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The effects of bisphenol A on adipose tissue development, metabolism, and endocrine function and the role it may play in the development of obesity

A Thesis Presented for the
Master of Science Degree

The University of Tennessee, Knoxville

Brantley Nelson Wyatt

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DEDICATION

To my wife, Julie

for making me incredibly happy

and the luckiest guy in the world

my parents, Harvey and Christy

for being my heroes and showing me that

you can get through anything with love

and my late sister, Shelby

for inspiring me to live life to the fullest

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ABSTRACT

While diet and sedentary lifestyle remain important factors in the development of obesity, recent findings have shown the possible involvement of environmental obesogens, chemicals that can disrupt homeostatic energy balance and increase adiposity. Bisphenol A (BPA) is a compound used in the manufacturing of plastics as a hardening agent and is ubiquitous in the environment due to its widespread use. BPA has been shown to be an endocrine disruptor through its ability to mimic estrogen, which is now known to play important roles in adipose tissue growth and metabolism. In fact, a small but compelling number of studies have shown that mice exposed to BPA *in utero* or postnatally are fatter as adults.

We hypothesized that BPA exposure exerts effects on adipose tissue, promoting adipogenesis and inflammation, and altering energy homeostasis in a manner that promotes obesity. We tested our hypothesis using both *in vitro* and *in vivo* models. First, we found that low concentrations of BPA increased the expression of the inflammatory genes, *Il-6* and *Tnfa*, approximately 1.5-3.0 fold in mouse adipose tissue explants. We also found a 3-fold increase in the expression of the lipogenic gene, *Fasn*. BPA also altered the adipose tissue metabolism, increasing the levels of a number of glycolytic and TCA cycle metabolites, suggesting that BPA may disrupt energy homeostasis. We also found that BPA exposure increased proliferation of mesenchymal stem cells approximately 1.2-fold, which are potential adipocyte precursors. The study was expanded using two different strains of mice, C57BL/6 and DBA/2J, chronically exposing them to BPA through drinking water for six weeks. A

moderate concentration of BPA increased the perigonadal fat pad mass in males. This increase in adiposity was associated with adipocyte hypertrophy and decreased serum adiponectin levels. There were also changes in the expression of some genes with BPA treatment, including a 1.4-fold increase in Leptin and decreases in some Cytochrome P450 genes; however the genes differentially expressed were different between the two strains. Our results suggest that childhood exposure to low doses of BPA, in lieu of any developmental exposure, may contribute to childhood obesity.

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LIST OF ABBREVIATIONS

BMI	Body mass index
BPA	Bisphenol A
DES	Diethylstilbestrol
E2	estradiol
EDCs	Endocrine disrupting chemicals
ER	Estrogen receptor
FASN	Fatty acid synthase
GTT	Glucose tolerance test
IL-6	Interleukin 6
LC-MS/MS	Liquid chromatography tandem mass spectrometry
NHANES	National health and nutrition examination survey
PPARγ	Peroxisome proliferator activated receptor gamma
QPCR	quantitative polymerase chain reaction
RXR	Retinoic acid receptor
SRM	selected reaction monitoring
TNFα	Tumor necrosis factor alpha
WAT	White adipose tissue

CHAPTER I

LITERATURE REVIEW

Obesity and the Role of Adipose Tissue

The incidence of obesity has increased dramatically over the past 25 years. More than 300 million people, worldwide, are obese, and the majority of developed and rapidly developing countries have seen dramatic increases in obesity (James, 2004, James et al., 2001). Obesity occurs when body fat becomes excessive and adversely affects other organs such as the heart and liver. This obesity epidemic has garnered much attention in recent years due to the overwhelming burden that obesity and its comorbidities (diabetes, cardiovascular disease, cancer, etc.) have put on the healthcare system. The estimated annual healthcare cost for obesity was 147 billion dollars in 2008, or up to 9.1% of annual healthcare cost compared to 6.5% in 1998 (Finkelstein et al., 2009). Recently, there was passage of a new healthcare bill in the United States. During this debate, there was a fair amount of discussion about ways to cut cost. Curbing the obesity trends in this country would certainly be one solution to reduce healthcare spending.

Obesity occurs as a result of a disruption of the body's energy homeostasis. When caloric intake far exceeds energy expenditure, excess energy is stored as fat in adipose tissue. The disruption of the body's energy balance equation has become inherently easier to incur as a result of the modern Western lifestyle. The average American diet consists of energy dense meals that have high amounts of fat, sugar, and

sodium. Many of the factory and industrial jobs that required physical labor have been replaced with office jobs, which have led many Americans to become much more sedentary. The combination of diet and inactivity has created an obesogenic environment in the United States and other western countries, and there is evidence that similar trends are occurring in developing nations, such as China and India (Cecchini et al., 2010).

Body fat, or adipose tissue, is the main repository for excess energy. The primary function of adipose tissue is to store the body's excess energy in the form of triglycerides. Adipose tissue is primarily made up of fat cells, or adipocytes, but it also contains other cells types such as macrophages and fibroblasts. Adipose tissue also contains preadipocytes that can undergo adipogenesis, or differentiate into mature adipocytes. Adipogenesis is regulated by the transcription factors peroxisome proliferator activate receptor gamma (PPARγ) and CCAAT-enhancing-binding proteins (C/EBPs), which, once activated, increase transcription of genes that change cell morphology and signal the adipocyte to begin storing lipids (Rosen et al., 2000).There are many different depots of adipose tissue throughout the body, and they each have different metabolic profiles. One of the most well known depots is the visceral fat that accumulates around the abdomen. Excessive abdominal fat is the depot most commonly associated with obesity and its comorbidities. Obesity can proceed through a combination of adipocyte hypertrophy, hyperplasia, or combination of both (de Ferranti and Mozaffarian, 2008). As energy intake far outweighs energy expenditure, adipocytes expand, increasing intracellular lipids. As the adipocytes continue to

expand, they signal preadipocytes to proliferate and undergo adipogenesis, thus increasing the number of mature adipocytes (Avram et al., 2007)

While the primary role of adipose tissue is the storage of excess energy, it is now known that adipose tissue acts as an endocrine organ (Siiteri, 1987, Zhang et al., 1994). Along with producing and secreting traditional hormones such as estrogen, adipose tissue produces and secretes a number of adipose-derive hormones. These hormones include the appetite regulator, Leptin, and glucose regulator, Adiponectin, as well as the inflammatory cytokines, tumor necrosis factor, alpha (TNFα) and interleukin-6 (IL-6). A chronic state of inflammation is associated with excess body fat, which may be a contributing factor to the diseases associated with obesity such as insulin resistance and cardiovascular disease (Shoelson et al., 2007). Furthermore, the inflammatory cytokines released from adipose tissue may promote adipocyte proliferation and expansion, creating a vicious cycle where adipose tissue expansion promotes inflammation, which, in turn, promotes adipose expansion (Avram et al., 2007).

Environmental Obesogens

The main driving force promoting obesity is disruption of the body's energy homeostasis, i.e. caloric intake far outweighing energy expenditure. Many believe that humans evolved to be able to sustain long periods of time with little food, and the modern Western lifestyle (high calorie diet, sedentary lifestyle) has led to the increased prevalence of obesity (Hales and Barker, 2001). While this theory certainly makes sense, many believe it doesn't fully explain the alarming rate of increase seen in the last

thirty years. Human exposure to synthetic chemicals has also increased dramatically over the past half century, concurrently with the increase in obesity (Baillie-Hamilton, 2002). A number of these chemicals are produced during many manufacturing and agricultural processes. Some are commonly used in many household items making it difficult to limit human exposure. The "environmental obesogen" hypothesis proposes that a number of environmental chemicals can disrupt homeostatic energy balance, lipid metabolism, and adipogenesis, leading to increasing adiposity (Grun and Blumberg, 2007, Grun and Blumberg, 2009a). The term "obesogen" was first used by Dr. Bruce Blumberg of the University of California Irvine in 2006, and a Pubmed search of "obesogen" produces only nine hits, so the idea that synthetic chemicals in the environment can promote obesity is a fairly novel one.

While the term "environmental obesogen" is a new one, the number of chemicals with possible obesogenic properties is not low. A number of these chemicals are environmental pollutants, such as phthalates, organotins, bisphenol A (BPA), and perfluoroalkyl compounds (PFCs) (Grun and Blumberg, 2009a). Other sources of possible environmental obesogens include nutritional components, such as phytoestrogens, and pharmaceutical components, such as diethylstilbestrol (DES) and thiazolidinediones (TZDs) (Newbold et al., 2007, Heindel and vom Saal, 2009).

Perhaps the most compelling evidence for environmental factors contributing to obesity is the increase in the number of overweight infants; the prevalence of overweight infants under 6 months old has increased 73% since 1980 (Kim et al., 2006). Because there is no official definition of overweight for children younger than 24

months, overweight was defined as weight-for-length greater than the 95th percentile. While there are a number of possible explanations for increased adiposity in newborns (maternal diet, epigenetics, etc.), *in utero* exposure to environmental pollutants is one certainly of great concern. Mothers can control their diets, but many of these chemicals are ubiquitous in the environment making it difficult to limit human exposure (Vandenberg et al., 2007). The concept of environmental obesogens is closely linked with Barker's hypothesis, or "the fetal basis of adult disease" theory. David Barker proposed this hypothesis in the 1980's to explain the correlation between low birth weight and a number of chronic conditions later in life, including cardiovascular disease and diabetes (Barker et al., 1989). Barker hypothesized that poor nutritional conditions *in utero* lead the fetus to adapt to an environment limited in nutrients. The fetus is essentially programmed for survival in a limited nutrient environment, but is born into an environment where nutrients are far from limited. The fetal basis for adult disease paradigm has been validated for obesity by a number of other *in utero* and perinatal conditions, such as maternal smoking, that result in increased adiposity later in life. The effect that *in utero* exposure to these chemicals may have on development is widely unknown, but a number of these chemicals are endocrine disruptors, compounds that can mimic and disrupt the body's natural hormones, specifically estrogen (Grun and Blumberg, 2009b).

Endocrine Disruptors

Endocrine disruptors are exogenous chemicals that mimic and disrupt the function of the body's natural hormones, and prenatal exposure to these chemicals is

believed to adversely affect gene expression and development of a number of systems (Colborn et al., 1993). Furthermore, exposure to these chemicals at other critical times in one's lifespan could have detrimental effects on systems that require hormonal signal to function properly (i.e. sexual development, reproduction, etc.). Reproductive problems, early puberty, behavioral problems, and even some cancers are some of the health problems that have been attributed to endocrine disruptors (Birnbaum and Fenton, 2003, Safe, 2004). These chemicals not only mimic the hormones of humans, but they can also have the same adverse effects on wildlife that are exposed, raising concerns about the impact that endocrine disruptors in the environment may have on a variety of organisms (Kloas, 2002).

An endogenous hormone binds its specific or group of specific hormone receptors to mediate its signaling and action. Endocrine disruptors, which generally share some structural similarity with the endogenous hormone, can also interact with the hormone receptor leading to disruption of the process. An endocrine disruptor may not be biologically active leading to attenuation of the signaling, or it may be more biologically active than the endogenous hormone leading to increased signaling. Endogenous hormones are also regulated by the body in a number of ways, including feedback inhibition, none of which would have an effect on exogenous endocrine disruptors (Zoeller, 2005, Ghisari and Bonefeld-Jorgensen, 2005).

The effect that endocrine disruptors have on the function of sex steroids, such as estrogen and androgen, is of particular concern. Dysfunction in the secretion or action of these hormones can lead to male reproductive dysfunction in wildlife and humans,

alike (Safe, 2004, Kloas, 2002). While the primary function of adipose tissue is to store energy in the form of fat, it is now known that adipose tissue also acts as an endocrine organ, synthesizing and secreting its own set of hormones known as adipokines(Kershaw and Flier, 2004). Adipocytes also express and secrete sex hormones, such as estrogen, and their receptors, and these hormones regulate adipose tissue development (Siiteri, 1987). Therefore, endocrine disrupting chemicals could potential disrupt adipocyte metabolism and energy homeostasis. Knockout models for certain components of the sex steroid pathway (aromatase, estrogen receptor, etc.) can result in dysregulation of adipocyte size and number and affect the sexually dimorphic deposition of adipose tissue (Heine et al., 2000, Murata et al., 2002). Loss of estrogen in mice via ovariectatomy results in weight gain and obesity, and menopausal women tend to gain body fat (Demir et al., 2008). Taken together, these data suggest that sex hormone dysregulation can have pro-adipogenic effects.

There are also a number of nuclear hormone receptors that act as regulators of a number of pathways important to adipose tissue, such as lipid metabolism and adipocyte proliferation and differentiation. Peroxisome proliferator activated receptors (PPARs) are the most important of these receptors, particularly the gamma isoform (PPARγ). Activated PPARγ increases the expression of key adipogenic genes that promote the storage of fatty acids and decreases the expression of lipolysis genes (Ferre, 2004). PPARs form heterodimers with 9-cis retinoic acid receptor (RXR) that can function as metabolic sensors for a variety of lipophilic hormones. The formation of preadipocytes from mesenchymal stem cells, as well as differentiation of preadipocytes,

is dependent on the activation of RXR-PPARγ (Rosen et al., 1999). The anti-diabetic drugs rosiglitazone and pioglitazone are PPARγ agonists that improve insulin sensitivity but result in increased weight gain (Larsen et al., 2003). The fact that a pharmaceutical agent can act as an obesogen through the activation of PPARγ would suggest the possible existence of other chemicals with similar effects. Recent findings have shown that some organic pollutants that are endocrine disrupting chemicals can also activate both the RXRs and PPARγ (Grun et al., 2006).

Bisphenol A

Bisphenol A (BPA) is an organic compound containing two equivalent phenol groups that was first synthesized in 1891 (Figure 1-1). In the 1930's, it was discovered that BPA has estrogenic properties, however its use as a synthetic estrogen was abandoned in favor of diethylstilbestrol (DES), which was determined to have more potent effects. DES was prescribed to women to help with difficult pregnancies, but it was discovered after it had been given to millions of women, over 30 years later, that DES was a carcinogen and could lead to reproductive defects in girls born to mothers taking DES. If DES could have such an adverse effect, it stands to reason that a chemical with similar chemical structure and properties such as BPA could have similar toxic effects.

In the 1940's, BPA began to be used in the production of plastics. BPA is used as a hardening agent in consumer plastics and is found in a number of different types, including widely used polycarbonate plastic. It is also used in dental sealants and

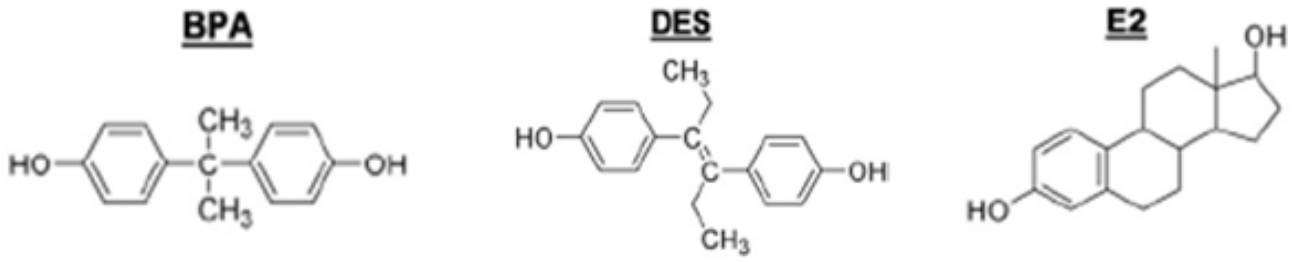


Figure 1-1. Chemical structures of Bisphenol A (BPA), diethylstilbestrol (DES), and estradiol (E_2).

fillings, CDs/DVDs, and household electronics. The epoxy resins that are used in canned food and beverage linings contain BPA, and it has commonly been used in the plastics that make baby bottles. The widespread use of BPA has led it to be one of the most produced chemicals in the world, at 2 million tons a year (Lang et al., 2008). The increased production and prevalent use has made BPA a ubiquitous chemical in theenvironment. The population is directly and chronically exposed to BPA through consumption due to its use in food and beverage packaging and storage containers, with exposure increased due to the fact that BPA leaches from plastics when exposed to heat or acidic environments (Le et al., 2008, Vandenberg et al., 2007).

Many of the effects that BPA has on health were first discovered by accident. In 1993, it was discovered that BPA leached from autoclaved polycarbonate flasks, leading to increased proliferation of breast cancer cells (Krishnan et al., 1993). Accidental exposure to BPA due to damaged polycarbonate cages led to disruption of meiosis in the oocyte of control female mice (Hunt et al., 2003). A study from the vom Saal lab tested the effects of BPA on blood serum and found that BPA had a higher binding affinity in serum than estradiol, indicating greater estrogenic bioactivity (Nagel et al., 1997). Furthermore, exposing pregnant mice to small amounts of BPA (2μg/kg/day) resulted in significantly larger prostates in male offspring compared to controls. This dose was 25,000 times lower than the toxic threshold set by the EPA (http://www.epa.gov/IRIS/subst/0356.htm).

The concern over BPA has grown over the last few years; however, there is still much debate over the potential effects of BPA exposure. One issue is the lack of

consistency in findings, and a number of studies contradict one another. Perhaps some of the dispute can be traced to the chemical companies that have an interest in protecting their multi-billion dollar industry. Over 90% of independently funded studies have found that BPA exposure at biologically relevant doses result in adverse health effects. However, every industry-funded study has found that BPA has no effect on health (vom Saal and Myers, 2008).

While chemical companies are trying to protect their own interest, there is legitimate debate and differences of opinion over data interpretation and the potential toxicity of BPA. For decades, a traditional toxicological approach was taken to study the effects of BPA. Extremely high doses of BPA were found to have adverse effects such as organ failure and cancer, but doses below a certain threshold were safe. There is now evidence that low doses, well below the threshold, have a completely different set of effects, which can effect development and metabolism. Proponents of BPA still dispute these findings, saying that the doses used in many of these studies (micromolar range) are still higher than environmentally relevant doses (low nanomolar range) (Ben-Jonathan et al., 2009). Another issue is the non-monotonic dose response of BPA. Most studies lack a linear dose-dependent curve of BPA effects, making it difficult to extrapolate the action of BPA. While the debate remains open, it is clear that BPA can act as an endocrine disruptor that can possibly disrupt a number of metabolic processes.

Bisphenol A and Obesity

BPA's endocrine disrupting properties, and its abundance in the environment has led it to be categorized as a potential environmental obesogen. The potential for BPA to increase adiposity was also first discovered by accident in 2001. The Rubin lab was studying the reproductive effects that BPA exposure had on pregnant rats. There had been some studies on the effect of BPA exposure in adult rodents but very few had examined the effects of perinatal exposure, which could produce more detrimental and irreversible effects. They found that the rats treated with a high dose (1.2 mg/kg body weight (bw)/day) or low dose (0.1 mg/kg bw/day) of BPA via drinking water produced heavier offspring compared to controls, and this phenotype persisted through adulthood (Rubin et al., 2001). Interestingly, the offspring from the lower dose were slightly heavier than the offspring of the high dose, indicating that the lower doses may be more detrimental in regards to adiposity than higher doses. They also found that female offspring exposed to the high dose had altered estrous cycles and decreased levels of plasma luteinizing hormone.

There have been a limited number of studies that further explored these effects of BPA, and a number of them have used extremely high doses. Some *in vitro* studies have shown that BPA can promote adipogenesis, increase expression of adipogenic genes, and promote inflammation. Hugo and colleagues studied the effects that low doses of BPA and E_2 have on secretion of adiponectin from adipose explants and mature adipocytes. They found that low nanomolar doses (0.1nM and 1nM) inhibited

Table 1-1. Summary of studies: BPA and its role as an environmental obesogen

Model	BPA Dose	Physiological Effect	Reference
In vitro studies			
murine macrophages	10nM, 100nM, 1μM	↑ production of IL-6, TNFα, MCP-1	Yamashita et al. 2005
3T3-L1 preadipocytes	80μM	↑ expression of adipogenic transcription factors	Phrakonkham et al. 2008
Human adipose explants/isolated adipocytes	0.1nM, 1nM	↓ secretion of adiponectin	Hugo et al. 2008
3T3-L1 preadipocytes	1μmol/L	↑ activation of glucocorticoid receptor, lipid accumulation	Sargis et al. 2009
human granulosa cells	40, 60, 80, 100μM	↓ E_2 secretion, ↑ PPARγ activation	Kwintkiewicz et al. 2010
In vivo studies			
Pregnant CF-1 mice	2.4μg/kg bw/day (gestation day 11-19)	↑ female offspring body weight, early onset of puberty	Howdeshell et al. 1999
Pregnant Sprague-Dawley rats	1mg/L, 10mg/L	↑ offspring body weight	Rubin et al. 2001
Pregnant Sprague-Dawley rats	1mg/L (gestation day 6-lactation)	↑ offspring body weight, parametrial WAT 3-fold (females), expression of lipogenic genes (females), adipocyte size (females)	Somm et al. 2009
Epidemiological studies			
NHANES 2003/04 survey (1453 adults)	NA	↑ urinary BPA associated with CVD, diabetes, and higher BMI	Lang et al. 2008
NHANES 2005/06 survey (1493 adults)	NA	↑ urinary BPA associated with coronary heart disease	Melzer et al. 2010

adiponectin secretion from adipose tissue and mature adipocytes, alike (Hugo et al., 2008). They also found that BPA increased the secretion of the pro-inflammatory cytokines, tumor necrosis factor α (TNFα) and interleukin-6 (IL-6). Sargis and colleagues tested the ability of a number of endocrine disrupting chemicals (EDCs) to stimulate the glucocorticoid receptor (GR) due to its central role adipocyte differentiation (Sargis et al., 2009). They treated 3T3-L1 preadipocytes with a variety of EDCs and assessed the ability of each to activate GR using a luciferase reporter construct. They found that BPA was one of four chemicals that stimulated activationof GR at a concentration of 1 μmol/L. BPA also increased lipid accumulation in differentiating adipocytes and increased expression of adipogenic proteins.

Howdeshell and colleagues fed pregnant mice BPA at a dose of 2.4μg/kg bw between days 11 and 17 of gestation. The offspring had similar birth weights. However, after weaning, female mice treated with BPA were significantly heavier than female controls (~10.5g compared to 9.25g). The results were very similar in the male counterparts (Howdeshell et al., 1999). They also found that the female mice exposed to BPA began ovulation earlier. To our knowledge, there has been only one study that has addressed BPA's effect on fat tissue. The Somm laboratory sought to determine the effect that perinatal exposure to BPA has on early adipose storage. Pregnant rats were exposed to BPA through drinking water at a dose of 1mg/L at day 6 of gestation through the end of lactation. The offspring were weaned and sacrificed at day 21 to study the perigonadal adipose tissue. They found that the birth weight for both males and females exposed to BPA were increased, however the body weight was only

increased in the females at the time of sacrifice. They also found that gestational exposure to BPA increased parametrial white adipose tissue (WAT) in female rats 3-fold. The increased pWAT was attributed to adipocyte hypertrophy; females exposed to BPA had significantly larger adipocytes (Somm et al., 2009). The exposed females also had increased expression of lipogenic genes, such as *Pparg* and fatty acid synthase (*Fasn*). These studies indicate that BPA exposure, particularly early in life, may alter adipose energy homeostasis in a way that is promoting fat storage and obesity, however the effect of postnatal and early childhood exposure remains unknown.

BPA is a lipophilic compound, therefore, it can accumulate in fat. Obesity is associated with chronic low-level inflammation that can contribute to further complications, such as cardiovascular disease (Grundy, 2004, Xu et al., 2003). BPA has been shown to upregulate important inflammatory cytokines and adipokines in adipose tissue explants, and a few *in vitro* studies have shown that it increases expression of pro-inflammatory cytokines, such as TNFα and IL-6 in macrophages (Yamashita et al., 2005, Yan et al., 2008, Hugo et al., 2008). Mice exposed to low levels of BPA *in utero* exhibited increased production of T-cell activating cytokines and diminished numbers of regulatory T-cells as adults, favoring inflammation (Yan et al., 2008). In humans, there have been significant correlations shown between urinary BPA levels and markers for inflammation (Melzer et al., 2010), and in post- but not pre-menopausal women, significant correlations have been shown between urinary BPA concentrations and markers of oxidative stress and inflammation (including C-reactive protein), suggesting interactions with estrogen-related signaling (Yang et al., 2009).

Both adipocytes and the stromal vascular fraction of cells in adipose tissue secrete pro-inflammatory cytokines. Studies now indicate that the inflammatory process of obesity begins before there are large changes in adipose tissue mass due to the infiltration of macrophages and CD8+ T-cells. These cells disrupt adipocyte metabolism and insulin sensitivity by secreting cytokines and promoting local inflammation within the adipose tissue (Greenberg and Obin, 2006, Cinti et al., 2005). If BPA increases adipose mass through adipocyte hypertrophy or hyperplasia, there would be an expected parallel increase in inflammatory adipokines. This indirect effect along with the direct effect that BPA may have on expression of TNF-α and IL-6 could have great consequences, specifically the manifestation of the metabolic syndrome.

Along with promoting inflammation, there is concern that developmental exposure to BPA may increase the number of adipocyte precursors and promote adipocyte differentiation. 3T3-L1 fibroblasts treated with BPA increased expression of important adipogenic genes, however, the doses used were well outside the biologically relevant range (Phrakonkham et al., 2008, Vandenberg et al., 2009). If BPA increases preadipocyte proliferation, adipogenesis, and inflammation at doses similar to human exposure (low nanomolar range), it could be an important contributing factor to the metabolic syndrome and the current obesity epidemic (Figure 1-2).

There have been a number of epidemiological studies to determine the level of exposure to BPA in humans and the possible adverse health effects of exposure. Perhaps the most widely known is the data acquired from the National Health and Nutrition Examine Survey (NHANES). The NHANES 2003/2004 survey provided the

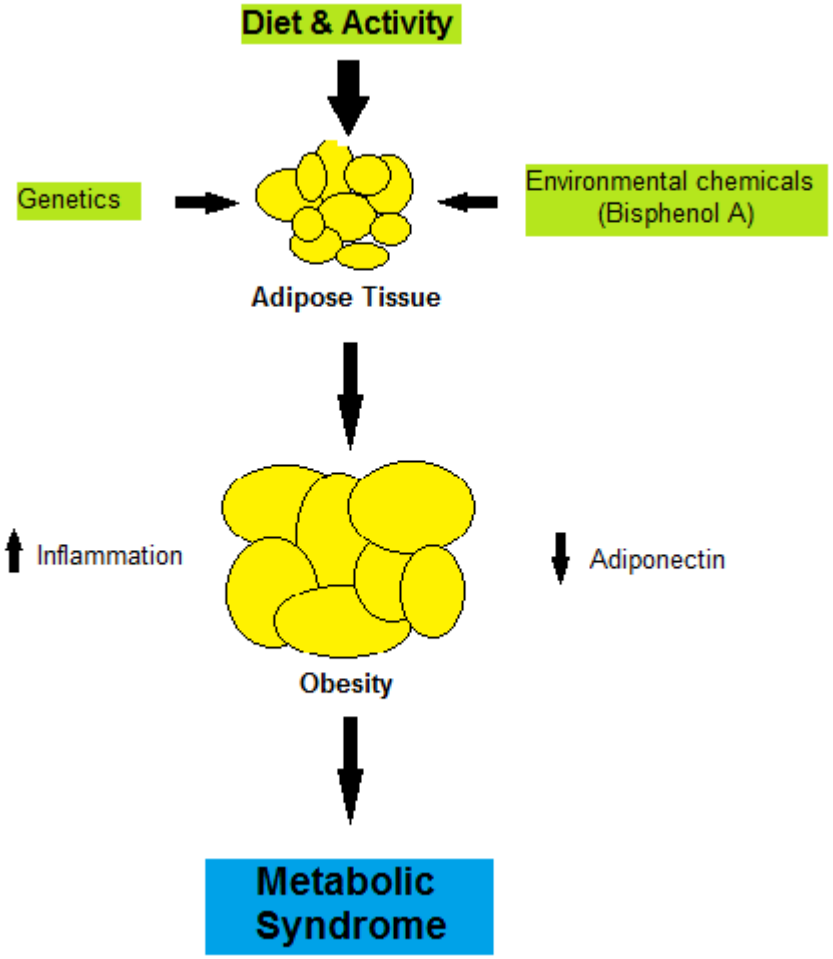


Figure 1-2. Model depicting various factors contributing to obesity and the metabolic syndrome. BPA decreases secretion of Adiponectin which results in decreases in insulin sensitivity. BPA also increases secretion of inflammatory cytokines from adipocytes which promotes insulin resistance as well as atherosclerosis and hypertension, all of which are part the metabolic syndrome (Ben-Jonathan et al., 2009).

first large scale view of BPA levels in the US population. Approximately 1500 adult participants, ages 18 to 74, with measured urinary BPA were assayed for chronic disease diagnosis as well as markers of liver function, glucose homeostasis, inflammation, and lipid changes. BPA was detected in the urine of over 92% of participants. This study found that higher urinary BPA levels were associated with increased incidence of coronary heart disease, even after regression models are adjusted for sex, age, smoking, BMI and other known variables that affect cardiovascular disease (Lang et al., 2008). Higher BPA concentrations were also associated with diabetes, and individuals with BMI's over 35 had nearly twice the level of BPA compared to individuals within the normal range (6.93 ng/mL compared to 3.91ng/mL). This study also suggested correlation between C-reactive protein levels and urinary BPA , although the relationship did not reach statistical significance when the model was fully adjusted for all potential covariates.

In a more recent study, Melzer and colleagues (Melzer et al., 2010) conducted a similar cross-sectional analysis with data from the NHANES 2005/06 study as well as data pooled across both studies. Despite average BPA levels being 30% lower in the 2005/06 group compared to the 2003/04 group, they also found that higher urinary BPA levels were associated with coronary heart disease in the 2005/06 cohort, as well as the pooled data. However, association with diabetes was not significant in the 2005/06 data, but remained significant in the pooled data. The decrease in average BPA levels is most likely due to increased consumer awareness and the decreased use of certain plastics. One group absent from the previously mentioned studies is children. Another

study to assess BPA exposure in the US general population included children of 6 years of age or greater (Calafat et al., 2008). They found that children ages 6-11 had the highest levels of urinary BPA of any age group, indicating a disproportionate level of accumulation or exposure in children. This is especially troubling considering children may be the group most susceptible to BPA's potential toxic effects.

Metabolic actions of BPA

While it is unknown how BPA mediates its action on adipose tissue and other systems, there are a few of potential interactions that could explain BPA's effect (Ben-Jonathan et al., 2009). The most well known interaction occurs between BPA and the classical estrogen receptors ERα and ERβ. BPA has been shown to interact with these receptors (Gould et al., 1998, Kuiper et al., 1998), and it's possible that BPA disrupts the endogenous estrogen signaling. However BPA binds these receptors much weaker than estradiol and should not be able to compete with endogenous estrogen at the low levels that humans are exposed, yet it does (Gould et al., 1998). There are a number of speculations to explain the disparity. It is thought that BPA may bind to the estrogen receptors differently than endogenous estradiol as a result of some interaction between BPA and serum proteins or some co-ligands. These interactions allow BPA to bind the ERs near the same strength of estradiol and compete in the nanomolar range (Welshons et al., 2006, Safe et al., 2002). BPA may also elicit a rapid response by binding non-classical membrane estrogen receptors (ncmERs) (Alonso-Magdalena et al., 2005) or estrogen-related receptors (ERRs) (Ben-Jonathan et al., 2009, Takayanagi et al., 2006). In fact, Nadal et al. found that, in pancreatic islets, BPA may mimic the

actions of E_2 on blood glucose homeostasis through two different pathways: a rapid pathway via ncmER and a prolonged pathway via ER. Overstimulation of these receptors by BPA on the β-cells increased insulin biosynthesis and secretion leading to insulin resistance.

There have been few studies looking at the effects of BPA on adipose tissue *in vivo*; however many of these have conflicting data, most likely due to the wide range of doses used. Some studies have found that BPA exposure increases adipose mass in rats (Somm et al., 2009), and others find that certain doses have no effect or actually decrease body weight (Nunez et al., 2001, Seidlova-Wuttke et al., 2005). This is a major issue with attributing BPA to a number of adverse effects. As stated previously, Hugo et al. found that BPA at low nanomolar concentrations inhibited the release of adiponectin from isolated human adipocytes and stimulated the release of IL-6 and TNFα, a direct effect of BPA that would be predicted to diminish insulin sensitivity (Hugo et al., 2008). There is also evidence that BPA may activate the key adipogenic regulator PPARγ. BPA treatment significantly up-regulated PPARγ in a dose dependent manner in human granulose cells, resulting in reduced insulin-like growth factor 1 (IGF-1) and aromatase expression and E_2 secretion (Kwintkiewicz et al., 2010).

It is clear that BPA has endocrine disrupting properties and can have adverse effects on health, especially at extremely high doses. The effects that BPA has at low doses are not quite as clear. Along with studying the effects that nanomolar doses of BPA has on adipocyte endocrine function, gene expression, and overall adiposity, we are also interested in how BPA affects adipocyte metabolism. Our goal is to determine

if biologically relevant levels of Bisphenol A (BPA) effect the development, endocrine function, and metabolism of adipose tissue in a way that promotes obesity.

CHAPTER II

GENERAL EXPERIMENTAL DESIGN

Our goal is to determine if biologically relevant levels of Bisphenol A (BPA) effect the development, endocrine function, and metabolism of adipose tissue in a way that promotes obesity. The ultimate purpose is to identify the potential metabolic pathways and genes that mediate response to BPA and ultimately understand the effects of BPA on adiposity. The research employed both *in vitro* and *in vivo* methods to meet these goals.

We began by assessing the effects of BPA using *in vitro* systems. Adipose tissue ex-plants were exposed to varying doses of BPA and analyzed for gene expression and changes in metabolism. We also used mesenchymal stem cells (MSCs) to assess the effects that BPA has on proliferation and adipogenesis (Part 1).

Secondly, we used two strains of mice (C57BL/6J and DBA/2J) to look at the effects that biologically relevant levels of BPA has on overall adiposity and adipose tissue gene expression, endocrine function, and metabolism (Part 2). BPA was delivered orally, via drinking water, which is the most common route of exposure in humans. Mice were exposed to BPA from 4 weeks of age to 12 weeks of age. Intraperitoneal glucose tolerance tests (GTTs) were conducted at 8 weeks of age. Adipose tissue was collected and analyzed for changes in gene expression (Illumina®), metabolism (mass spectrometry), and protein levels (Luminex®). We also collected serum to look at fasting insulin levels.

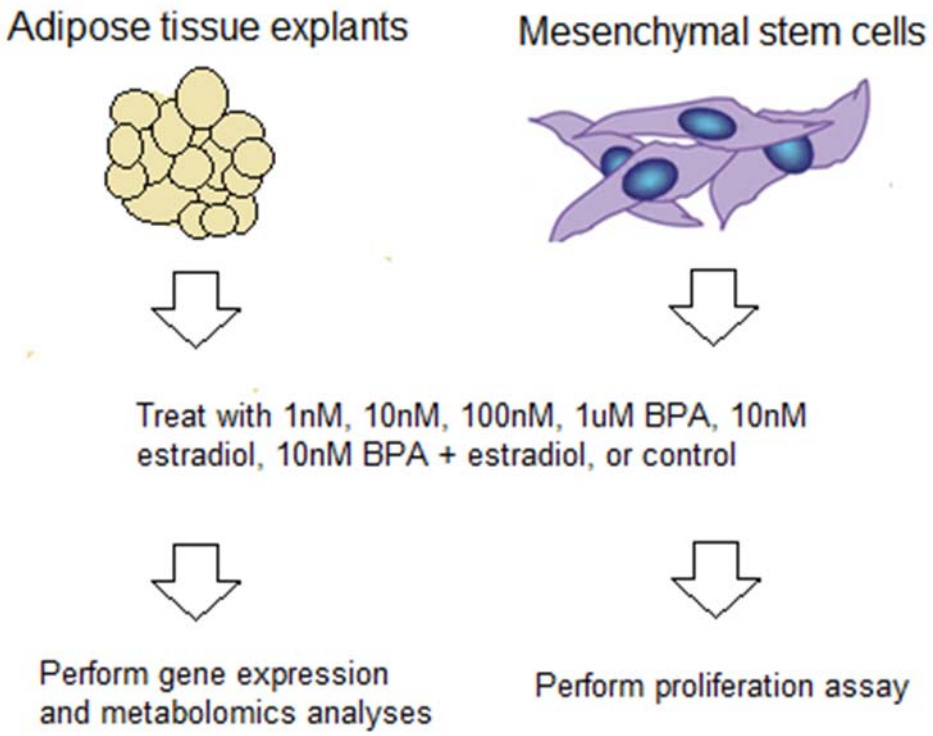


Figure 2-1. Experimental design: *in vitro* experiments. Adipose tissue explants from 16 BXD mice were exposed to various concentrations of BPA and estradiol for 24 hrs. Tissue was then snap frozen and pulverized for metabolic and gene expression analyses. Mouse mesenchymal stem cells were treated with various concentrations of BPA and estradiol for 24 or 48 hours and proliferation rate was determined using the CellTiter 96 AQueous One Proliferation Assay (Promega.com).

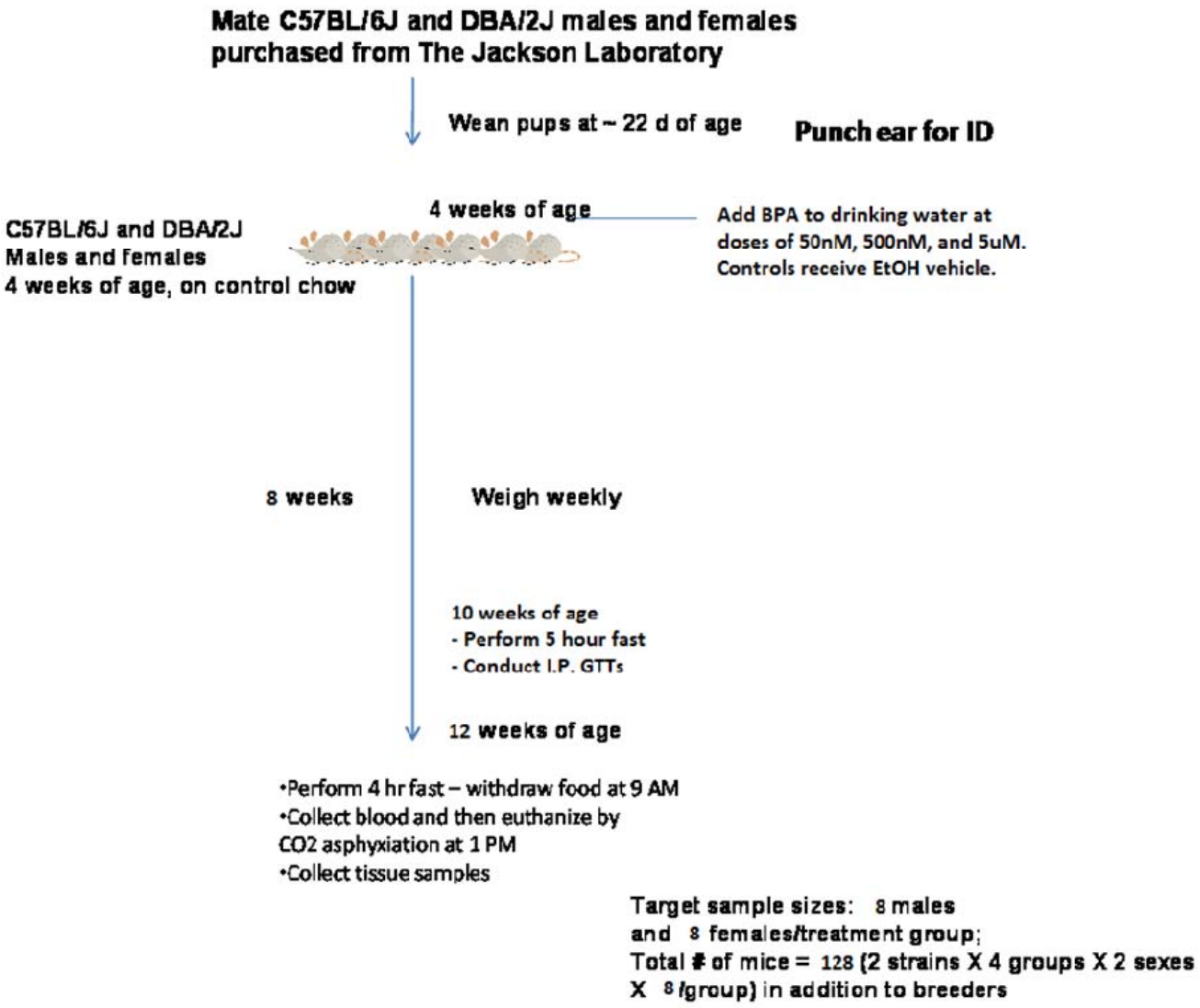


Figure 2-1. Experimental design: *in vivo* experiment. Mice were exposed to various doses of BPA and sacrificed after 8 weeks of treatment. Adipose tissue was harvested an snap frozen or placed in *RNAlater.* Tissue was used to analyze gene expression, metabolomics, and protein levels.

CHAPTER III

EFFECTS OF BISPHENOL A ON ADIPOCYTE GENE EXPRESSION, METABOLISM, AND PROLIFERATION

Introduction

Bisphenol A (BPA) is a chemical found in many items that humans use daily, including polycarbonate plastics. BPA is structurally similar to the potent synthetic estrogen, diethylstilbestrol (DES), and it is clear that BPA has endocrine disrupting properties as well (Alonso-Magdalena et al., 2005, Krishnan et al., 1993). Adipose tissue has a critical role as an endocrine organ, along with its role of energy storage. It not only responds to traditional hormones, but it also produces and secretes its own set of peptides, adipokines, which play an important role in regulating physiology and inflammation (Kershaw and Flier, 2004). Adipose tissue also produces and secretes sex hormones (Siiteri, 1987). Therefore, a known endocrine disruptor such as BPA could have an adverse effect on the endocrine function and metabolism of adipocytes. We hypothesize that low doses of BPA, similar to levels exposed to humans, can disrupt normal gene expression and endocrine function in a way that would promote adipocyte expansion and inflammation.

Adipose tissue is primarily made up of adipocytes, but it also consists of a variety of other cell types including fibroblasts and macrophages (Balistreri et al., 2010, Gaetani, 2008). BPA has been shown to increase the secretion of the pro-inflammatory

cytokine, TNFα, from adipose tissue (Ben-Jonathan et al., 2009). If BPA increases adipose tissue mass, there were would a parallel increase in inflammation. However, if BPA directly increases expression and secretion of pro-inflammatory adipokines, this could increase the number of infiltrating macrophages which would further exacerbate the inflammation and increase the risk of developing cardiovascular disease or type II diabetes (Hugo et al., 2008, Fantuzzi, 2005). We measured the expression of two important inflammatory adipokines, TNFα and IL-6, in adipose tissue explants exposed to BPA. There is also a strong correlation between TNFα production, adiposity, and insulin resistance (Ryden and Arner, 2007). . If BPA directly increases production of inflammatory adipokines, this would support the idea that BPA and other endocrine disruptors can regulate inflammatory adipokines independent of adipocyte hypertrophy (Newbold et al., 2007),

We also measured the expression of the adipogenic regulator, PPARGγ, and the lipogenic gene, FASN. Adipogenesis is the process of preadipocytes differentiating to mature adipocytes (Rosen et al., 1999). This process is regulated by the transcription factor PPARγ, and there is evidence that BPA may activate this adipogenic regulator (Chen et al., 2009a, Grun et al., 2006). Fatty acid synthase (FASN) is a large multi-enzyme complex responsible for the synthesis of long-chain fatty acids (Sul et al., 2000). If BPA was to increase the expression of one or both of these genes, it would indicate that BPA may promote adipose expansion through increased adipogenesis and lipogenesis.

Adiposity can increase through adipocyte expansion (hypertrophy), proliferation (hyperplasia), or a combination of both. The number of adipocytes remains mostly constant throughout adulthood for both lean and obese individuals, which indicates that the number of adipocytes is determined at a young age (Spalding et al., 2008). Developmental exposure during gestation to BPA has been shown to increase birth weight which persist into adulthood and is associated with increased fat mass (Somm et al., 2009). We believe that a possible explanation for this is that developmental exposure to BPA increases the proliferation of adipocyte precursors which leads to an increase in adipose tissue capacity later in life. Mesenchymal stem cells (MSCs) are a major source for adipocyte progenitors, as well as bone and cartilage cells and are, therefore, a good model to study the effects that BPA has on adipocyte precursor proliferation. To determine if BPA exposure could increase the number of adipocyte precursors, we treated mouse mesenchymal stem cells with BPA assayed the proliferation rate compared to controls. An increase in proliferation of MSCs with BPA treatment would indicate that developmental exposure could possibly program an individual for increased fat mass.

Our overall goal for this study was to determine if low levels of BPA creates an adipose tissue phenotype that is sensitized to expansion through increased proliferation of adipocyte precursors, altered energy metabolism, and changes in gene expression that favor obesity. We chose to use mouse adipose tissue explants as a model rather than 3T3-L1 fibroblasts, which is a typical cell model used to study adipocytes, because

the sensitivity of estrogen receptors to estrogens has been questioned (Rubin and Soto, 2009). This model also provided a better representation of adipose tissue *in vivo*.

Methods

Materials

Stock solutions of BPA (Invitrogen, Inc.) were prepared in the Campagna lab (Chemistry Dept., UTK) using distilled ethanol. The ethanol was distilled to prevent contamination with additional estrogenic compounds leaching from the plastic bottles. The mouse mesenchymal stem cells were obtained from Invitrogen Life Technologies, Inc. (Gaithersburg, MD) and were isolated from the bone marrow of C57BL/6 mouse at ≤ 8 weeks after gestation. DMEM/F-12 media, Phenol-free DMEM and fetal bovine serum (FBS) were also obtained from Invitrogen Life Technologies, Inc. All quantitative PCR (QPCR) primers were predesigned QuantiTect, and the primers and master mix were obtained from Qiagen, Inc. (Valencia, CA).

Adipose tissue explants and treatment

Perigonadal white adipose tissue (pWAT) was harvested from 16 surplus BXD recombinant inbred mice and pooled together in phenol-free DMEM (Invitrogen) supplemented with 10% charcoal-stripped FBS to minimize endogenous estrogen exposure. The adipose tissue was cultured in glass plates to prevent outside sources of BPA. After 24 hours, the adipose tissue was split into six treatment groups plus a control group (4 plates/treatment; n=4): control, 1nM, 10nM, 100nM, and 1μM BPA,

10nM E_2, and 10nM E_2 + 10nM BPA. The tissue was pooled together and split into treatment groups to avoid confounding effects. After an additional 24 hours of treatment, the tissue was snap frozen and pulverized using a mortar and pestle for metabolic and gene expression analysis.

Metabolomics

Approximately 15 mg of each adipose tissue sample was weighed into 1.5 mL centrifuge tubes. Cold methanol (500 µL at -78^{o}C) and the internal standards, tris (5 µL of 4.7mM in H_2O) and benzoic acid (5 µL of 1.7 mM in H_2O), were added to each tube, and then mixed by vortexing for 2 min. The metabolites were extracted for 30 min at 4^{o}C on ice, which precipitates the enzymes, followed by centrifugation (5 min, 13.2 rpm, 16.1 rcf, 4^{o}C). Supernatants were split into two autosampler vials each. One vial was placed directly in the autosampler for analysis, and the other was frozen at -80^{o}C for analysis in the opposite mode the following day. High performance liquid chromatography (HPLC) was performed utilizing a quaternary pump to generate a gradient for the elution of compounds from the stationary phase. For all samples, 10 µL was injected onto the column via an autosampler cooled to 4 °C. Eluent was directly introduced into the electrospray ionization (ESI) chamber of the triple quadrupole mass spectrometer. Samples were analyzed using selected reaction monitoring (SRM), and the scan time for each SRM was 0.05 s with a scan width of 1 m/z. Raw files for each sample were first processed using mzRock, and once the data set was narrowed, Xcalibur Quan Browser was used to visualize and manually integrate metabolite peaks over a set time window.

RNA isolation and quantitative PCR

Total RNA was extracted from adipose tissue using the RNeasy™ Lipid Tissue Mini Kit (Qiagen, Inc.). The RNA quality and concentration were assessed using the Nanodrop™ 2000 spectrophotometer (Thermo Scientific, Inc.). We reverse transcribed 500ng total RNA using the iScript™ cDNA synthesis kit (Bio-Rad) which uses a modified MMLV-derived reverse transcriptase and a combination of oligo(dT) and random hexamer primers. The relative expression of transcripts coding for mouse *Tnfa* (tumor necrosis factor alpha), *Il6* (interleukin-6), *Fasn* (fatty acid synthase), and *Pparg* (peroxisome proliferator-activated receptor gamma) were measured by quantitative real-time PCR (QPCR) using a CFX96™ Real-Time PCR Detection System (Bio-Rad) and were normalized using the housekeeping gene hypoxanthine guanine phosphoribosyl transferease (*Hprt)*. All samples were analyzed in triplicate. We quantified the expression using the QuantiTect SYBR Green PCR kit and QuantiTect Primer Assays (Qiagen, Inc.), and relative gene expression was determined using the $\Delta\Delta$Ct method and the Relative Expression Software Tool (REST) (Pfaffl et al., 2002).

Proliferation Assay

Mouse mesenchymal stem cells (MSCs) were plated in a 96-well culture plate at a density of ~5,000 cells per well and grown in DMEM/F-12 media. The cells were allowed to attach and grow for 24 hours, then the media was replaced containing the one of the following: vehicle control (EtOH), 1nM BPA, 10nM BPA, 1μM BPA, 10nM E_2, or 10nM E_2 + 10nM BPA. The cells were treated for either 24 or 48 hrs. Cell

proliferation was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Kit (Promega). Briefly, the AQueous One Solution contains an MTS tetrazolium compound which is reduced by living cells to a soluble, colored formazan product. This product's absorbance can be measured at 490nm and is directly proportional to the number of living cells in culture.

Statistical analysis

The QPCR data were analyzed using REST which uses a mathematical model based on the correction for exact PCR efficiencies and the mean threshold value (Ct) deviation between treatment groups and the control group. The expression ratios were then tested for significance using a Pair Wise Fixed Reallocation Randomisation Test©. Differences in proliferation were analyzed using one-way analysis of the variance (ANOVA), and once significance was determined, post hoc tests (t-tests and LSD) were performed.

Results

Adipose tissue explants were treated with BPA or estradiol for 24 hours. Following treatment, we analyzed the gene expression of some inflammatory markers as well as lipogenic/adipogenic genes. Our data suggest that exposure to BPA increases expression of inflammatory cytokines (Figure 3-1). There were statistically significant (p<0.05) increases in *Tnfa* expression at 10nM and 1μM concentrations and significant increases in *Il*-6 at the 1μM dose. *Tnfa* was increased 2.2-fold and 1.98-fold in the 10nM and 1μM treatment, respectively. *Il-6* expression was increased 2.76-fold

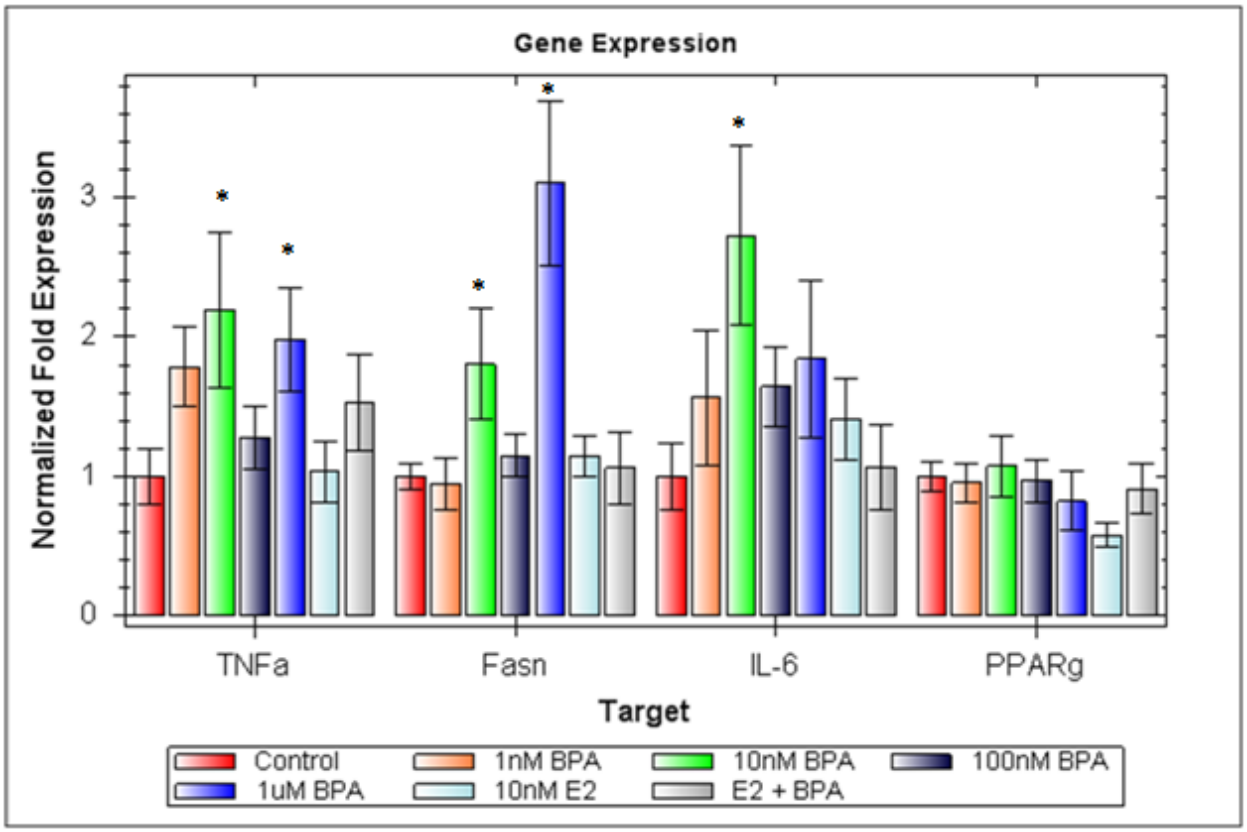


Figure 3-1. QPCR results for selected genes in mouse adipose tissue explants. Data were analyzed using REST and results are expressed as means ± SEM. (*) indicates significant difference from control ($p<0.05$).

in the 10nM treatment. There were also significant increases in the expression of *Fasn* at 10nM and 1μM concentrations (1.79-fold and 2.98-fold, respectively), which would suggest a possible increase in lipogenesis activity. *Pparg* expression was unchanged, indicating that BPA is not enhancing adipogenesis. However, BPA may possibly increase the activity of *Pparg* without affecting its expression. Estradiol did not appear to alter the expression of any of the genes. Our sample size was small (n=4) and could explain a lack of significant results at additional treatments, however these results are consistent with experiments previously conducted in our lab as well as by others (Yan et al., 2008).

Technical advances in recent years have advanced the ability to use global metabolomics to query changes in profiles of small molecule metabolites in serum and tissues. It has been proposed that BPA alters adipose tissue energy metabolism which leads to an increase in adipose mass (Chen et al., 2009b). After the adipose tissue explants were treated with BPA for 24 hours, metabolites were extracted from the tissue and analyzed using mass spectrometry. The metabolite ratios were calculated after correcting ion counts for mass and signal difference. The data shown represent ratios of select metabolites in treated vs. control adipose tissue. Due to the small sample size, there were no statistically significant differences between any treatments and control, however there was a trend for increased glycolytic and TCA cycle intermediates with BPA treatment, which are important pathways in energy metabolism (Figure 3-2). These data indicate that BPA may alter energy metabolism in adipose tissue.

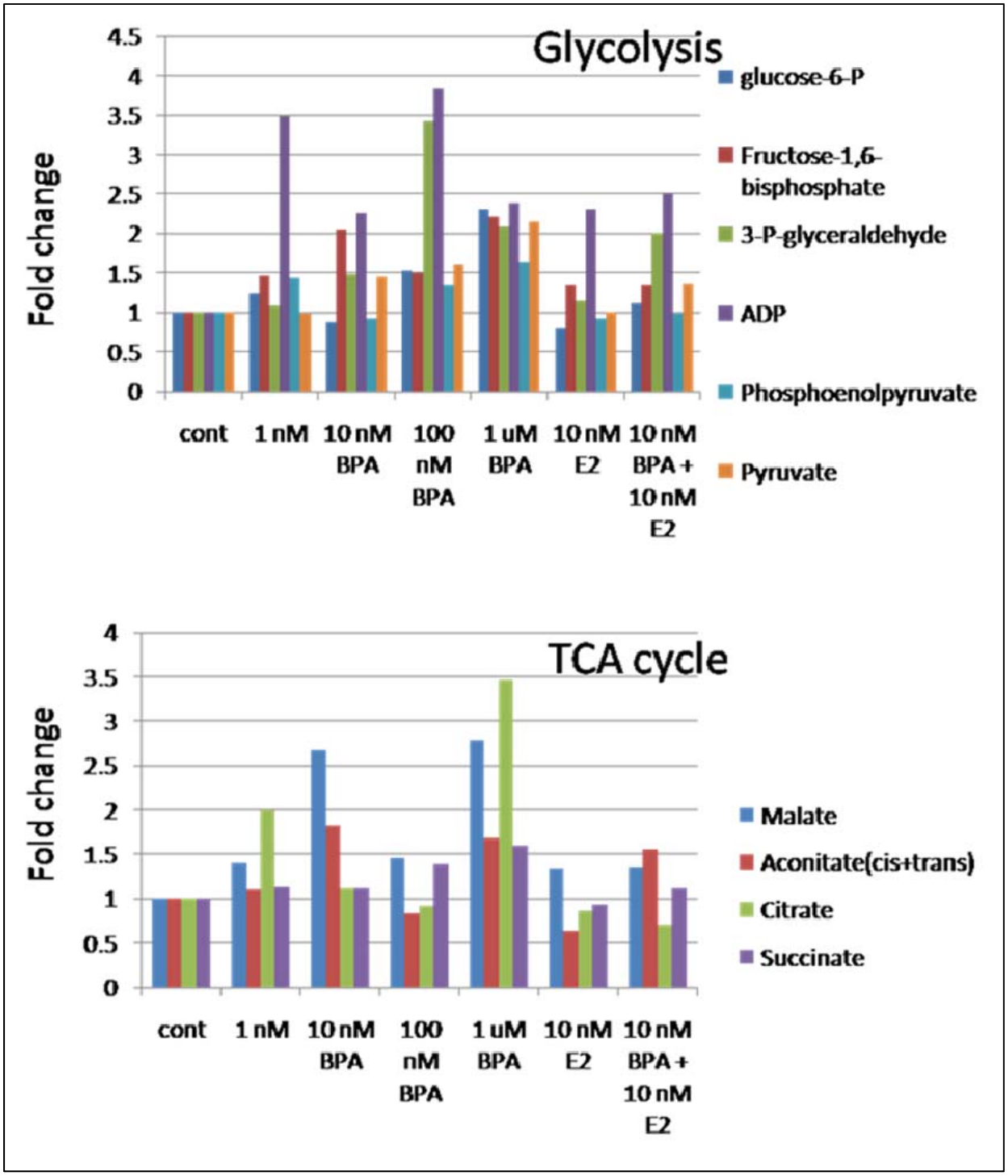


Figure 3-2. Metabolic profiling of BPA-treated adipose tissue. LC-MS/MS was used to measure metabolite levels in white adipose tissue treated with various concentrations of BPA or estradiol.

Mouse mesenchymal stem cells (MSCs) were treated with BPA or estradiol for 24 or 48 hours to analyze the effect on proliferation. We believe that *in utero* and perinatal exposure to BPA can possibly "program" a person to be obese by increasing proliferation of adipocyte precursors which would increase the adipose capacity later in life. The CellTiter 96 AQueous One Proliferation Assay was used which measures the formation of formazan from a tetrazolium compound and is correlated with the number of viable cells in culture. We found that all three concentrations of BPA used modestly but significantly increased proliferation (1.18-1.21-fold increase, $p<0.05$) of MSCs at both 24 and 48 hours (Figure 3-3). The estradiol treatment did not increase proliferation after 24 hours, but it did increase proliferation similar to BPA after 48 hours. The combination of BPA and estradiol did not have a significant effect on proliferation.

Discussion

To start to study the effects that BPA has on adipose tissue development and function, we began by using *in vitro* and *ex* vivo models. We used a wide range of concentrations of BPA (low nM – low μM) because the effects of BPA are often non-monotonic (Ben-Jonathan et al., 2009). Just because there is an effect at a specific dose doesn't mean that effect is going to be exacerbated at higher doses, and vice versa. Traditional toxicological studies generally take a "dose makes the poison" approach, but BPA may actually have a greater effect at lower levels, in some cases. Since BPA is known to have estrogenic properties, we also used a 10nM concentration of estradiol and a combination of 10nM BPA and 10nM estradiol to compare the effects of a natural hormone and a chemical that mimics the natural hormone. Adipose tissue

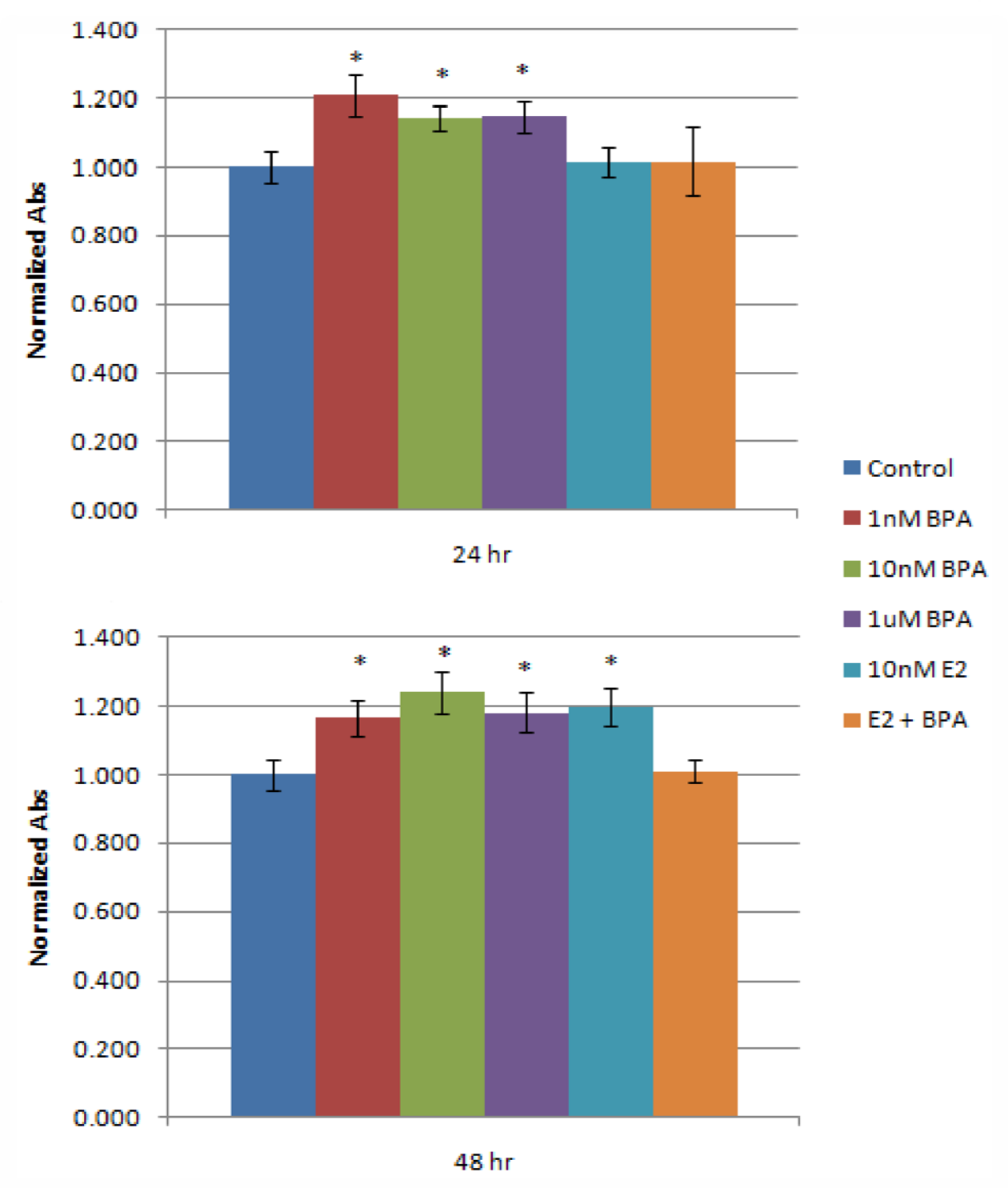


Figure 3-3. Effects of BPA and E_2 on proliferation of mouse mesenchymal stem cells. Mouse MSCs were treated with various concentrations of BPA or E_2 for 24 or 48 hours. Proliferation was assayed using the CellTiter 96 AQueous One Proliferation Assay (Promega). (n=22/treatment, * $p<0.05$).

explants were chosen to be used because they are a better representation of adipose tissue *in vivo*, and there is evidence that the adipocyte cell line, 3T3-L1, which we normally use as a model of adipose tissue may underexpress ERs, which are candidate mediators of BPA action (Rubin and Soto, 2009).

The gene expression data shown in Figure 3-1 indicate that the expression levels of the pro-inflammatory cytokines *Tnfa* and *Il-6* are increased in some treatments of BPA. The 10nM concentration of BPA significantly increased the expression of *Tnfa* and *Il6* 2.19-fold and 2.67-fold, respectively. The 1μM concentration of BPA also significantly increased *Tnfa* expression by 1.95-fold. While some of the other concentrations of BPA increased the expression of these cytokines, they were not significantly different from the control treatment, which may be a result of our small sample size (n=4). We also measured the expression of *Fasn* and *Pparg* as markers of lipogenesis and adipogenesis, respectively. While there was no change in expression of *Pparg*, *Fasn* expression increased 1.8-fold and 3-fold in 10nM BPA and 1μM BPA, respectively. This increase in fatty acid synthase expression would indicate the possibility that BPA promotes enhanced *de novo* lipogenesis.

There are some additional interesting things to take from these data. The gene expression data across the doses of BPA creates an inverted 'U' shape curve, specifically for the cytokines. The 10nM dose of BPA seems to increase the expression of *Tnfa* and *Il6*, yet this effect isn't enhanced in the 100nM or 1μM dose, but, rather diminished. This result is consistent with the non-monotonic effects often seen in BPA studies (Ben-Jonathan et al., 2009). The estradiol treatment did not seem to affect the

gene expression of any of the genes tested, however the combination treatment of 10nM BPA and 10nM estradiol seemed to attenuate the effects seen in the 10nM BPA treatment alone. This could reflect a competition between the two molecules for the same receptor(s), possibly ERs.

Along with our collaborator, Dr. Shawn Campagna, we performed global metabolic profiling using small molecule metabolic mass spectrometry to query changes in metabolite pools from adipose tissue treated with BPA. It is believed that BPA mediates its action through ERs, which are now known to play a key role in energy metabolism (Chen et al., 2009a, Gao et al., 2007). Disruption of adipose energy balance has been proposed as a mechanism through which BPA increases adipose mass (Chen et al., 2009b). The data presented in Figure 3-2 represent ratios of glycolytic and TCA cycle metabolites in treated vs. control adipose tissue; ratios were calculated after correcting ion counts for mass and signal differences. These data indicate a trend for increasing doses of BPA to alter adipose energy metabolism, particularly intermediates of glycolysis. Due to the method used, traditional statistical methods were not used to analyze the metabolomics data, rather we looked at the fold-change: a fold-change of 1.5 or greater was considered interesting. Once again there is no monotonic response to increasing BPA concentration, but changes to metabolites spread throughout doses in both glycolytic and TCA cycle intermediates. The 1μM concentration of BPA was the only dose that had a 1.5 fold or greater increase in all the metabolites shown.

Glucose is broken down via glycolysis into 2 molecules of pyruvate which then enter the mitochondria, are converted to acetyl-CoA, and enter the tricarboxylic acid (TCA) cycle which forms important reducing agents necessary to produce energy. However, intermediates of the TCA cycle, specifically citrate, can be exported out of the mitochondria to the cytosol, converted to acetyl-CoA, and used for fatty acid synthesis (Wakil et al., 1983). We believe that the increase in glycolytic and TCA cycle intermediates seen in some of the BPA treatments may be an indication that BPA increases glycolysis and the TCA cycle which leads to increased fatty acid synthesis. This would be consistent with our finding that BPA may increase fatty acid synthase expression, especially in the 1μM BPA treatment. To determine if BPA alters adipocyte energy metabolism in a way that promotes *de novo* lipogenesis, a metabolic flux experiment must be conducted. Metabolic flux profiling allows the tracking of labeled isotopes into specific metabolic pathways

To determine if developmental exposure to BPA could possibly increase the number of adipocyte precursors, we assayed the proliferation of mouse MSCs treated with various concentrations of BPA. The data in Figure 3-3 show that all three BPA treatments (1nM, 10nM, 1μM) significantly increased the proliferation of MSCs compared to control (p=0.013, 0.032, 0.038, respectively) after 24 hours of treatment. There is one less BPA treatment in this experiment due to spacing issue in the 96-well plate; we omitted the 100nM because we were more interested in the low nanomolar effects and comparing them to the 1μM treatment, which appeared to be having the greatest impact. We saw similar effects after 48 hours of treatment, however the 10nM

estradiol treatment had now increased proliferation of the MSCs. There is evidence that BPA may mediate its effects in two different ways. First, it may elicit a rapid response via an interaction with ncmERs. It may also then elicit a slower, prolonged response via an interaction with classical ERs (Alonso-Magdalena et al., 2005). Our data may support these findings. The increase in proliferation seen in the first 24 hours only occurs in cells treated with BPA (possibly via ncmERs). The increase seen after 48 hours also occurs in cells treated with estradiol, which would indicate a slower response via the classical ERs. Once again, the cells treated with both BPA and estradiol did not show any change in proliferation rate compared to control, which would further indicate a competition between the molecules.

In summary, we have shown that BPA can increase the expression of important inflammatory cytokines in adipose tissue. Inflammation plays an important role in a number of the comorbidities associated with obesity, and BPA will only exacerbate the vicious circle of inflammation and obesity. We also showed that BPA may increase glycolysis and the TCA cycle which leads to enhanced *de novo* lipogenesis. These findings go hand-in-hand with our finding that some concentrations of BPA increase expression of fatty acid synthase. Finally, we also showed that BPA can increase the proliferation of MSCs, thus increasing the number of adipocyte precursors and increasing the fat potential later in life. These findings are interesting, however the experiments do not represent the likely route of exposure in humans. To truly study the effects that BPA has on adipose tissue, we must look at the effects it has in the context of an organism via the most likely route of exposure, food and drink.

CHAPTER IV

CHRONIC EXPOSURE TO BISPHENOL A IN JUVENILE MICE AND THE EFFECTS ON ADIPOSE FUNCTION AND METABOLISM

Introduction

There have been some studies that have shown that BPA has adverse effects *in vivo,* but at very high doses (Snyder et al., 2000, Kwon et al., 2000). BPA has been detected in the urine of over 92% of the population, and higher BPA levels were associated with cardiovascular disease and type II diabetes, but these results do not prove causality (Melzer et al., 2010). A small number of studies have shown that BPA affects the metabolism, insulin sensitivity, and adiposity of mice or rats exposed *in utero* (Alonso-Magdalena et al., 2010, Somm et al., 2009). These findings are extremely worrisome and show that BPA can have adverse effects on developing life. An analysis of the 2003-04 NHANES data found that children ages 6-11 had the highest intake of BPA based on bodyweight (Lakind and Naiman, 2008), and children obviously continue to develop after birth and have important developmental periods through puberty. In fact, this time period is a critical time for adipose tissue development in which both adipocyte hyperplasia and hypertrophy can occur (Spalding et al., 2008). Therefore, BPA exposure during early childhood through puberty could have similar effects to those seen in perinatal exposure.

There are a number of methods to administer BPA to mice (osmotic pump, injection), but the most likely route of exposure in humans is food and drink. Once ingested, BPA can be metabolized in the liver to the conjugated form Bisphenol A-glucuronide, but it can also be deconjugated throughout the body (Ginsberg and Rice, 2009, Nishikawa et al., 2010). Complex chemistry can take place in the body and, therefore, adding BPA to the drinking water or food of the mice is the most relevant method of exposure, however, this method makes measuring daily intake much more difficult.

We added BPA to the drinking water of C57BL/6 and DBA/2J mice at three concentrations (50nM, 500nM, and 5μM) as well as vehicle control starting at four weeks of age. They were weighed weekly, and glucose tolerance tests were performed at 8-9 weeks of age. Mice were sacrificed at 11 weeks of age and gonadal fat pads were dissected for gene expression profiling, metabolomics analysis, and adipocyte sizing. Blood was also collected for additional assays. We also had additional mice that were treated with BPA for 6 more weeks on a high fat diet These mice were sacrificed at 17 weeks of age and fat pads were dissected for the same analyses as the other mice.

Methods

Animals

C57BL/6J and DBA/2J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were housed and maintained in the Animal Facility and Office of Laboratory Animal Care in Walters Life Sciences Building at the University of Tennessee. Mice received a standard chow diet and water *ad libitum*. The housing conditions were maintained at 70 ± 2 °F and 40-60% humidity. These stocks were used to propagate offspring for this study. Sixty-four mice (32 males and 32 females) of each strain were weaned at four weeks of age and randomized to a treatment group to avoid litter effect. These mice were housed in special BPA-free polypropylene cages, and water was provided in glass bottles to limit exposure to BPA or other estrogenic compounds from the environment. BPA was administered to the mice via drinking water, which was available *ad libitum*, at the concentrations of 50nM, 500nM, or 5μM. The control groups received distilled ethanol, which was used to dissolve the BPA, at a dose equal to the highest concentration of BPA. The mice were weighed weekly. At 11 weeks of age, mice were fasted for ~5 hours and sacrificed using CO_2 asphyxiation and cervical dislocation. The perigonadal fat pads were dissected and blood was collected to measure fasting insulin levels. A portion of the fat pad was put in RNAlater for RNA extraction, a portion was put in 4% paraformaldehyde for sectioning, and a portion was snap frozen in liquid nitrogen for metabolomics analysis. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Tennessee.

Glucose Tolerance Tests

At between 8 and 9 weeks of age (14 and 15 for mice on high-fat diet), mice were fasted for ~5 hours and injected intraperitoneally with glucose (1mg/g bw). Blood was collected from the tail vein at 0, 15, 30, 60, and 120 mins after the injection and measured using a glucometer. The measurements were averaged and plotted, and the area under the curve (AUC) was determined.

RNA isolation

Total RNA was extracted from adipose tissue using the RNeasy™ Lipid Tissue Mini Kit (Qiagen, Inc., Valencia, CA). The RNA quality and concentration were assessed using the Nanodrop™ 2000 spectrophotometer (Thermo Scientific, Inc.) and Experion RNA StdSens Chips on the Experion™ automated electrophoresis system (Bio-Rad, Hercules, CA) to ensure excellent quality. RNA samples were stored at -80ºC until needed.

Expression profiling

RNA from 24 male mice (6 500nM BPA and 6 Control per strain) was sent to McGill University and Génome Québec Innovation Centre for gene expression profiling using Illumina BeadArray™ technology. Total RNA (500ng) was amplified, synthesized to cDNA, and hybridized to the MouseWG-6 v2 Expression BeadChip (Illumina, Inc.). The BeadChip was then scanned using the iScan reader.

Quantitative PCR

We reverse transcribed 500ng total RNA using the iScript™ cDNA synthesis kit (Bio-Rad) which uses a modified MMLV-derived reverse transcriptase and a combination of oligo(dT) and random hexamer primers. The relative expression of transcripts coding for select genes were measured by quantitative real-time PCR (QPCR) using a Quantitect primers on a CFX96™ Real-Time PCR Detection System (Bio-Rad) and were normalized using the housekeeping gene *Hprt*. The genes measured included *Fasn, Lep, Pparg,* Estrogen receptor alpha (*Esr1*), Adiponectin (*Adipoq*), Estrogen related receptor gamma, (*Esrrg*), Paternally expressed gene 3 (*Peg3*), *Serpine1*, Calcium and integrin binding protein 2 (*Cib2*), Copine 8 (*Cpne8*), Gap junction alpha 5 (*Gja5*), Lactate dehydrogenase (*Ldhd*), and Thrombospondin 2 (*Thbs2*). We quantified the expression using the QuantiTect SYBR Green PCR kit and Primer Assays (Qiagen, Inc.), and relative gene expression was determine using the $\Delta\Delta$Ct method with the Relative Expression Software Tool (Pfaffl et al., 2002).

Luminex analysis of adipokine levels

Adipose tissue concentrations of insulin, leptin, resistin, IL-6, TNFα, monocyte chemotactic protein 1 (MCP-1), and plasminogen activator inhibitor 1 (PAI-1) were analyzed by a multiplexed flow cytometric assay using a mouse serum adipokine Milliplex® map kit (MADPK-71K, Millipore, Billerica, MA) on a Luminex® 200 system (Luminex Inc., Austin, TX). The multiplexed assay had intra- and inter-assay variation of < 4.5% and < 10.3%, respectively. All samples were analyzed in duplicate. The

assay was measured in pg/mL and normalized by measuring the total protein using a BCA protein assay (Thermo Scientific) giving a final measurement of pg/μg total protein.

Serum insulin and adiponectin assays

Blood samples were collected at the time of sacrifice after a 5 hour fast and centrifuged at 4ºC for 15 minutes at 2,000 x *g*. Serum aliquots were stored at -80ºC. Fasting serum insulin levels were measured using a commercially available ultra sensitive mouse insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL). Serum adiponectin levels were measured using a commercially available mouse adiponectin enzyme immunoassay kit (SPI-bio Bertin Pharma, Montigny le Bretonneux, France).

Histological analysis of adipocyte size

A portion of the perigonadal fat pad was fixed for 24 hours in 4% paraformaldehyde then washed in PBS. The samples were embedded in paraffin, cut into 5μm sections, and stained with hematoxylin and eosin. Adipocyte size was measured using a previously established method (Chen and Farese, 2002). Briefly, the tissue sections were viewed at 10X magnification, and images were obtained. The illumination of the images was adjusted using CellProfiler cell image analysis software (www.cellprofiler.org). After the illumination was adjusted, the images were converted to a binary image in ImageJ (NIH; rsbweb.nih.gov/ij/) using the following commands: Gray Mode and Threshold. The binary images were further adjusted using Despeckle, Watershed, Erode, and Paintbrush. The cross-sectional area of the adipocytes was

measured using the “Analyze particles” command. Approximately 200 adipocytes per mouse were measured.

Metabolomics

Approximately 15 mg of each adipose tissue sample was weighed into 1.5 mL centrifuge tubes. Cold methanol (500 µL at -78^{o}C) and the internal standards, tris (5 µL of 4.7mM in H_2O) and benzoic acid (5 µL of 1.7 mM in H_2O), were added to each tube, and then mixed by vortexing for 2 min. The samples were extracted for 30 min at 4^{o}C on ice prior to centrifugation (5 min, 13.2 rpm, 16.1 rcf, 4^{o}C). Supernatants were split into two autosampler vials each. One vial was placed directly in the autosampler for analysis, and the other was frozen at -80^{o}C for analysis in the opposite mode the following day. High performance liquid chromatography (HPLC) was performed utilizing a quaternary pump to generate a gradient for the elution of compounds from the stationary phase. For all samples, 10 µL was injected onto the column via an autosampler cooled to 4 °C. Eluent was directly introduced into the electrospray ionization (ESI) chamber of the triple quadrupole mass spectrometer. Samples were analyzed using selected reaction monitoring (SRM), and the scan time for each SRM was 0.05 s with a scan width of 1 m/z. Raw files for each sample were first processed using mzRock, and once the data set was narrowed, Xcalibur Quan Browser was used to visualize and manually integrate metabolite peaks over a set time window.

Statistical analysis

Differences in body weights were analyzed using repeated measures one-way ANOVA and Tukey HSD post hoc test using R 2.12.0 or SYSTAT 13. Differences in adiposity, glucose tolerance, adipose adipokines, serum insulin, serum adiponectin, and adipocyte size were analyzed using a one-way ANOVA, and post hoc tests (t-test and LSD) were performed once significance was determined. Results are shown as means ± SEM. A p-value < 0.05 was considered significant. Metabolomics data was also analyzed using ANOVA, however a change of 1.5-fold or higher was considered interesting and significant. All of the QPCR data was analyzed using REST which uses a mathematical model based on the correction for exact PCR efficiencies and the mean crossing point deviation between treatment groups and the control group. The expression ratios are then tested for significance using a Pair Wise Fixed Reallocation Randomisation Test©.

Statistical analysis of the Illumina microarray probe profile data was performed using R 2.12.0. Data were processed based on detection p-values of at least 20 arrays out of 24 arrays less than 0.05 using Genefilter package in Bioconductor, otherwise, they were assumed to be low expression data and were filtered out. Quantile normalization was applied to the data to make intensities consistent within each array. The significance of gene expression was analyzed by ANOVA and empirical bayes statistics (ebayes) using limma package. The differentially expressed genes with p-value < 0.005 were identified.

Results

Effect of BPA on body weight and adiposity

C57BL/6 and DBA/2J mice were exposed to BPA via drinking water after weaning for seven weeks, and the mice were weighed weekly beginning at 4 weeks of age. There was no difference in bodyweight between any of the treatments for females of either strain (data not shown). However, there was a significant difference in the body weight of male C57BL/6 mice treated with 50nM BPA compared to control starting at 7 weeks of age that persisted until the time of sacrifice (Figure 4-1). Male C57BL/6 mice were 1.5-2.0g heavier (10 weeks of age: 500nM = 26.1g, control = 24.3, $p<0.05$) from 7 weeks of age to the time of sacrifice. Male DBA/2J mice treated with 500nM BPA were significantly heavier than control, but only near the end of the study at 10 weeks of age (500nM = 26g, control = 25g, $p<0.05$).

At the end of the study, gonadal fat pads were weighed and normalized to body weight to determine adiposity. Again, there was no difference between any of the treatments in females of either strain (data not shown). Adiposity was increased in the 500nM BPA treatment approximately 25% in the C57BL/6 males compared to control (1.65 fat pad percentage in 500nM treatment compared to 1.32 in control, $p=0.028$) (Figure 4-2). There was a smaller and less significant increase in adiposity of 8% in DBA/2J male mice treated with 500nM BPA compared to control (2.26 fat pad percentage in 500nM treatment compared to 2.09 in control, $p=0.3$). These data

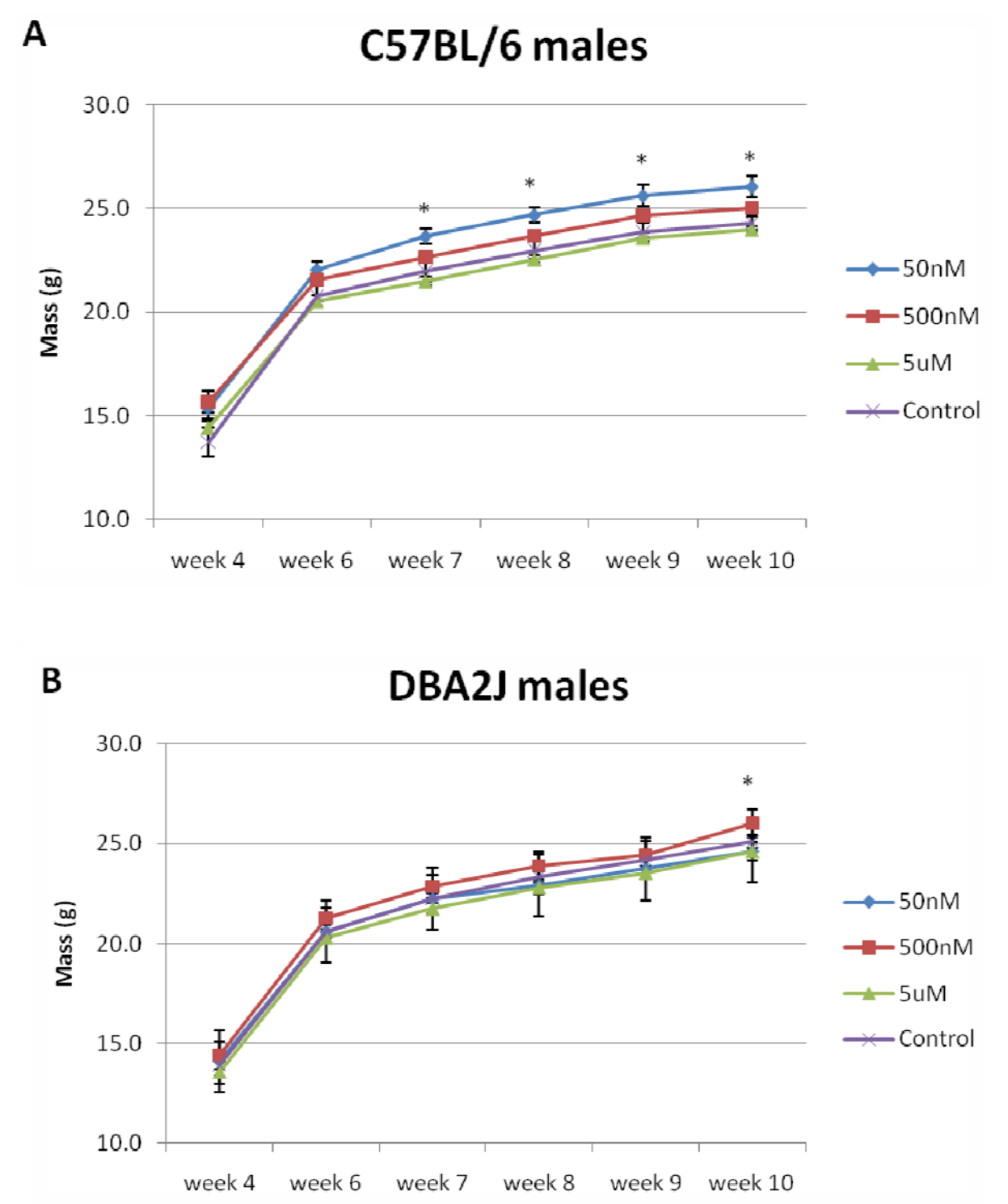


Figure 4-1. Effects of BPA on bodyweight in male mice. (A) C57BL/6 male mice and (B) DBA/2J male mice. Mice were exposed to various doses of BPA via drinking water. Results are means ± SEM. (*) $p<0.05$.

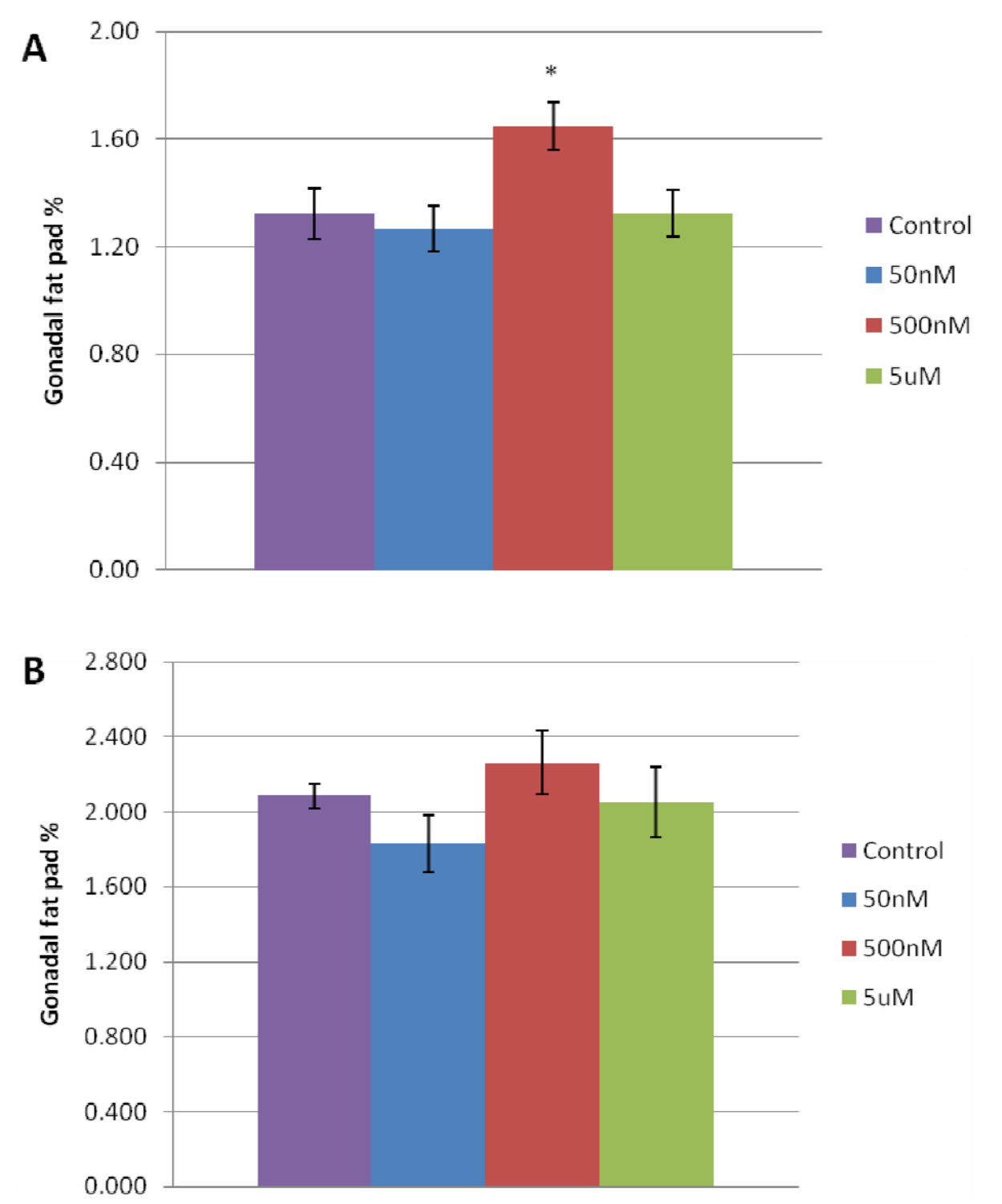


Figure 4-2. Effects of BPA on adiposity in male mice. (A) C57BL/6 males and (B) DBA/2J males. Gonadal fat pads were weighed and expressed as a percentage of total body weight. Results are shown as means ± SEM. (*) $p<0.05$.

suggest that the increase in body weight is associated with an increase in adipose tissue.

Glucose tolerance tests

BPA has been shown to disrupt glucose homeostasis in pregnant mice as well as their male offspring once they reached adulthood (Alonso-Magdalena et al., 2010). Therefore, glucose tolerance tests were performed to determine if BPA has an effect on insulin sensitivity and the ability to clear glucose. Due to the large of number of mice, GTTs were performed over a two week span. There was a clear strain difference in the sensitivity to this assay. Normally, following an i.p. injection of a glucose bolus, blood glucose levels spike and then gradually decrease over the next 2 hours. We found that the DBA/2J strain had a minimal spike and returned to pre-injection levels within 30 minutes. A previous study showed that DBA/2J mice have higher serum insulin levels and generally lower blood glucose levels, so these results weren't surprising (Alexander et al., 2006). For this reason, we were unable to detect any difference between the treatments for the DBA/2J mice (data not shown). The C57BL/6 mice had a more traditional response to the glucose injection. Blood glucose measurements were changed from mg/dL units to mmol/L and plotted. The area under the curve (AUC) was determined to assay differences in glucose tolerance. We found that the AUC measurements were higher for the mice exposed to BPA in both the males and females of the C57BL/6 strain, however, only the 500nM treatment in the males was close to being statistically significant ($p=0.06$) (Figure 4-3).

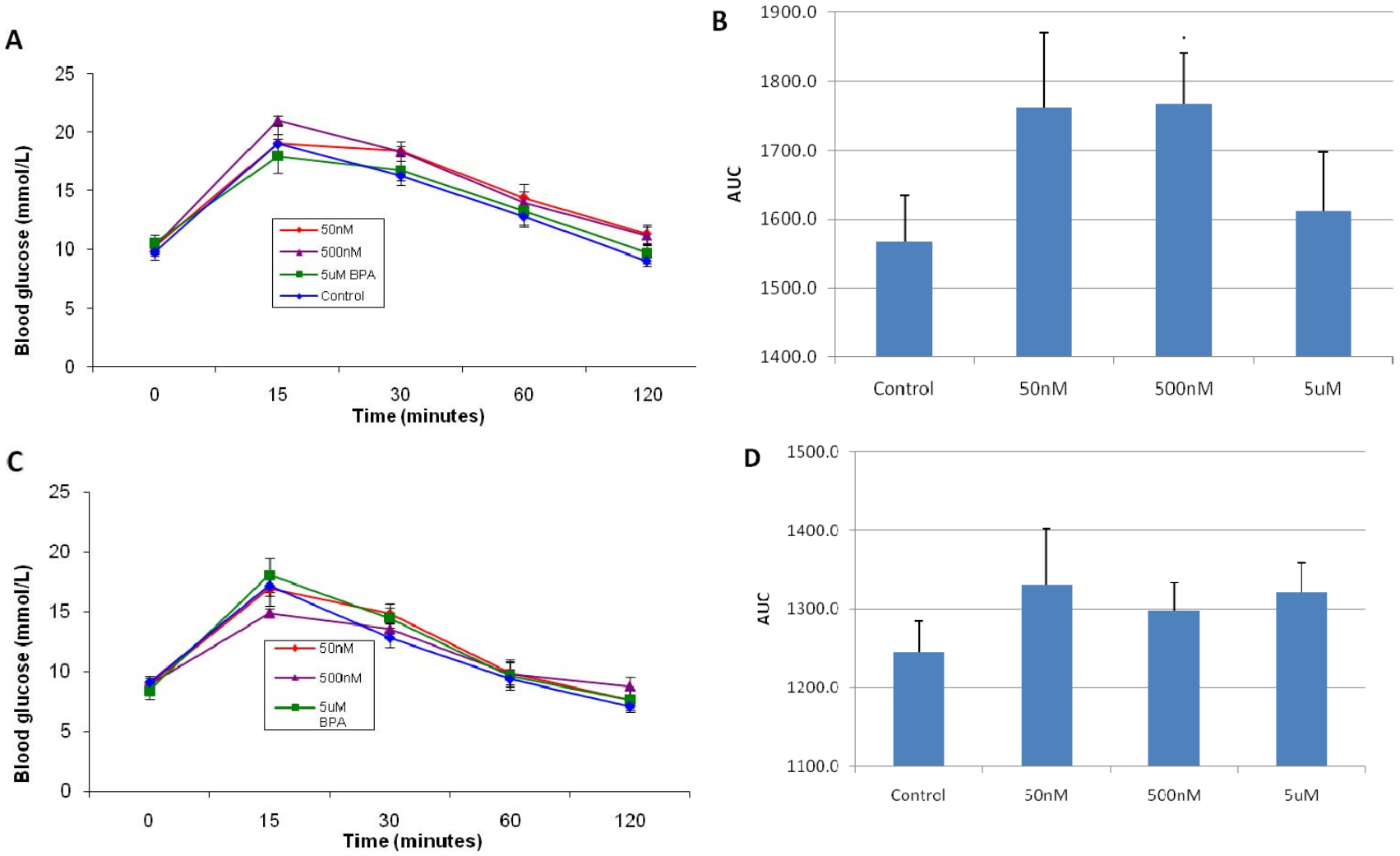


Figure 4-3. Glucose tolerance test results for C57BL/6 mice treated with BPA. Males (A, B) treated with 50nM or 500nM BPA showed slower clearance rates compared to controls. The difference was not as great in females (C, D). Results are shown as means ± SEM. (·) $p<0.1$.

Serum insulin and adiponectin levels

We collected blood to measure fasting serum insulin levels to determine the effect that BPA has on circulating insulin levels. Interestingly, we found no significant differences in the males of either strain. However, we did see differences in the females. All three treatments of BPA showed significantly higher serum insulin levels in female C57BL/6 mice with the 5μM treatment having the greatest difference compared to control (Figure 4-4). Surprisingly, we found the opposite result in female DBA2 mice. All three treatments of BPA had significantly lower serum insulin levels compared to controls, illustrating not only a physiological difference between the two strains but a difference in the way each strain is affected by BPA. Due to limited resources, some of the experiments and assays were only done for the specific groups that showed differences in adiposity, i.e. the males of the 500nM BPA treated group and control group. Measurement of serum adiponectin was one of these assays. We found that the serum adiponectin levels were significantly ($p=0.0012$) lower in 500nM BPA group of C57BL/6 males and were nearly significant ($p=0.08$) for the DBA/2J males (Figure 4-5).

Adipose adipokine profiles of BPA treated mice

The quantity of six adipokines were assayed in the adipose tissue of mice treated with BPA using the mouse serum adipokine Milliplex® map kit on a Luminex® 200 system. The measurements for each adipokine were normalized using total protein content and are expressed as pg/μg of total protein. We found much higher levels of

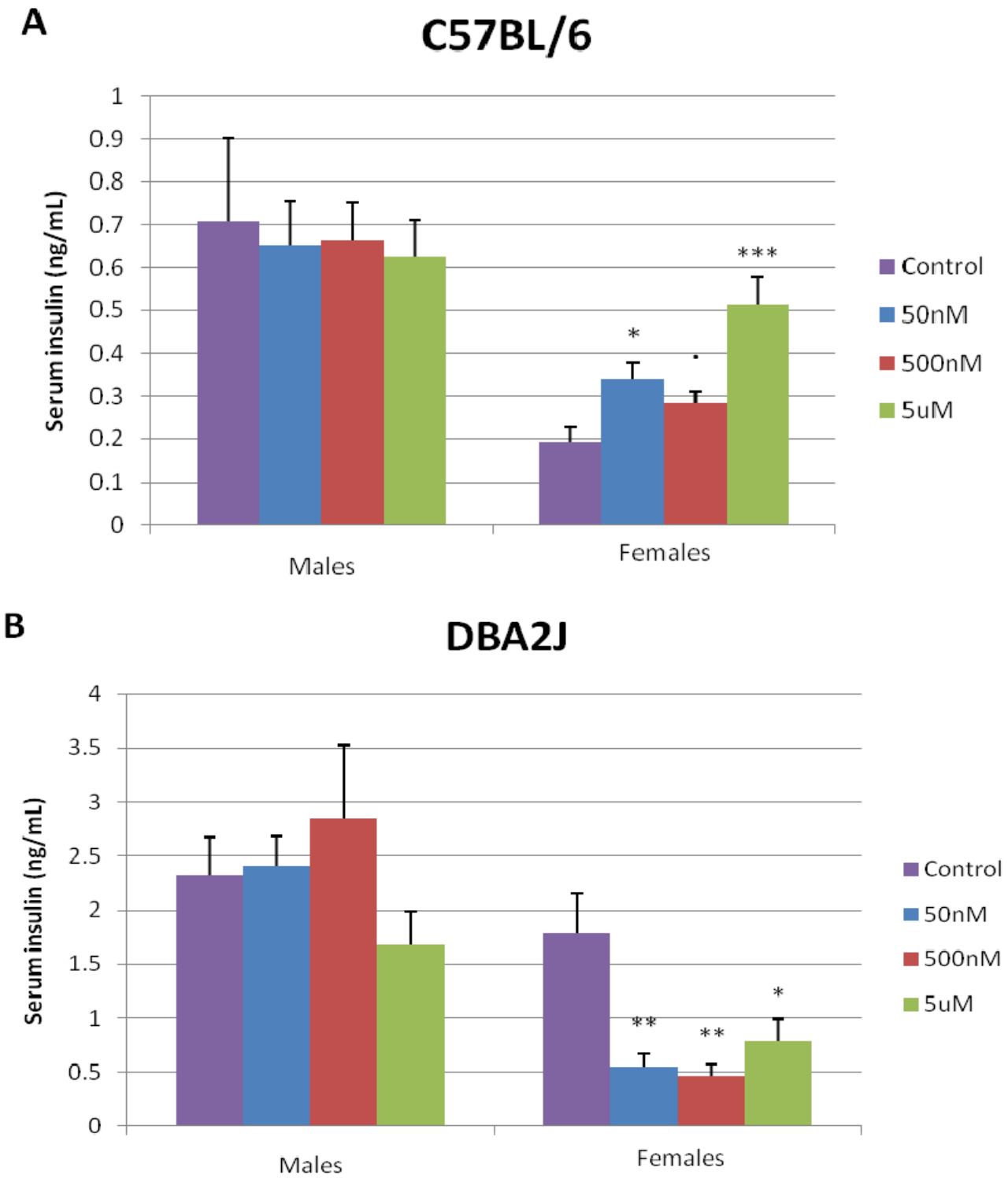


Figure 4-4. Fasting serum insulin levels in mice treated with BPA. Mice were fasted for ~5hrs and blood was drawn at sacrifice. Insulin was measured using a commercially available ELISA kit. (·) $p<0.1$, (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$.

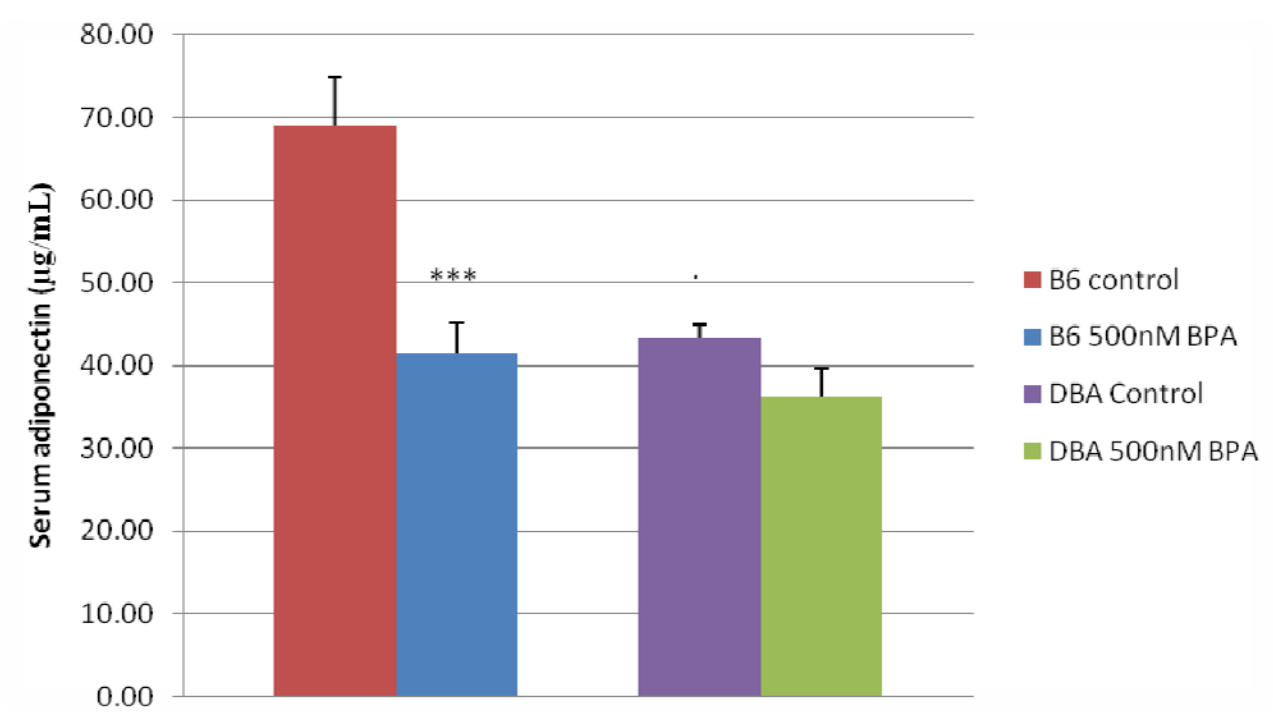
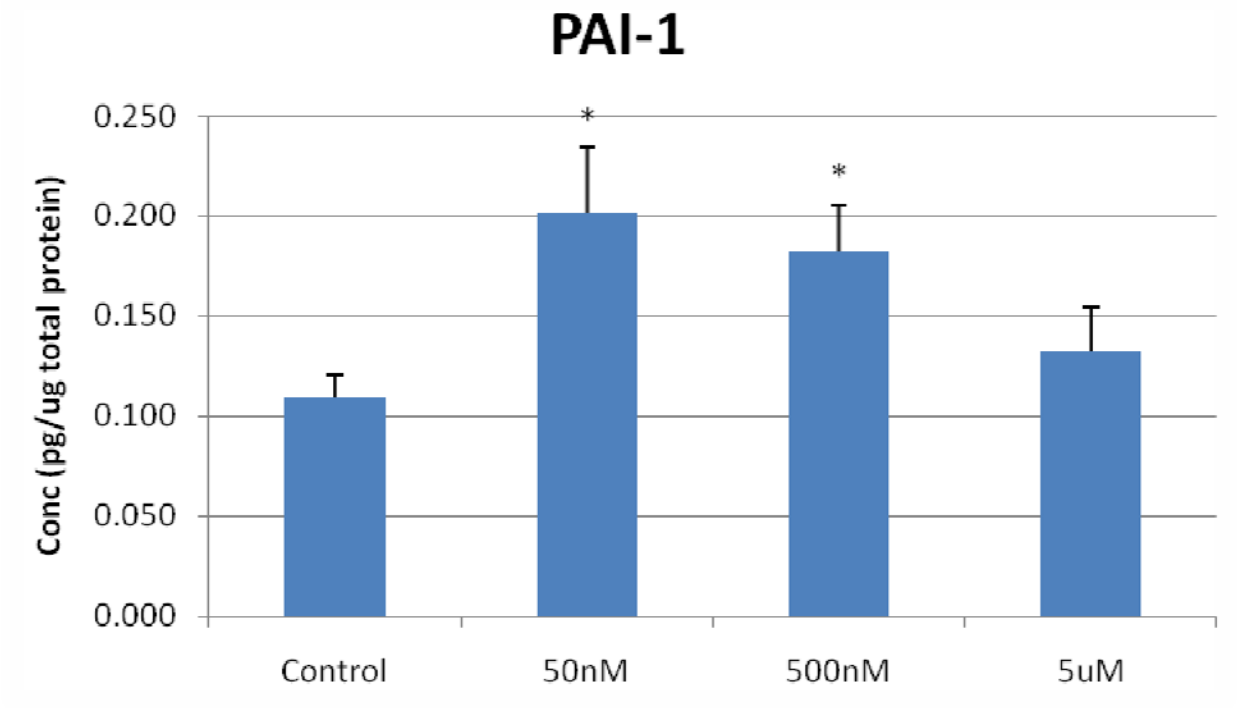


Figure 4-5. Serum adiponectin levels in males treated with BPA. Serum adiponectin levels were determined using a commercially available EIA kit. (***) $p<0.001$.

resistin compared to the other adipokines. There were no differences between treatments for MCP-1, IL-6, TNFα, or resistin. There were also no extreme differences between strains for any of the adipokines. The only adipokine that was significantly altered by BPA was plasminogen activator inhibitor 1 (PAI-1). C57BL/6 males treated with 50nM and 500nM had approximately twice the levels of PAI-1 compared to controls ($p=0.018$, 0.012 for 50nM and 500nM, respectively) (Figure 4-6). We also found that the 500nM dose of BPA slightly increased leptin in both C57BL/6 males and DBA/2J males (data not shown), but the results were not significant.

Gene expression profiling and QPCR

Our results indicate that male mice exposed to 500nM BPA were significantly fattier than their control counterparts. To identify changes in gene expression associated with the increased adiposity, global gene expression profiling was performed for these mice on the Illumina platform (Genome Quebec Innovation Center). Due to the distribution of the data and the lack of a large set of expression changes, we were unable to do FDR and extract q-values. Therefore, we were conservative in selecting genes and used a p-value < 0.005. We found 95 genes differentially expressed in C57BL/6 males and 71 genes differentially expressed in DBA/2J males. Of all these genes, only 2 genes, Leptin and Heparan sulfate proteoglycan 2 (*Hspg2*), were differentially expressed in both strains. Leptin was increased 1.45-fold and 1.41-fold and *Hspg2* was increased 1.25-fold and 1.18-fold in C57BL/6 and DBA/2J, respectively. Generally, there were no large increases or decreases in expression that were



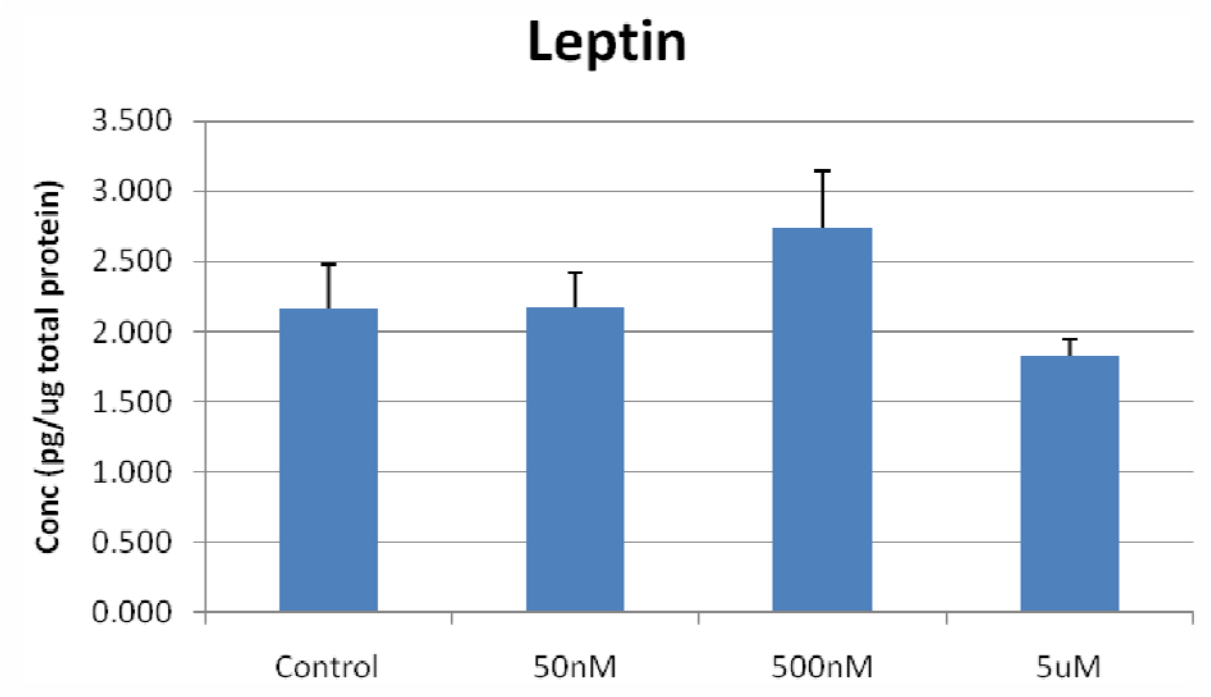


Figure 4-6. Adipose levels of PAI-1 and Leptin in C57BL/6 males. Adipokine levels were measured in adipose tissue using a Milliplex map kit and Luminex system. Results shown as mean ± SEM. (*) $p<0.05$.

statistically significant. The genes with the greatest significant change for each strain are listed below (Table 4-1 and 4-2). There was a 2.56-fold decrease in Cytochrome P450 2f2 for C57/BL6 treated compared to control and a 3.85-fold decrease in Immunoglobulin kappa constant for DBA/2J. Transmembrane glycoprotein NMB increased 1.7-fold in BPA treated C57BL/6 mice compared with control, and S100 calcium binding protein 8 increased 1.96-fold in DBA/2J mice.

We also conducted QPCR for a number of genes. We selected the adipogenic and lipogenic genes, *Pparg* and *Fasn*. We also tested a number of other genes of interest as well as some genes of interest identified from the initial array data. We found no statistically significant difference in *Fasn, Pparg, Esr1, Esrrg, or Adipoq* for the C57BL/6 males treated with BPA compared to control. There was a slight increase in leptin expression, which matches with our array data, but the change was not statistically significant (p=0.17) (Figure 4-7). For genes identified from the array data, we confirmed statistically significant increases in *Peg3* (2.25-fold increase, p=0.028), *Gja5* (1.75-fold increase, p=0.011), and *Thbs2* (2.046-fold increase, p=0.005). We also found a decrease in *Ldhd* expression (1.48-fold decrease) but the result weren't statistically significant (p= 0.09).

For the DBA/2J mice, there were also no significant changes in the expression of *Fasn, Pparg, Esr1, Essrg, or Adipoq*. There was, however, a 1.40-fold increase in the expression of Leptin (p=0.02) (Figure 4-8). For the genes chosen from the array data,

Table 4-1. Top genes differentially expressed in C57BL/6 male mice treated with 500nM BPA compared to control.

Gene symbol	Gene name	Fold change	p-value
Gpnmb	Transmembrane glycoprotein NMB	1.7	0.0028
Mmp12	Matrix metalloproteinase 12	1.69	0.0012
Sfrp5	Secreted frizzled-related protein 5	1.66	0.0018
Thbs2	Thrombospondin 2	1.64	0.0070
Peg3	Paternally expressed gene 3 protein	1.62	0.0048
Emp3	Epithelial membrane protein 3	1.57	0.0030
Lep	Leptin	1.45	0.0018
Fbn1	Fibrillin-1	1.45	0.0020
Itih5	Inter-alpha (globulin) inhibitor H5	1.44	0.0059
Myl4	Myosin light chain 4	1.4	0.0031
Cyp2f2	Cytochrome P450 2F2	-2.56	0.0007
Tst	Thiosulfate sulfurtransferase	-1.89	0.0023
Net1	Neuroepithelial cell-transforming 1	-1.64	0.0002
Ldhd	Lactate dehydrogenase	-1.54	0.0029
Fos	FBJ osteosarcoma oncogene	-1.47	0.0077
Cyp2b10	Cytochrome P450 2B10	-1.35	0.0072

Table 4-2. Top genes differentially expressed in DBA/2J male mice treated with 500nM BPA compared to control.

Gene symbol	Gene name	Fold change	p-value
S100a8	S100 calcium binding protein 8	1.96	0.0040
Cdkn1a	Cyclin-dependent kinase inhibitor 1	1.59	0.0050
Dusp1	Dual specificity protein phosphatase 1	1.52	0.0066
Klf6	Krueppel-like factor 6	1.48	0.0015
Lep	Leptin	1.41	0.0033
Vldlr	Very low density lipoprotein receptor	1.35	0.0024
Serpina1b	Serine peptidase inhibitor A1B	1.34	0.0033
Eif5	Eukaryotic translation initiation factor 5	1.32	0.0060
Adap2	ArfGAP w/ dual PH domains 2	1.3	0.0041
Osgin1	Oxidative stress induced growth factor 1	1.3	0.0035
Igkc	Immunoglobulin kappa constant	-3.85	0.0013
Cxcl13	CXC motif chemokine 13	-3.03	0.0002
Igkv5	Immunoglobulin kappa variable 5	-2.78	0.0011
Faim3	Fas apoptotic inhibitory molecule 3	-1.49	0.0045
Cyp2e1	Cytochrome P450 2E1	-1.41	0.0011
Aebp1	Adipocyte enhancer binding protein 1	-1.39	0.0099

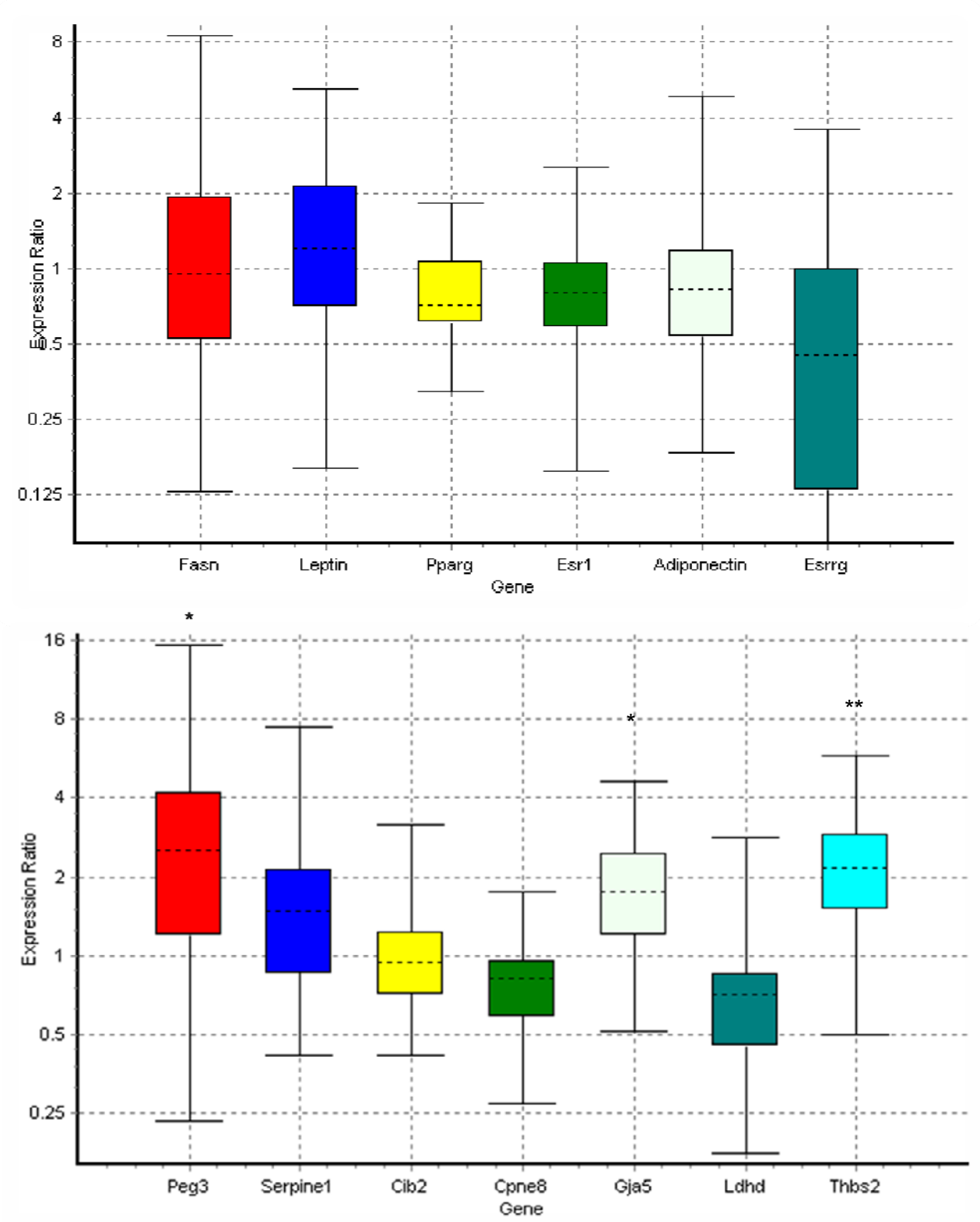
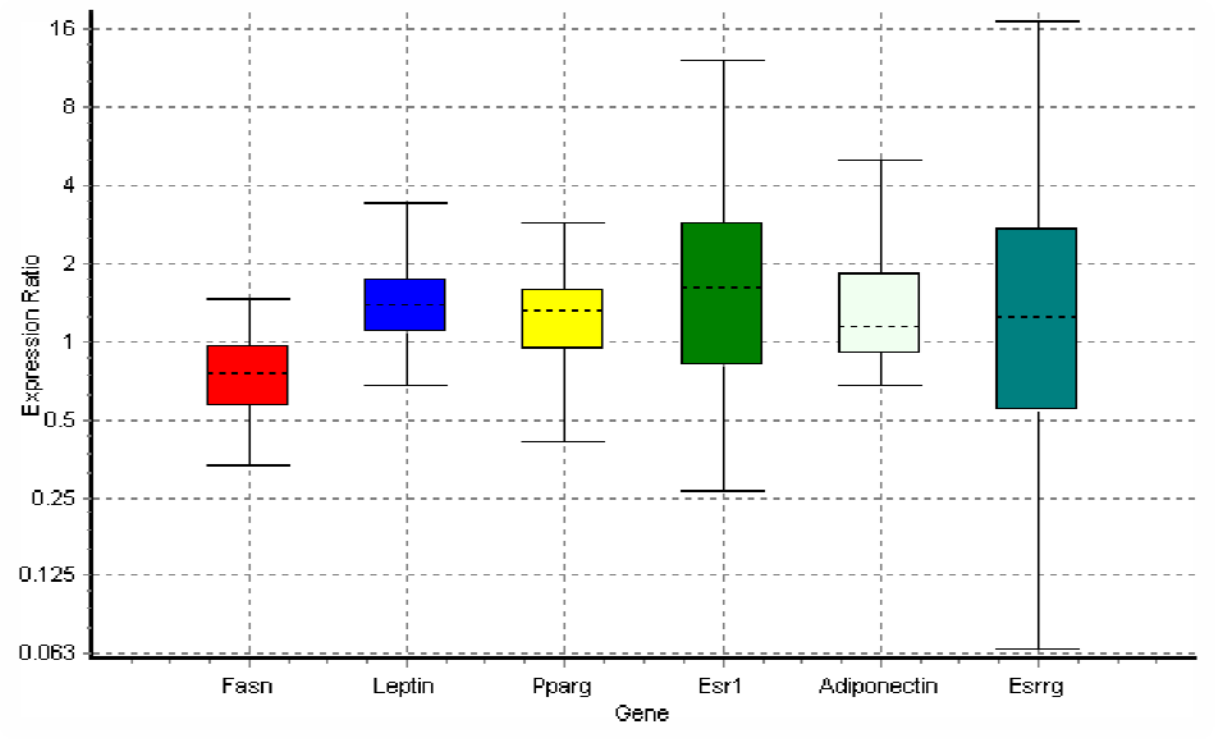


Figure 4-7. QPCR results for selected genes in C57BL/6 male mice. Mice exposed to 500nM BPA are compared to Controls. Results are expressed as expression ratios determined using the $\Delta\Delta Ct$ method. (*)$p<0.05$, (**)$p<0.01$.



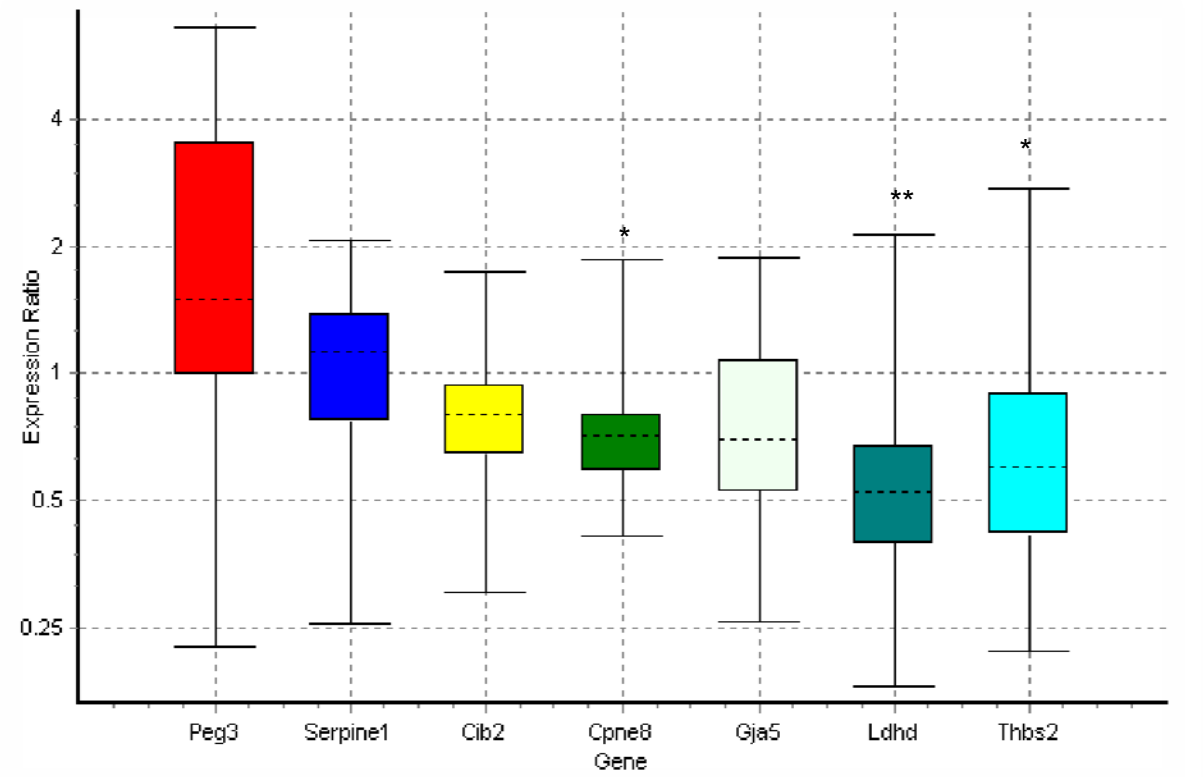


Figure 4-8. QPCR results for selected genes in DBA/2J male mice. Mice exposed to 500nM BPA are compared to Controls. Results are expressed as expression ratios determined using the ΔΔCt method. (*)$p<0.05$, (**)$p<0.01$.

we confirmed significant changes in the expression of *Cpne8* (1.39 decrease, p=0.018), *Ldhd* (1.82 decrease, p=0.008), and *Thbs2* (1.6 decrease, p=0.04).

Adipocyte size measurement

To determine if BPA exposure has an effect on adipocyte expansion, we used a portion of the gonadal fat pad for histological analysis and measured average adipocyte differences between mice exposed BPA versus control mice. We used a variation of the method previously described (Chen and Farese, 2002) which uses computer image analysis software to measure adipocyte size. The image conversion process results in some small artifacts, therefore, we set a lower limit threshold that would allow us to measure approximately 200 adipocytes per animal/slide. Once again, only the males treated with 500nM or control were used for histological analysis. We found that the males treated with 500nM had significantly larger adipocytes compared to control (Figure 4-9). C57BL/6 males treated with 500nM BPA had an average adipocyte size of 10859 μm^2 compared to 9143 μm^2 in control (p=0.048). The DBA/2J males showed similar results with an average adipocyte size of 13392 μm^2 and 10081 μm^2 in 500nM BPA treatment and control, respectively (p=0.041).

Metabolomics

A portion of the gonadal fat pad was subjected to global metabolic profiling to determine if *in vivo* exposure to BPA changes adipose tissue metabolism, which could explain the changes in adiposity. We previously used this technique in adipose tissue explants treated with BPA and found and trend for increased glycolytic and TCA cycle

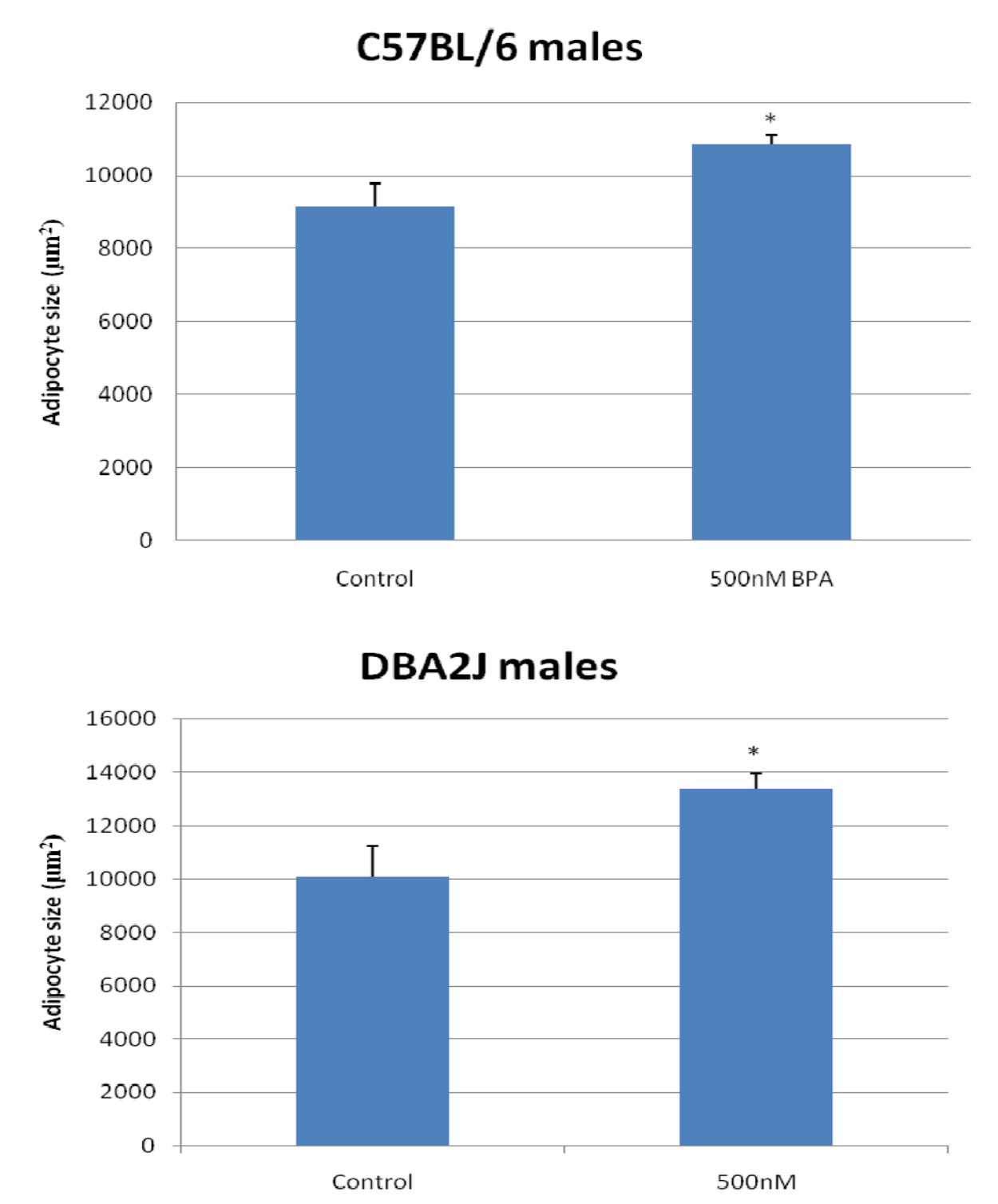


Figure 4-9. Effects of BPA on adipocyte size. Mice treated with 500nM BPA are compared to control. Results are shown as means ± SEM. (*)$p<0.05$

intermediates, indicating a change in adipose tissue energy metabolism. We expected to see more subtle yet similar results for our *in vivo* treatment. We only used male mice to measure the changes in metabolite levels since the changes in adiposity were seen in males. Due to the variance in metabolite measurements from mouse to mouse, we looked for metabolites with 1.5-fold difference between a BPA treatment and control. We did not see many large changes in glycolytic metabolites or TCA cycle metabolites. There was a decrease in the glycolytic metabolites fructose-6-phosphate (50nM = -1.89-fold, 500nM = -2.35-fold) and phosphoenolpyruvate (5μM = -1.55-fold). Citrate is the metabolite in the TCA cycle that can be shuttled out of the mitochondria and used for fatty acid synthesis, and we saw a decrease in this metabolite as well (50nM = -2.12-fold, 500nM = -2.01-fold) (Figure 4-10).

Discussion

We studied the effects that chronic exposure to BPA has on adipose tissue in an organism using two different inbred strains of mice, C57BL/6 and DBA/2J. These are the parental strains for the BXD recombinant inbred line, and both are routinely used in cardiovascular and obesity studies (Campen et al., 2004, Gallou-Kabani et al., 2007). We chose our doses of BPA based on previous research (Somm et al., 2009). Somm and colleagues used a treatment of 1 mg/L BPA in the drinking water of pregnant Sprague-Dawley rats and found increased birth weight, as well as increased adiposity and adipogenesis in females at weaning. The highest concentration that we used, 5μM, is 1.14 mg/L or approximately the same dose used in the Somm study. Because rats

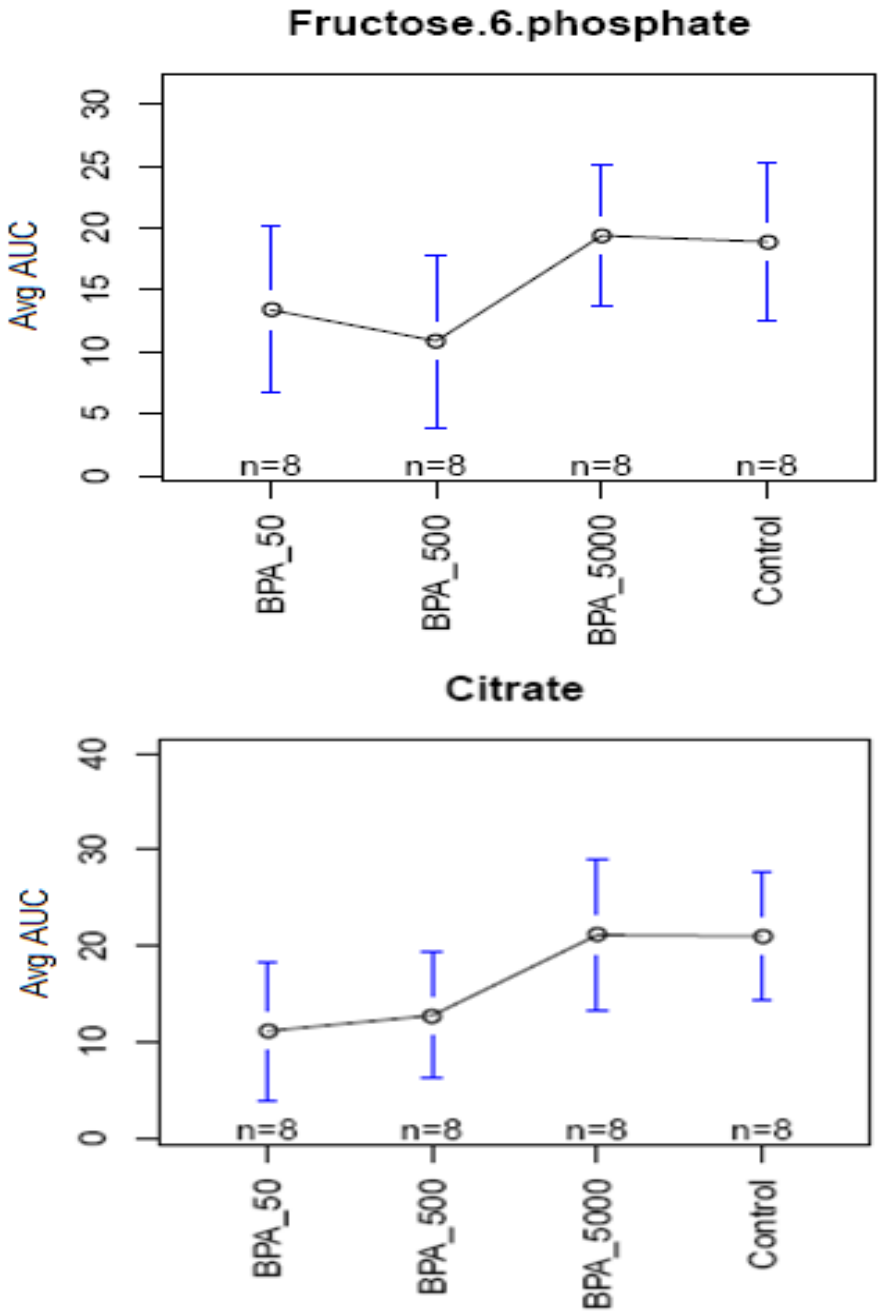


Figure 4-10. Glycolytic and TCA cycle intermediates levels in adipose tissue. Fructose-6-phosphate and citrate were the two intermediates from glycolysis and TCA cycle, respectively, with the greatest fold-change. Results are shown as means ± SEM.

are larger than mice, we chose to use the smaller, as well as more biologically relevant, concentrations of 500nM and 50nM BPA. Based on an estimated daily intake of 6-7mL water for each mice, we estimated that the daily intake of BPA for the 5μM, 500nM and 50nM treatment was 350μg/kg bw/day, 35μg/kg bw/day, and 3.5μg/kg bw/day, respectively. This estimation was determined based on weekly water consumption and does not account for spillage, therefore, the actual intake may be slightly lower than the estimation. All three concentrations are lower than the No-observed-adverse-effect-level (NOAEL) of 50mg/kg bw/day, and the low and middle concentration are below the acceptable daily intake (ADI) for humans of 50μg/kg bw/day set by the EPA (http://www.epa.gov/iris/subst/0356.htm).

We found no difference in bodyweight or adiposity for females exposed to BPA compared to control. The results in Figure 4-1 and 4-2 show, however, that we did find differences in males for both strains. In C57BL/6, males treated with lowest dose of BPA were significantly heavier beginning at 7 weeks of age and continuing throughout the study, but the only difference in adiposity occurred in the 500nM treatment. It's possible that the B6 males treated with 50nM BPA had greater fat gain in other depots, however we only dissected gonadal fat pads which are representative of abdominal fat in humans. DBA/2J males treated with 500nM were significantly heavier than controls by the time of sacrifice. They also had increased adiposity, however, the results were not statistically significant ($p=0.08$). There was a clear difference between the effects seen in males and females. Because BPA is an endocrine disruptor and mimics estrogen, it isn't surprising to find that males and females may react differently to the

treatments. It was also not surprising to find that the 500nM was the only treatment that increased adiposity due to the lack of monotonic results in many other studies. Based on these results, we decided to focus on males for a number of the other experiments, and specifically the 500nM treatment of BPA.

Not surprisingly, we saw a significant difference in fasting blood glucose levels between the sexes. What was surprising was the great difference between the strains. The blood glucose levels in DBA/2J mice barely spiked after injection (data not shown), indicating an ability in the DBA/2J strain to quickly regulate blood glucose levels. C57/BL6 mice reacted as expected (Figure 4-3), and we found that male mice treated with 50nM and 500nM cleared glucose from the blood slower than control, but the results were no significant. We also measured fasting insulin levels to determine if BPA may affect insulin synthesis and secretion. Surprisingly, we found significant difference in females for both strains, but opposite results (Figure 4-4). C57BL/6 females had higher fasting insulin levels in all treatments compared to control, but the DBA/2J females had lower serum insulin levels in all treatments. There were no significant differences in the males for either strain. These results, once again indicate a physiologically difference between the two strains, and that the two strains may be having different physiologically responses to BPA.

Low nanomolar concentrations of BPA have been shown to inhibit secretion of adiponectin from adipose tissue (Hugo et al., 2008). We used an adiponectin EIA kit to measure serum adiponectin levels in males treated with 500nM BPA or control. Our findings are consistent with findings described by Hugo and colleagues (Hugo et al.,

2008). Adiponectin levels were significantly lower in the serum of mice exposed to 500nM BPA (Figure 4-5). The decreases were greater and more significant in C57BL/6 mice than DBA/2J. We also measured the levels of other adipokines in the adipose tissue of the male mice treated with BPA using the mouse serum adipokine Milliplex xmap kit on the Luminex platform. While the Luminex system gave us a large amount of data, we only found a significant difference in one analyte, PAI-1. C57BL/6 male mice treated with 50nM or 500nM BPA had significantly higher levels of PAI-1 compared to control (Figure 4-6). PAI-1, or plasminogen activator inhibitor 1, is a serine protease inhibitor, and higher serum levels of PAI-1 have been detected in patients with coronary heart disease (Kohler and Grant, 2000). It has also been linked to obesity and insulin resistance (Juhan-Vague and Alessi, 1997). PAI-1 is also known as Serpine1, and the expression of many Serpines have been found to be elevated in fatty chickens (Larry Cogburn, personal communication). Interestingly, the elevated PAI-1 levels were found in the mice that were significantly heavier (50nM) and fatter (500nM) compared to control.

We conducted expression profiling for the male mice treated with 500nM BPA and control to try to identify the genes that were differentially expressed, which would shed some insight on the molecular basis of BPA increasing adiposity. The list of genes differentially expressed between treatments was fairly small, which prevented us from performing FDR and extracting q-values. The mice were treated with lower doses and at a less critical time of development compared to perinatal development, which could explain more subtle changes in gene expression. We found that Leptin

expression was higher in treated which is consistent with our finding that the 500nM treatment increased adiposity. There were some interesting genes identified. One of particular interest was *Peg3*, or paternally expressed gene 3. We found that *Peg3* was increased 1.62-fold in C57BL/6 males treated with BPA ($p=0.0048$), and these results were validated using QPCR (2.25-fold increase). A past study showed that a targeted mutation in *Peg3* led to increased body fat in mice and is a candidate gene for regulation of fat content (Curley et al., 2005). It has also been shown to regulate energy during gestation. *Peg3* expression has also been shown to activate the TNF/NF-κB pathway in kidney cells (Nogueira et al., 2009). Perhaps BPA increases *Peg3* expression leading to disruption of energy homeostasis in adipose tissue, promoting increased fat mass and inflammation. We also found a few Cytochrome P450 genes that were down-regulated in the treated mice for both C57BL/6 and DBA/2J. We also found that Serpinea1b expression was increased in DBA/2J males treated with BPA which is consistent with the finding that Serpines have higher expression in fattier chickens.

It is thought that obesity proceeds through both expansion of adipocytes and proliferation of preadipocytes, however adipocyte hypertrophy occurs first (Spalding et al., 2008). Adipocyte size is also positively correlated with insulin resistance and inflammation (Skurk et al., 2007).Therefore, we believed that if BPA increased adiposity and inflammation, we would see a difference in adipocyte size. We found that the adipocytes of mice treated with 500nM BPA were slightly larger than those of mice treated with the vehicle control (1.2x larger in C57BL6, 1.3x larger in DBA/2J), and

these results were statistically significant (Figure 4-9). The changes were subtle and not easily visible to the naked eye. There is evidence that smaller adipocytes are more insulin sensitive than the larger adipocytes, and there is a greater population of smaller adipocytes in leaner individuals (Skurk et al., 2007). Due to the image conversion method, we were only able to measure the 200 largest cells per frame and could not measure the smaller adipocytes.

The changes in metabolite levels in mice exposed to BPA compared to control were more subtle than the changes observed in the adipose tissue explants, which was not surprising. However, what was somewhat surprising was to find that the levels of some glycolytic and TCA cycle intermediates were lower in treated mice, which was the opposite of our findings in the *ex vivo* study (Figure 4-11). Fructose-6-phosphate, an intermediate of glycolysis, was decreased in mice treated with 50nM and 500nM BPA (1.89-fold and 2.35-fold change, respectively. Citrate, the TCA cycle intermediate that is used in lipogenesis, was also decreased for these treatments (2.12-fold and 2.01-fold change, respectively). A low metabolite pool can indicate that the metabolic pathway is decreased or it could mean that the metabolic pathway is so active that the metabolites do not have a chance to form a pool before being used in another pathway. It's possible that the metabolites of glycolysis and the TCA cycle were able to pool in the adipose tissue explants, however in the live mice the metabolites get used by another pathway much more rapidly. If this is the case, then this would indicate that glycolysis and the TCA cycle are still increased, and perhaps citrate is being shuttled out of the mitochondria rapidly to form acetyl-CoA and undergo lipogenesis (Figure 4-11).

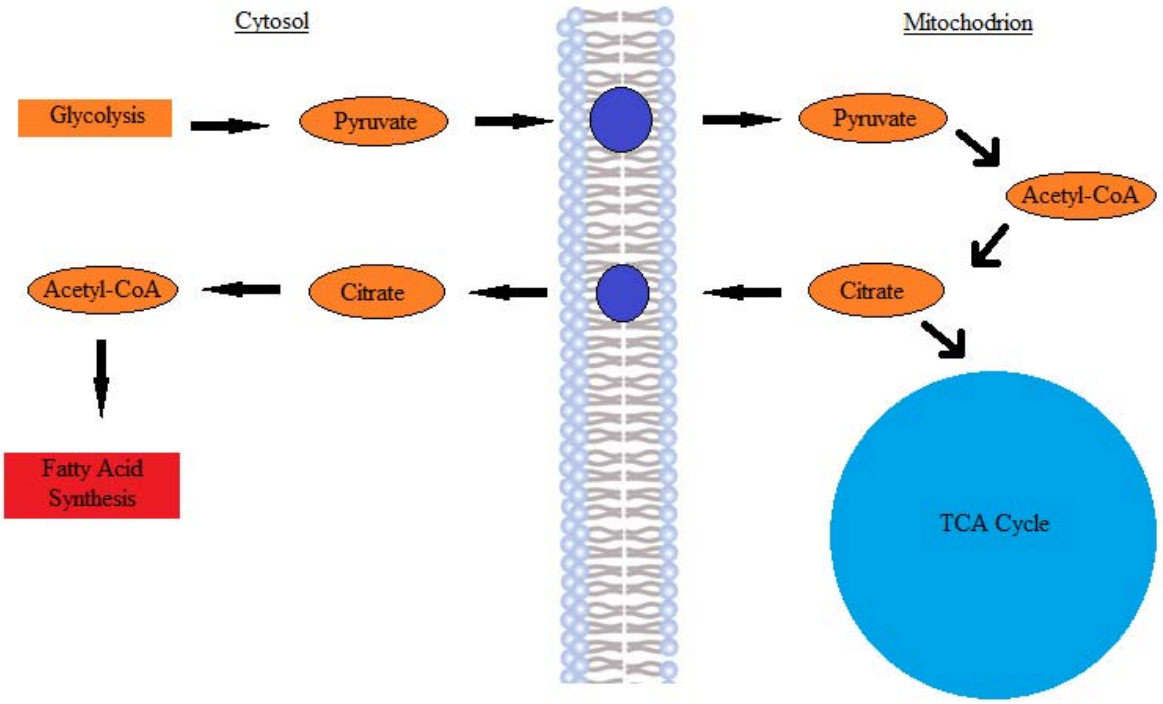


Figure 4-11. Diagram of the TCA cycle and citrate shuttle. Pyruvate from glycolysis enters the mitochondria to enter the TCA cycle. An intermediate, citrate can be shuttled to the cytosol to be converted to acetyl-CoA and begin fatty acid synthesis (Garrett and Grisham. *Biochemistry*. 2nd Ed. 1999).

Conclusion

There are definitely genetic and phenotypic differences between sex and strain, and these differences appear to affect the impact that BPA has on an individual. The BPA studies to date for adiposity are few, and almost all have focused on the effects of *in utero* exposure. We essentially modeled childhood exposure, independent of any "programming" that may occur during embryonic development. We showed a modest but significant increase in fatness with consumption of a reasonable level of BPA over a seven week period. The bodyweight and adiposity of the males were increased for both C57BL/6 and DBA/2J treated with BPA. The serum insulin levels were unchanged in the males but were changed in females for both strains, however BPA had an opposite effect in each strain. A different set of genes were differentially expressed between C57BL/6 and DBA/2J. The adipocytes were slightly larger for both strains exposed to BPA, and some glycolytic and TCA cycle intermediates were lower in treated indicating that those metabolic pathways may have been more active. Taken together these data indicate that BPA disrupts energy homeostasis and gene expression in adipose tissue in way that promotes weight gain, however the severity of its action is unclear. Our results imply that BPA consumed in children may contribute to childhood obesity by promoting adipocyte expansion, independent of any developmental effects of BPA. In conclusion, we showed that BPA exposure below the ADI may increase adiposity and promote weight gain, and although the effects are probably not as severe as those that occur from gestational and perinatal exposure, they can further exacerbate the effects incurred *in utero.*

CHAPTER V

FUTURE STUDIES

We have demonstrated that BPA can disrupt adipose tissue metabolism, gene expression, and endocrine function in a way that promotes weight gain, however, the effects were modest and not dose-dependent. These non-monotonic results are consistent with other results commonly seen in studies of endocrine disrupting chemicals (Grun and Blumberg, 2009a, Vandenberg et al., 2009). The method of delivery was also a possible source of variability. Controlling the dosage is much more difficult when administering orally via drinking water due to the variability of water intake between animals. Our estimates of daily intake are based on daily water intake per cage and don't account for spillage, so it is possible that some of the treatments were lower from cage to cage. However, this is necessary to replicate the most likely route of exposure in humans.

A future study that is planned is to determine if BPA treatment exacerbates weight gain and the inflammatory effects of a high fat diet. We conducted a preliminary experiment that studied the effect that a high fat diet combined with BPA exposure has on body weight and adiposity, and these results can be found in the appendix of this thesis. The typical consumption pattern in the United States would be that of a high dietary fat intake coupled with significant BPA exposure, and it is quite possible that these two things in concert have led to the obesity epidemic.

There are also plans to conduct a study in which we will treat pregnant mice with BPA via drinking water and continue treatment in the pups after birth. Our future *in vivo* BPA studies should focus on one strain, C57BL/6. This strain appeared to be more sensitive to BPA than the DBA/2J strain and is commonly used in obesity studies (Gallou-Kabani et al., 2007, Schreyer et al., 1998). Humans are commonly exposed to low nanomolar doses, therefore, we should use a low nanomolar dose as well. In the next study, we should replace the 5μM dose with a 5nM dose. For these future studies, along with the measurement we performed previously (body weight, adiposity, adipocyte size, etc.), we should measure fasting glucose and the circulating levels of adipokines rather than adipokine levels in the tissue. We should also examine the effects that BPA has on the liver and pancreas. A study that combines perinatal BPA exposure and continues treatment to adolescence would best represent the level of exposure to BPA in the US, especially those in lower income environments. Those in lower income communities are most likely not educated on the potential dangers of BPA. A study that exposed mice to low levels of BPA during gestational and perinatal development through adulthood would likely represent the levels of exposure for a large portion of humans.

We found that BPA did not alter expression of the adipogenic regulator, PPARγ, however, it is possible that BPA may enhance the activity of PPARγ. A mutation in the this gene that results in constitutively active PPARγ results in extreme obesity, and there is evidence that some endocrine disruptors affect PPARγ signaling (Ristow et al., 1998, Grun et al., 2006). It is possible that the increase in adiposity observed in our

study may be due to enhanced activity of PPARγ. To study the effects that BPA has on PPARγ activity, we would use a PPARγ response element plasmid. Briefly, PPRE X3-TK-luc plasmid would be transfected into differentiated 3T3-L1s, or another cell line that adequately expresses PPARγ. The cells would then be treated with doses of BPA ranging from 1nM to 1μM. If BPA enhances the activity of PPARγ, the transcription factor will bind the PPRE resulting in luciferase expression and a light signal. Rosiglitazone would be used as a positive control, and the expression of luciferase would be proportional to the PPARγ activity. This experiment would allow us to determine if biologically relevant concentrations of BPA may promote adipogenesis through activation of PPARγ.

The metabolomic experiments we conducted allowed us to measure pools of metabolites in adipose tissue and detect changes that may be a result of BPA treatment. While these experiments provide insight to the metabolic changes that occur as a result of BPA, they only provide a snapshot of the metabolism at the time of sacrifice. To determine the rate at which these metabolites are being used or produced, we must conduct a metabolic flux analysis. This type of experiment can be done using adherent mammalian cells, and we would use differentiated 3T3-L1s (Yuan et al., 2008). Cells are grown in unlabeled media for a period of time before being switched to media containing C-13 labeled glucose or glutamine. Metabolites are extracted from the cells at various time points from 0 mins to 4 hrs. Metabolites are measured using LC-MS/MS to measure the rate that a certain metabolite is produced. In our experiment, cells would be treated with 1nM, 10nM, 1μM, or control (EtOH) for 24 hrs

before being switched to the labeled media. The treatment would continue during the labeled media switch. This would allow us to definitively determine if the increase or decrease in glycolytic and TCA cycle metabolites is a result of changes in these metabolic pathways, and if citrate is being funneled into *de novo* lipogenesis.

LIST OF REFERENCES

ALEXANDER, J., CHANG, G. Q., DOURMASHKIN, J. T. & LEIBOWITZ, S. F. 2006. Distinct phenotypes of obesity-prone AKR/J, DBA2J and C57BL/6J mice compared to control strains. *Int J Obes (Lond),* 30**,** 50-9.

ALONSO-MAGDALENA, P., LARIBI, O., ROPERO, A. B., FUENTES, E., RIPOLL, C., SORIA, B. & NADAL, A. 2005. Low doses of bisphenol A and diethylstilbestrol impair Ca2+ signals in pancreatic alpha-cells through a nonclassical membrane estrogen receptor within intact islets of Langerhans. *Environ Health Perspect,* 113**,** 969-77.

ALONSO-MAGDALENA, P., VIEIRA, E., SORIANO, S., MENES, L., BURKS, D., QUESADA, I. & NADAL, A. 2010. Bisphenol A exposure during pregnancy disrupts glucose homeostasis in mothers and adult male offspring. *Environ Health Perspect,* 118**,** 1243-50.

AVRAM, M. M., AVRAM, A. S. & JAMES, W. D. 2007. Subcutaneous fat in normal and diseased states 3. Adipogenesis: from stem cell to fat cell. *J Am Acad Dermatol,* 56**,** 472-92.

BAILLIE-HAMILTON, P. F. 2002. Chemical toxins: a hypothesis to explain the global obesity epidemic. *J Altern Complement Med,* 8**,** 185-92.

BALISTRERI, C. R., CARUSO, C. & CANDORE, G. 2010. The role of adipose tissue and adipokines in obesity-related inflammatory diseases. *Mediators Inflamm,* 2010**,** 802078.

BARKER, D. J., OSMOND, C., GOLDING, J., KUH, D. & WADSWORTH, M. E. 1989. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ,* 298**,** 564-7.

BEN-JONATHAN, N., HUGO, E. R. & BRANDEBOURG, T. D. 2009. Effects of bisphenol A on adipokine release from human adipose tissue: Implications for the metabolic syndrome. *Mol Cell Endocrinol,* 304**,** 49-54.

BIRNBAUM, L. S. & FENTON, S. E. 2003. Cancer and developmental exposure to endocrine disruptors. *Environ Health Perspect,* 111**,** 389-94.

CALAFAT, A. M., YE, X., WONG, L. Y., REIDY, J. A. & NEEDHAM, L. L. 2008. Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003-2004. *Environ Health Perspect,* 116**,** 39-44.

CAMPEN, M. J., TAGAITO, Y., LI, J., BALBIR, A., TANKERSLEY, C. G., SMITH, P., SCHWARTZ, A. & O'DONNELL, C. P. 2004. Phenotypic variation in cardiovascular responses to acute hypoxic and hypercapnic exposure in mice. *Physiol Genomics,* 20**,** 15-20.

CECCHINI, M., SASSI, F., LAUER, J. A., LEE, Y. Y., GUAJARDO-BARRON, V. & CHISHOLM, D. 2010. Tackling of unhealthy diets, physical inactivity, and obesity: health effects and cost-effectiveness. *Lancet,* 376**,** 1775-84.

CHEN, H. C. & FARESE, R. V., JR. 2002. Determination of adipocyte size by computer image analysis. *J Lipid Res,* 43**,** 986-9.

CHEN, J. Q., BROWN, T. R. & RUSSO, J. 2009a. Regulation of energy metabolism pathways by estrogens and estrogenic chemicals and potential implications in obesity associated with increased exposure to endocrine disruptors. *Biochim Biophys Acta,* 1793**,** 1128-43.

CHEN, J. Q., CAMMARATA, P. R., BAINES, C. P. & YAGER, J. D. 2009b. Regulation of mitochondrial respiratory chain biogenesis by estrogens/estrogen receptors and physiological, pathological and pharmacological implications. *Biochim Biophys Acta,* 1793**,** 1540-70.

CINTI, S., MITCHELL, G., BARBATELLI, G., MURANO, I., CERESI, E., FALOIA, E., WANG, S., FORTIER, M., GREENBERG, A. S. & OBIN, M. S. 2005. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res,* 46**,** 2347-55.

COLBORN, T., VOM SAAL, F. S. & SOTO, A. M. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect,* 101**,** 378-84.

CURLEY, J. P., PINNOCK, S. B., DICKSON, S. L., THRESHER, R., MIYOSHI, N., SURANI, M. A. & KEVERNE, E. B. 2005. Increased body fat in mice with a targeted mutation of the paternally expressed imprinted gene Peg3. *FASEB J,* 19**,** 1302-4.

DE FERRANTI, S. & MOZAFFARIAN, D. 2008. The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences. *Clin Chem,* 54**,** 945-55.

DEMIR, B., OZTURKOGLU, E., SOLAROGLU, A., BASKAN, B., KANDEMIR, O., KARABULUT, E. & HABERAL, A. 2008. The effects of estrogen therapy and estrogen combined with different androgenic progestins on carbohydrate and lipid metabolism in overweight-obese younger postmenopausal women. *Gynecol Endocrinol,* 24**,** 347-53.

FANTUZZI, G. 2005. Adipose tissue, adipokines, and inflammation. *J Allergy Clin Immunol,* 115**,** 911-9; quiz 920.

FERRE, P. 2004. The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. *Diabetes,* 53 Suppl 1**,** S43-50.

FINKELSTEIN, E. A., TROGDON, J. G., COHEN, J. W. & DIETZ, W. 2009. Annual medical spending attributable to obesity: payer-and service-specific estimates. *Health Aff (Millwood),* 28**,** w822-31.

GAETANI, S. 2008. A sideways glance: does the color matter? A revised model of the origin of white and brown fat cells. *Genes Nutr,* 3**,** 99-100.

GALLOU-KABANI, C., VIGE, A., GROSS, M. S., RABES, J. P., BOILEAU, C., LARUE-ACHAGIOTIS, C., TOME, D., JAIS, J. P. & JUNIEN, C. 2007. C57BL/6J and A/J mice fed a high-fat diet delineate components of metabolic syndrome. *Obesity (Silver Spring),* 15**,** 1996-2005.

GAO, Q., MEZEI, G., NIE, Y., RAO, Y., CHOI, C. S., BECHMANN, I., LERANTH, C., TORAN-ALLERAND, D., PRIEST, C. A., ROBERTS, J. L., GAO, X. B., MOBBS, C., SHULMAN, G. I., DIANO, S. & HORVATH, T. L. 2007. Anorectic estrogen mimics leptin's effect on the rewiring of melanocortin cells and Stat3 signaling in obese animals. *Nat Med,* 13**,** 89-94.

GHISARI, M. & BONEFELD-JORGENSEN, E. C. 2005. Impact of environmental chemicals on the thyroid hormone function in pituitary rat GH3 cells. *Mol Cell Endocrinol,* 244**,** 31-41.

GINSBERG, G. & RICE, D. C. 2009. Does rapid metabolism ensure negligible risk from bisphenol A? *Environ Health Perspect,* 117**,** 1639-43.

GOULD, J. C., LEONARD, L. S., MANESS, S. C., WAGNER, B. L., CONNER, K., ZACHAREWSKI, T., SAFE, S., MCDONNELL, D. P. & GAIDO, K. W. 1998. Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. *Mol Cell Endocrinol,* 142**,** 203-14.

GREENBERG, A. S. & OBIN, M. S. 2006. Obesity and the role of adipose tissue in inflammation and metabolism. *Am J Clin Nutr,* 83**,** 461S-465S.

GRUN, F. & BLUMBERG, B. 2007. Perturbed nuclear receptor signaling by environmental obesogens as emerging factors in the obesity crisis. *Rev Endocr Metab Disord,* 8**,** 161-71.

GRUN, F. & BLUMBERG, B. 2009a. Endocrine disrupters as obesogens. *Mol Cell Endocrinol,* 304**,** 19-29.

GRUN, F. & BLUMBERG, B. 2009b. Minireview: the case for obesogens. *Mol Endocrinol,* 23**,** 1127-34.

GRUN, F., WATANABE, H., ZAMANIAN, Z., MAEDA, L., ARIMA, K., CUBACHA, R., GARDINER, D. M., KANNO, J., IGUCHI, T. & BLUMBERG, B. 2006. Endocrine-

disrupting organotin compounds are potent inducers of adipogenesis in vertebrates. *Mol Endocrinol,* 20**,** 2141-55.

GRUNDY, S. M. 2004. Obesity, metabolic syndrome, and cardiovascular disease. *J Clin Endocrinol Metab,* 89**,** 2595-600.

HALES, C. N. & BARKER, D. J. 2001. The thrifty phenotype hypothesis. *Br Med Bull,* 60**,** 5-20.

HEINDEL, J. J. & VOM SAAL, F. S. 2009. Role of nutrition and environmental endocrine disrupting chemicals during the perinatal period on the aetiology of obesity. *Mol Cell Endocrinol,* 304**,** 90-6.

HEINE, P. A., TAYLOR, J. A., IWAMOTO, G. A., LUBAHN, D. B. & COOKE, P. S. 2000. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proc Natl Acad Sci U S A,* 97**,** 12729-34.

HOWDESHELL, K. L., HOTCHKISS, A. K., THAYER, K. A., VANDENBERGH, J. G. & VOM SAAL, F. S. 1999. Exposure to bisphenol A advances puberty. *Nature,* 401**,** 763-4.

HUGO, E. R., BRANDEBOURG, T. D., WOO, J. G., LOFTUS, J., ALEXANDER, J. W. & BEN-JONATHAN, N. 2008. Bisphenol A at environmentally relevant doses inhibits adiponectin release from human adipose tissue explants and adipocytes. *Environ Health Perspect,* 116**,** 1642-7.

HUNT, P. A., KOEHLER, K. E., SUSIARJO, M., HODGES, C. A., ILAGAN, A., VOIGT, R. C., THOMAS, S., THOMAS, B. F. & HASSOLD, T. J. 2003. Bisphenol a exposure causes meiotic aneuploidy in the female mouse. *Curr Biol,* 13**,** 546-53.

JAMES, P. T. 2004. Obesity: the worldwide epidemic. *Clin Dermatol,* 22**,** 276-80.

JAMES, P. T., LEACH, R., KALAMARA, E. & SHAYEGHI, M. 2001. The worldwide obesity epidemic. *Obes Res,* 9 Suppl 4**,** 228S-233S.

JUHAN-VAGUE, I. & ALESSI, M. C. 1997. PAI-1, obesity, insulin resistance and risk of cardiovascular events. *Thromb Haemost,* 78**,** 656-60.

KERSHAW, E. E. & FLIER, J. S. 2004. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab,* 89**,** 2548-56.

KIM, J., PETERSON, K. E., SCANLON, K. S., FITZMAURICE, G. M., MUST, A., OKEN, E., RIFAS-SHIMAN, S. L., RICH-EDWARDS, J. W. & GILLMAN, M. W. 2006. Trends in overweight from 1980 through 2001 among preschool-aged children enrolled in a health maintenance organization. *Obesity (Silver Spring),* 14**,** 1107-12.

KLOAS, W. 2002. Amphibians as a model for the study of endocrine disruptors. *Int Rev Cytol,* 216**,** 1-57.

KOHLER, H. P. & GRANT, P. J. 2000. Plasminogen-activator inhibitor type 1 and coronary artery disease. *N Engl J Med,* 342**,** 1792-801.

KRISHNAN, A. V., STATHIS, P., PERMUTH, S. F., TOKES, L. & FELDMAN, D. 1993. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology,* 132**,** 2279-86.

KUIPER, G. G., LEMMEN, J. G., CARLSSON, B., CORTON, J. C., SAFE, S. H., VAN DER SAAG, P. T., VAN DER BURG, B. & GUSTAFSSON, J. A. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology,* 139**,** 4252-63.

KWINTKIEWICZ, J., NISHI, Y., YANASE, T. & GIUDICE, L. C. 2010. Peroxisome proliferator-activated receptor-gamma mediates bisphenol A inhibition of FSH-stimulated IGF-1, aromatase, and estradiol in human granulosa cells. *Environ Health Perspect,* 118**,** 400-6.

KWON, S., STEDMAN, D. B., ELSWICK, B. A., CATTLEY, R. C. & WELSCH, F. 2000. Pubertal development and reproductive functions of Crl:CD BR Sprague-Dawley rats exposed to bisphenol A during prenatal and postnatal development. *Toxicol Sci,* 55**,** 399-406.

LAKIND, J. S. & NAIMAN, D. Q. 2008. Bisphenol A (BPA) daily intakes in the United States: estimates from the 2003-2004 NHANES urinary BPA data. *J Expo Sci Environ Epidemiol,* 18**,** 608-15.

LANG, I. A., GALLOWAY, T. S., SCARLETT, A., HENLEY, W. E., DEPLEDGE, M., WALLACE, R. B. & MELZER, D. 2008. Association of urinary bisphenol A concentration with medical disorders and laboratory abnormalities in adults. *JAMA,* 300**,** 1303-10.

LARSEN, T. M., TOUBRO, S. & ASTRUP, A. 2003. PPARgamma agonists in the treatment of type II diabetes: is increased fatness commensurate with long-term efficacy? *Int J Obes Relat Metab Disord,* 27**,** 147-61.

LE, H. H., CARLSON, E. M., CHUA, J. P. & BELCHER, S. M. 2008. Bisphenol A is released from polycarbonate drinking bottles and mimics the neurotoxic actions of estrogen in developing cerebellar neurons. *Toxicol Lett,* 176**,** 149-56.

MELZER, D., RICE, N. E., LEWIS, C., HENLEY, W. E. & GALLOWAY, T. S. 2010. Association of urinary bisphenol a concentration with heart disease: evidence from NHANES 2003/06. *PLoS One,* 5**,** e8673.

MURATA, Y., ROBERTSON, K. M., JONES, M. E. & SIMPSON, E. R. 2002. Effect of estrogen deficiency in the male: the ArKO mouse model. *Mol Cell Endocrinol,* 193**,** 7-12.

NAGEL, S. C., VOM SAAL, F. S., THAYER, K. A., DHAR, M. G., BOECHLER, M. & WELSHONS, W. V. 1997. Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol. *Environ Health Perspect,* 105**,** 70-6.

NEWBOLD, R. R., PADILLA-BANKS, E., SNYDER, R. J. & JEFFERSON, W. N. 2007. Perinatal exposure to environmental estrogens and the development of obesity. *Mol Nutr Food Res,* 51**,** 912-7.

NISHIKAWA, M., IWANO, H., YANAGISAWA, R., KOIKE, N., INOUE, H. & YOKOTA, H. 2010. Placental transfer of conjugated bisphenol A and subsequent reactivation in the rat fetus. *Environ Health Perspect,* 118**,** 1196-203.

NOGUEIRA, E. F., BOLLAG, W. B. & RAINEY, W. E. 2009. Angiotensin II regulation of adrenocortical gene transcription. *Mol Cell Endocrinol,* 302**,** 230-6.

NUNEZ, A. A., KANNAN, K., GIESY, J. P., FANG, J. & CLEMENS, L. G. 2001. Effects of bisphenol A on energy balance and accumulation in brown adipose tissue in rats. *Chemosphere,* 42**,** 917-22.

PFAFFL, M. W., HORGAN, G. W. & DEMPFLE, L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res,* 30**,** e36.

PHRAKONKHAM, P., VIENGCHAREUN, S., BELLOIR, C., LOMBES, M., ARTUR, Y. & CANIVENC-LAVIER, M. C. 2008. Dietary xenoestrogens differentially impair 3T3-L1 preadipocyte differentiation and persistently affect leptin synthesis. *J Steroid Biochem Mol Biol,* 110**,** 95-103.

RISTOW, M., MULLER-WIELAND, D., PFEIFFER, A., KRONE, W. & KAHN, C. R. 1998. Obesity associated with a mutation in a genetic regulator of adipocyte differentiation. *N Engl J Med,* 339**,** 953-9.

ROSEN, E. D., SARRAF, P., TROY, A. E., BRADWIN, G., MOORE, K., MILSTONE, D. S., SPIEGELMAN, B. M. & MORTENSEN, R. M. 1999. PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol Cell,* 4**,** 611-7.

ROSEN, E. D., WALKEY, C. J., PUIGSERVER, P. & SPIEGELMAN, B. M. 2000. Transcriptional regulation of adipogenesis. *Genes Dev,* 14**,** 1293-307.

RUBIN, B. S., MURRAY, M. K., DAMASSA, D. A., KING, J. C. & SOTO, A. M. 2001. Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. *Environ Health Perspect,* 109**,** 675-80.

RUBIN, B. S. & SOTO, A. M. 2009. Bisphenol A: Perinatal exposure and body weight. *Mol Cell Endocrinol,* 304**,** 55-62.

RYDEN, M. & ARNER, P. 2007. Tumour necrosis factor-alpha in human adipose tissue -- from signalling mechanisms to clinical implications. *J Intern Med,* 262**,** 431-8.

SAFE, S. 2004. Endocrine disruptors and human health: is there a problem. *Toxicology,* 205**,** 3-10.

SAFE, S. H., PALLARONI, L., YOON, K., GAIDO, K., ROSS, S. & MCDONNELL, D. 2002. Problems for risk assessment of endocrine-active estrogenic compounds. *Environ Health Perspect,* 110 Suppl 6**,** 925-9.

SARGIS, R. M., JOHNSON, D. N., CHOUDHURY, R. A. & BRADY, M. J. 2009. Environmental Endocrine Disruptors Promote Adipogenesis in the 3T3-L1 Cell Line through Glucocorticoid Receptor Activation. *Obesity (Silver Spring)*.

SCHREYER, S. A., WILSON, D. L. & LEBOEUF, R. C. 1998. C57BL/6 mice fed high fat diets as models for diabetes-accelerated atherosclerosis. *Atherosclerosis,* 136**,** 17-24.

SEIDLOVA-WUTTKE, D., JARRY, H., CHRISTOFFEL, J., RIMOLDI, G. & WUTTKE, W. 2005. Effects of bisphenol-A (BPA), dibutylphtalate (DBP), benzophenone-2 (BP2), procymidone (Proc), and linurone (Lin) on fat tissue, a variety of hormones and metabolic parameters: a 3 months comparison with effects of estradiol (E2) in ovariectomized (ovx) rats. *Toxicology,* 213**,** 13-24.

SHOELSON, S. E., HERRERO, L. & NAAZ, A. 2007. Obesity, inflammation, and insulin resistance. *Gastroenterology,* 132**,** 2169-80.

SIITERI, P. K. 1987. Adipose tissue as a source of hormones. *Am J Clin Nutr,* 45**,** 277-82.

SKURK, T., ALBERTI-HUBER, C., HERDER, C. & HAUNER, H. 2007. Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab,* 92**,** 1023-33.

SNYDER, R. W., MANESS, S. C., GAIDO, K. W., WELSCH, F., SUMNER, S. C. & FENNELL, T. R. 2000. Metabolism and disposition of bisphenol A in female rats. *Toxicol Appl Pharmacol,* 168**,** 225-34.

SOMM, E., SCHWITZGEBEL, V. M., TOULOTTE, A., CEDERROTH, C. R., COMBESCURE, C., NEF, S., AUBERT, M. L. & HUPPI, P. S. 2009. Perinatal exposure to bisphenol a alters early adipogenesis in the rat. *Environ Health Perspect,* 117**,** 1549-55.

SPALDING, K. L., ARNER, E., WESTERMARK, P. O., BERNARD, S., BUCHHOLZ, B. A., BERGMANN, O., BLOMQVIST, L., HOFFSTEDT, J., NASLUND, E., BRITTON, T., CONCHA, H., HASSAN, M., RYDEN, M., FRISEN, J. & ARNER, P. 2008. Dynamics of fat cell turnover in humans. *Nature,* 453**,** 783-7.

SUL, H. S., LATASA, M. J., MOON, Y. & KIM, K. H. 2000. Regulation of the fatty acid synthase promoter by insulin. *J Nutr,* 130**,** 315S-320S.

TAKAYANAGI, S., TOKUNAGA, T., LIU, X., OKADA, H., MATSUSHIMA, A. & SHIMOHIGASHI, Y. 2006. Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor gamma (ERRgamma) with high constitutive activity. *Toxicol Lett,* 167**,** 95-105.

VANDENBERG, L. N., HAUSER, R., MARCUS, M., OLEA, N. & WELSHONS, W. V. 2007. Human exposure to bisphenol A (BPA). *Reprod Toxicol,* 24**,** 139-77.

VANDENBERG, L. N., MAFFINI, M. V., SONNENSCHEIN, C., RUBIN, B. S. & SOTO, A. M. 2009. Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. *Endocr Rev,* 30**,** 75-95.

VOM SAAL, F. S. & MYERS, J. P. 2008. Bisphenol A and risk of metabolic disorders. *JAMA,* 300**,** 1353-5.

WAKIL, S. J., STOOPS, J. K. & JOSHI, V. C. 1983. Fatty acid synthesis and its regulation. *Annu Rev Biochem,* 52**,** 537-79.

WELSHONS, W. V., NAGEL, S. C. & VOM SAAL, F. S. 2006. Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. *Endocrinology,* 147**,** S56-69.

XU, H., BARNES, G. T., YANG, Q., TAN, G., YANG, D., CHOU, C. J., SOLE, J., NICHOLS, A., ROSS, J. S., TARTAGLIA, L. A. & CHEN, H. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest,* 112**,** 1821-30.

YAMASHITA, U., SUGIURA, T., YOSHIDA, Y. & KURODA, E. 2005. Effect of endocrine disrupters on macrophage functions in vitro. *J UOEH,* 27**,** 1-10.

YAN, H., TAKAMOTO, M. & SUGANE, K. 2008. Exposure to Bisphenol A prenatally or in adulthood promotes T(H)2 cytokine production associated with reduction of CD4CD25 regulatory T cells. *Environ Health Perspect,* 116**,** 514-9.

YANG, Y. J., HONG, Y. C., OH, S. Y., PARK, M. S., KIM, H., LEEM, J. H. & HA, E. H. 2009. Bisphenol A exposure is associated with oxidative stress and inflammation in postmenopausal women. *Environ Res,* 109**,** 797-801.

YUAN, J., BENNETT, B. D. & RABINOWITZ, J. D. 2008. Kinetic flux profiling for quantitation of cellular metabolic fluxes. *Nat Protoc,* 3**,** 1328-40.

ZHANG, Y., PROENCA, R., MAFFEI, M., BARONE, M., LEOPOLD, L. & FRIEDMAN, J. M. 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature,* 372**,** 425-32.

ZOELLER, R. T. 2005. Environmental chemicals as thyroid hormone analogues: new studies indicate that thyroid hormone receptors are targets of industrial chemicals? *Mol Cell Endocrinol,* 242**,** 10-5.

APPENDIX

Effects of Bisphenol A on Body Weight and Adiposity in Combination with a High Fat Diet

We performed a small preliminary experiment to study the effect that BPA has in combination with a high fat diet. There were 8 C57BL/6 mice (4 males and 4 females) and 8 DBA/2J mice were exposed to 5μM BPA beginning at 4 weeks of age, as well as 8 control mice per strain. At 11 weeks of age, all mice were switched from standard chow to a high fat diet (58% fat). Mice were weighed weekly and sacrificed at 17 weeks of age. We dissected gonadal fat pads to assess adiposity. We found that both C57BL/6 males and females treated with BPA were significantly heavier compared to control by the end of the study (37.4g vs 33.2g in males, p=0.035; 28.7g vs 24.8g in females, p=0.014) (Figure A-1). There was no significant difference in the DBA/2J mice. We also found that both C57BL/6 males and females treated with BPA were fattier (6.8% vs 5.94% in males, p=0.065; 4.21% vs 2.99%, p=0.074) (Figure A-2). The C57BL/6 males exposed to BPA also had significantly higher serum insulin levels compared to controls, but there was no difference in females (Figure A-3). These data suggest that BPA may exacerbate the effects of a high fat diet and further experiments and analysis are necessary.

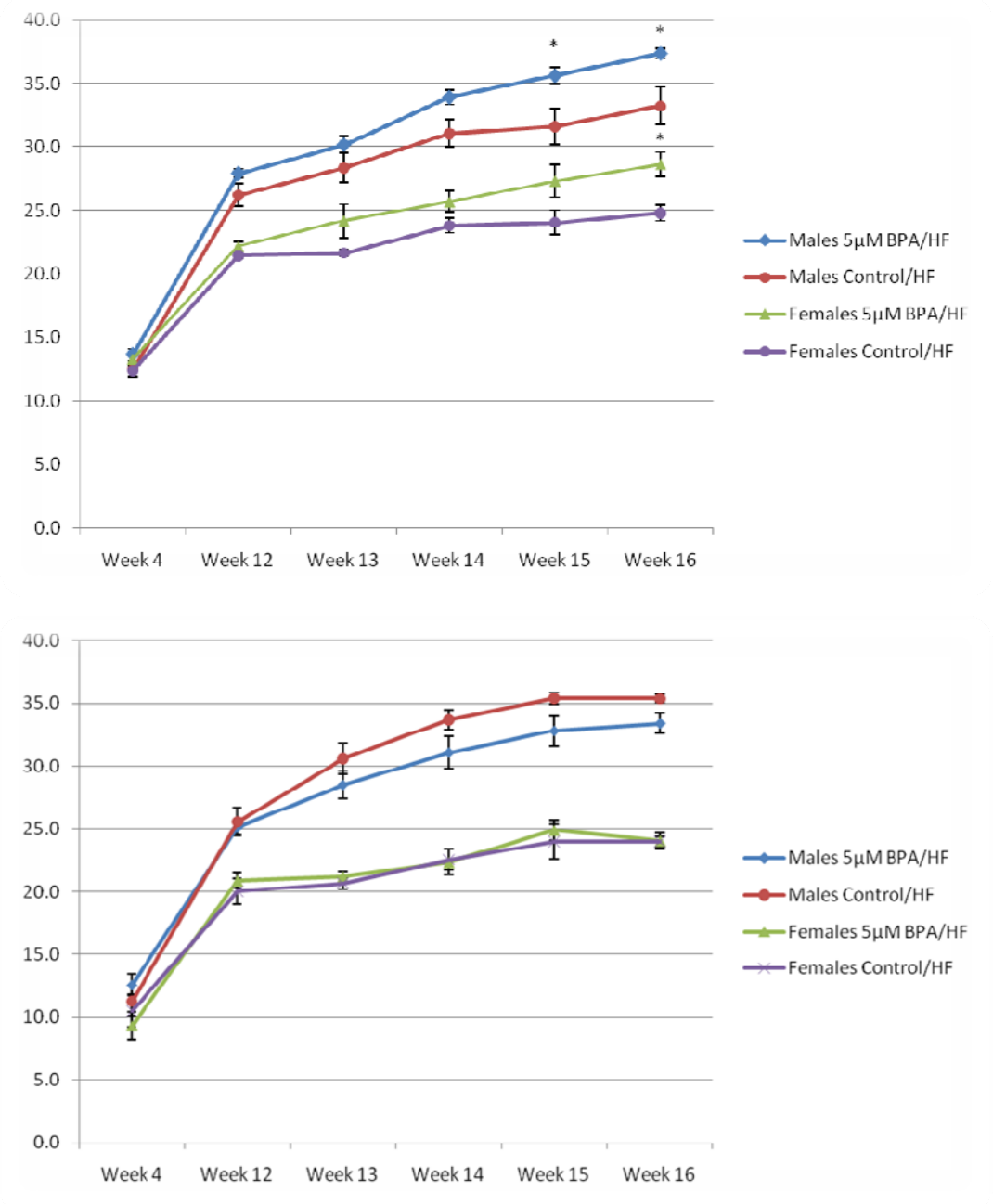


Figure A-1. Bodyweight data of mice on a high fat diet treated with BPA. C57BL/6 mice (above) and DBA/2J mice (below) were treated with 5uM BPA or control from 4 weeks of age to 17 weeks of age. They were switched to a high fat diet at 12 weeks of age. Results are means ± SEM. (*)$p<0.05$.

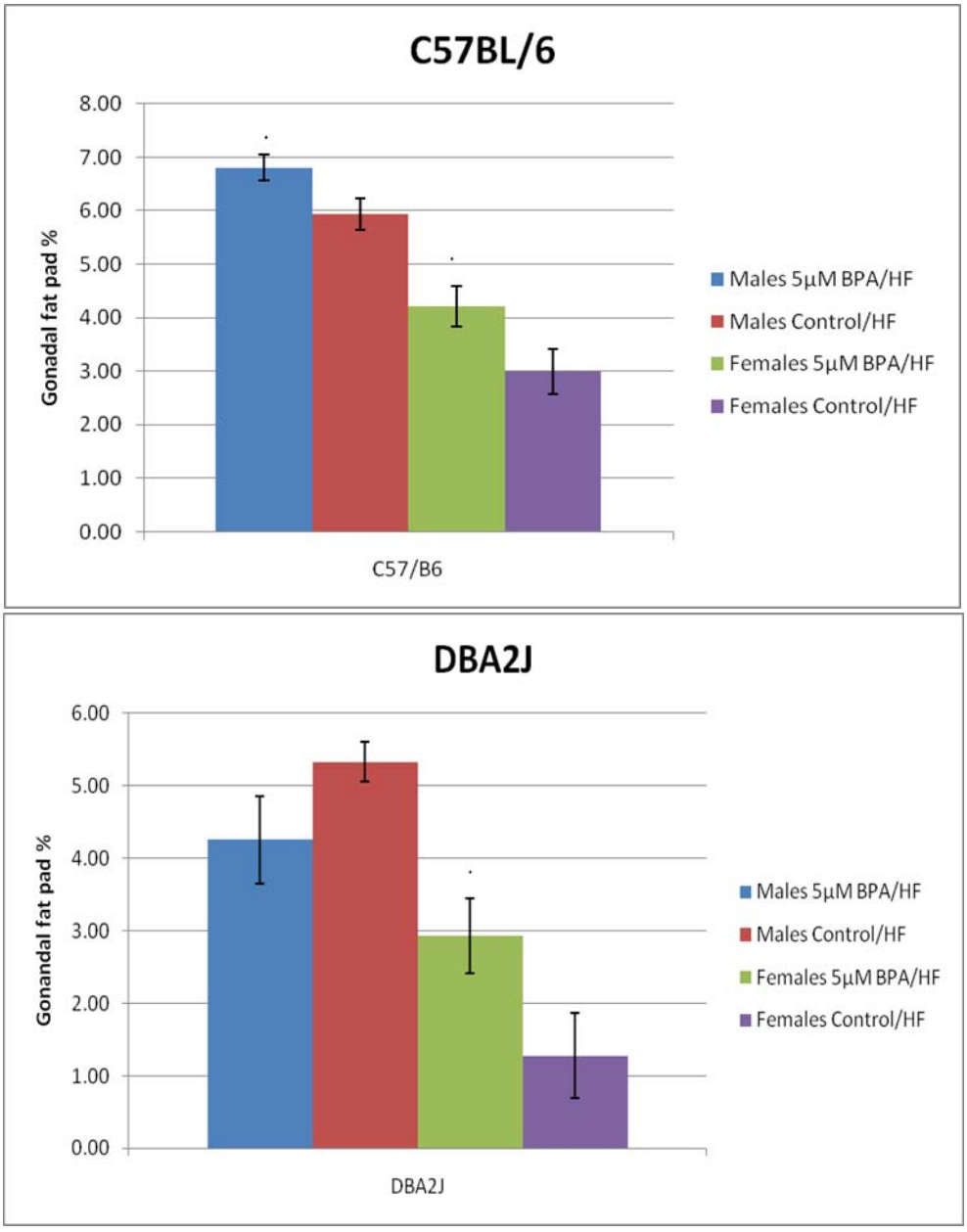


Figure A-2. Effects of BPA on adiposity in mice on a high fat diet. Gonadal fat pads were dissected from C57BL/6 (above) and DBA/2J (below) and divided by total body weight as a measurement of adiposity. Results are means ± SEM. (·)$p<0.1$.

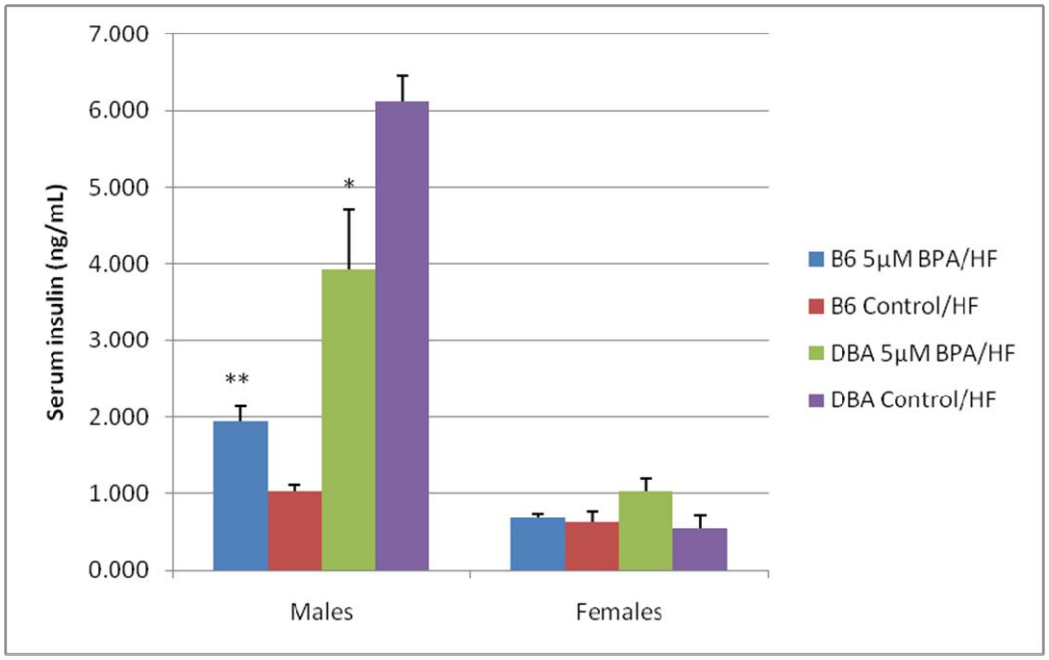


Figure A-3. Serum insulin levels in mice on a high fat diet treated with BPA. Insulin levels are higher in C57BL/6 males treated with BPA and lower in DBA/2J males treated with BPA. Results are means ± SEM. (*)$p<0.05$, (**)$p<0.01$.

Table A-1. Genes significantly changed in C57BL/6 males treated with 500nM BPA compared to control.

Gene symbol	Gene name	Fold change	p-value
GPNMB	glycoprotein (transmembrane) nmb (Gpnmb), mRNA.	1.703	0.00279
MMP12	matrix metallopeptidase 12 (Mmp12), mRNA.	1.686	0.00119
SFRP5	secreted frizzled-related sequence protein 5 (Sfrp5), mRNA.	1.655	0.00184
PEG3	paternally expressed 3 (Peg3), mRNA.	1.620	0.00481
EMP3	epithelial membrane protein 3 (Emp3), mRNA.	1.568	0.00299
HIST1H2AI	histone cluster 1, H2ai (Hist1h2ai), mRNA.	1.491	0.00262
LEP	leptin (Lep), mRNA.	1.449	0.00178
FBN1		1.447	0.00204
MYL4	myosin, light polypeptide 4 (Myl4), mRNA.	1.402	0.00309
SLC11A1	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1 (Slc11a1), mRNA.	1.387	0.00485
EIF4E3	eukaryotic translation initiation factor 4E member 3 (Eif4e3), mRNA.	1.365	0.00397
RTN2	reticulon 2 (Z-band associated protein) (Rtn2), transcript variant B, mRNA.	1.362	2.00E-04
SEMA7A	sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A (Sema7a), mRNA.	1.358	0.00254
HIST1H2AN	histone cluster 1, H2an (Hist1h2an), mRNA.	1.354	0.00215
9130211I03RIK	RIKEN cDNA 9130211I03 gene (9130211I03Rik), mRNA.	1.351	0.00213
PALM	paralemmin (Palm), mRNA.	1.345	0.00199
GJA5	gap junction membrane channel protein alpha 5 (Gja5), mRNA.	1.314	0.00134
RCN3	reticulocalbin 3, EF-hand calcium binding domain (Rcn3), mRNA.	1.309	0.00477
MYO1G	myosin IG (Myo1g), mRNA.	1.305	0.0011
PFTK1	PFTAIRE protein kinase 1 (Pftk1), mRNA.	1.303	0.00242
ADSSL1	adenylosuccinate synthetase like 1 (Adssl1), mRNA.	1.296	0.00393
MFSD7A	major facilitator superfamily domain containing 7A (Mfsd7a), mRNA.	1.281	0.0019
SLC25A19	solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19 (Slc25a19), nuclear gene encoding mitochondrial protein, mRNA.	1.277	0.00341
PRELP	proline arginine-rich end leucine-rich repeat (Prelp), mRNA.	1.275	0.00085
HEY1	hairy/enhancer-of-split related with YRPW motif 1 (Hey1), mRNA.	1.275	0.00234
TMEM160	transmembrane protein 160 (Tmem160), mRNA.	1.269	0.00348
ARFGAP2	ADP-ribosylation factor GTPase activating protein 2 (Arfgap2), mRNA.	1.267	0.00193
PQLC3	PQ loop repeat containing (Pqlc3), mRNA.	1.264	0.00478
PLD3	phospholipase D family, member 3 (Pld3), mRNA.	1.259	0.00112

Table A-1 (continued)

Gene symbol	Gene name	Fold change	p-value
CSNK1G2	casein kinase 1, gamma 2 (Csnk1g2), mRNA.	1.249	0.00204
IL27RA	interleukin 27 receptor, alpha (Il27ra), mRNA.	1.247	0.002
UCHL1	ubiquitin carboxy-terminal hydrolase L1 (Uchl1), mRNA.	1.244	0.0032
GLTP	glycolipid transfer protein (Gltp), mRNA.	1.243	0.00478
2410008K03RIK	RIKEN cDNA 2410008K03 gene (2410008K03Rik), mRNA.	1.241	0.00062
FOXO1		1.234	0.00438
1810008A18RIK	RIKEN cDNA 1810008A18 gene (1810008A18Rik), mRNA.	1.226	0.00026
KIF1C	kinesin family member 1C (Kif1c), mRNA.	1.225	0.00355
CAMK1	calcium/calmodulin-dependent protein kinase I (Camk1), mRNA.	1.222	8.00E-05
NECAP2	NECAP endocytosis associated 2 (Necap2), mRNA.	1.218	0.00479
PEA15		1.216	0.00152
RUSC2	RUN and SH3 domain containing 2 (Rusc2), transcript variant 1, mRNA.	1.211	0.00037
PACS2	phosphofurin acidic cluster sorting protein 2 (Pacs2), mRNA.	1.210	0.00147
MARVELD1	PREDICTED: MARVEL (membrane-associating) domain containing 1 (Marveld1), misc RNA.	1.208	0.00114
HPCAL1	hippocalcin-like 1 (Hpcal1), mRNA.	1.207	0.00317
ESAM	endothelial cell-specific adhesion molecule (Esam), mRNA.	1.198	0.00219
D930038O18RIK		1.193	0.00436
D330027H18RIK	PREDICTED: RIKEN cDNA D330027H18 gene (D330027H18Rik), mRNA.	1.185	0.00374
LHX6		1.180	0.00277
LEPRE1	leprecan 1 (Lepre1), transcript variant 3, mRNA.	1.179	0.00126
NFKB2		1.176	0.0038
2810043G13RIK		1.174	0.00468
ROD1	ROD1 regulator of differentiation 1 (S. pombe) (Rod1), transcript variant 1, mRNA.	1.172	0.00178
ZBTB3	zinc finger and BTB domain containing 3 (Zbtb3), mRNA.	1.171	0.00489
FLOT1	flotillin 1 (Flot1), mRNA.	1.169	0.0017
CTSS	cathepsin S (Ctss), mRNA.	1.159	0.00464
ABCA3	ATP-binding cassette, sub-family A (ABC1), member 3 (Abca3), transcript variant 1, mRNA.	1.157	0.0046
2410127L17RIK	RIKEN cDNA 2410127L17 gene (2410127L17Rik), mRNA.	1.150	0.00172
BATF3	basic leucine zipper transcription factor, ATF-like 3 (Batf3), mRNA.	1.150	0.00477
ZFP473	zinc finger protein 473 (Zfp473), mRNA.	1.138	0.00375
STRN		1.120	0.00463
RBMY1A1	RNA binding motif protein, Y chromosome, family 1	-1.125	0.00453

Table A-1 (continued)

Gene symbol	Gene name	Fold change	p-value
NDUFA4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4 (Ndufa4), mRNA.	-1.148	0.00069
DUSP6	dual specificity phosphatase 6 (Dusp6), mRNA.	-1.149	0.00195
LOC100046744	PREDICTED: similar to Serine/arginine repetitive matrix protein 2 (LOC100046744), mRNA.	-1.153	0.00054
4930487N19RIK		-1.157	0.00319
RBM3	RNA binding motif protein 3 (Rbm3), mRNA.	-1.160	0.00184
CAMK2D	calcium/calmodulin-dependent protein kinase II, delta (Camk2d), transcript variant 2, mRNA.	-1.173	0.00484
CML1	camello-like 1 (Cml1), mRNA.	-1.179	0.00323
MYOG	myogenin (Myog), mRNA.	-1.188	0.00142
BAG4	BCL2-associated athanogene 4 (Bag4), mRNA.	-1.206	0.00245
5530401J07RIK		-1.223	0.00269
RAF1		-1.224	0.00493
MYO1B	myosin IB (Myo1b), mRNA.	-1.227	0.00425
ZFAND6	zinc finger, AN1-type domain 6 (Zfand6), mRNA.	-1.228	0.0049
UQCRC2	ubiquinol cytochrome c reductase core protein 2 (Uqcrc2), mRNA.	-1.239	7.00E-04
NKG7	natural killer cell group 7 sequence (Nkg7), mRNA.	-1.240	0.00341
DCXR	dicarbonyl L-xylulose reductase (Dcxr), mRNA.	-1.244	0.00412
PDE4DIP	phosphodiesterase 4D interacting protein (myomegalin) (Pde4dip), transcript variant 1, mRNA.	-1.266	0.00199
CAR1	carbonic anhydrase 1 (Car1), transcript variant 1, mRNA.	-1.271	0.00403
TTC33	tetratricopeptide repeat domain 33 (Ttc33), mRNA.	-1.276	0.00439
PRDX3		-1.287	0.00205
MAOB	monoamine oxidase B (Maob), nuclear gene encoding mitochondrial protein, mRNA.	-1.298	0.00297
SCL33870.2_144		-1.299	4.00E-05
4631423B10RIK		-1.308	0.00021
RSPO3	R-spondin 3 homolog (Xenopus laevis) (Rspo3), mRNA.	-1.312	0.00252
LUC7L2	LUC7-like 2 (S. cerevisiae) (Luc7l2), mRNA.	-1.319	0.00177
SCL000408.1_6		-1.371	0.00099
SCL33870.2_144		-1.436	4.00E-04
NET1	neuroepithelial cell transforming gene 1 (Net1), transcript variant 1, mRNA.	-1.506	0.00028
LDHD	lactate dehydrogenase D (Ldhd), nuclear gene encoding mitochondrial protein, mRNA.	-1.545	0.00287
NET1	neuroepithelial cell transforming gene 1 (Net1), transcript variant 2, mRNA.	-1.636	0.00015
TST	thiosulfate sulfurtransferase, mitochondrial (Tst), nuclear gene encoding mitochondrial protein, mRNA.	-1.897	0.00231
CYP2F2	cytochrome P450, family 2, subfamily f, polypeptide 2 (Cyp2f2), mRNA.	-2.558	7.00E-04

Table A-2. Genes significantly changed in DBA/2J males treated with 500nM BPA compared to control

Gene symbol	Gene name	Fold change	p-value
S100A8	S100 calcium binding protein A8 (calgranulin A) (S100a8), mRNA.	1.963	0.00401
KLF6	Kruppel-like factor 6 (Klf6), mRNA.	1.475	0.00154
LEP	leptin (Lep), mRNA.	1.411	0.00329
2310036D22RIK		1.402	5.00E-05
VLDLR	very low density lipoprotein receptor (Vldlr), mRNA.	1.345	0.0024
SCL0002315.1_12		1.342	0.00198
SERPINA1B	serine (or cysteine) preptidase inhibitor, clade A, member 1b (Serpina1b), mRNA.	1.337	0.00331
HIST2H3C1	histone cluster 2, H3c1 (Hist2h3c1), transcript variant 1, mRNA.	1.306	0.00474
ADAP2	ArfGAP with dual PH domains 2 (Adap2), mRNA.	1.305	0.00414
OSGIN1	oxidative stress induced growth inhibitor 1 (Osgin1), mRNA.	1.301	0.00347
FABP9	fatty acid binding protein 9, testis (Fabp9), mRNA.	1.287	0.00303
IQGAP1		1.286	0.00076
NFIC		1.256	0.00491
JUN	Jun oncogene (Jun), mRNA.	1.255	0.00331
C130073O12RIK		1.243	0.00362
CCDC53	coiled-coil domain containing 53 (Ccdc53), mRNA.	1.238	0.0038
SERPINA1A	serine (or cysteine) peptidase inhibitor, clade A, member 1a (Serpina1a), mRNA.	1.235	3.00E-04
KCNB1	potassium voltage gated channel, Shab-related subfamily, member 1 (Kcnb1), mRNA.	1.233	0.00304
PI4K2B	phosphatidylinositol 4-kinase type 2 beta (Pi4k2b), transcript variant 2, mRNA.	1.226	4.00E-05
BET1	blocked early in transport 1 homolog (S. cerevisiae) (Bet1), mRNA.	1.221	0.00118
1500034J01RIK	PREDICTED: RIKEN cDNA 1500034J01 gene (1500034J01Rik), mRNA.	1.213	0.0018
SMEK2	SMEK homolog 2, suppressor of mek1 (Dictyostelium) (Smek2), mRNA.	1.211	0.0034
PRKRIR	protein-kinase, interferon-inducible double stranded RNA dependent inhibitor, repressor of (P58 repressor) (Prkrir), mRNA.	1.207	0.00393
IGSF21	immunoglobin superfamily, member 21 (Igsf21), mRNA.	1.206	0.00052
IMPAD1	inositol monophosphatase domain containing 1 (Impad1), mRNA.	1.203	0.00288
TTC14		1.202	0.00024
N6AMT1	N-6 adenine-specific DNA methyltransferase 1 (putative) (N6amt1), mRNA.	1.197	0.00479
CALU	calumenin (Calu), transcript variant 2, mRNA.	1.191	0.00491
NACC2	nucleus accumbens associated 2, BEN and BTB (POZ) domain containing (Nacc2), transcript variant 1, mRNA.	1.184	0.00256
2610524H06RIK	RIKEN cDNA 2610524H06 gene (2610524H06Rik), mRNA.	1.183	0.00304
HSPG2	PREDICTED: perlecan (heparan sulfate proteoglycan 2) (Hspg2), mRNA.	1.179	0.00348
HAS2	hyaluronan synthase 2 (Has2), mRNA.	1.170	0.00407
OLFR1085	olfactory receptor 1085 (Olfr1085), mRNA.	1.165	0.00206
OPN1MW	opsin 1 (cone pigments), medium-wave-sensitive (color blindness, deutan) (Opn1mw), mRNA.	1.161	0.00285
LYPD6B	LY6/PLAUR domain containing 6B (Lypd6b), mRNA.	1.153	0.00482

Table A-2 (continued)

Gene symbol	Gene name	Fold change	p-value
LRSAM1	leucine rich repeat and sterile alpha motif containing 1 (Lrsam1), mRNA.	1.145	0.00257
IGHV1S20_K02153_IG_HEAVY_VARIABLE_1S20_37		1.142	0.00238
AMIGO3	adhesion molecule with Ig like domain 3 (Amigo3), mRNA.	1.137	0.00162
LOC380854		1.110	0.00401
4933406P04RIK		-1.113	0.00416
LOC100044698	PREDICTED: similar to Unknown (protein for MGC:117841) (LOC100044698), mRNA.	-1.128	0.00206
AOC2	amine oxidase, copper containing 2 (retina-specific) (Aoc2), mRNA.	-1.135	0.00483
FGD6		-1.139	0.00198
6030441H18RIK		-1.143	0.00185
TCEAL6	transcription elongation factor A (SII)-like 6 (Tceal6), mRNA.	-1.149	0.0045
PSMB4	proteasome (prosome, macropain) subunit, beta type 4 (Psmb4), mRNA.	-1.155	0.00475
1810009O10RIK		-1.162	0.00377
MRAS	muscle and microspikes RAS (Mras), mRNA.	-1.177	0.00219
MTMR11	myotubularin related protein 11 (Mtmr11), mRNA.	-1.180	0.00424
PHF13	PHD finger protein 13 (Phf13), mRNA.	-1.189	0.00125
KLC2	kinesin light chain 2 (Klc2), mRNA.	-1.193	0.00193
MUP3	major urinary protein 3 (Mup3), mRNA.	-1.204	0.00359
BC039093		-1.207	0.0026
AFG3L1	AFG3(ATPase family gene 3)-like 1 (yeast) (Afg3l1), mRNA.	-1.208	0.00445
E030027L17		-1.209	0.00349
GIMAP7	GTPase, IMAP family member 7 (Gimap7), mRNA.	-1.213	0.00217
BC030499	cDNA sequence BC030499 (BC030499), mRNA.	-1.221	0.00219
FBXL6	F-box and leucine-rich repeat protein 6 (Fbxl6), mRNA.	-1.223	0.00333
TRF	transferrin (Trf), mRNA.	-1.236	0.00222
LOC100047815	PREDICTED: similar to CD79A antigen (immunoglobulin-associated alpha) (LOC100047815), mRNA.	-1.251	0.00276
FCGRT	Fc receptor, IgG, alpha chain transporter (Fcgrt), mRNA.	-1.254	0.00427
RHBDL1	rhomboid, veinlet-like 1 (Drosophila) (Rhbdl1), mRNA.	-1.267	0.00072
CYP2E1	cytochrome P450, family 2, subfamily e, polypeptide 1 (Cyp2e1), mRNA.	-1.418	0.00107
FAIM3	Fas apoptotic inhibitory molecule 3 (Faim3), mRNA.	-1.503	0.00452
LOC381774		-2.553	0.00128
LOC100047899	PREDICTED: similar to NF-180 (LOC100047899), mRNA.	-2.578	0.00235
IGK-V5	PREDICTED: immunoglobulin kappa chain variable 5 (V5 family) (Igk-V5), mRNA.	-2.761	0.00108
CXCL13		-3.059	2.00E-04
IGK-C		-3.918	0.00128
LOC100047628	PREDICTED: similar to Chain L, Structural Basis Of Antigen Mimicry In A Clinically Relevant Melanoma Antigen System, transcript variant 3 (LOC100047628), mRNA.	-4.252	0.0011

VITA

Brantley Wyatt was born in Jackson, TN, to the parents of Harvey and Christy Wyatt. He is the oldest of three children and has two sisters: Kellye and Shelby. He attended North Side High school, and upon graduation in 2003 started attending the University of Tennessee in Knoxville. Brantley majored in Biological Sciences with a concentration in Biochemistry, Cellular and Molecular Biology and graduated *magna cum laude* in 2007 with a grade point average of 3.74. He decided to continue his studies and was accepted to the Graduate School of Genome Science and Technology at the University of Tennessee. In June 2008, Brantley married his long time girlfriend, Julie Nguyen. He graduated with a Master of Science degree in May 2011. Upon graduation, Brantley and Julie will be moving to Atlanta, GA where he has accepted a position with Medical Neurogenetics, LLC.