quantities in serum, urine, saliva, and breast milk in the majority of study participants (Vandenberg et al., 2007). Levels of BPA detected in biologic samples vary from individual to individual, but can also vary from day to day for a single individual (Ye et al., 2011; Christensen et al., 2012; Braun et al., 2012). Most studies report biologic BPA levels in humans centering around the low nanomolar range. For example, typical total BPA levels in adult serum and urine range from 0.2-20 ng/mL and 1.12-2.82 ng/mL, respectively (Vandenberg et al., 2007). Furthermore, quantification of BPA in infant cord blood (Ikezuki et al., 2002), amniotic fluid (Edlow et al., 2012), placenta (Schonfelder et al., 2002), and fetal liver tissues (Nahar et al., 2013) reveal higher levels of free BPA than found in adult samples. The higher fetal burden of BPA may result from the developing liver’s altered capacity for metabolism, enterohepatic recirculation, and elimination of xenobiotics (Ginsberg et al., 2004; Vandenberg et al., 2009; Nahar et al., 2013).

The half-life of BPA in humans has been estimated to be very short – on the order of six hours – though new evidence questions the accuracy of this observation (Pritchett et al., 2002; Volkel et al., 2002; Ye et al., 2005). Human studies conducted in the early
2000s by Volkel et al., Ye et al., and Pritchett et al. examined BPA metabolism and toxicokinetics of administered doses. These reports described efficient BPA glucuronidation and sulfonation leading to rapid excretion with relatively low body burden (Pritchett et al., 2002; Volkel et al., 2002; Ye et al., 2005). However, the presence of free BPA excreted in urine suggests incomplete metabolism (Calafat et al., 2005; Liao and Kannan, 2012; Mendonca et al., 2012). Other reports have suggested that high levels of BPA in certain tissues, for example placental tissue (Schonfelder et al., 2002), umbilical tissue (Ikezuki et al., 2002), and possibly adipose tissue (Fernandez et al., 2007), indicate bioaccumulation in part due to BPA being lipophilic. Additionally, BPA may be deconjugated in the intestines (Sakamoto et al., 2002) and possibly other tissues (Stowell et al., 2006), thus potentially lengthening the biological half-life. Moreover, a study by Stahlhut et al. using 2003-2004 National Health and Nutrition Examination Survey (NHANES) data compared total BPA levels in urine with reported fasting times of nearly 1,500 participants (Stahlhut et al., 2009). This study did not report a rapid decline in urinary BPA levels up to 24 hours of fasting, thus suggesting that the half-life of BPA in the body is longer than originally thought, or that non-food exposures have a sizable impact on biologic BPA levels, or both. Conversely, a similar study by Christensen et al. that monitored five fasting volunteers reported declined total BPA levels in urine 12-24 hours after fasting that remained low up to 48 hours (Christensen et al., 2012).

Even though a consensus on the biological half-life of BPA has not been reached, there is agreement that human exposure to BPA occurs on a daily basis for most
individuals. Even with a hypothetical rapid excretion of BPA from the body, daily consumption of BPA through tainted food perpetuates biologic levels causing complete elimination of BPA from the body to be very difficult, if not impossible (Vandenberg et al., 2007). Based on literature quantifying BPA contamination in food, water, air, and soil, Kang et al. estimated the daily human BPA intake for adults to be 1 μg/kg body weight/day (Kang et al., 2006), while the European Commission’s estimate of human BPA intake from only food sources ranges from 0.48-1.6 μg/kg body weight/day (European Commission Scientific Committee on Food, 2002). Neonates are exposed to the highest amounts of BPA due to lower body weight, possible higher concentrations of BPA in breast milk that in other bodily fluids, and use of BPA-containing plastic bottles. Estimates suggest that infants less than three months old are exposed to 24 μg BPA/kg body weight/day (Wong et al., 2005).

Reliance on BPA quantification in foodstuffs as a way to estimate BPA intake is very subjective since levels of BPA can vary from the type of food, the type of container, the manufacturer, and the time of manufacture (Vandenberg et al., 2007). For example, D’Antuono et al. observed that heating water in polycarbonate baby bottles to 100 °C for 30 minutes result in a mean BPA leachate level of 1.2 ng/mL (D’Antuono et al., 2001). However, Brede et al. demonstrated that unused baby bottles leached an average of 0.23 ng/mL of BPA after being heated to 100 °C for 1 hour, while bottles washed 51 times and brushed 13 times leached an average of 8.4 ng/mL of BPA and bottles washed 169 times and brushed 23 times leached and average of 6.7 ng/mL of BPA (Brede et al., 2003). Similarly, Brontons et al. examined BPA leachate in water heated to 125 °C for 30
minutes in 20 different food cans and reported BPA levels ranging from 4-23 μg/can (Brontons et al., 1995). Kang et al. reported that the contents of canned foods also influence concentration of BPA leachate, with vegetable oil and salt solutions having greater BPA leachate than water or glucose (Kang et al., 2003). Quantification of BPA in paper towels conducted by Vinggaard et al. indicated almost no detectable BPA in virgin paper towels, but yielded BPA levels of 0.55-24.1 mg/kg in paper towels made from recycled paper products (Vinggaard et al., 2000). Another study examining the portion of paper and cardboard takeout containers in direct contact with food found detectable levels of BPA in 47% of paper containers and 38% of cardboard containers, with higher levels in the cardboard containers (Lopez-Espinosa et al., 2007).

**BPA Endocrine Disruption**

The distinguishing structural feature of the BPA monomer is two protruding phenol groups, which create a structure similar to estradiol (E2) as well as other synthetic estrogens (Figure 1.3). This similarity allows for BPA molecules to fit into estrogen receptor (ER) binding pockets and alter normal endocrine signaling, thereby behaving as an endocrine-disrupting chemical (EDC). Additionally, BPA has been demonstrated to bind to the thyroid hormone receptor (TR) (Zoeller et al., 2005), peroxisome proliferator-activated receptor-γ (PPAR-γ) (Kwintkiewicz et al., 2010), estrogen-related receptor-γ (ERR-γ) (Matsushima et al., 2007), human pregnane X receptor (Sui et al., 2012), and G-protein Coupled Estrogen Receptor-1 (GPER, also GPR30) (Dong et al., 2011; Pupo et al., 2012).
Kinetic studies of BPA-ER binding have reported that BPA binds to both ER alpha (ERα) and ER beta (ERβ), with an approximate 10-fold higher affinity toward ERβ (Gould et al., 1998; Matthews et al., 2001; Routledge et al., 2000). However, early kinetic studies labeled BPA as a “weak estrogen” due to the observation that BPA-ER binding affinity was approximately 10,000-fold weaker than E2-ER affinity (Kuiper et al., 1998; Andersen et al., 1999; Fang et al., 2000). More recent evidence has demonstrated that low doses of BPA (femtomolar to nanomolar) promote E2-mediated responses at similar or greater strength than E2 (Alonso-Magdalena et al., 2005; Hugo et al., 2008; Zsarnovszky et al., 2005). Higher BPA-ER binding affinity at low doses has been explained in part by BPA binding to ERs differently than E2 (Gould et al., 1998). For example, Washington et al. demonstrated that BPA binds to the low-affinity type II estrogen binding site where it outcompetes E2 at concentrations up to 10-15 μM (Washington et al., 2001). Higher BPA-ER binding affinity at low doses has also been explained by BPA eliciting responses through non-classical estrogen receptors (Alonso-Magdalena et al., 2012). In addition, BPA activity has also been demonstrated to act in a tissue-specific manner (Welshons et al., 2006), with high activity especially noted in the prostate (Gupta, 2000).

Over many years, numerous in vivo studies have demonstrated estrogenic activity of BPA through alterations to female reproductive tissues. In 1936, Dodds and Lawson were the first to show BPA estrogenicity when rats injected twice daily for a total of four days with 100 mg BPA had increased uterine wet weight by the end of the exposure (Dodds and Lawson, 1936). The impact of this finding, however, was minimal since use
of BPA in plastics manufacturing did not begin until the 1950s. More recently, rats treated with 400 mg/kg BPA a day for three days (Ashby and Tinwell, 1998) and rats treated with 10 mg/kg and 30 mg/kg BPA once a day for four days (Dodge et al., 1996) exhibited increased uterine wet weight by the end of the exposure period. Proliferation of vaginal and uterine epithelial cells from 2-3 layers to 6-8 layers and increased uterine luminal epithelial cell height following 0.3 mg/kg/day BPA exposure in rats has also been reported (Steinmetz et al., 1998). Furthermore, proliferative activity of mammary gland epithelium following 11 days of 0.1 mg/kg/day and 0.5 mg/kg/day BPA exposure in rats was seen to increase 143% and 220%, respectively (Colerangle and Roy, 1997).

Evidence implicating BPA as an EDC has also been displayed in vitro using the estrogen-sensitive human breast cancer cell lines MCF-7, T-47D, and ZR-75-1. When exposed to BPA at quantities equal to or greater than 1 μM for one week, cells display enhanced proliferation (Schafer et al., 1999).

Controversy continues to surround the perceived toxicity of BPA (Vandenberg et al., 2009). A toxicological profile of BPA conducted by Morrissey et al. established the maximum tolerated dose (or lowest observed adverse effect level (LOAEL)) for BPA to be 1000 mg/kg body weight/day in rats and mice (Morrissey et al., 1987). Using the LOAEL, the EPA established a reference dose of 50 μg/kg body weight/day by buffering with a safety factor of 1000 (Vandenberg et al., 2009); however, the reference dose is typically calculated based on the no observed adverse effect level (NOAEL). Due to the low-dose effects and nonmonotonic dose response curves seen in a variety of endpoints
resulting from BPA exposure, a NOAEL has not been established. Thus, a “safe” level for human exposure remains undefined.

**BPA Epigenetic Modification**

In addition to BPA acting as an EDC, evidence also supports that BPA acts as an epigenetic modifier by altering the methylation state of cytosine-phosphate-guanine (CpG) sites in DNA (Rubin, 2011). Methylation of DNA is a normal biological process used to silence specific genes in certain cells when appropriate. When methyl groups are present on DNA, they alone or through proteins that specifically bind methylated CpG sites act as a steric hindrance preventing transcription factors from interacting with DNA. Thus, alteration of the methylation profile by BPA results in misregulation of gene transcription and activation (Dolinoy et al., 2007; Bromer et al., 2010). The mechanism of BPA-induced DNA methylation alterations is unclear (Ooi and Bestor, 2008).

Theories postulate that BPA may alter the activity of DNA methyltransferases, a group of enzymes responsible for catalyzing the transfer of a methyl group onto DNA, or Tet methylcytosine dioxygenases, a group of enzymes that catalyze the conversion of 5-methylcytosine to 5-hydroxymethylcytosine (Szyf, 2012). Another theory suggests that BPA may directly interact with DNA methyl groups.

Early evidence linking BPA exposure and DNA hypomethylation was reported by Dolinoy et al. (Dolinoy et al., 2007). Using the viable yellow agouti mouse model, which has distinct phenotype dictated by DNA methylation status of the metastable allele $A^{vy}$. 
Dolinoy et al. showed that maternal exposure to BPA caused a shift in offspring coat color from brown to yellow corresponding to increased hypomethylation of the interstitial A particle in the agouti gene (Dolinoy et al., 2007). Additionally, it was shown that dietary supplementation with methyl-donating compounds was able to counteract the DNA hypomethylating effect of BPA (Dolinoy et al., 2007). Subsequent studies have demonstrated BPA-induced hypomethylation or hypermethylation for several genes with diverse functions (Singh and Li, 2012). For example, Anderson et al. demonstrated that perinatal exposure to multiple environmentally relevant doses of BPA through the maternal diet increased global DNA methylation and increased DNA methylation at the CDK5 activator-binding protein metastable epiallele (Anderson et al., 2012).

While most works examining the epigenetic modification potential of BPA have been focused on DNA methylation, newer studies have also demonstrated that BPA plays a role in histone modification and microRNA (miRNA) expression. Doherty et al. demonstrated in MCF-7 cells and mouse mammary glands that BPA treatment induces histone H3 trimethylation of lysine 27, which was linked to increased expression of the histone methyltransferase EZH2 (Doherty et al., 2010). Meanwhile, Avissar-Whiting et al. showed that BPA exposure in immortalized human cytotrophoblast cell lines causes overexpression of the miRNA miR-146a resulting in decreased cell proliferation (Avissar-Whiting et al., 2010). Through use of a microarray, Cho et al. also showed 2-fold upregulation or downregulation of 37 miRNAs following BPA exposure in an immortalized mouse Sertoli cell line (Cho et al., 2010).
The *in utero* and early postnatal development periods are believed to be highly sensitive to chemical insults that induce epigenetic modifications. Embryogenesis is an especially sensitive period since the DNA synthesis rate is high and elaborate DNA methylation patterning and chromatin organization take place (Bernal and Jirtle, 2010). In accordance with the developmental origins of health and disease hypothesis, several studies suggest that epigenetic changes sustained in early development resulting from BPA exposure can have permanent effects on health into adulthood (Kundakovic and Champagne, 2011). Additionally, BPA exposure during gestational development has been demonstrated to alter gene expression transgenerationally (Wolstenholme et al., 2012). Some epigenetic modifications, specifically DNA methylation and miRNA expression, have been demonstrated to be mitotically and meiotically heritable changes in gene expression (Robertson, 2005).

**BPA and Inflammation Associated with Asthma**

Epidemiological evidence supports the idea that E2 contributes to asthma disease status. First, the prevalence of asthma and other allergic diseases are three times more common in women during middle adulthood compared to men (De Marco et al., 2002; Vink et al., 2010; Leynaert et al., 2012). Also, patients undergoing estrogen replacement therapy have a high occurrence of new onset asthma either during or after therapy (Barr et al., 2004; Dratva, 2010). Thirdly, some women with an existing asthma problem report worsened symptoms corresponding to ovulation, which is a time when E2 levels become upregulated (Vrieze et al., 2003; Thornton et al., 2012). Lastly, the ER
polymorphisms IVS1-397CT and IVS1-397TT have been associated with the presence of airway hyperresponsiveness in females diagnosed with asthma, while the polymorphisms IVS1-351AA, exon1+30CT, and exon1+30TT have been associated with lung function decline in females diagnosed with asthma (Dijkstra et al., 2006). In addition to epidemiologic studies, in vitro studies have determined that E2 and progesterone may contribute to pulmonary inflammation associated with asthma by activating mast cells (Zaitsu et al., 2007; Jensen et al., 2010). Importantly, mast cell activation has also been reported for several EDCs including endosulfan, dieldrin, dichlorodiphenyl dichloroethylene (DDE), nonylphenol, Aroclor 1242, Aroclor 1254, and 4-tert-octylphenol (Narita et al., 2007; Kennedy et al., 2012). Evidence also suggests that BPA exposure may induce mast cell activation; limited studies examining mast cell activation following exposure to a high dose of BPA (50 μM) have reported increased histamine, IL-4, IL-6, TNF-α, and IFN-γ release (Shim and Lim, 2009; Park and Lim, 2010; Lee and Lim, 2010; Lee et al., 2012).

Evidence indicates that epigenetic modifications, including DNA methylation and histone modification, are critical regulatory mechanisms for proper immune function (Mostoslavsky and Bergman, 1997; Teitell and Richardson, 2003). Additionally, new studies focusing on regulation of mast cell function via DNA methylation (Kuramasu et al., 1998; Walczak-Drzewiecka et al., 2010) and miRNA expression (Ishizaki et al., 2011; Mayoral et al., 2011; Molnar et al., 2012) have begun emerging. Thus, researchers have speculated that epigenetic dysregulation may play a role in immune disorders (Richardson, 2003; Robertson, 2005), especially asthma and other allergic sensitivities.
(Miller et al., 2008; Shaheen and Adcock, 2009; Durham et al., 2011; North and Ellis, 2011; Kabesch and Adcock, 2012; Yang and Schwartz, 2012). With the previously mentioned understanding that sensitivity to epigenetic dysregulation is highest during gestation and that dysregulation can lead to disease status later in life, several reports have supported the developmental origins of adult asthma and other allergic diseases (Martino and Prescott, 2011; Henderson and Warner, 2012; Duijts, 2012; De Luca et al., 2010; Hong and Wang, 2012). Meanwhile, experimental approaches seeking to link epigenetic regulation through environmental exposure with asthma and allergic disease have begun to emerge (Kohli et al., 2012; Nadeau et al., 2010; Ho, 2010).

Limited studies have examined a connection between BPA exposure and asthma. An epidemiologic investigation by Spanier et al. demonstrated an association with total BPA in maternal urine at 16 weeks gestation and incidence of infant wheezing from age 6 months to 3 years (Spanier et al., 2012). Similarly, Vaidya and Kulkarni using NHANES data established that higher total BPA in urine from adult females corresponded to a higher likelihood of having asthma and predicted an asthmatic episode over the past 12 months (Vaidya and Kulkarni, 2012). An experimental study by Midoro-Horiuti et al. reported that neonatal mice exposed to 10 μg/mL BPA through maternal drinking water beginning 1 week before fertilization and ending on postnatal day (PND) 21 and subsequently sensitized to ovalbumin (OVA) display worsened asthma-like pulmonary inflammation as detected by increased pulmonary eosinophil infiltration and greater airway hyperresponsiveness after OVA challenge (Midoro-Horiuti et al., 2010). Using a similar experimental model, Nakajima et al. demonstrated that neonatal mice exposed to
10 μg/mL BPA through maternal drinking water during gestation only (gestational day (GD) -7 to GD 20) or during gestation and lactation (GD -7 to PND 21) displayed increased eosinophil infiltration and greater airway hyperresponsiveness, whereas mice free of BPA exposure or mice exposed to BPA during maternal lactation only (PND 0 to PND 21) did not display changes in eosinophil numbers nor airway hyperresponsiveness (Nakajima et al., 2012). Bauer et al. examined pulmonary inflammation in an adult allergen-induced mouse model using intraperitoneal sensitization with OVA following in utero and early life BPA exposure through maternal diet (Bauer et al., 2012). In this study, female offspring exposed to BPA at doses of 0.5, 5, or 50 μg/kg/day beginning on GD 6 and ending on PND 21 displayed significantly lowered pulmonary eosinophilia after challenge compared to controls, while animals exposed to 500 μg/kg/day had the same level of eosinophilia as controls. Additionally, BPA-exposed offspring displayed lower levels of serum IgE than controls (Bauer et al., 2012).

**Summary**

The correspondence between the increased prevalence of asthma over the past 40 years and the increased manufacture and usage of BPA-containing products suggests a possible connection between the two. Additionally, the suggested regulation of atopic asthma through both epigenetic modification and endocrine signaling makes BPA an ideal compound to study in asthma research, since BPA behaves as both an EDC and an epigenetic modifier. Limited epidemiologic studies have indicated an association between BPA exposure and severity of asthma symptoms and likelihood of having...
asthma; though, experimental studies have produced mixed results on whether gestational and/or early-life BPA exposure alters allergen-induced bronchial inflammation and hyperresponsiveness in murine models of asthma. Still, any mechanistic explanation of BPA increasing atopic asthma pathogenesis or worsening asthma-related inflammation has not been examined. Mast cell stimulation by BPA may be one possible mechanistic explanation of BPA increasing asthma prevalence and worsening pulmonary inflammation, since evidence indicates that mast cells are sensitive to activation by E2 and other EDCs. This research will test the hypothesis that exposure to environmentally relevant levels of BPA induces subclinical changes that enhance the release of pro-inflammatory mediators associated with allergic airway disease in mast cells, and results in worsened pulmonary inflammation in an allergen-induced rodent model of asthma. This hypothesis will be tested through the following aims:

(1) Determine the short-term effect of BPA exposure on BMMC activation through quantification of histamine and CysLT release.

(2) Assess the long-term effect of BPA exposure through maternal diet on BMMC production of pro-inflammatory mediators associated with asthma (CysLTs, PGD₂, IL-4, IL-5, IL-6, IL-13, TNF-α, histamine).

(3) Characterize the impact of BPA exposure through maternal diet on severity of inflammation in adult mice by using an allergen-induced asthma model to measure cellular recruitment, pulmonary cytokines and eicosanoids, splenocyte cytokines, and serum IgE.
This work will add to the growing literature regarding BPA exposure and adverse health outcomes. Especially relevant is the inclusion of environmental BPA levels, perinatal exposure, and physiologic delivery of BPA to the developing fetus via the maternal diet. Collectively, studies of BPA exposure and adverse health issues have prominence as the question regarding potential regulation of BPA production continues to be discussed, and the limited number of studies on BPA exposure and inflammation associated with asthma has prompted more research in this area.
Figure 1.1: BPA monomer is formed through a condensation reaction between phenol and acetone in a 2-to-1 ratio.
Figure 1.2: The polymerization reaction to form polycarbonate plastic (a) involves a reaction catalyzed by HCl in the presence of a 1-to-1 ratio of BPA monomer and phosgene, while polymerization to form epoxide resin (b) involves a reaction catalyzed by NaOH in the presence of BPA monomer and epichlorohydrin, usually in a 1-to-1 ratio.
Figure 1.3: Comparison of the molecular structures of BPA \((a)\) and E2 \((b)\). Notice the similarity between the two protruding phenol groups on BPA and the one protruding phenol group and one protruding hydroxide on E2.


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CHAPTER 2

BISPHENOL A AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS ENHANCES HISTAMINE AND CYSTEINYL LEUKOTRIENE RELEASE FROM BONE MARROW- Derived MAST CELLS

Abstract

Bisphenol A (BPA), a monomer of polycarbonate plastics and epoxide resin, acts as an endocrine-active compound and has been shown to enhance the inflammatory response to allergen challenge. Previous reports in rodents have demonstrated that perinatal BPA exposure alters airway inflammation following sensitization and challenge to ovalbumin in juvenile and adult offspring. Since mast cells play an important role in allergen-induced airway inflammation, the effect of BPA exposure on mast cell activation was examined. Primary murine bone marrow-derived mast cells (BMMCs) produced from femurs of female C57BL/6 mice were stimulated with BPA or estradiol (E2) in vitro to assess the effects on histamine and cysteinyl leukotriene (CysLT) release. Both BPA and E2 were observed to increase BMMC histamine release over a range of nanomolar concentrations (1-1000 nM). Estrogen receptor alpha (ERα) antagonism using ICI 182,780 partially blocked the ability of E2, but not BPA, to elevate histamine release. BPA also increased CysLT release, which was not abrogated by ERα inhibition. It was also observed that the ability of BPA to enhance histamine and CysLT release was inhibited by blocking the extracellular signal-regulated kinase (ERK) pathway with
U0126 or by chelating extracellular calcium ions (Ca$^{2+}$) using EGTA. In summary, these experiments demonstrated that acute BPA exposure enhances mast cell histamine and CysLT release in vitro. This effect was not blocked by ER$\alpha$ antagonism, indicating the effect of BPA on histamine and CysLT release is not dependent on an ER$\alpha$-mediated mechanism. Instead, BPA-induced mast cell histamine and CysLT release may be mediated via the ERK pathway and extracellular Ca$^{2+}$ concentrations. In conclusion, these data suggest that exposure to BPA at environmentally relevant levels may provoke an acute inflammatory response in atopic individuals via mast cell activation.

**Introduction**

The prevalence of asthma, a chronic inflammatory disease of the airways characterized by wheezing, shortness of breath, chest tightness, and coughing (Murdoch and Llyod, 2010), has steadily increased since the 1970s (Lai et al., 2009; Anandan et al., 2010; To et al., 2012). Current estimates suggest 300 million individuals have been diagnosed with asthma globally, and that asthma accounts for nearly 300,000 deaths yearly (Holgate et al., 2007; WHO, 2007). Asthma pathogenesis arises from a complex interplay of genetic predisposition and environmental exposure (Holgate et al., 2007). The observation that asthma prevalence tends to be highest in industrialized areas suggests that one or more products of industrialization may be contributing to asthma pathogenesis (Masoli et al., 2004; Anandan et al., 2010; Asher, 2010; Crinnion, 2012; To et al., 2012).
Observational epidemiologic studies have suggested that endogenous and exogenous estrogen may contribute to the pathogenesis and severity of asthma (Bonds and Midoro-Horiuti, 2013); asthma prevalence among adults is higher in women than in men (Leynaert et al., 2012; Vink et al., 2010), and peaks in estrogen and progesterone corresponding to the ovulation cycle have been associated with worsened asthma symptoms (Vrieze et al., 2003; Thornton et al., 2012). Additionally, a higher prevalence of adult-onset asthma has been reported in patients undergoing hormone replacement therapy (Barr et al., 2004; Dratva, 2010). Lab-based approaches also indicate that estradiol, progesterone, and synthetic xenoestrogens can enhance mast cell degranulation (Narita et al., 2007; Zaitsu et al., 2007; Jensen et al., 2010; Kennedy et al., 2012), and since mast cells play a central role in atopic asthma, it has been hypothesized that widespread exposure to synthetic xenoestrogens (Yang et al., 2006; Phillips and Foster, 2008; Latini et al., 2010) may be contributing to the increased prevalence of asthma by enhancing mast cell activation.

Mast cells play a critical role in the development of atopic asthma and other allergic diseases by initiating an acute inflammatory response (Amin, 2012). Activated mast cells release preformed mediators stored in cytoplasmic secretory granules including histamine, leukotriene (LT) C4, and prostaglandin (PG) D2, which are capable of inducing bronchoconstriction, mucus secretion, and edema, as well as pro-inflammatory cytokines (IL-4, IL-5, IL-13, and TNF-α), which contribute to IgE production and eosinophil recruitment (Boyce, 2003; Bradding et al., 2006).
Recently, the endocrine-active compound, BPA, a monomer of polycarbonate plastics and epoxide resin, has been associated with worsened asthma symptoms in humans (Spanier et al., 2012; Vaidya and Kulkarni, 2012; Donohue et al., 2013) and the development of asthma in juvenile mice (Midoro-Horiuti et al., 2010; Nakajima et al., 2012). Human exposure to BPA is widespread (Vandenberg et al., 2007), with National Health and Nutrition Examination Survey (NHANES) data revealing detectable levels of total BPA in the urine of 95% of participants (Calafat et al., 2005). BPA is capable of disrupting normal endocrine signaling by weakly binding to steroid receptors including the ERs and thyroid hormone receptor (Zoeller et al., 2005). BPA has also been demonstrated to bind to the membrane-bound G-protein coupled ER 1 (GPER, also GPR30) (Bouskine et al., 2009; Sheng and Zhu, 2011), as well as the nuclear receptor estrogen-related receptor-\(\gamma\) (ERR-\(\gamma\)) (Matsushima et al., 2007). BPA levels in adult human samples including urine, serum, blood, and saliva can vary depending on sample type, study population, and detection method, though reported levels repeatedly focus around low nanomolar concentrations (Vandenberg et al., 2007; Vandenberg et al., 2012). Previous reports conducted using a high level of BPA have shown that BPA can enhance mast cell activation (Shim and Lim, 2009; Park and Lim, 2010; Lee and Lim, 2010; Lee et al., 2012).

This study tests the hypothesis that BPA at levels relevant to human exposure enhances BMMC release of histamine and CysLTs. Furthermore, the requirements of ER\(\alpha\), the ERK pathway, and extracellular Ca\(^{2+}\) in BPA-induced mediator increases were investigated. It was observed that BPA exposure increased histamine and CysLT release
from mast cells, and that these responses could not be attenuated by inhibiting ERα. Yet, BPA-induced histamine and CysLT increases were found to require ERK signaling and extracellular Ca^{2+}. These observations suggest that BPA exposure may exacerbate inflammation mediated by enhanced mast cell degranulation in atopic individuals.

Materials and Methods

Animals

Wild type female C57BL/6 mice, 8 weeks of age, were purchased from Charles River (Wilmington, MA) and housed in a University of Michigan animal facility. Animals were treated according to National Institutes of Health guidelines for the use of experimental animals with approval of the University of Michigan Committee for the Use and Care of Animals.

Generation and Culture of BMMCs

Following euthanasia by CO₂ inhalation, femurs were obtained from mice and lavaged with RPMI (Life Technologies, Invitrogen, Carlsbad, CA). Primary BMMCs were generated by culturing bone marrow cells in RPMI containing 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) supplemented with 10 ng/mL murine IL-3 (Sigma, St. Louis, MO) and 10 ng/mL murine stem cell factor (Sigma) at 37 °C in 5% CO₂. Throughout incubation, culture media and culture flasks were changed once weekly. After 4 weeks in culture, cells were cytospun onto glass slides using a cytocentrifuge (STAT SPIN, Norwood, MA), and the mast cell phenotype was confirmed.
when 95% of the cells were positive for c-kit. During immunocytostaining, c-kit was probed using an anti-c-kit antibody produced in rabbit (Cell Signaling, Beverly, MA), secondarily probed using a goat anti-rabbit avidin biotin complex kit (Vector, Burlingame, CA) and visualized using a diaminobenzidine kit (Vector) according to the manufacturer’s instructions.

*Stimulation of BMMCs for Pro-inflammatory Mediator Release*

Differentiated BMMCs were collected by centrifugation, resuspended in RPMI containing 1% penicillin/streptomycin, enumerated using a hemocytometer, and plated in flat-bottom 96-well plates at a concentration of 2.0 x 10^5 cells per well. Plated BMMCs were treated with vehicle control (0.01% ethanol), BPA (0.1, 1, 10, 100, or 1000 nM) (National Toxicology Program standard), or 17β-estradiol (E2) (0.1, 1, 10, 100, or 1000 nM) (Sigma) for 30 minutes at 37 °C with 5% CO_2 to induce BMMC release of histamine and CysLTs. As an internal control, cells were also treated with 1 μM of the calcium ionophore A23187 (Sigma) to induce mass release of pro-inflammatory mediators. In subsequent experiments, plated BMMCs were pretreated with the ER antagonist ICI 182,780 (referred to as ICI) (0.1, 1, or 10 μM) (Sigma), the ERK1/2 inhibitor U0126 (10 μM) (Sigma), or the Ca^{2+} chelator ethylene glycol tetraacetic acid (EGTA) (3 mM) (Sigma) for 1 hour at 37 °C with 5% CO_2 before treatment with 10 nM BPA or E2 for 30 minutes. After the allotted time, cell culture media were collected and stored at -80 °C until analysis.

*Histamine Determination*
Analysis of histamine was conducted according to the protocol previously described by Zhao et al. (Zhao et al., 2001). Briefly, 30 μL from collected supernatants were distributed on 384-well plates. 6 μL of 1 M NaOH and 1.5 μL of 10 mg/mL o-Phthaldialdehyde (Sigma) prepared in methanol were added to each sample to induce histamine derivatization. After incubation at room temperature for 4 minutes, 5 μL of 3 M HCl were added to each well to halt histamine derivatization. Fluorescence was read at 530 nm (360 nm excitation, 450 nm emission). Values are expressed as % release of the vehicle control.

CysLT Determination

The levels of CysLTs produced by BMMCs following stimulation were determined by commercially available enzyme immunoassay (EIA) kits (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions.

Statistical Analysis

Data are expressed as mean ± SEM. Analyses were conducted in Prism Graph Software using one-way analysis of variance with the Bonferroni test (Figures 2.1 and 2.2a) or Student’s t-test (Figures 2.2b and 2.3) for separation of the means. In all cases, a p-value of <0.05 was considered statistically significant.

Results

BPA Enhances BMMC Histamine and CysLT Release
To determine if short-term BPA exposure affects the release of pro-inflammatory mediators from mast cells, BMMC release of histamine and CysLTs was determined following treatment with varying concentrations (0.1 to 1000 nM) of BPA for 30 minutes (Figure 2.1). Cells exposed to 1 nM ($p=0.001$), 10 nM ($p<0.0001$), and 100 nM ($p=0.030$) BPA displayed significantly increased histamine release compared to vehicle control, with cells in the 10 nM exposure group displaying the highest mean increase in histamine release (9.0%) among BPA-exposed cells (Figure 2.1a). Increased histamine release was not significant following exposure to BPA at 0.1 nM ($p=0.076$) or 1000 nM ($p=0.061$). As a comparison, cells exposed to E2 also displayed increased histamine release in 10 nM ($p=0.0004$) and 100 nM ($p=0.046$) exposure groups, as well as at 1000 nM ($p=0.024$), compared to vehicle control. Treatment with 0.1 nM ($p=0.145$) and 1 nM ($p=0.191$) E2 did not result in significantly increased histamine release. Cells in the 10 nM E2 exposure group displayed the highest mean increase in histamine release (13.5%) among E2-treated cells. There was no difference in histamine release between BPA- and E2-exposed cells treated with matching concentrations. CysLT release from treated BMMCs was increased at 10 nM ($p=0.013$), 100 nM ($p=0.029$), and 1000 nM ($p=0.002$) BPA compared to vehicle control, with no statistical differences between concentrations (Figure 2.1b).

**ERα Antagonism on BPA-induced Histamine and CysLT Release from BMMCs**

BMMCs have previously been demonstrated to express only the ERα subunit, and not to express the ER beta (ERβ) subunit (Zaitsu et al., 2007; Jensen et al., 2010). Thus, the role of ERα in BPA-induced increased release of histamine and CysLTs from BMMCs
was investigated using the antagonist ICI (Figure 2.2). Pretreatment with 0.1 μM ($p=0.178$), 1 μM ($p=0.627$), or 10 μM ($p=0.674$) ICI before treatment with 10 nM BPA for 30 minutes, did not alter histamine release compared to BPA treatment alone (Figure 2.2a). However, pretreatment with ICI at 0.1 μM ($p=0.034$), 1 μM ($p=0.048$), or 10 μM ($p=0.049$) before treatment with 10 nM E2 decreased E2-induced histamine release from BMMCs compared with E2 alone, but did not completely abolish the response (Figure 2.2a). Pretreatment with 1 μM ICI before treatment with 10 nM BPA did not alter CysLT release ($p=0.463$) from BMMCs compared to BPA treatment alone (Figure 2.2b).

**ERK Inhibition on BPA-induced Histamine and CysLT Release from BMMCs**

To examine the role of the ERK pathway in BPA-induced increases in histamine and CysLT release, cells were pretreated with the inhibitor U0126 (Figure 2.3). Pretreatment with U0126 followed by treatment with 10 nM BPA for 30 minutes nearly completely inhibited the increase in histamine release from BMMCs observed with 10 nM BPA alone ($p=0.003$) (Figure 2.3a). A similar response was observed with CysLT release. Pretreatment with U0126 followed by treatment with 10 nM BPA resulted in decreased CysLT release compared to 10 nM BPA alone ($p=0.004$) (Figure 2.3b).

**Extracellular Ca$^{2+}$ Chelation on BPA-induced Histamine and CysLT Release from BMMCs**

The requirement of extracellular Ca$^{2+}$ needed for BPA-induced increases in histamine and CysLT release from mast cells was studied by pretreating cells and media with EGTA before treatment with 10 nM BPA (Figure 2.3). Pretreatment with the Ca$^{2+}$
chelator followed by BPA treatment nearly completely inhibited increased histamine release compared to 10 nM BPA alone ($p=0.025$) (Figure 2.3a). Similarly, EGTA pretreatment followed by 10 nM BPA treatment inhibited CysLT release compared to BPA alone ($p=0.037$), bringing EGTA-pretreated CysLT levels in line with vehicle control CysLT levels ($p=0.427$) (Figure 2.3b).

Discussion

The results of this study are the first to demonstrate that environmentally relevant concentrations of BPA stimulate BMMCs to release the pro-inflammatory mediators histamine and CysLTs. In this study, upregulated levels of histamine induced by BPA treatment match closely to the increased levels of histamine induced by E2, both of which produced nonmonotonic dose response curves for increased histamine release. Meanwhile, increased CysLT release from mast cells was observed to be monotonic in response to BPA treatment. Since BPA is a known xenoestrogen, the hypothesis that BPA (and E2) may be acting through ERα to cause changes in mediator release was tested by antagonizing ERα with ICI. The results do not support a role for ERα in BPA-induced changes in histamine or CysLT release, although, data suggest that E2-induced histamine increase is partially dependent on ERα. Additionally, the role of the ERK pathway in the BPA-induced responses was examined, since this pathway is integral for mast cell production of CysLTs and other lipid mediators (Kambayashi and Koretzky, 2007) and is also a common pathway used in ER-initiated signaling (Levin, 2011). The requirement of the ERK pathway in BPA-induced mast cell stimulation was tested by
pretreating cells with the inhibitor U0126, which actively inhibits ERK kinase 1/2 (MEK1/2), and thus passively inhibits ERK1/2 and gene transcription downstream. Pretreatment with the inhibitor resulted in diminished BMMC histamine and CysLT release compared to 10 nM BPA alone, thus supporting a novel role of the ERK pathway in BPA-mediated increases in histamine and CysLT release. Lastly, the role of extracellular Ca\(^{2+}\) on the ability of BPA to alter mediator release was examined, since cytoplasmic influx of Ca\(^{2+}\) is necessary for mast cells to induce exocytosis of granular contents (Kambayashi and Koretzky, 2007). Pretreatment with the extracellular Ca\(^{2+}\) chelator EGTA reduced histamine and CysLT release from BMMCs exposed to 10 nM BPA, suggesting a novel requirement for extracellular Ca\(^{2+}\) in the BPA-mediated response. Collectively, this study indicates that environmentally relevant levels of BPA can activate BMMCs to increase histamine and CysLT release, and do so in an ERα-independent, ERK-dependent, and Ca\(^{2+}\)-dependent manner.

While other studies have demonstrated that BPA can enhance histamine release from HMC-1 (a human mast cell line) and RBL-2H3 (a rat mast cell line) cells, this report is the first to demonstrate that BPA increases BMMC release of CysLTs and histamine and does so at levels that are in the range of human exposures. Previous reports have observed that physiologic levels of E2 (Zaitsu et al., 2007; Jensen et al., 2010) and progesterone (Jensen et al., 2010) can enhance the release of β-hexosaminidase (β-hex), a degranulatory enzyme, and LTC\(_4\) from mast cells. In partial agreement with the current study, Zaitsu et al. reported that \textit{in vitro} treatment of mast cells with E2 resulted in nonmonotonic dose responses curves for increased β-hex and LTC\(_4\) release.
Additionally, treatment of mast cells with synthetic xenoestrogens including endosulfan, dieldrin, dichlorodiphenyldichloroethylene (DDE), nonylphenol, Aroclor 1242, Aroclor 1254 (Narita et al., 2007), and 4-tert-octylphenol (Kennedy et al., 2012) has been demonstrated to increase release of β-hex.

Some studies have examined the effect of BPA exposure on mast cell mediator release, but have done so using BPA concentrations above the environmentally relevant range (Shim and Lim, 2009; Park and Lim, 2010; Lee and Lim, 2010; Lee et al., 2012). Shim and Lim demonstrated that treatment of HMC-1 cells with 50 μM BPA resulted in increased histamine, IL-4, and IFN-γ release and upregulated phosphorylation of ERK1/2 (Shim and Lim, 2009), while Lee and Lim reported increased release of IL-6 and TNF-α under the same conditions (Lee and Lim, 2010). Increased release of histamine, IL-4, and IFN-γ following treatment with the same BPA concentration was also reported in RBL-2H3 cells (Park et al., 2010). Lee et al. reported increased release of IL-6 and TNF-α and increased phosphorylation of ERK1/2 in RBL-2H3 cells following treatment with 50 μM BPA, and also reported increased in vivo levels of β-hex and histamine in BALB/c mice dosed with 5 mg BPA/kg BW/day for four weeks (Lee et al., 2012).

The requirement of ERα for E2-induced mast cell release of mediators has been clearly demonstrated by Zaitsu et al. (Zaitsu et al., 2007). Pretreatment with the ER antagonist tamoxifen followed by E2 treatment in both RBL-2H3 cells and HMC-1 cells resulted in diminished β-hex and LTC4 release compared to E2 alone (Zaitsu et al., 2007). In addition, BMMCs from ERα knockout (KO) mice did not exhibit upregulated β-hex or LTC4 release displayed by BMMCs from wild-type (WT) mice when treated with various
concentrations of E2 (Zaitzu et al., 2007). However, the requirement of ERα for enhanced mediator release induced by synthetic xenoestrogens, including BPA, remains unclear. Of the synthetic xenoestrogens examined by Narita et al., one displayed no difference in β-hex release between BMMCs of WT mice and ERα KO mice (Arocolr 1242), while another displayed a difference at only one particular dose of E2 (Arocolr 1252, 1 nM) (Narita et al., 2007). Interestingly, β-hex release from mast cells following treatment with DDE, dieldrin, or nonylphenol resulted in U-shaped dose response curves indicating that ERα was required at low and high concentrations of E2, but not at middle-range doses (Narita et al., 2007). One synthetic xenoestrogen even displayed significantly greater E2-stimulated β-hex release in the ERα KO BMMCs compared to WT BMMCs for one dose of E2 (endosulfan, 1 nM) (Narita et al., 2007).

Considering the variability in requirement of ERα for synthetic xenoestrogens to stimulate mast cells (Narita et al., 2007), it’s not surprising that no effect on ERα antagonism with regard to histamine and CysLT release following treatment with 10 nM BPA was observed.Variable ERα requirements by synthetic xenoestrogens including BPA may be explained by interactions with nonclassical ERs, especially GPER. At environmentally relevant doses, BPA has been demonstrated to interact with GPER (Bouskine et al., 2009; Sheng and Zhu, 2011). Interestingly, Dong et al. reported that BPA induced activation of ERK in ERα/β-negative breast cancer cell lines, and that siRNA blocking GPER inhibited BPA-induced ERK activation (Dong et al., 2011).

The requirement of ERK activation and Ca²⁺ influx for mast cell activation and degranulation are well understood. Allergen-mediated mast cell activation occurs when
IgE molecules bound to high-affinity Fcε receptors (FcεRI) on the mast cell surface crosslink with a single ligand. Downstream signaling rapidly induces ERK1/2 phosphorylation, thereby helping to mediate mast cell activation by initiating synthesis of lipid mediators (Kambayashi and Koretzky, 2007). Similarly, IgE crosslinking and subsequent signaling result in a critically important influx of Ca\(^{2+}\) that mediates cellular degranulation (Kambayashi and Koretzky, 2007). The novel findings that mast cell mediator release enhanced by BPA is blocked by ERK inhibition and Ca\(^{2+}\) influx inhibition suggest that BPA relies on these critical mechanisms to stimulate mast cells.

The new findings that BPA, at levels relevant to human exposure, can enhance mast cell release of histamine and CysLTs suggests that exposure to this endocrine-active chemical can exacerbate symptoms associated with allergen-mediated inflammation. Due to the current environmental situation where humans are regularly exposed to multiple synthetic estrogens (Yang et al., 2006; Phillips and Foster, 2008; Latini et al., 2010), it will be important for future studies to examine the effect of exposure to multiple xenoestrogens at the same time on mast cell activation and function. Likewise, future \textit{in vivo} studies should examine adverse health outcomes like asthma and other allergic inflammatory diseases that may be augmented following exposure to multiple xenoestrogens.
Figure 2.1: Percent increase in histamine release (a) from BMMCs following 30-minute treatment with E2 (open bars) or BPA (solid bars), and CysLT release (b) following 30-minute treatment with BPA. Bars represent mean ± SEM. *p<0.05 and **p≤0.001 compared to untreated cells.
Figure 2.2: Percent increase in histamine release (a) from BMMCs pretreated with ICI for 1 hour before treatment with 10 nM E2 (open bars) or 10 nM BPA (solid bars) for 30 minutes, and CysLT release (b) from BMMCs pretreated with (shaded bar) or without (solid bar) 1 μM ICI before treatment with 10 nM BPA. Bars represent mean ± SEM. *p<0.05 compared to no ICI treatment.
Figure 2.3: Percent increase in histamine release (a) and CysLT release (b) from BMMCs pretreated with 10 μM U0126 (shaded bar) or 3 mM EGTA (checkered bar) for 1 hour before treatment with 10 nM BPA for 30 minutes. Bars represent mean ± SEM. *p<0.05 and **p<0.005.
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CHAPTER 3

PERINATAL BISPHENOL A EXPOSURES INCREASE PRODUCTION OF PRO-INFLAMMATORY MEDIATORS IN BONE MARROW- DERIVED MAST CELLS OF ADULT MICE

Abstract

Bisphenol A (BPA) is a widely used monomer of polycarbonate plastics and epoxide resin that has been implicated in asthma pathogenesis when exposure occurs to the developing fetus. However, few studies have examined the relationship between perinatal BPA exposure and asthma pathogenesis in adulthood. This study used an isogenic mouse model to examine the influence of perinatal BPA exposure via maternal diet on inflammatory mediators associated with asthma in 6-month-old adult offspring by measuring bone marrow-derived mast cell (BMMC) production of lipid mediators (cysteinyl leukotrienes and prostaglandin D$_2$), cytokines (IL-4, IL-5, IL-6, IL-13, and TNF-α), and histamine. Global DNA methylation levels in BMMCs from adult offspring were also determined to gauge the permanence of BPA-induced changes in the production of pro-inflammatory mediators. Four environmentally relevant BPA exposure doses were tested: low (50 ng BPA/kg diet, n=5), medium (50 μg BPA/kg diet, n=4), high (50 mg BPA/kg diet, n=4), and control (n=3). Following BMMC activation, increases in cysteinyl leukotriene ($p<0.01$) and TNF-α ($p<0.05$) production were observed in all BPA-exposure groups, and increases in prostaglandin D$_2$ ($p<0.01$) and IL-
13 (p<0.01) production were observed in the high exposure group. Additionally, BMMCs from adult mice in all exposure groups displayed a decrease in global DNA methylation compared to control animals. Thus, perinatal BPA exposure displayed a long-term influence on mast cell-mediated production of pro-inflammatory mediators associated with asthma and global DNA methylation levels, suggesting a potential for mast cell dysregulation, which could affect pulmonary inflammation associated with allergic airway disease into adulthood.

**Introduction**

There is an accumulating body of literature supporting the developmental origins of adult asthma and other lung diseases (Duijts, 2012; Henderson and Warner, 2012; Harding and Maritz, 2012). For example, the worldwide prevalence of asthma, a chronic inflammatory disease of the airways characterized by wheezing, coughing, chest tightness, and airway obstruction (Murdoch and Lloyd, 2010), has been steadily increasing over the past 40 years (Lai et al., 2009; Anandan et al., 2010; To et al., 2012); current estimates suggest nearly 300 million individuals are diagnosed with asthma globally (WHO, 2007; Holgate et al., 2007). Furthermore, observational reports revealing that asthma prevalence is highest among industrialized countries suggest that one or more byproducts of industrialization present in the environment may be contributing to asthma pathogenesis, thus stressing the important role that environmental influences can have on disease status (Masoli et al., 2004; Anandan et al., 2010; Asher et al., 2010; To et al., 2012).
Bisphenol A (BPA), a monomer of polycarbonate plastics and epoxide resin, is a high production volume chemical that has been implicated in asthma pathogenesis when exposure occurs to the developing fetus (Midoro-Horiuti et al., 2010; Nakajima et al., 2012). BPA routinely leaches into the food and water supply from consumer products including food and liquid storage containers, baby bottles, and linings of aluminum cans. While the main route of human exposure to BPA is through indigestion of contaminated food and water, possible exposure through inhalation and dermal routes may exist due to the presence of BPA in dental sealants and thermal paper (Vandenberg et al., 2007; Marquet et al., 2011). Human exposure to BPA is widespread with National Health and Nutrition Examination Survey (NHANES) data revealing detectable amounts of BPA in the urine of 95% of study participants (Calafat et al., 2005; Vandenberg et al., 2012). Quantification of BPA in infant cord blood (Vandenberg et al., 2007), amniotic fluid (Edlow et al., 2012), placenta (Schonfelder et al., 2002), and fetal liver tissues (Nahar et al., 2013) shows higher quantities of free BPA than found in adult samples. The higher fetal burden of estrogenic BPA may result from the developing liver’s altered capacity for detoxification, enterohepatic recirculation, and elimination of xenobiotics (Ginsberg et al., 2004; Vandenberg et al., 2009).

Asthma pathogenesis arises from a complex interplay of genetic susceptibility and environmental exposures (Holgate et al., 2007), resulting in abnormal inflammatory responses led by T helper type 2 (Th2) lymphocytes. Mast cells play an unequivocal role in the pathogenesis of atopic asthma and other allergic diseases, and contribute to airway and lung tissue inflammation by secreting cytokines (TNF-α, IL-4, IL-5, IL-6, and IL-13)
and vasoactive agents such as histamine and lipid mediators (leukotrienes and prostaglandins). Observational epidemiologic studies indicate that mast cells are sensitive to activation or dysregulation by estradiol (E2) (Bonds and Midoro-Horiuti, 2013), and it has long been known that asthma prevalence is two to three times higher in women than in men (Vink et al., 2010; Leynaert et al., 2012). More recent studies have shown that peaks in E2 during ovulation (Vrieze et al., 2003; Thornton et al., 2012) or hormone replacement therapy (Barr et al., 2004; Dratva, 2010) are associated with worsened asthma symptoms in women. Additionally, E2 (Zaitsu et al., 2007) and other xenoestrogens (Narita et al., 2007; Kennedy et al., 2012) are capable of activating human or rat mast cell lines in vitro in the absence of IgE crosslinking.

It has been suggested that heightened sensitivity to industrialization exposures, such as BPA, during critical windows of development may influence the pathogenesis of asthma or other allergic airway diseases in the exposed individual (Ahmed, 2000; Teitell and Richardson, 2003; Clayton et al., 2011). Perinatal exposures to BPA have resulted in altered Th2 allergic responses using the ovalbumin-induced asthma model in mice (Midoro-Horiuti et al., 2010; Bauer et al., 2012; Nakajima et al., 2012). Additionally, at μM levels, BPA has been demonstrated to increase the release of TNF-α, IL-6, and histamine in both HMC-1 cells (human mast cell line) (Lee and Lim, 2010) and RBL-2H3 cells (rat mast cell line) (Lee et al., 2012). Using an isogenic mouse model, this study sought to determine if exposures to BPA through the maternal diet increase pro-inflammatory cytokine and lipid mediator production and alter global DNA methylation levels in bone marrow-derived mast cells (BMMCs) from adult mouse offspring.
Interestingly, it was observed that cysteinyl leukotrienes (CysLTs), prostaglandin D$_2$ (PGD$_2$), TNF-$\alpha$, and IL-13 production were increased at 6 months of age following perinatal BPA exposure. A decrease in global DNA methylation levels in BMMCs from BPA-exposed animals was also observed. Thus, perinatal exposure to BPA results in enhanced pro-inflammatory mediator synthesis and altered DNA methylation in BMMCs from adult mice.

**Materials and Methods**

*Animals*

Breeders were obtained from a University of Michigan A$^{vy}$ breeding colony maintained on a genetically identical C57BL/6 and C3H/HeJ background (Waterland and Jirtle, 2003; Anderson et al., 2012). At 6 weeks old, virgin female breeders with an a/a genotype were randomly assigned to one of four modified phytoestrogen-free BPA-supplemented diets with 7% corn oil substituted for 7% soybean oil (Harland, Madison, WI): 50 ng BPA/kg diet (low dose, diet 09798), 50 µg BPA/kg diet (medium dose, diet 09797), 50 mg BPA/kg diet (high dose, diet 09518), or BPA-free control diet (diet 95092). All diet ingredients were supplied by Harland, except for BPA (National Toxicology Program standard). Female breeders were maintained on the assigned diet for two weeks before being paired with a heterozygous A$^{vy}$/a male. Dams and offspring remained on the assigned diet throughout gestation and lactation until weaning on post
natal day 21. At weaning, all offspring were group-housed by sex and fed the BPA-free control diet and subsequently aged to 6 months. Offspring generated from the mating paradigm were 50% a/a and 50% A<sup>vy</sup>/a, though only a/a offspring were used in this study to avoid bias introduced by the A<sup>vy</sup> retroelement, which induces ectopic Agouti expression and varies dramatically among isogenic A<sup>vy</sup>/a mice (Miltenberger et al., 1997; Waterland and Jirtle, 2003). In this study, the control, 50 ng BPA/kg diet, 50 µg BPA/kg diet, and 50 mg BPA/kg diet groups each had 3, 5, 4, and 4 subjects, respectively. Due to experimental limitations, all animals in both the 50 ng BPA/kg diet group and the 50 µg BPA/kg diet group were female, while all animals in the 50 mg BPA/kg diet group were male, and animals in the control group were one male and two females. Additionally, offspring in the control, 50 ng BPA/kg diet, 50 µg BPA/kg diet, and 50 mg BPA/kg diet groups were generated from 1, 4, 2, and 3 litters, respectively. Animals were housed in a University of Michigan animal facility and treated according to National Institutes of Health guidelines for the use of experimental animals with approval of the University of Michigan Committee for the Use and Care of Animals.

*Generation and Culture of BMMCs*

Following euthanasia with CO<sub>2</sub> inhalation, femurs were obtained from mice and lavaged with RPMI (Life Technologies, Invitrogen, Carlsbad, CA). Primary BMMCs were generated by culturing bone marrow cells in RPMI containing 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) supplemented with 10 ng/mL murine IL-3 and 10 ng/mL murine stem cell factor (Sigma, St. Louis, MO) at 37 °C in 5% CO<sub>2</sub>. Throughout incubation, culture media and culture flasks were changed once
weekly. After 4 weeks in culture, cells were cytospun onto glass slides using a
cytocentrifuge (STAT SPIN, Norwood, MA), and the mast cell phenotype was confirmed
when 95% of the cells were positive for c-kit. During immunocytostaining, c-kit was
probed using an anti-c-kit antibody produced in rabbit (Cell Signaling, Beverly, MA),
secondarily probed using a goat anti-rabbit avidin biotin complex kit (Vector,
Burlingame, CA), and visualized using a diaminobenzidine kit (Vector) according to the
manufacturer’s instructions.

Stimulation of BMMCs for Pro-inflammatory Mediator Production

BMMCs were pelleted by centrifugation, resuspended in RPMI, enumerated using a
hemocytometer, and plated in 96-well plates at a concentration of 2.0 x 10^5 cells per well.
BMMCs were cultured with vehicle (0.001% ethanol) alone (as a control) or with 100
ng/mL anti-DNP IgE antibody (Sigma) for 1 hour at 37 °C with 5% CO₂ before the
addition of 10 ng/mL DNP-BSA (Sigma) for 30 minutes to activate the cells via IgE
crosslinking (Narita et al., 2007). After stimulation, cell culture media were collected
and stored at -80 °C until analysis.

CysLT and PGD₂ Determinations

The levels of CysLTs and PGD₂ (measured after methoximation (PGD₂-MOX)) produced
by mast cells following stimulation were determined by commercially available enzyme
immunoassay (EIA) kits (Cayman Chemical, Ann Arbor, MI) according to the
manufacturer’s instructions.

Cytokine Determinations
Analyses of cytokines TNF-α, IL-4, IL-5, IL-6, and IL-13 in cell culture supernatants were conducted by the University of Michigan Immunology Core Facility using commercial EIA kits (DuoSet, R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Values below the limit of detection (4.1 pg/mL) are reported as zero.

**Histamine Determination**

Analysis of histamine was conducted according to the protocol previously described by Zhao et al. (Zhao et al., 2001). Briefly, 30 μL from collected supernatants were distributed on 384-well plates. 6 μL of 1 M NaOH and 1.5 μL of 10 mg/mL o-phthaldehyde (Sigma) prepared in methanol were added to each sample to induce histamine derivatization. After incubation at room temperature for 4 minutes, 5 μL of 3 M HCl were added to each well to halt histamine derivatization. Fluorescence was read at 530 nm (360 nm excitation, 450 nm emission). Values are expressed as % release of the control.

**Global DNA Methylation Determination**

Unstimulated BMMCs obtained from culture were collected by centrifugation, washed twice in cold PBS, and stored at -80 °C. Frozen cells were thawed, and DNA was extracted using a DNeasy Blood and Tissue Kit for purification of total DNA according to the manufacturer’s instructions (Qiagen, Valencia, CA). Samples were processed by the automated QIAcube platform (Qiagen). Isolated DNA was quantified using a micro-volume nucleic acid spectrophotometer NanoDrop2000 (Thermo Fisher Scientific,
Waltham, MA). Analysis of global DNA methylation was conducted on 50 ng of isolated sample using a commercial EIA kit that detects 5-methylcytosine (5-mC) (Epigentek, Farmingdale, NY) according to the manufacturer’s instructions.

**Statistical Analyses**

Data analyses were conducted using a one-way analysis of variance with a post-hoc Bonferroni test for separation of the means in Prism Graph Software. In all cases, a $p$-value of <0.05 was considered statistically significant.

**Results**

*Perinatal BPA Exposure Increases Adult BMMC CysLT and PGD$_2$ Levels after IgE Crosslinking*

To test whether perinatal exposure to BPA upregulates the production of pro-inflammatory lipid mediators known to contribute to the pathogenesis of allergic airway disease, BMMC secretion of eicosanoids CysLTs and PGD$_2$ following IgE crosslinking was assessed (Figure 3.1). Following activation, 3- to 4-fold increases in the secretion of CysLTs from the low ($p=0.013$), medium ($p=0.005$), and high ($p=0.036$) BPA exposure groups compared to the control group were observed, with no differences between BPA exposure groups (Figure 3.1a). Additionally, a 2-fold increase in production of PGD$_2$ in the high BPA exposure group ($p=0.009$), without increased PGD$_2$ levels in the low ($p=0.542$) and medium ($p=0.120$) BPA exposure groups, was observed (Figure 3.1b).
Perinatal BPA Exposure Enhances Adult BMMC IL-13 and TNF-α Production after IgE Crosslinking

Next, the impact of BPA exposure on IgE crosslinking-induced BMMC cytokine release was assessed (Figure 3.2). In these experiments, the levels of TNF-α, IL-4, IL-5, IL-6, and IL-13 were determined since these mast cell-derived cytokines contribute to allergic airway inflammation (Walls et al., 2001; Boyce, 2003; Bradding et al., 2006; Amin, 2012). IgE mediated activation resulted in a 3- to 4-fold increase in BMMC secretion of TNF-α from low (p=0.018), medium (p=0.008), and high (p=0.019) BPA exposure groups compared with the control group (Figure 3.2a). Furthermore, IL-13 release was also upregulated in the high BPA exposure group (p=0.0005) (Figure 3.2b). However, there were no statistically significant differences in IL-4, IL-5, or IL-6 between the control group and BPA exposure groups following BMMC stimulation (Figures 3.2c, 3.2d, 3.2f).

Effect of Perinatal BPA Exposure on Adult BMMC Histamine Release

The effect of BPA exposure on IgE crosslinking-induced histamine release was measured (Figure 3.3). Compared to controls, a statistical increase in histamine release among BMMCs of BPA-exposed animals was not observed, despite a nearly 2-fold higher release of histamine from the low (p=0.213) and medium (p=0.333) BPA exposure groups, compared to the control group.

Perinatal BPA Exposure Decreases Global DNA Methylation in Adult BMMCs
The observed increases in pro-inflammatory mediator secretion from BMMCs of animals exposed to BPA perinatally suggest increased gene transcription of cytokines and proteins involved in the synthesis of eicosanoids, which may be regulated by DNA methylation changes (Richter et al., 2007). To test this hypothesis, the relative concentrations of 5-mC in DNA extracts from BMMCs of BPA-exposed and control animals were determined by EIA. As shown in figure 3.4, global DNA methylation levels in extracts of BMMCs from the BPA-exposed animals were decreased compared to controls, although, statistical significance was observed only in the low BPA exposure group ($p=0.014$).

**Discussion**

Since little is known regarding the long-term effect of perinatal BPA exposure on mast cell function, the current study measured mediator production from BMMCs obtained from 6-month-old mice exposed perinatally to four doses of BPA (low, medium, high, and control) through the maternal diet. Enhanced lipid mediator synthesis (CysLTs and PGD$_2$) and cytokine release (TNF-$\alpha$ and IL-13) following BMMC activation via IgE crosslinking were observed. In association with these increases in pro-inflammatory mediator production, a decrease in global DNA methylation was observed. Thus, these results highlight the potential for persistent epigenetic modification by the endocrine-active monomer BPA, resulting in lasting consequences on mast cell-mediated pro-inflammatory lipid mediator and cytokine production in adulthood that could potentially worsen inflammation associated with allergic airway disease.
An important and novel finding of this study is that the increases in pro-inflammatory mediator production and changes in DNA methylation states were observed in cells obtained from adult animals that were exposed to BPA perinatally. Thus, effects of BPA exposure persisted for over five months following termination of exposure, supporting a role for early environmental exposures in mast cell dysregulation of pro-inflammatory mediator production. Several previous reports have demonstrated long-term effects from early exposures to BPA (Richter et al., 2007), especially when dealing with reproductive physiology (Markey et al., 2005; Newbold et al., 2009; Edlow et al., 2012). Additionally, there have been significant findings in newly focused epigenetic studies directed toward transgenerational outcomes in the F3 generation and beyond following an environmental exposure in the F0 generation (Skinner and Guerrero-Bosagna, 2009; Skinner et al., 2010). However, few in vivo studies have investigated long-term effects on mast cell function, or other immune functions for that matter, after perinatal BPA exposure from an epigenetic standpoint as the current has done.

CysLTs and PGD$_2$ are arachidonic acid metabolites, with CysLTs generated via the 5-lipoxygenase (5-LO) pathway and PGD$_2$ generated via cyclooxygenase-1 and -2 (COX1/2) pathway. While data from this study indicate that perinatal BPA exposure resulted in increased CysLT and PGD$_2$ release from BMMCs of adult animals, a clear mechanism explaining increased CysLT and PGD$_2$ release has yet to be determined. The observed global hypomethylation in BPA exposure groups compared to the control group may lead to increased transcription of genes whose promoter regions were less methylated following exposure (Dolinoy et al., 2007; Anderson et al., 2012).
Dysregulation of the mitogen-activated protein kinase (MAPK) pathway, which is upstream of where the 5-LO and COX1/2 pathways converge, may explain the observed responses. Yi and Krieg demonstrated that DNA demethylation resulted in increased c-Jun, p38, MAPK, and activator protein-1 (AP-1) activation (Yi and Krieg, 1998). Additionally, Lee et al. showed in head and neck cancer cell lines that silencing of suppressor of cytokine signaling-1 (SOCS-1) by DNA hypermethylation resulted in increased downstream activation of signal transducer and activator of transcription 3 (STAT3) through activation of Janus kinase 1 and 2 (JAK1/2) and extracellular signal-regulated kinase (ERK) (Lee et al., 2006). Future work examining gene-specific methylation changes to the promoter regions of the genes described here will be needed to identify the mechanisms of increased BMMC lipid mediator secretion following perinatal BPA exposure.

Following perinatal BPA exposure, the current study also observed an increase in BMMC release of the cytokines IL-13, which contributes to eosinophil recruitment and IgE production in the asthmatic lung, and TNF-α, which increases airway hyperresponsiveness and sputum neutrophils (Chung and Barnes, 1999). Future work will focus on identifying possible DNA methylation changes to the promoter regions of the Il13 gene, which may explain increased BMMC IL-13 production. Recently, Yu et al. demonstrated in a model of constitutive DNA methyltransferase 3a (Dnmt3a) and 3b (Dnmt3b) knockout (KO) mice that IL-13 cytokine production is modifiable depending on gene methylation state, with increased IL-13 production observed in KO mice compared to wild-type mice (Yu et al., 2012). Likewise, future work will examine
methylation states of the \textit{Tnf} promoter to help explain BMMC increases in TNF-\(\alpha\) production. Several reports have described age-related or disease-related CpG methylation changes to the \textit{Tnf} promoter (Gowers et al., 2011; Han et al., 2011; Wang et al., 2012), and Kamei et al. observed that overexpression of Dnmt3a in white adipose tissue of obese mice increased TNF-\(\alpha\) gene expression compared to wild-type mice (Kamei et al., 2010). In addition to the cytokines examined in this report, future research should also examine the T helper cell type 1 (Th1) cytokine INF-\(\gamma\) and the Th2 cytokine IL-4. Yoshino et al. showed that fetal exposure to BPA through maternal consumption of 300 \(\mu\)g BPA/kg BW/day from days 0 to 17 of gestation resulted in increased production of INF-\(\gamma\) and IL-4 in stimulated splenocytes (Yoshino et al., 2004). Likewise, Yan et al. demonstrated similar results by showing that maternal exposure to 100 nM BPA in drinking water two weeks before mating and during the first week of gestation increased adult offspring production of IL-4 and INF-\(\gamma\) from stimulated splenocytes (Yan et al., 2008).

It is important to note that limitations to the exposure model used in the current study are the number, litter distribution, and sex of animals included as test subjects. Animals observed in this study were generated from a larger heterozygous breeding scheme of \textit{A\textsuperscript{vy}} mice (Anderson et al., 2012; Anderson et al., 2013) and were selected based on \textit{a/a} genotype and age. Heterozygous \textit{A\textsuperscript{vy}/a} offspring were not included in the study group due to the inheritance of metabolic disorders, obesity, and tumorigenesis (Miltenberger et al., 1997; Morgan et al., 1999). Thus, the animals examined in this study represent skewed sex and litter distributions described in the Materials and
Methods section. In terms of pro-inflammatory mediator production from cells, some studies suggest sex differences in cellular production of Th2 cytokines, specifically TNF-α, and CysLTs. Spitzer reported increased TNF-α production from female rat alveolar macrophages and Kupffer cells following \textit{in vivo} ethanol treatment compared to males (Spitzer, 1999). Bouman et al. also reported that production of TNF-α, IL-12, and IL-1β was increased in healthy males compared to healthy females, but suggested that higher cytokine quantity in males was the result a higher initial number of circulating monocytes in the study participants (Bouman et al., 2004). Similarly, Pergola et al. reported a decrease in leukotriene production from peripheral monocytes, neutrophils, and whole blood of healthy males compared to healthy females, which was attributed to lower baseline expression of 5-LO in cells from males and inhibition of phospholipase D by 5α-dihydrotestosterone (Pergola et al., 2008; Pergola et al., 2011). In the current study, robust CysLT and TNF-α upregulation from BMMCs in all BPA exposure groups was observed compared to the control group, with no significance between exposure groups. Thus, it is suspected that sex differences in cytokine production are minimal, if present, for CysLT and TNF-α production in this model. On the other hand, possible mitigating influences of sex with IL-13 and PGD₂ production cannot be ruled out. For these makers, significant IL-13 and PGD₂ increases were observed only in the highest BPA exposure group, which is a group composed entirely of male animals. Future research should examine possible baseline differences in cytokine and lipid mediator production between sexes in mice and humans.
Ideally, sera BPA levels in dams or neonate offspring used in this study would be quantified to validate BPA dosing through the supplemented diets. However, the study design and limited subjects precluded these measurements. A study conducted by Jasarevic et al. reported conjugated and unconjugated (free) BPA levels in sera of deer mice dams chronically (~12 months) fed the same high BPA diet (50 mg BPA/kg diet) used in the current study (Jasarevic et al., 2013). Conjugated BPA levels ranged from 1.6 ng/mL to 157 ng/mL with a mean of ~60 ng/mL, while free BPA levels ranged from 0.79 to 19.3 ng/mL with a mean of ~5.5 ng/mL (Jasarevic et al., 2013) – values that are within the range of human exposure (Vandenberg et al., 2007). Comparatively, dams fed the control diet had conjugated or free BPA sera levels ranging from the limit of detection (0.1 ng/mL) to 0.79 ng/mL (Jasarevic et al., 2013). Jasarevic et al. also note that, based on the linear response curves for BPA pharmacokinetics (Doerge et al., 2010; Doerge et al., 2011; Taylor et al., 2011), dams chronically fed the medium or low BPA diets are expected to have free BPA sera levels below the limit of detection (Jasarevic et al., 2013). BPA levels in liver of 22-day-old offspring generated from this breeding scheme have been reported previously (Anderson et al., 2012). Offspring exposed to the high BPA diet displayed a mean conjugated BPA level of 278 ng/g and a mean free BPA level of 164 ng/g (Anderson et al., 2012). However, offspring in the medium and low exposure groups had much lower mean conjugated BPA (0.3 and 1.0 ng/g, respectively) and mean free BPA (1.8 and 1.8 ng/g, respectively) levels, which did not differ from controls (conjugated: 0.6 ng/g, unconjugated 3.7 ng/g) but were within the range of human environmental exposure (Anderson et al., 2012).
While the debate regarding removal of BPA from consumer products persists, a strength of this study is its focus on environmentally relevant levels of BPA using an exposure model that includes low (50 ng BPA/kg diet), medium (50 µg BPA/kg diet), and high (50 mg BPA/kg diet) doses, where the medium dose is the closest to the consumption levels seen in humans (Anderson et al., 2012; Anderson et al., 2013). It is important to note that significant increases in pro-inflammatory mediator secretion were observed at the lowest BPA dose, which is well below the average human consumption levels, indicating the necessity of continuing relevant BPA research, especially at low doses (Volkel et al., 2002; Tominaga et al., 2006). The current report has established an novel association between perinatal BPA exposure through maternal diet and mast cell dysregulation, as evidenced by increased CysLT, PGD$_2$, TNF-α, and IL-13 release from BMMCs of adult offspring with perinatal BPA exposures. These responses occurred in conjunction with decreased global DNA methylation in BMMCs. Due to the important role of mast cells in the development of atopic asthma, BPA-induced mast cell dysregulation could have implications on asthma pathogenesis. Future studies focusing on the developmental origins of asthma will help to lay a better groundwork for reduction of environmental exposures to the developing fetus.
Figure 3.1: Levels of CysLTs (a) and PGD$_2$ (b) secreted after IgE crosslinking from BMMCs of animals exposed perinatally to BPA. Concentrations of CysLTs and PGD$_2$ were determined in cell culture supernatants after 30 minutes of cellular activation. Bars represent mean ± SEM for $n=3-5$ mice per group. **$p<0.01$ compared to control.
Figure 3.2: Levels of TNF-α (a), IL-13 (b), IL-4 (c), IL-5 (d), and IL-6 (e) secreted after IgE crosslinking from BMMCs of animals exposed perinatally to BPA. Concentrations of TNF-α, IL-13, IL-4, IL-5, and IL-6 were determined in cell culture supernatants after 30 minutes of cellular activation. Bars represent mean ± SEM for n=3-5 mice per group. *p<0.05 and **p<0.01 compared to control.
Figure 3.3: Percentage increase in histamine release after IgE crosslinking from BMMCs of animals exposed perinatally to BPA, as compared to IgE-free control. Relative fluorescence units were determined in cell culture supernatants after 30 minutes of cellular activation. Bars represent mean ± SEM for n=3-5 mice per group.
Figure 3.4: Decreased levels of global DNA methylation in BMMCs from adult animals with perinatal BPA exposures. DNA was isolated from cultured BMMCs of adult animals, and 5-mC concentration was measured. Bars represent mean arbitrary OD units ± SEM for n=3-5 mice per group. **p<0.01 compared to control.
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CHAPTER 4

PERINATAL BISPHENOL A EXPOSURES ENHANCE ALLERGEN SENSITIZATION, BUT NOT PULMONARY INFLAMMATION, IN ADULT MICE

Abstract

Bisphenol A (BPA), a monomer of polycarbonate plastics and epoxide resin, is a high-production-volume chemical that has been implicated in asthma pathogenesis when exposure occurs to the developing fetus. However, few studies have examined the effect of in utero and early-life BPA exposure on the pathogenesis of asthma in adulthood. In this study, the influence of perinatal BPA exposure through maternal diet on allergen sensitization and pulmonary inflammation was examined. Two weeks before mating, BALB/c dams were randomly assigned to a phytoestrogen-free, BPA-free control diet or phytoestrogen-free diets containing 50 ng, 50 μg, or 50 mg of BPA/kg of rodent chow. Dams remained on the assigned diet throughout gestation and lactation until postnatal day 21 when offspring were weaned onto the BPA-free diet. Twelve-week-old offspring were sensitized to ovalbumin (OVA) with alum by intraperitoneal injection and subsequently challenged with aerosolized OVA. Sera, splenocytes, bronchoalveolar lavage fluid, and whole lungs were recovered to assess allergen sensitization and pulmonary inflammation 24 hrs after the last OVA challenge. Serum anti-OVA IgE levels were increased 2-fold in offspring of mice exposed to 50 μg and 50 mg BPA/kg.
diet as compared with the control diet. In addition, production of IL-13 and IFN-γ was increased in OVA-stimulated splenocytes recovered from mice exposed to BPA. However, pulmonary inflammation, as indicated by total and differential leukocyte counts, cytokines, and pulmonary histopathology inflammatory scores, were either not different or reduced in mice exposed to BPA through the maternal diet. While these data suggest that perinatal BPA exposure enhances allergen sensitization by increasing serum IgE and splenocyte cytokine production, a substantial impact of BPA on OVA-induced pulmonary inflammation in adulthood was not observed.

Introduction

The increase in global asthma prevalence observed since the 1970s, especially among developed countries (Lai et al., 2009; Anandan et al., 2010; To et al., 2012), has given rise to the belief that industrialization and associated environmental exposures play a role in asthma pathogenesis (Holgate et al., 2007). Human exposure to synthetic xenoestrogens, a group of chemicals structurally similar to estrogen and known to interfere with estrogen receptor signaling, has been implicated in the pathogenesis of asthma (Dodson et al., 2012). Furthermore, epidemiologic studies suggest that estrogen plays a role in asthma development and severity of symptoms (Haggerty et al., 2003; Tam et al., 2011; Bonds and Midoro-Horiuti, 2013) as evidenced by the higher prevalence of asthma in adult females compared to adult males (Vink et al., 2010; Leynaert et al., 2012), worsened asthma symptoms corresponding to peaks in estrogen and progesterone corresponding to the ovulation cycle (Vrieze et al., 2003; Thornton et
al., 2012), and an increased risk of developing adult-onset asthma in patients undergoing hormone replacement therapy (Barr et al., 2004; Dratva, 2010). Recently, human exposure to the ubiquitous xenoestrogen BPA has been associated with worsened asthma symptoms (Spanier et al., 2012; Vaidya and Kulkarni, 2012; Donohue et al., 2013).

BPA is a synthetic monomer produced in high quantities on a global scale (Vandenberg et al., 2009), and it is a regular component of polycarbonate plastic and epoxide resin products such as baby bottles, water bottles, food storage containers, and linings of metal cans (Vandenberg et al., 2007). BPA is also a component or precursor in thermal receipt paper (Biedermann et al., 2010), dental sealants (Joskow et al., 2006), and flame retardants (Meerts et al., 2001). Human exposure to BPA mainly occurs through ingestion of tainted food and drink, though exposure through inhalation and dermal absorption are possible exposure routes as well (Vandenberg et al., 2007). National Health and Nutrition Examination Survey (NHANES) data report detectable amounts of BPA and BPA metabolites in the urine of 95% of participants, indicating that BPA exposure is widespread (Calafat et al., 2005; Vandenberg et al., 2012).

Research on the developmental origins of asthma (Henderson and Warner, 2012; Duijts, 2012) has recently focused on in utero and early postnatal exposures to BPA (Midoro-Horiuti et al., 2010; Nakajima et al., 2012; Bauer et al., 2012). This study seeks to build upon existing literature by examining the effect of perinatal BPA exposure at doses relevant to human exposure on allergen-induced pulmonary inflammation in adulthood. Through use of the OVA sensitization model in BALB/c mice, markers of inflammation and allergen sensitization including cellular recruitment, cytokine and
chemokine production, lipid mediator production, lung histopathology scoring, sera anti-OVA IgE levels, and splenocyte cytokine production were assessed. The current study reports that BPA exposure enhanced OVA sensitization as indicated by elevated serum IgE and splenocyte cytokine production, but did not enhance pulmonary inflammation. These data suggest that early life exposures to BPA in humans may contribute to enhanced allergen sensitization in adulthood.

Methods and Materials

Animals

Eight-week-old male and female BALB/c breeders were obtained from Charles River (Wilmington, MA). Dams were randomly assigned to one of four modified, BPA-supplemented diets with 7% corn oil substituted for 7% soybean oil (Harland, Madison, WI): 50 ng, 50 µg, or 50 mg BPA/kg diet (diets 09798, 09797, and 09518, respectively), or a BPA-free control diet (diet 95092). All diet ingredients were supplied by Harland, except for BPA which was provided by the National Toxicology Program. Female breeders were maintained on the assigned diet for two weeks before being paired with a BALB/c sire. Dams and offspring remained on the assigned diet throughout gestation and maternal lactation until weaning at postnatal day (PND) 21. At weaning, all offspring were group-housed by sex and fed the BPA-free control diet and subsequently aged to 12 weeks. Animals were housed in a University of Michigan animal facility and treated according to National Institutes of Health guidelines for the use of experimental
animals with approval of the University of Michigan Committee for the Use and Care of Animals.

Induction of Allergic Asthma

Twelve-week-old offspring from all of the dietary treatment groups were sensitized to OVA with a single 200-μL intraperitoneal injection of a PBS solution containing 20 μg OVA (Sigma, St. Louis, MO) with 2 mg of Al(OH)₃ from Imject Alum® (Thermo Fisher Scientific, Waltham, MA) as an adjuvant. One week after sensitization, offspring were challenged twice, with 24 hours in between, by exposure to an aerosol of 3% OVA in PBS for 20 minutes using an ultrasonic nebulizer (ICEL US-800) delivering particles of 0.5-10 nm in diameter at 0.75 mL/min. Twenty-four hours after the second OVA challenge, lungs, sera, and spleens were collected from euthanized animals.

Lung Leukocyte Recovery by Bronchoalveolar Lavage (BAL) and Enumeration

Lungs were removed en bloc from euthanized mice, cannulated through the trachea, and lavaged twice with 1 mL of ice cold HEPES buffer, as previously described (Mancuso et al., 1998). The maximum amount of BAL fluid (BALF) retrievable was collected (1.6 mL on average). The total number of cells suspended in BALF was enumerated by counting on a hemacytometer under a light microscope. Differential counts were determined after cells had been cytospun onto glass slides using a StatSpin Cytofuge 2 Centrifuge (Iris Sample Processing, Westwood, MA) and stained using a modified Wright-Giemsa stain (Differential Quik Stain, Thermo Fisher Scientific). A total of 200 cells were counted in randomly chosen fields under a light microscope (×1000) by a
single observer (EO). The total number of cells per mL of a particular leukocyte subset was determined by multiplying the percentage of the population by the total number of lung leukocytes per mL collected from the same mouse, as previously described (Mancuso et al., 2002).

**Splenocyte Culture**

Excised spleens were homogenized in 2 mL of cold PBS and passed through a 40-μm filter. Suspended splenocytes were centrifuged at 1500 RPMs and 4 °C for 5 minutes, then resuspended in 1 mL of RPMI (Life Technologies, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Cells in suspension were enumerated on a hemacytometer under a light microscope and plated in 96-well culture plates at a concentration of 5.0 x 10⁵ cells/well. Cells were stimulated with or without 15 μg OVA/well for 72 hours to elicit cytokine production, after which supernatants were collected and stored at -80 °C until analysis. Due to small sample size (n=4), male and female splenocyte cytokine data were combined.

**Sera IgE Determination**

Sera were collected at the time of dissection and stored at -80 °C until analysis. The levels of anti-OVA IgE in sera were determined by a commercially available enzyme immunoassay (EIA) kit (Cayman Chemical) according to the manufacturer’s instructions. IgE measurements below the limit of detection (3.12 ng/mL) are reported as zero.

**Cytokine and Chemokine Determinations**
Measurement of the cytokines and chemokines TNF-α, INF-γ, IL-4, IL-5, IL-13, RANTES (CCL5), MCP-1 (CCL2), MIP-3 (CCL20), and eotaxin-1 (CCL11) in BALF, lung homogenates, and/or splenocyte supernatants were conducted by the University of Michigan Immunology Core Facility using commercially available EIA kits (DuoSet, R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Cytokine measurements below the limit of detection (4.1 pg/mL) are recorded as zero. Cytokine and chemokine values in lung homogenates were normalized by total protein content of samples.

*Cysteinyl Leukotriene (CysLT) and Prostaglandin D₂ (PGD₂) Determinations*

The levels of CysLTs in BALF and PGD₂ after methoximation (PGD₂-MOX) in lung homogenates were determined by commercially available EIA kits (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions. PGD₂ values in lung homogenates were normalized by total protein content of samples.

*Histology*

Excised lungs were fixed in a solution of 60% ethanol (Thermo Fisher Scientific), 30% chloroform (Sigma), and 10% glacial acetic acid (Thermo Fisher Scientific). Samples were parafilm embedded, sectioned onto slides, and stained with hematoxylin and eosin (H&E) by the University of Michigan Research Histology & Immunoperoxidase Core Facility. Lung sections were scored by a histopathologist, who was blinded to the identity of the samples, in the University of Michigan Unit for Laboratory Animal Medicine (Dr. Ingrid L. Bergin) for severity of inflammation in the following categories:
peribronchiolar inflammation (0: none; 1: mild; 2: moderate; 3: severe; 4: marked), perivascular inflammation (0: none; 1: mild; 2: moderate; 3: severe; 4: marked), and alveolar inflammation (0: absent; 1: few foci present; 2: many foci present). Each lung section was individually scored in all three categories. Categories were then summed across the parameters to give a total inflammatory score (maximum score of 10). The criteria dictating the numeric scores for peribronchiolar and perivascular inflammation were as follows: 0: none; 1: thin inflammatory peribronchiolar/perivascular infiltrate (<3 cell layers) confined to central lung; 2: dense inflammatory peribronchiolar/perivascular infiltrate (≥3 cell layers) confined to central lung; 3: thin (<3 cell layers) to dense (≥3 cell layers) inflammatory peribronchiolar/perivascular infiltrate extending to peripheral (smaller diameter) airways/vessels; 4: dense (≥3 cell layers) inflammatory peribronchiolar/perivascular infiltrate extending to pleural surface.

Statistical Analyses

All data are expressed as mean ± SEM. Analyses for data in table 4.1 were conducted using a one-way analysis of variance with a post-hoc Bonferroni test for separation of the means in Prism Graph Software. Each data point in figures 4.1-4.5 represents a measurement from a single offspring. Data in figures 4.1-4.5 that were normally distributed were analyzed using a categorical mixed model to test the significance of BPA-exposed groups to the control group where measure = intercept + beta1*(bpadose1=1) + beta2*(bpadose2=2) + beta3*(bpadose3=3). Within-litter correlation was adjusted by including a random intercept by litter with BPA exposure group as a fixed effect. Data in figures 4.1-4.5 with skewed distributions were analyzed
using generalized estimating equations with Poisson distribution. Within-litter correlation was adjusted by using a compound symmetric covariance matrix. Analyses for figures 4.1-4.5 were conducted in statistical analysis system (SAS) software. Data from female and male offspring were analyzed separately. In all cases, a $p$-value of <0.05 was considered statistically significant.

Results

Impact of BPA Exposure on Offspring

Consistent with previous reports, prenatal exposure to 50 ng BPA/kg (4 litters, 20 offspring), 50 μg BPA/kg (4 litters, 23 offspring), or 50 mg BPA/kg (4 litters, 18 offspring) did not significantly alter litter size ($p=0.790$) or offspring survival ($p=0.603$), compared to control offspring (4 litters, 19 offspring) (Table 4.1) (Anderson et al., 2012; Anderson et al., 2013). Interestingly, BPA exposure increased the percentage of female offspring per litter among dams fed the 50 mg BPA/kg diet compared to control dams ($p=0.036$). The mean percentage of female offspring per litter was approximately 80% in 50 mg BPA/kg diet litters, compared to 44% in control litters. There was no significant difference in the percentage of female offspring per litter in 50 ng BPA/kg diet ($p=0.171$) or 50 μg BPA/kg diet ($p=0.402$) exposure groups compared to controls.

Anti-OVA IgE Sera Levels
To determine the impact of BPA exposure on systemic OVA sensitization, anti-OVA IgE levels in sera were measured (Figure 4.1). A modest increase in sera anti-OVA IgE levels in female (\(p=0.016\)) and male (non-significant increase, \(p=0.096\)) offspring exposed to the 50 ng BPA/kg diet was observed, while mean IgE levels increased 2-fold in sera obtained from both female and male offspring exposed to the 50 μg (female: \(p<0.0001\), male: \(p=0.021\)) and 50 mg BPA/kg (female: \(p<0.0001\), male: \(p=0.038\)) diets, compared to respective controls.

*OVA-stimulated Splenocyte Cytokine Production*

Since the balance of T helper cell type 1 (Th1) and Th2 cytokines plays an important role in driving the production of IgE, cytokine production in splenocytes obtained from mice was examined in order to determine if BPA exposure influences Th1-Th2 balance (Figure 4.2). As shown in figure 4.2a, the Th2 cytokine IL-13, known to promote immunoglobulin class switching to IgE, was increased in OVA-stimulated splenocytes obtained from mice exposed to the 50 μg (\(p=0.004\)) and 50 mg BPA/kg (\(p=0.028\)) diets, compared to controls. Interestingly, IFN-\(\gamma\) production was also increased in OVA-stimulated splenocytes obtained from offspring exposed to the 50 ng (\(p<0.0001\)), 50 μg (\(p<0.0001\)), and 50 mg BPA/kg (\(p<0.0001\)) diets (Figure 4.2b). There was no difference in IL-4, IL-5, or TNF-\(\alpha\) production from OVA-stimulated splenocytes between any treatment groups (data not shown). Additionally, there was no difference in the production of any cytokine (IL-4, IL-5, IL-13, IFN-\(\gamma\), or TNF-\(\alpha\)) from splenocytes that were not stimulated with OVA (data not shown).

*Leukocyte Recruitment following OVA Challenge*
The influence of perinatal BPA exposure on leukocyte recruitment to the lungs following OVA challenge was examined as one endpoint indicative of altered pulmonary inflammation (Figure 4.3). As shown in figure 4.3a, the total number of leukocytes obtained from BALF of male offspring exposed to 50 ng (\(p<0.0001\)) or 50 mg BPA/kg (\(p=0.0003\)) were decreased compared to male controls. Additionally, females exposed to 50 mg BPA/kg displayed a modest decreased (\(p=0.047\)) in total leukocytes, compared to female controls. Total eosinophil cell counts revealed a decrease among female mice exposed to 50 mg BPA/kg (\(p<0.0001\)) and males exposed to 50 ng BPA/kg (\(p=0.041\)) (Figures 4.3b, 4.3c). Total polymorphonuclear neutrophils (PMNs) were also decreased in male mice exposed to 50 ng (\(p<0.0001\)), 50 μg (\(p=0.018\)), and 50 mg BPA/kg (\(p<0.0001\)), while total lymphocytes were decreased in males exposed to 50 mg BPA/kg (\(p<0.0001\)) and total macrophages were decrease in males exposed to 50 ng BPA/kg diet (\(p=0.027\)) (Figure 4.3c). Total PMNs, lymphocytes, and macrophages did not differ between exposure groups in females (Figure 4.3b). In addition, the percentage of lymphocytes recovered from both female (\(p=0.001\)) and male (\(p=0.015\)) mice exposed to the 50 ng PBA/kg diet were increased, compared to respective control offspring (Figures 4.3d, 4.3e). Female offspring exposed to 50 mg BPA/kg also displayed a modest decrease in the percent airway leukocytes that were eosinophils (\(p=0.045\)), which was accompanied by an increases in percentage of PMNs (\(p=0.019\)) (Figure 4.3d). The percent of airway leukocytes that were macrophages did not differ between exposure groups among males or females.

_Cytokines and CysLTs in BALF following OVA Challenge_
Cytokine and CysLT concentrations in BALF were measured as one way to determine if perinatal BPA exposure effects pulmonary inflammation following OVA challenge (Figure 4.4). The concentrations of IL-4, IL-13, and TNF-α in BALF collected from female offspring were significantly lower among animals exposed to the 50 ng BPA/kg (IL-4: \( p=0.007 \), IL-13: \( p=0.002 \), TNF-α: \( p=0.007 \)) and 50 mg BPA/kg (IL-4: \( p=0.001 \), IL-13: \( p=0.040 \), TNF-α: \( p=0.027 \)) diets compared to female controls (Figure 4.4a). Changes in BALF concentrations of IL-4, IL-13, and TNF-α among males did not differ between exposure groups (Figure 4.4b). Compared with their respective controls, BALF levels of IL-17 were lower for both female and male mice in all BPA exposure groups: 50 ng BPA/kg (female: \( p=0.004 \), male: \( p<0.0001 \)), 50 μg BPA/kg (female: \( p=0.006 \), male: \( p=0.001 \)), and 50 mg BPA/kg (female: \( p=0.001 \), male: \( p<0.0001 \)) (Figures 4.4a, 4.4b). Additionally, CysLTs levels were decreased in BALF from females (\( p<0.0001 \)) and males (\( p=0.003 \)) exposed to the 50 mg BPA/kg diet (Figure 4.4c). Perinatal BPA exposure had no effect on eotaxin-1 levels in BALF following OVA challenge (data not shown).

Lung Homogenate Cytokines, Chemokines, and PGD\(_2\) following OVA Challenge

Lungs were homogenized following OVA challenge in order to measure production of cytokines, chemokines, and the lipid mediator PGD\(_2\) (Figure 4.4d). An increase in RANTES production was observed in female offspring exposed to 50 ng BPA/kg diet, compared to controls (\( p=0.006 \)). However, there was no difference in the levels of TNF-α, IFN-γ, IL-4, IL-5, IL-13, MCP-1, MIP-3, eotaxin-1, or PGD\(_2\) between any BPA exposure group and controls (data not shown).
Lung sections were examined and scored as a means to directly quantify the severity of pulmonary inflammation. Examples of tissues and scoring are shown in figures 4.5a, 4.5b, and 4.5c. There was no difference in total inflammatory score among female BPA-exposed and control offspring (Figure 4.5d). Likewise, there was no difference in inflammation score between female offspring within individual parameters (i.e. peribronchiolar inflammation, perivascular inflammation, and alveolar inflammation) (data not shown). In contrast, the total inflammatory score was lower than the control for male offspring exposed to the 50 mg BPA/kg diet ($p=0.003$) (Figure 4.5e). The lower total inflammation score among 50 mg BPA/kg diet males was not driven by any one individual parameter since this exposure group consistently exhibited a significantly lower score within each inflammatory parameter (data not shown).

Discussion

The current study examined the effect of in utero and early-life BPA exposure on allergic airway inflammation in adult BALB/c mice through use of the conventional OVA sensitization-aerosol challenge model. This study was conducted using BALB/c mice since this strain is considered susceptible to allergen sensitization due to it having a Th2 dominant immune response. Offspring exposure to BPA began two weeks before fertilization and ended on PND 21. All of embryogenesis was included in the exposure window due to the considerable amount of epigenetic reprogramming that occurs during
in utero development, especially early on after fertilization (Morgan et al., 2005; Jirtle and Skinner, 2007), and the potential for BPA to act as an epigenetic disruptor by altering DNA methylation (Dolinoy et al., 2007; Bernal and Jirtle, 2010; Doshi et al., 2011; Fernandez et al., 2012; Singh and Li, 2012; Zhang et al., 2012; Patel et al., 2013). Methyl groups on DNA act as steric hindrances to silence gene transcription, and modification of the methylation state during in utero and early postnatal development could lead to long-lasting improper gene transcription and activation into adulthood. Previously, two studies using the OVA model examined the influence of in utero and early-life BPA exposure on allergic inflammation in neonate offspring (Midoro-Horiuti et al., 2010; Nakajima et al., 2012), and one study examined allergic inflammation in adult offspring (Bauer et al., 2012).

A novel observation in this study was that perinatal BPA exposure enhanced allergic sensitization to OVA in adult mice, as evidenced by increased sera anti-OVA IgE levels. This result was similar to a report by Midoro-Horiuti et al. which found elevated IgE levels in sera of juvenile BALB/c mice exposed to BPA perinatally through maternal drinking water at a concentration of 10 μg/mL and sensitized to a “suboptimal” dose of OVA with alum (5 μg OVA, 1 mg alum) on PND 4 (Midoro-Horiuti et al., 2010). In the current study, mice reached an adult age (12 weeks) prior to OVA sensitization indicating the early-life exposure to BPA may have long-term consequences on hypersensitivity responses. In addition, a study by Lee et al. also reported that BPA exposure enhances allergen sensitization (Lee et al., 2003). In the study conducted by Lee et al., adult female BALB/c mice sensitized to keyhole limpet haemocyanin (KLH, simulates
shellfish allergy) with alum and subsequently treated with 25 mg/kg BPA or saline once every other day for one week displayed elevated levels of anti-KLH IgE in sera compared to saline-treated animals (Lee et al., 2003). In contrast, Bauer et al. revealed that exposure to 0.5, 5, or 500 μg BPA/kg BW/day through maternal gavage in C57BL/6 mice from gestational day (GD) 6 through PND 21, followed by intraperitoneal OVA sensitization and subsequent challenge in adulthood, decreased anti-OVA IgE levels, compared to controls (Bauer et al., 2012). Differences in IgE responses following perinatal BPA exposure and OVA sensitization may be reflective of the timing of BPA exposure. In the current study and the report by Midoro-Horiuti et al., BPA exposure occurred through maternal diet or drinking water prior to pregnancy, throughout gestation, and throughout nursing until day 21 after birth. In contrast, Bauer et al. exposed dams to BPA beginning on GD 6 until PND 21. This detail is significant since BPA is capable of altering DNA methylation prior to implantation of the fertilized embryo and during early post-implantation development when the genome is most vulnerable to epigenetic reprogramming (Reik et al., 2001; Chao et al., 2012). Differences in mouse strains may have also influenced the levels of OVA-specific IgE, which has been demonstrated to be variable between different strains of mice (Shinagawa and Kojima, 2003) and rats (Pauwels et al., 1979).

Enhanced production of IL-13 and IFN-γ from OVA-stimulated splenocytes of mice exposed to BPA perinatally through the maternal diet and subsequently sensitized and challenged to OVA is also a novel observation of the current study. IL-13 is a classical Th2 cytokine known to promote the growth and differentiation of allergen-
specific B cells that elaborate IgE (Ingram and Kraft, 2012), while IFN-γ is a classical Th1 cytokine that can inhibit eosinophilia and IgE production during asthma (Chung and Barnes, 1999; Teixeira et al., 2005). Enhanced splenocyte production of IL-13 and IFN-γ observed in the current study is similar to a report by Yoshino et al., which observed that male mice exposed to 300 or 3000 μg BPA/kg BW/day through maternal drinking water prior to fertilization until GD 18 and sensitized to OVA as adults displayed increased IL-4 and IFN-γ production following OVA stimulation of cultured splenocytes (Yoshino et al., 2004). Combined, these results suggest that BPA exposure upregulates both Th1 and Th2 responses (Yoshino et al., 2004). This effect has also been reported in mice exposed to BPA as adults (Yoshino et al., 2003), and a similar response was observed for male mice exposed to 10 or 100 nM BPA through maternal drinking water from fertilization until GD 7 and sensitized to *Leishmania major* at 10 weeks old (Yan et al., 2008).

BPA exposure has been shown to influence Th1 and Th2 cell populations in allergen-induced inflammation models (Yoshino et al., 2004; Yan et al., 2008). A very modest increase in lymphocyte recruitment among animals in the 50 ng BPA/kg diet exposure group was also observed. This increase in lymphocyte recruitment was associated the novel observation that lung homogenate levels of RANTES, a chemoattractant known to recruit T cells to the lung in response to allergen challenge (Palmqvist et al., 2007), were increased in animals from the 50 ng BPA/kg diet group. In contrast, Bauer et al. observed a nonsignificant increased in lymphocyte recruitment to airways among adult female mice exposed to 500 μg BPA/kg BW/day via maternal gavage and sensitized with OVA or OVA with lipopolysaccharide intratracheally.
However, increased lymphocyte recruitment was not observed in females exposed to 0.5, 5, or 50 μg BPA/kg BW/day or among BPA-exposed males (Bauer et al., 2012). Likewise, neither male nor female offspring receiving the same BPA exposures but being sensitized to OVA intraperitoneally displayed changes in airway lymphocyte recruitment compared to controls (Bauer et al., 2012). Collectively, these results suggest that increases in lymphocyte recruitment, and other endpoints for that matter, could depend on a variety of factors including BPA dose, BPA exposure window, sex, age (Pilegaard and Madsen, 2004), animal strain (Gueders et al., 2009; Zhu and Gilmour, 2009), OVA type (Huntington and Stein, 2001), OVA sensitization route (Bauer et al., 2012), and OVA challenge quantity (Stumm et al., 2011). Future research should examine the influence of BPA exposure on lymphocyte recruitment and production of lymphocyte chemoattractants.

The conclusions from this study in regard to pulmonary inflammation reflect those summarized by Bauer et al. (Bauer et al., 2012). Evidence does not suggest that perinatal BPA exposure results in worsened allergen-induced pulmonary inflammation in adulthood; pulmonary inflammation, as measured by leukocyte recruitment; BALF cytokines and CysLTs; lung homogenate cytokines, chemokines, and PGD$_2$; and histopathology scores, appeared to be unaffected or possibly dampened among BPA-exposed animals. The enhanced eosinophilia and airway hyperreactivity observed in animals exposed perinatally to BPA in studies by Midoro-Horiuti et al. and Nakajima et al. are likely complemented by the young age at which offspring were sensitized and challenged (Midoro-Horiuti et al., 2010; Nakajima et al., 2012). Juvenile sensitivity to
hormone dysregulation may possibly influence airway inflammation outcomes (Bauer et al., 2012), but as neonates age into adulthood in the absence of the original BPA exposure, the body burden of BPA that is present at weaning will be gradually reduced as well. Thus, body burden of BPA at the time of sensitization and challenge may also affect pulmonary inflammation outcomes. However, this concept of reduced BPA body burden does not factor in possible epigenetic dysregulation occurring in early development as a result of BPA exposure, which could affect immune responses into adulthood (Teitell and Richardson, 2003).

OVA sensitization in mice provokes the recruitment of eosinophils to the lung and is used as a model of atopic asthma. Using a “suboptimal” model for OVA sensitization, Midoro-Horiuti et al. demonstrated increased eosinophil counts in the lungs of mice exposed to BPA during perinatal development (Midoro-Horiuti et al., 2010). However, in the current study, mice exposed to BPA through the maternal diet displayed no difference or a decrease in eosinophil counts. This discrepancy was most likely due to the differences in OVA sensitization. In the current study, mice were challenged with enough OVA to induce a robust influx of eosinophils into the lung (100,000 to 300,000 eosinophils per mL of BALF). In contrast, the suboptimal OVA sensitization model used by Midoro-Horiuti et al. resulted in a very modest number of eosinophils in BALF (<2,000 cells per mL of BALF) (Midoro-Horiuti et al., 2010). Therefore, if BPA exposure induces a subtle effect on eosinophil-mediated pulmonary inflammation, this may not be observed using a more robust OVA-sensitization model.
The current study reports the finding that IL-4, IL-13, and TNF-α levels in BALF from female offspring exposed to 50 ng and 50 mg BPA/kg diet were decreased compared to control females, a response not seen in males. In contrast, lung inflammatory scores were reduced in male offspring exposed to the 50 mg BPA/kg diet compared with control males, yet inflammatory score was unchanged among females. These examples of sex differences in inflammatory endpoints, while not uncommon (Blacquiere et al., 2010; Regal et al., 2006), may be caused by sex hormones known to play a role in development and severity of asthma (Balzano et al., 2001) and interaction with the endocrine disruptor BPA (Bauer et al., 2012).

A limitation of this work is the exclusion of experimental investigation into physiologic alterations in lung function following perinatal BPA exposure and subsequent allergen challenge. Previously, it was reported that in utero (Nakajima et al., 2012) and perinatal (Midoro-Horiuti et al., 2010; Nakajima et al., 2012) BPA exposure enhanced airway hyperresponsiveness after allergen challenge in juvenile mice as determined through whole-body barometric plethysmography and/or forced oscillation with methacholine challenge. However, perinatal BPA exposure did not influence airway hyperresponsiveness as measured by forced oscillation in offspring undergoing allergen challenge as adults (Bauer et al., 2012). The unchanged or dampened measures of pulmonary inflammation in adult offspring with perinatal BPA exposure observed in the current study and the report by Bauer et al. suggest a low likelihood that there would be enhancement of airway hyperresponsiveness were it assessed in the current study (Bauer et al., 2012).
The current study observed that litters born to dams fed the 50 mg BPA/kg diet were significantly more female than control litters, an effect that has not been previously reported. Colleagues using the same BPA exposure model in a mouse strain with mixed C57BL/6 and C3H/HeJ backgrounds did not observe the same effect (Anderson et al., 2012; Anderson et al., 2013). The highest dose of BPA used in this study was designed to be an order of magnitude lower than the established maximum non-lethal threshold in rodents (Takahashi and Oishi, 2003), and is above the range of normal human BPA exposure (Vandenberg et al., 2007). Future studies should seek to clarify if this is a real effect and possibly identify differences between mouse strains.

In conclusion, the current study is the first to demonstrate that exposure to BPA throughout all of gestation and early postnatal development via the maternal diet enhances allergen sensitization in adulthood. While BPA exposure was not observed to worsen pulmonary inflammation following allergen challenge, this study is the first to report on stimulated splenocyte cytokine production, cytokine levels in BALF, CysLT levels in BALF, and cytokine; chemokine; and PGD₂ levels in lung homogenates as markers of inflammation after challenge. This study suggests that BPA promotes hypersensitivity responses in adults that are exposed to this chemical in early development. BPA-induced hypersensitivity could be mediated through endocrine-disruptive mechanisms or epigenetic modification of genes that regulate Th2 cytokines and IgE-mediated allergic responses, or a combination of both. Based on these findings, developmental BPA exposures may play a role in the asthma pathogenesis, while not worsening pulmonary inflammation in adulthood.
<table>
<thead>
<tr>
<th>Exposure</th>
<th>Number of litters</th>
<th>Mean Litter Size</th>
<th>Mean Offspring Survival Rate</th>
<th>Mean Percent of Female Offspring</th>
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<tr>
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<td>4.75 ± 1.25</td>
<td>1.00 ± 0.00</td>
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</tr>
<tr>
<td>50 ng BPA/kg diet</td>
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<td>6.33 ± 0.33</td>
<td>0.90 ± 0.10</td>
<td>55.8 ± 6.72</td>
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<tr>
<td>50 μg BPA/kg diet</td>
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<td>0.91 ± 0.06</td>
<td>53.5 ± 10.48</td>
</tr>
<tr>
<td>50 mg BPA/kg diet</td>
<td>4</td>
<td>5.00 ± 0.58</td>
<td>1.00 ± 0.00</td>
<td>79.2 ± 14.43 *</td>
</tr>
</tbody>
</table>

* *p<0.05

**Table 4.1:** Number of litters, mean litter size, mean offspring survival rate, and mean percent of female offspring displayed for each exposure group.
Figure 4.1: Anti-OVA IgE measured in sera of female (a) and male (b) offspring with developmental exposure to BPA and subsequent OVA challenge. Note that the y-axes in (a) and (b) are the same to facilitate comparison between female and male offspring. Bars represent mean ± SEM. *p<0.05 and **p<0.0001 compared to respective control (open bar).
Figure 4.2: Production of cytokines IL-13 (a) and IFN-γ (b) from stimulated splenocytes of male and female offspring with developmental exposure to BPA and subsequent OVA challenge. Cytokines are measured in collected cell culture media. Bars represent mean ± SEM. *p<0.05, **p<0.005, and †p<0.0001 compared to respective control (open bar).
Figure 4.3: Airway influx of total leukocytes as measured in BALF of female and male offspring (a), airway influx of macrophages; PMNs; lymphocytes; and eosinophils as measured in BALF of female (b) and male (c) offspring, and percent composition of macrophages; PMNs; lymphocytes; and eosinophils among total leukocytes in BALF from female (d) and male (e) offspring all with developmental BPA exposure and subsequent OVA challenge. Bars represent mean ± SEM. *p<0.05, **p<0.005, and †p<0.0001 compared to respective control (open bar).
Figure 4.4: Airway production of cytokines IL-4, IL-13, TNF-α, and IL-17 measured in BALF of female (a) and male (b) offspring, airway production of CysLTs (c) measured in BALF of female and male offspring, and lung concentration of RANTES (d) measured in tissue homogenates from female offspring all with perinatal exposure to BPA and subsequent OVA challenge. Note that the y-axes in (a) and (b) are the same to facilitate comparison between female and male offspring. Bars represent mean ± SEM. *p<0.05, **p<0.01, †p<0.005, and ‡p≤0.001 compared to respective control (open bar).
Figure 4.5: Representative lung sections from OVA-challenged mice stained with H&E (a-c) depicting (a) no inflammation, (b) thin inflammatory infiltrate around the bronchiole and in the interbronchiolar-interarteriolar space (arrows), and (c) dense peribronchiolar inflammation (arrow). Bar = 100 μm. Total inflammatory score of lung sections from female (d) and male (e) offspring with perinatal exposure to BPA and subsequent OVA challenge. Total inflammatory score represents the summation of individual scores for peribronchiolar, perivascular, and alveolar inflammation. Bars represent mean ± SEM. *p<0.005 compared to male control (open bar).


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113


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CHAPTER 5

SUMMARY AND CONCLUSION

Summary of Research

The research presented here tested the hypothesis that exposure to environmentally relevant levels of bisphenol A (BPA) induces subclinical changes that enhance the release of pro-inflammatory mediators associated with allergic airway disease in mast cells, leading to worsened pulmonary inflammation in an allergen-induced rodent model of asthma. Both short-term and long-term changes in mast cell release of pro-inflammatory mediators were examined following BPA exposure. Chapter 2 examined the short-term effect of \textit{in vitro} exposure to BPA at environmentally relevant doses on mediator release from bone marrow-derived mast cells (BMMCs). Thirty minutes after treatment, BPA at concentrations of 1, 10, and 100 nM increased histamine release, with maximal increased histamine release observed at 10 nM. A similar response was observed when BMMCs were treated with exogenous estradiol (E2). E2 concentrations of 10, 100, and 1000 nM caused increased histamine release compared to vehicle control, with maximal histamine release observed at 10 nM. Additionally, BPA treatment at 10, 100, and 1000 nM increased cysteinyl leukotriene (CysLT) release from BMMCs, compared to vehicle control. The requirements of estrogen receptor alpha (ER\textsubscript{\alpha}), extracellular signal-regulated kinase (ERK) signaling, and extracellular calcium
ion (Ca$^{2+}$) in BPA-induced histamine and CysLT increases were individually tested by pretreating BMMCs with the ER antagonist ICI 182,780, the ERK inhibitor U0126, or the Ca$^{2+}$ chelator EGTA, respectively, before treatment with 10 nM BPA. ERα antagonism did not alter BPA-induced increases in histamine or CysLT release; though, ERα antagonism partially inhibited E2-induced increased histamine release. BPA-induced increases in histamine and CysLT release were blocked by ERK inhibition and extracellular Ca$^{2+}$ chelation (Figure 5.1).

The long-term effect of BPA exposure on mast cell release of pro-inflammatory mediators was tested in chapter 3 using an in vivo BPA exposure model. Mice were exposed to low (50 ng BPA/kg diet), medium (50 µg), and high (50 mg) doses of BPA through the maternal diet during gestation and maternal lactation. After weaning on postnatal day (PND) 21, animals were transferred onto the control diet and subsequently aged to 6 months old. BMMCs from offspring were activated by IgE crosslinking to induce release of pro-inflammatory mediators. BMMCs from offspring in the low, medium, and high BPA exposure groups displayed increased CysLT and TNF-α release compared to controls. Additionally, BMMCs from animals in the high BPA exposure group exhibited increased prostaglandin D$_2$ (PGD$_2$) and IL-13 release. However, BMMC release of histamine, IL-4, IL-5, and IL-6 did not differ between BPA-exposed and control groups. Global DNA methylation in BMMC lysates was quantified as a preliminary investigation into a possible epigenetic mechanism that could explain the long-term effect of BPA exposure on mast cell mediator release. Methylation analysis revealed decreased global DNA methylation in BMMCs from BPA-exposed animals.
compared to controls, though significance was only observed for the low exposure group (Figure 5.1).

Severity of pulmonary inflammation and allergen sensitization following BPA exposure were examined in chapter 4 using the same in vivo BPA exposure model that was used in chapter 3. At weaning on PND 21, BPA-exposed offspring were transferred onto the control diet and aged to 12 weeks old. At 12 weeks old, animals were sensitized to the allergen ovalbumin (OVA) with an adjuvant by intraperitoneal injection. One week after sensitization, animals were challenged to aerosolized OVA in an enclosed chamber for 20 minutes. The challenge was repeated 24 hours later, and samples were collected 24 hour after the second challenge. Quantification of IgE in sera indicated a robust increase in circulation of anti-OVA IgE in males and females from the medium and high BPA exposure groups. Additionally, stimulation of splenocytes with OVA resulted in increased IL-13 release among animals from medium and high exposure groups and increased IFN-γ release from animals in all BPA exposure groups. However, splenocyte release of IL-4, IL-5, and TNF-α did not differ among BPA-exposed and control animals. In general, leukocyte recruitment to the airways as measured in bronchoalveolar lavage fluid (BALF) was unchanged or dampened among BPA-exposed females and males, compared to respective controls. However, female and male offspring in the low exposure group displayed an increase in the percentage of lymphocytes among total leukocytes. Similarly, quantification of pro-inflammatory mediators in BALF revealed decreased levels of IL-4, IL-13, and TNF-α in females exposed to the low dose of BPA and decreased levels of IL-4, IL-13, TNF-α, and CysLTs
in females exposed to the high dose of BPA. Except for a decrease in CysLTs in the high exposure group, these observations were not seen in male offspring. Additionally, female and male offspring in the all BPA exposure groups displayed decreased levels of IL-17 compared to controls. Eotaxin-1 measured in BALF did not differ between BPA-exposed and control animals. RANTES was quantified in lung homogenates from female offspring and revealed increased levels in females in the low exposure group. However, levels of IL-4, IL-5, IL-13, TNF-α, IFN-γ, eotaxin-1, MCP-1, MIP-3, and PGD$_2$ in lung homogenates from female offspring did not differ between BPA-exposed and control animals. Lastly, severity of inflammation was quantified by histopathological scoring. Among female offspring, there was no difference in total inflammatory score between BPA-exposed and controls animals; however, male offspring in the high exposure group scored significantly lower than control males (Figure 5.1).

The studies conducted in chapters 2 and 3 revealed that exposure to environmentally relevant levels of BPA can result in short- and long-term changes that upregulate the release of pro-inflammatory mediators from mast cells, thus confirming the first part of the proposed hypothesis. Additionally, the short-term effects study indicated a requirement for mitogen-activated protein kinase (MAPK) signaling and extracellular Ca$^{2+}$, but did not support a requirement for ERα. Meanwhile, preliminary evidence from the long-term effects study supports a mechanism involving altered DNA methylation states, but needs further investigation. Enhanced mediator release from mast cells observed in chapters 2 and 3 did not result in worsened pulmonary inflammation in the allergen-induced asthma model conducted in chapter 4. Instead, markers of
pulmonary inflammation were generally unchanged or decreased among BPA-exposed animals compared to controls, suggesting a diminished role for mast cells in the inflammatory response. However, this study revealed that BPA-exposed animals have a greater allergen sensitization compared to control animals, indicating the possibility of an enhanced inflammatory response under chronic challenge conditions.

**Contributions to Existing Knowledge**

Prior to the current report, BPA as well as other synthetic xenoestrogens had been demonstrated to stimulate release of pro-inflammatory mediators from mast cells, though, critical regulatory mechanisms had not been identified (Narita et al., 2007; Shim and Lim, 2009; Park and Lim, 2010; Lee and Lim, 2010; Lee et al., 2012; Kennedy et al., 2012). The current report has contributed to the understanding of mast cell dysregulation by demonstrating that ERK signaling and extracellular Ca\(^{2+}\) influx are required by BPA to upregulate mast cell mediator release. These findings have bearing on E2-induced and other synthetic xenoestrogen-induced mast cell dysregulation as well.

The current report has further contributed to the understanding of mast cell dysregulation by also demonstrating that *in utero* and early postnatal exposure to BPA can lead to enhanced mast cell release of inflammatory mediators in adulthood, possibly stemming from BPA-induced DNA hypomethylation in the mast cell. This report is the first to have assessed the function of any immune cell from adult animals with continuous perinatal BPA exposure. The importance of enhanced mediator release from mast cells
of adult mice with developmental BPA exposure is not limited only to the mediators measured in this report nor only to mast cells. Individual production of each pro- and anti-inflammatory cytokine and chemokine is ultimately enabled by gene transcription, and there is the potential that mediators not measured in this report had their genes expressed as a result of BPA exposure. Additionally, production of inflammatory mediators occurs in many cell types, including both immune cells and non-immune cells, using the same genes for specific mediators. Thus, the observation of enhanced mediator release from mast cells of animals with perinatal BPA exposure has broader implications on BPA-induced immune dysfunction as a whole.

Broader implications of immune dysfunction stemming from perinatal BPA exposure were observed through the allergen challenge model of asthma in adult animals reported on in chapter 4. Though it went against the proposed hypothesis, several measures used to assess pulmonary inflammation displayed significantly lower values in BPA-exposed animals compared to controls. However, the study did not mechanistically explain the observed changes. Additionally, the allergen challenge study corroborated previous findings regarding pulmonary inflammation in adulthood following perinatal BPA exposure (Bauer et al., 2012) and has helped solidify the ideal that severity of asthma symptoms in individuals with in utero and early postnatal BPA exposure are highly dependent on age (Midoro-Horiuti et al., 2010; Nakajima et al., 2012).

An important contribution to existing knowledge of asthma pathogenesis came from the allergen challenge study conducted in chapter 4 when it indicated that BPA exposure throughout all of gestation enhanced allergen sensitization in adult animals.
Not only does this finding implicate perinatal BPA exposure in contributing to asthma pathogenesis in adulthood in accordance with the developmental origins of health and disease hypothesis, it also establishes a critical window of sensitivity to BPA during early postfertilization development. Previously, enhanced allergen sensitization in adulthood was not observed when BPA exposure began on gestational day 6 and ended on PND 21 (Bauer et al., 2012). However, in the current report, BPA exposure began two weeks before fertilization and included all of gestation, thus indicating that exposure during the first six days of gestation can have a very significant impact on disease status later in life.

In summary, this work has furthered existing knowledge on environmental exposure-induced mast cell dysfunction and pulmonary inflammation associated with asthma (Figure 5.2). Additionally, this work implicates perinatal BPA exposure in asthma pathogenesis – a finding which has bearing on other diseases with perceived developmental origins – and has established early postfertilization development as a critical window of sensitivity to endocrine-active and/or epigenetic-disruptive environmental compounds in relation to allergen sensitization. The latter suggests that limiting human BPA exposure during early gestation is important for preventing the development of asthma and other allergic diseases in adulthood.

**Future Investigations**

In chapters 3 and 4, offspring were perinatally exposed to BPA during a known window of sensitivity to epigenetic insults. As mentioned, the upregulation of pro-
inflammatory mediators observed in chapter 3 and the enhancement of allergic sensitization observed in chapter 4 may be due, in part, to BPA-induced epigenetic dysregulation of methylation profiles in the promoter regions of relevant genes (Salam et al., 2012; Karmaus et al., 2013). To confirm a role for epigenetic dysregulation in the observed effects, future research should investigate gene-specific DNA methylation using banked samples. Candidate genes include Alox5 (the gene for 5-lipoxygenase) and Ptgs1 (the gene for cyclooxygenase-1), since release of CysLT and PGD2 from mast cells of animals with perinatal BPA exposure was observed to be increased. Additionally, the genes Ccl5, Ifng, Il13, and Tnf are included as candidates due to observed increased levels of the chemokine RANTES and the cytokines IFN-γ, IL-13, and TNF-α from lung tissue, splenocytes, and/or BMMCs of animals exposed to BPA perinatally. Future research should also include investigation of BPA-induced alterations to other epigenetic mechanisms, including histone modification and microRNA expression, in relation to asthma and pulmonary inflammation endpoints.

Experimental investigations of altered pulmonary inflammatory responses to allergen challenge following perinatal exposure to synthetic xenoestrogens have been limited to studies of BPA alone. Yet, other synthetic xenoestrogens have been implicated in asthma pathogenesis, such as phthalates (Bornehag and Nanberg, 2010; Bertelsen et al., 2013) and alkylphenols (Dodson et al., 2012; Suen et al., 2012). Future research should investigate the effect of developmental exposure to other synthetic xenoestrogens on asthma pathogenesis and worsened inflammation associated with asthma, especially using juvenile models (Rigoli et al., 2011). Furthermore, future studies examining
mixtures of synthetic xenoestrogens at relevant environmental doses would better represent human-environment exposures than single-exposure models.

Interestingly, the investigation of perinatal BPA exposure on allergen-induced pulmonary inflammation discussed in chapter 4 indicated a dampening of intrabronchial inflammation following allergen challenge in animals exposed to low and high levels of BPA. This observation introduces a potential role for perinatal BPA exposure in inhibiting innate host defenses against bacterial or viral infection, something that few in vivo studies have examined (Rogers et al., 2013). One study by Sugita-Konishi et al. treated adult mice subcutaneously with 5 mg BPA/kg BW/day for 5 days before intraperitoneal administration of Escherichia coli K-12 (Sugita-Konishi et al., 2003). Compared to control animals, BPA-treated animals displayed diminished peritoneal bacterial clearance 24 hours after infection, reduced macrophage and lymphocyte recruitment to the infection site, and reduced neutrophil phagocytosis (Sugita-Konishi et al., 2003). Only one study has examined the influence of perinatal BPA exposure on host defense after pathogen exposure. Roy et al. reported that mice exposed to BPA perinatally and subsequently infected with influenza A virus exhibited moderate inhibition of innate pulmonary immune defenses including decreased TNF-α, IFN-γ, RANTES, IP-10, and iNOS production and decreased histopathological inflammatory scores, although survival after infection did not differ between BPA-exposed and control animals (Roy et al., 2012). Future research should not only focus on the effect of perinatal BPA exposure on immune disorders, but also on normal immune function as well.
Discussion on Limits of Detection

In chapters 2, 3, and 4, limits of detection are reported only for cases where a sample value fell below the detection limit for a specific assay. Thus, if the limit of detection for a measure is not reported, all sample values were above the detection limit. The limits of detection for measurements conducted in this report are very low (4.1 pg/mL or lower). Due to the increased intra-assay variability and resultant heightened inaccuracy of assessing values at these low levels, values that fell below the detection limit were reported as zero. Additionally, cytokine and IgE levels that fall below the respective detection limits are highly unlikely to have a physiologic bearing in asthmatic inflammatory responses, and such low levels would be considered to have the same physiologic response as a value of zero. Values below the detection limit did not influence significance, if any, whether they were reported as zero (lowest possible estimate) or reported as the approximated value based on the standard curve (high estimate). Furthermore, Croghan and Egeghy reported that substituting values below the detection limit with either zero, half the detection limit, or the square root of the detection limit introduced little to no bias when a small percentage (≤10%) of values fell below the detection limit (Croghan and Egeghy, 2003). However, when a large percent of values (≥75%) fell below the detection limit, all substitution methods introduced large biases and were equally considered “inadequate replacement techniques” (Croghan and Egeghy, 2003; Helsel, 2006).
BPA Controversy

In the mid-2000s, unfavorable media attention began focusing on human exposure to BPA and associated adverse health outcomes, so much so that writer Jerome Groopman referred to BPA as one of the most vilified chemicals in the world (Groopman, 2010). As considerable media reports continued to depict BPA as a detrimental chemical, advocacy groups, legislators, and everyday citizens moved to limit BPA exposure by putting pressure on manufacturers to cease BPA production and by petitioning for legislative measures restricting BPA usage. While there is currently no outright ban on BPA manufacture in any country, measures to prohibit production (and in some cases import, export, sale, or advertisement) of baby bottles and other items used for infants containing BPA have been implemented in Canada (Layton and Lee, 2008), the European Union (Koch, 2010), Australia, China, Denmark, France, Germany, Malaysia, and New Zealand (Mei, 2011). Similar measures have been implemented in the states of Connecticut, Delaware, Maine, Maryland, Massachusetts, Minnesota, New York, Vermont, Washington, Wisconsin (Shapley, 2011), and California (Mohan, 2011), and last year, the US Food and Drug Administration announced a ban on production of baby bottles and sippy cups containing BPA (Koch, 2012). In some case, stricter measures banning BPA have been implemented. For example, France has moved to prohibit manufacture, import, export, and marketing of all food packing containing BPA by 2015 (Goessl, 2012), while Suffolk County, New York, has prohibited the use of BPA-containing cash register receipts (Carducci, 2013).
Opinions on legislatively prohibiting manufacture of certain consumer products that contain BPA differ between agreement, disagreement, and neutral. In 2007, the National Toxicology Program (NTP) organized an expert panel to review the current literature regarding BPA exposure and human health. While the NTP report concluded that BPA exposure is of some concern for fetuses, infants, children, and pregnant women, it did not give a recommendation on limiting exposure (NTP, 2008). Similarly, a 2010 expert panel led by the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) prepared a comprehensive report on BPA exposure and human health, concluding that there may be concern regarding human BPA exposure while not commenting on restricting production (FAO/WHO, 2010). One proponent of restricting BPA manufacture is the American Medical Association (AMA) which stated in a July 2011 report that it “encourages actions to stop producing BPA-containing baby bottles and infant feeding cups and supports bans on the sale of such products” (AMA, 2011). Conversely, the American Chemistry Council (ACC) is one opponent of legislation at state, national, and international levels that seeks to restrict BPA use, and promotes the use of BPA in manufacturing by referencing multiple government and scientific bodies that “have declared that BPA is safe as used” (ACC, 2013).

In addition to legislative reform, negative media attention focused on BPA and subsequent consumer complaints have also led to the voluntary removal of the compound from manufacturing processes and distribution. In 2008, Nalgene, a producer of reusable plastic water bottles, announced it would discontinue the use of BPA in its products.
(Austen, 2008), while large retailers including Toys “R” Us and Wal-Mart opted to removed BPA-containing baby bottles and other infant products from distribution (Lee, 2008). In more drastic cases, petitions against specific companies have led to promises from executives that BPA will be phased out of products in order to appease consumers – such was the case with Campbell’s Soup. In 2011, a petition led by Healthy Child Healthy World on Change.org calling on Campbell’s Soup to remove BPA from the linings of soup cans resulted in a response from company leaders in March 2012 agreeing to phase out BPA usage (Westervelt, 2012; Change.org, 2013). However, other companies have taken a different approach to consumer concern over BPA usage. The beverage distributor Coca-Cola, for example, published its own assessment on BPA, which conveyed that the use of BPA in epoxide can linings is safe and will continue to be used in Coca-Cola containers (Bottemiller, 2011; Coca-Cola Company, 2013).

**BPA Substitutes**

While debates over voluntary and involuntary removal of BPA from consumer products and packaging continue to play out, new investigations are beginning to examine human exposure to BPA substitutes and potential adverse health outcomes. In plastics manufacturing, when BPA is removed from a product, it is usually replaced by another bisphenol. Commonly used BPA substitutes include bisphenol B (BPB), bisphenol F (BPF), bisphenol AF (BPAF), and bisphenol S (BPS) (Figure 5.2), though other bisphenols may be used as well. While there is no information available on the production volume of any bisphenol other than BPA, recently, BPB and BPF have been
detected in some canned beverages (Cunha et al., 2011; Gallart-Ayala et al., 2011), and BPB was also been detected in canned tomatoes (Grumetto et al., 2008). Additionally, BPS has been detected in various canned foods (Vinas et al., 2010) and a variety of paper products including receipts and currency (Liao et al., 2012).

Distribution into the environment has been observed for BPAF, BPF, and BPS. Detectable levels of BPAF have been reported in samples of river water, sediment, soil, indoor dust, and well water collected near industrial sites in China (Song et al., 2012), while BPF and BPS have been detected in soil samples collected from industrial sites in the United States, Japan, Korea (Liao et al., 2012), and Germany (Fromme et al., 2002). Additionally, household dust samples collected from homes in the United States, China, Japan, and Korea that were not near industrial sites contained measurable levels of BPF, BPAF, and BPS (Liao et al., 2012). Widespread human exposure to BPS was observed in a study conducted by Liao et al. (Liao et al., 2012). In this study, total BPS was quantified in human urine samples representative of the general populations in China, India, Japan, Korea, Kuwait, Malaysia, the United States, and Vietnam (Liao et al., 2012). BPS was detected in 81% of the samples, with detection observed for each country (Liao et al., 2012). In a separate study of healthy and endometriotic women conducted by Cobellis et al., total BPB was detected in the sera of 16 out of 58 endometriotic patients, while it was not detected in the sera of any health patients (Cobellis et al., 2009). Additionally, Cunha and Fernandes reported the detection of conjugated BPB in the urine of 2 out of 20 health individuals (Cunha and Fernandes, 2010).
Despite the few studies summarized above, very little is known regarding environmental exposure in humans to BPB, BPF, BPAF, BPS, or other bisphenols that may be used as substitutes for BPA. Thus, experimental-based studies examining potential estrogenicity and toxicity of these bisphenols are limited by not knowing what doses to consider “environmentally relevant.” However, estrogenicity and cytotoxicity have been implicated for several BPA alternatives. BPB has been reported to be estrogenic as measured by proliferation of estrogen-sensitive MCF-7 cells (a human breast cancer cell line) (Hashimoto et al., 2001; Kitamura et al., 2005; Pisapia et al., 2012) and dimerization with ERs using the yeast two-hybrid system (Chen et al., 2002). In one study, BPB was reported to be more estrogenic than BPA at equal concentrations (Kitamura et al., 2005). Additionally, BPB has also been demonstrated to inhibit androgenic activity of 5α-dihydrotestosterone in NIH3T3 cells (a mouse fibroblast cell line) (Kitamura et al., 2005).

Similar to BPB, estrogenicity of BPF has been detected through MCF-7 cell proliferation (Perez et al., 1998; Hashimoto and Nakamura, 2000; Hashimoto et al., 2001; Pisapia et al., 2012), dimerization with ERs (Hashimoto and Nakamura, 2000), and increased luciferase activity in Hep G2 cells (a human liver cancer cell line) transfected with hERα and/or hERβ (ER-responsive luminescent reporter genes) (Cabaton et al., 2009); although, BPF estrogenicity was reported to be weaker than that of BPA (Pisapia et al., 2012). BPF estrogenicity was also demonstrated in an in vivo study where 22-day-old female rats treated with BPF via gavage for four days resulted in increased vaginal epithelial cell cornification and uterine wet weight (Stroheker et al., 2003). Anti-
androgenic activity of BPF has also been noted in androgen-sensitive MDA-kb2 cells (a human breast cancer cell line) transfected with MMTV-neo-Luc (an androgen receptor-responsive luminescent reporter gene) when BPF treatment resulted in lower luciferase activity (Cabaton et al., 2009). BPF has been reported to be slightly genotoxic, as indicated by increased DNA fragmentation (Cabaton et al., 2009) and increased histone H2AX phosphorylation (indicative of DNA double-strand breaks) (Audebert et al., 2011) in Hep G2 cells.

Estrogenicity has also been reported for BPAF (Perez et al., 1998), and multiple studies have observed that BPAF acts as an ERα agonist and an ERβ antagonist in both MCF-7 cells (Kitamura et al., 2005) and HeLa cells (a human cervical cancer cell line) (Matsushima et al., 2010; Li et al., 2012). Additionally, an in vivo study where adult male rats were treated with BPAF via gavage for 14 days led to decreased testosterone levels and increased luteinizing hormone and follicle-stimulating hormone levels in serum (Feng et al., 2012). BPAF has also been reported to induce apoptosis in HT-22 cells (a mouse hippocampal neuronal cell line) and primary mouse neuronal cells via activation of the MAPK pathway (Lee et al., 2013).

BPS estrogenicity has been reported to be comparable to that of BPA as measured through MCF-7 cell proliferation (Hashimoto and Nakamura, 2000; Hashimoto et al., 2001; Kuruto-Niwa et al., 2005) and dimerization with ERs (Hashimoto and Nakamura, 2000; Chen et al., 2002). Additionally, a study conducted by the European Commission Institute of Health and Consumer Protection reported BPS estrogenicity comparable to BPA estrogenicity using MCF-7 cells transfected with ERE-βGlob-Luc-SVNeo (an ER-
responsive luminescent reporter gene) and BG-1 cells (a human ovarian cancer cell line) transfected with hERα and hERβ (Grignard et al., 2012). Although, in silico modeling predictions of BPS-ER binding suggested a 37-fold lower binding affinity than that of BPA (Grignard et al., 2012). Vinas and Watson also reported that treating GH3/B6/F10 cells (a rat pituitary cell line) with BPS resulted in increased ERK1/2 phosphorylation and cell proliferation, a response similarly observed with E2 (Vinas and Watson, 2013).

**BPA Free**

The question of whether or not to go “BPA free” is one that has been, and continues to be, debated by politicians, physicians, scientists, manufacturers, and ordinary individuals. Interestingly, only two studies have attempted to substantiate marketing claims of BPA-free products and packaging. One independent study seeking to confirm that four different types of reusable water bottles labeled as “BPA free” were indeed free of BPA leachate reported that BPA levels in water stored at room temperature for 120 hours or 100 °C water cooled to room temperature over 24 hours in the tested bottles were below the limit of detection (0.05 ng/mL), thus corroborating the BPA-free claim (Cooper et al., 2011). However, a study conducted by the non-profit group Consumers Union measured BPA leachate in canned foods produced by two different companies that both labeled their products as being BPA free and subsequently found detectable BPA levels of 1 and 20 ng/mL, respectively (Consumerreports.org, 2009). Furthermore, no investigation has been conducted to confirm that the removal of BPA
from consumer products that has already occurred, both legislatively and voluntarily, has been successful in reducing human BPA exposure.

It is important to point out that “BPA free” does not equate to “xenoestrogen free” or “free of estrogen activity.” Besides BPA, other estrogenic chemicals including other bisphenols, phthalates (Gonzales-Castro et al., 2011), alkylphenols (Amiridou and Voutsa, 2011; Gurt et al., 2011; Niu et al., 2012), and brominated flame retardants (Schecter et al., 2008; Schecter et al., 2010) have been reported as food contaminants. In fact, many plastic and epoxide products used in food packaging test positive for estrogenic activity using sensitive in vitro assays (Ogawa et al., 2006; Wanger and Oehlmann, 2009; Yang et al., 2011). The unfavorable media attention surrounding BPA use and the “BPA free” movement have done little to address the bigger issue of human exposure to synthetic xenoestrogens. Research of endocrine-active chemicals is a large field that will continue to grow as human exposure to synthetic xenoestrogens persists.
Figure 5.1: Conceptual summary of research aims.
**Figure 5.2:** A proposed model for BPA-induced mast cell dysregulation and the broader effect of perinatal BPA exposure on pulmonary inflammation after allergen challenge in adulthood. BPA upregulates the release of pro-inflammatory mediators from mast cells through ERK-dependent and extracellular Ca$_{2+}$-dependent signaling as well as through DNA hypomethylation induced during perinatal development. Changes in mast cell function resulting from BPA exposure are a partial contribution to adult airway inflammation after perinatal BPA exposure but are overshadowed by changes sustained in other cell types. Many cell types contribute to allergen-induced pulmonary inflammation, including (clockwise from top left) basophils, dendritic cells, B cells, mast cells, airway epithelial cells, T cells, airway smooth muscle cells, natural killer cells, macrophages, eosinophils, and polymorphonuclear neutrophils. While perinatal BPA exposure does not worsen pulmonary inflammation, it does enhance allergen sensitization through upregulating IgE production from B cells and enhancing T cell (splenocyte) cytokine production after allergen challenge.
Figure 5.3: Comparison of the molecular structures of BPA (a), BPB (b), BPF (c), BPAF (d), and BPS (e).
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