Fall 2011

The effect of in vivo PBDE treatment on hepatic phosphoenolpyruvate carboxykinase (PEPCK) enzyme kinetics in male Wistar rats

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THE EFFECT OF *IN VIVO* PBDE TREATMENT ON HEPATIC PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) ENZYME KINETICS IN MALE WISTAR RATS

BY

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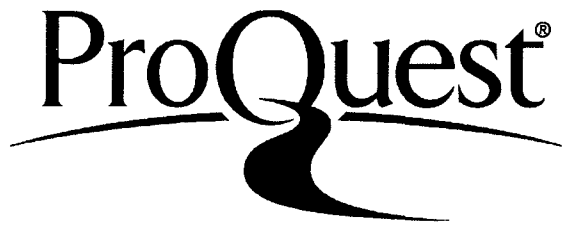
THESIS

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Master of Science
in
Nutritional Sciences

September 2011



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ACKNOWLEDGEMENTS

I am deeply grateful to all of those who made this accomplishment possible. First and foremost, I would like to thank my friends and family, my parents, in particular, for giving me endless support in more ways than I could have imagined. They provided motivation and encouragement for my success through love and adoration. My boyfriend, Anthony, for reminding me how important it is to laugh especially during difficult times and also allowing me to grow into the person I have become. I would like to thank Elyse for always being there whenever I needed her. I could not have asked for a better officemate or friend.

Anne Ronan was fundamentally helpful in completing this project. Half of this project would not exist if not for her knowledge and tips of lab techniques. I want to thank my committee members Dr. Anthony Tagliaferro and Dr. Paul Tsang. They have been an integral part of my success here by participating in the development of this research plan and also by lending a helping hand. Dr. Tsang in particular for his guidance through countless challenges seen during my first project.

Finally, I would like to thank my advisor, Dr. Gale Carey. She has immeasurable patience and never lost faith in me as a growing scientist. When the first project did not pan out, she was there to support and create a new direction to ensure I completed my goals. She didn't waiver and that allowed me to maintain confidence throughout it all. She also nominated me for the Graduate Student Teaching Award. I would not have received the award had she not been leading by example. I would not be the teacher or scientist I am today without her.

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ABSTRACT

THE EFFECT OF *IN VIVO* PBDE TREATMENT ON
HEPATIC PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK)
ENZYME KINETICS IN MALE WISTAR RATS

by

Jessica T. Nash

University of New Hampshire, September 2011

Polybrominated diphenyl ethers (PBDEs) are synthetic flame-retardant chemicals that enter the environment and mammalian body and may disrupt glucose metabolism. This study investigated the effect of PBDEs on a key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK). Forty-eight male Wistar rats were gavaged with corn oil or corn oil containing 14 mg/kg DE-71 for 3, 14 or 28 days (N = 8 per group). At each time point, fasting plasma glucose, insulin and C-peptide were measured and liver PEPCK enzyme activity was assayed. PBDEs significantly decreased PEPCK Vmax (μmol/min/g liver weight) at 3 days by 26%; this reduction persisted through 28 days. PBDEs also reduced total PEPCK activity (μmol/min/liver) by 18% (3 days) to 41% (28 days). Fasting plasma glucose levels remained unaffected by PBDE treatment. Findings demonstrate that PBDEs reduce hepatic PEPCK Vmax as early as three days of treatment; the implications for this reduction in glucose homeostasis remain to be determined.

CHAPTER I

LITERATURE REVIEW

Preface

This literature review is divided into five sections: chemical and physical properties of PBDEs including general characteristics, environmental presence and routes of exposure; metabolism of PBDEs including information about drug metabolizing enzymes; the metabolic effects of PBDEs including endocrine disruption and cytochrome P450 enzyme induction; the mechanism of PBDE action including PBDEs binding as a ligand to nuclear receptors; and phosphoenolpyruvate carboxykinase (PEPCK), xenobiotics and PBDEs including information about PEPCK activity following xenobiotic exposure.

Chemical and Physical Properties of PBDEs

Polybrominated diphenyl ethers (PBDEs) are a family of lipophilic, flame retardant chemicals introduced in the 1970's. PBDEs are used in consumer products including plastics, electronics, furniture and carpeting to slow the rate at which products burn. The structure of PBDEs is two phenyl rings attached by an ether bridge (Figure 1). The number of bromine atoms found on the phenyl rings can vary, generating 209 possible congeners (Darnerud et al. 2001). The three most common industrial mixtures are penta-BDE, used mostly in furniture, and deca-BDE and octa-BDE, used mostly in

electronics (Hale et al. 2003; McDonald 2002). Penta-BDEs contain 3 to 6 bromine atoms and include the congeners BDE-47, -99, -100, -153, -154 and -85. Octa-BDEs contain 6 to 8 bromine atoms and include the congeners BDE-183 and -153. Deca-BDEs contain 8 to 10 bromine atoms and include the congener BDE-209 (Hale et al. 2003).

Figure 1: General structure of PBDEs

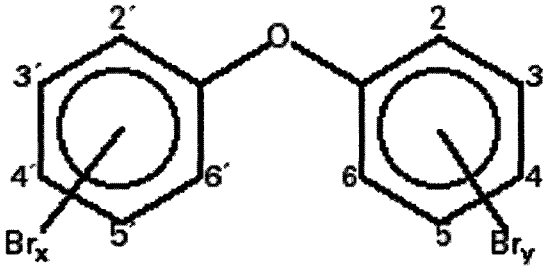


Figure 1: General structure of PBDEs. Location and number of bromine atoms theoretically forms 209 different congeners (Darnerud et al. 2001).

PBDEs can be incorporated in consumer products in one of two ways: via a chemical reaction or as an additive. When the flame retardant is chemically reacted with a product, a covalent bond is formed between the flame retardant and product making the PBDEs less likely to leach into the environment. Additive PBDEs are only mixed with the polymer resin. Therefore, as products break down, additive PBDEs have the ability to leach into the environment because they are not chemically bound to the product (Darnerud et al. 2001; Kim et al. 2006; McDonald 2002).

PBDEs are resistant to chemical and physical breakdown, therefore, once in the environment, they can bioaccumulate and even biomagnify within the food chain (Alaee and Wenning 2002; Costa et al. 2008; Hale et al. 2003). Originally, it was thought that deca-BDE was less bioaccumulative compared to other lower brominated congeners due to being fully brominated, poorly absorbed, rapidly excreted and having a high molecular

weight (McDonald 2002); (Huwe 2005; Norris et al. 1975). However, when exposed to ultraviolet light, deca-BDE can be converted to lower brominated congeners, increasing its bioaccumulative ability (Watanabe et al. 1987).

Environmental Presence

Since their introduction in the 1970s, PBDEs have been found in the environment and organisms worldwide including soil sediment, marine animals, fish-eating birds, human breast milk, serum and adipose tissue. In 1981, it was discovered that fish from the Viskan River in Sweden had PBDEs in their tissues (Andersson and Blomkviet 1981). In 1983, it was found that bird tissues from several U.S. locations and Ontario, Canada also contained PBDEs (Stafford 1983). PBDEs were found in fish-eating birds and marine animals in the Arctic region and in the same year, Watanabe et al., confirmed the same findings in the Pacific region (Jansson et al. 1987; Watanabe et al. 1987). More recently, PBDEs have been detected in human tissues including breast milk, serum and adipose, with levels in North Americans reportedly two to ten times higher than levels in Europeans and Asians (Dunn et al. 2010; Inoue et al. 2006; Lorber 2008; Petreas et al. 2003; Schecter et al. 2003).

Routes and Sources of Exposure

There are three major routes of PBDE exposure: inhalation, dermal absorption and ingestion. Dust can serve as a PBDE source for all three routes. Indoor house dust was analyzed in cities in Canada, New Zealand, United Kingdom and United States and showed median concentrations of PBDEs ranging from 59 to 2,800 ng/g (Harrad et al. 2008). A positive correlation was found between the levels of PBDEs in house dust and the levels of PBDEs in breast milk of mothers in Massachusetts (Wu et al. 2007).

Dust not only accounts for exposure via inhalation but also via dermal absorption (Lorber 2008). Blood samples from computer-dismantle workers in Sweden contained a variety of PBDEs. Congener BDE – 183 was the highest detected at 7.8 ng/g lipid weight whereas the control group had only 0.12 ng/g lipid weight (Sjodin et al. 1999). However, one *in vitro* study showed that only 3.13% of an applied dose of tetrabromodiphenyl ether was absorbed through human skin membranes and concluded that the risk of dermal absorption of PBDEs is lower than via inhalation and ingestion (Roper et al. 2006).

Diet acts as another source of exposure to PBDEs. PBDEs have been found in a variety of marine animals consumed by humans, but they have also been detected in dairy products. A survey of various foods in the United States have shown a total concentration of greater than 6,000 pg/g in butter and over 900 pg/g and 1,000 pg/g in salmon and canned sardines, respectively. PBDE congener BDE-47 was detected in 30 of the 31 food samples tested and BDE-99 was detected in 28 of 31 food samples (Schecter et al. 2010).

Breast milk has been analyzed in the U.S. with median levels ranging from 6.1 – 419 ng/g lipid (Schecter et al. 2003). Breast milk also serves as a source of PBDE exposure in infants. One case study analyzed serum levels of PBDEs in a family of four. The researchers found that the breast-fed infant had serum PBDE levels two to five times higher than his parents (Fischer et al. 2006). The sum of 6 PBDE congeners measured was 418 ng/g lipid weight (lw), while the toddler had 247 ng/g lw, mother 106 ng/g lw and the father 64 ng/g lw. The researchers concluded that the two to five-fold differences in PBDE levels seen in the younger children could be attributed to breast milk and dust as main sources of exposure for the infant and toddler, respectively.

Metabolism of PBDEs

Once in the body, the target organs for PBDE toxicity are the liver, kidney and thyroid gland (Costa et al. 2008). One study sought to explore the level of toxicity of BDE-209 in neonatal rats. Sprague-Dawley rats were administered 100-600 mg of BDE-209/kg body weight daily from postnatal day (PND) 10 to PND 46. Hypertrophy of the liver and thyroid gland, as absolute weight, were seen in the high dose group (Lee et al. 2010).

Another study investigated the toxicity of DE-71, a commercial mixture of mostly penta-BDE congeners. Male and female F344/N rats and B6C3F1 mice were administered a range of 0 – 500 mg/kg/day for 5 days/week for 13 weeks. The researchers found an increase in liver weights and liver lesions of rats receiving doses of 5 mg/kg or higher and mice receiving 50 mg/kg or higher. They also saw hepatocyte hypertrophy in both rats and mice receiving doses of 50 mg/kg or higher (Dunnick and Nyska 2009).

Metabolism studies of PBDEs have been mostly limited to rodents. von Meyerinck et al. (1990) administered a single dose of 300 mg/kg of Bromkal-70, a commercial mixture of penta-BDEs, to male and female rats. The highest concentration was seen in the adipose tissue four days after exposure (von Meyerinck et al. 1990). Orn and Klasson-Wehler demonstrated that after a single dose of 14.5 mg/kg body weight of ^{14}C-labeled BDE-47, 86% remained in the adipose, liver, lung, kidney, brain and plasma after 5 days while only 14% was excreted through the feces and <0.5% was excreted through the urine. The highest concentration was found to be in the adipose tissue (Orn and Klasson-Wehler 1998).

It has been shown PBDE metabolites can form within the body. The majority of metabolites found in the previous study were hydroxylated compounds: *ortho*-OH-tetrabromoDE, *meta*-OH-tetrabromoDE and *para*-OH-tetrabromoDE (Orn and Klasson-Wehler 1998). The purpose of adding an -OH group is to create a more hydrophilic compound making excretion of the toxin more feasible.

Cytochrome P-450

The detoxification of xenobiotics, specifically aromatic or aliphatic compounds such as PBDEs, occurs with the insertion of an oxygen atom (Poulos 1988, 2005).

$$R - H + O_2 + 2e^- \rightarrow R - OH$$

A majority of hydroxylation occurs in the liver via drug metabolizing enzymes. One specific family of drug metabolizing enzymes is cytochrome P450 (CYP). There are 22 known gene families of CYPs in mammals, denoted as CYP1, CYP2, CYP3 etc. and subfamilies denoted as CYP1A, CYP1B, CYP1C, etc. (Hakk and Letcher 2003; Nelson et al. 1996). The CYP1, CYP2 and CYP3 families are responsible for the majority of detoxification of xenobiotics (Nedelcheva and Gut 1994). CYPs are comprised of a single polypeptide chain containing a noncovalently bound heme. CYPs are between 45,000 and 55,000 Daltons and are highly expressed in the liver and intestines (Poulos 1988, 2005).

Metabolic Effects of PBDEs

Many studies have demonstrated an increase of CYP gene expression and enzyme activity following exposure to PBDEs. It has been reported that the minimum dose of PBDEs necessary to induce CYPs ranges 3 – 10 mg/kg (Hakk and Letcher 2003). Sanders et al. (2005) administered treatments via gavage of DE – 71, PCBs and individual congeners of PBDEs in varying amounts of 0.005 – 150 mg/kg/day to male

F344 rats for 3 days. DE – 71 up-regulated gene expression of CYP1A1 by up to 3500-fold at 150 mg/kg/day. When congeners BDE-47, -99 and -153 were administered between 57 and 150 mg/kg/day, CYP1A1 gene expression increased by 2.4, 8.1 and 19-fold, respectively. Similar increase of gene expression was seen for CYP2B and CYP3A (Sanders et al. 2005). Exposure of human hepatocytes to BDE-99 and BDE-209 increased gene expression of CYP1A2 by up to 2 x 10^5 fold, and CYP3A4 up to 10 fold (Stapleton et al. 2009). A significant increase (fold increase was not reported) in protein expression of CYP1A2, CYP3A1 and CYP2B1 was also observed in a study that administered doses of 100, 300 or 600 mg/kg body weight of BDE-209 (Lee et al. 2010).

PBDEs have been categorized as endocrine disrupting chemicals as they interfere with hormones including thyroid and the estrogen family. Hallgren and Darnerud (2002) found that free thyroxine (T_4) was decreased by 61% compared to control rats following exposure to BDE-47 (Hallgren and Darnerud 2002). Zhou et al. (2001) found that weanling female rats, when exposed to varying commercial mixtures of PBDEs for 4 days, had a dose-dependent decrease in total thyroxine (T_4) (Zhou et al. 2001). The same researchers found that exposing pregnant female rats to the commercial penta mixture of PBDEs, DE-71, also decreased total T_4 levels in the fetuses in a dose-dependent manner (Zhou et al. 2002).

Studies have also demonstrated the ability of PBDEs to disrupt estrogen homeostasis. In one study, breast cancer cells and ovarian cancer cells were exposed to DE-71 and six synthesized hydroxylated PBDE metabolites. DE-71 failed to displace estradiol from the estrogen receptor alpha (ER-α), however, all six hydroxylated metabolites were able to displaced estradiol binding from ER-α (Mercado-Feliciano and

Bigsby 2008). Another study treated pregnant Wistar rats with 140 or 700 μg/kg body of BDE-47 beginning on gestation day 6 through postnatal day 21. The researchers found a significant decrease in ovarian weight in the lower dose group and a decrease in serum estradiol concentrations in the higher dose group. (Talsness et al. 2008)

PBDEs have also been implicated in disrupting adipocyte metabolism. Male Sprague-Dawley rats were gavaged 14 mg/kg of a penta-BDE mixture daily for 2 and 4 weeks. Adipocytes were isolated and lipolysis and glucose oxidation was measured. Isoproterenol-induced lipolysis was 30% higher and insulin-stimulated glucose oxidation was 59% lower in cells of 4 week- treated rats compared to control (Hoppe and Carey 2007).

Mechanism of PBDE Action

Similar to many other xenobiotics, PBDEs act by binding to nuclear receptors (NRs). NRs are a family of transcription factors that can be activated by a number of ligands including pharmaceutical drugs, endogenous compounds and environmental chemicals. Some examples of NRs include constitutive androstane receptor (CAR), pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR). Xenobiotics, such as PBDEs, will enter a cell either by passive diffusion or active transport and can bind to NRs in the cytosol (Figure 2). Once bound by a ligand, the nuclear receptor-xenobiotic compound will enter the nucleus. The nuclear receptor will form a heterodimer with other receptors, such as retinoid X receptor (RXR), and bind to a response element on the DNA, triggering gene transcription and, eventually, translation of the corresponding CYP enzyme (Hewitt et al. 2007; Tompkins and Wallace 2007).

Figure 2: Cell model of CYP Induction

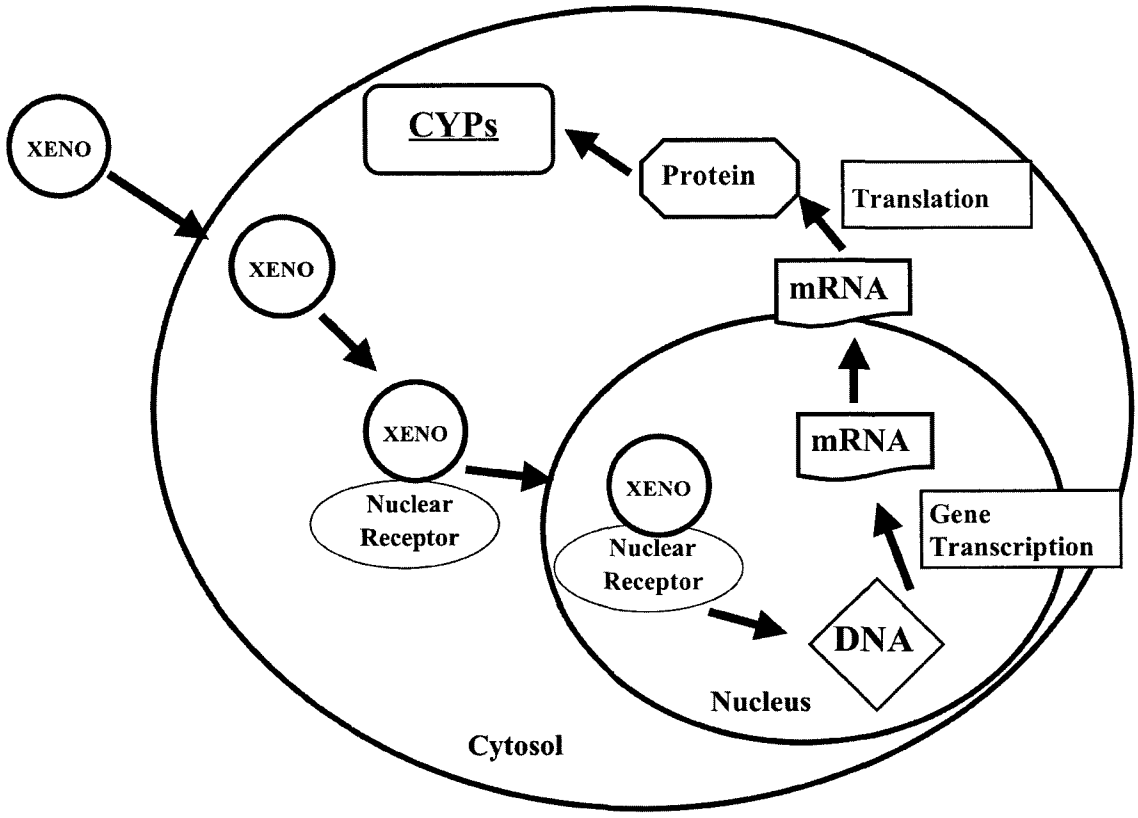


Figure 2: General induction process of the drug metabolizing enzymes, CYPs, via gene transcription following exposure to a xenobiotic. (Denoted in the figure as "xeno"; adapted from (Hewitt et al. 2007).

PBDEs as a Ligand

PBDEs have been confirmed as ligands and activators of PXR, but not for CAR or AhR (Sakai et al. 2009; Wahl et al. 2010). Pacyniak et al. (2007) used human HepG2 cells and transfected them with a number of plasmids including cDNA for mouse PXR bound to luciferase vectors. Cells were exposed to PBDEs for 24-hours. Luciferase activity significantly increased in a dose-dependent response to varying PBDE congeners demonstrating that PBDEs bind as a ligand to PXR.

The researchers wanted to explore if there was a similar response *in vivo* and used two groups of mice, wild type and PXR knockout, to investigate this question. The mice were injected with a mixture of 100 mg/kg/day of BDE-47, -99, and -209 for 4 days. There was an increase in CYP3A and 2B RNA and protein by 2 to 2.5 fold in the wild type, as expected, and an increase, but to a lesser degree, of the same CYP RNA and protein in the PXR knockout mice. The authors suggested that there might be other ways in which PBDEs induce CYPs (Pacyniak et al. 2007).

In a similar study, human HepG2 and rat hepatoma H4IIE cells were transfected with an XREM reporter gene (a plasmid that is transactivated by PXR) containing luciferase as the reporter. Cells were exposed to a technical mixture of pentaBDEs for 48 hours. The pentaBDE mixtures increased CYP3A1 and CYP3A4 mRNA by 1 to 4 fold in both cell types (Fery et al. 2009), demonstrating that PBDEs act as a ligand for PXR in human and rat liver cells *in vitro*.

PXR Crosstalks with Gluconeogenesis

PXR has direct transcriptional regulation over drug metabolizing enzymes but can also crosstalk with other transcription factors to alter expression of other metabolic genes involved in gluconeogenesis (GNG) (Ihunnah et al. 2011). GNG is the conversion of substrates including lactate, glycerol and pyruvate, to glucose, as the brain needs a constant supply of glucose to sustain function. GNG occurs during fasted states such as overnight when no dietary glucose is being supplied. Consumption of a high-carbohydrate diet will slow GNG to cessation until the next fasted state. GNG is a regulated process and the regulation is three-fold: via substrates, by hormones such as

insulin and glucagon and by key regulatory enzymes, one of which is phosphoenolpyruvate carboxykinase (PEPCK) (Harvey and Ferrier 2011).

Studies have shown that activated PXR can repress specific genes found in the GNG pathway, including PEPCK. Zhou et al. (2008) used wild type mice or transgenic mice that expressed an activated form of PXR in the liver. In the transgenic mice, RNA expression of PEPCK was reduced compared to the wild type (Zhou et al. 2006). Bhalla et al. (2004) found similar results *in vitro*. HepG2 cells were transfected with human PXR vector and then exposed to 10 μM rifampicin, a known PXR ligand, or dimethyl sulfoxide (Me_2SO) as control for 24 hours. RNA expression of PEPCK was decreased in the rifampicin-treated cells compared to cells treated with Me_2SO (Bhalla et al. 2004). These results demonstrate that when PXR is activated, PEPCK gene expression is suppressed.

PEPCK, Xenobiotics and PBDEs

Phosphoenolpyruvate Carboxykinase (PEPCK)

PEPCK converts oxaloacetate to phosphoenolpyruvate and uses guanosine triphosphate (GTP)- or inosine triphosphate (ITP)- as a co-substrate (Figure 3). PEPCK is present in the liver, kidney, lung, heart, small intestine and adipose tissue and resides in both the mitochondria and cytosol of most mammals, although subcellular distribution varies by species. For example, in humans, 60% of PEPCK is found in the mitochondria with 40% in the cytosol. In rats, only 10% is found in the mitochondria while 90% is in the cytosol (Hanson and Garber 1972; Hanson and Reshef 1997).

Cytosolic PEPCK is highly regulated by diet and hormones including thyroid hormone, glucagon, glucocorticoids and insulin. During fasting, activity of cytosolic PEPCK increases by two- to eight-fold while the mitochondrial isoform remains virtually

Figure 3: Gluconeogenesis Pathway

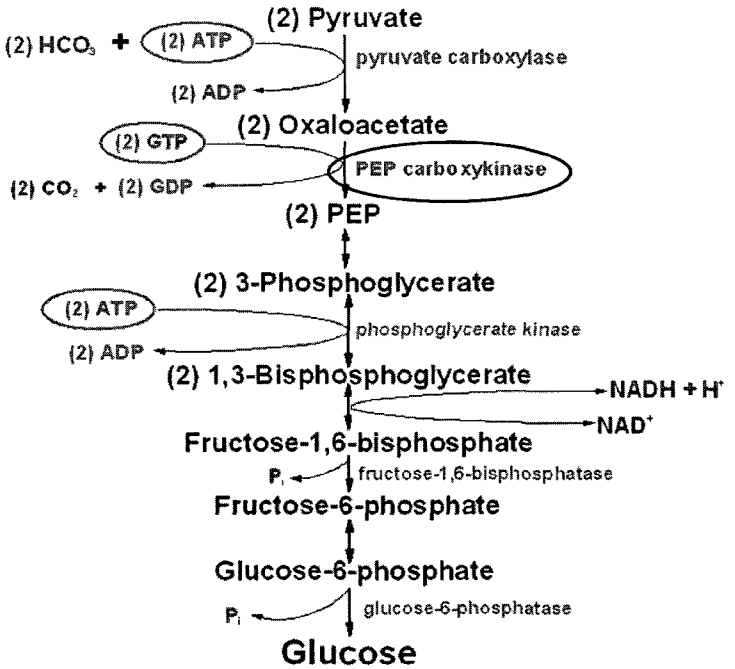


Figure 3: The pathway of gluconeogenesis showing PEPCK (circled), dependent on GTP, to convert oxaloacetate to phosphoenolpyruvate (PEP) (King 2011).

unaffected, suggesting distinct isoform regulation (Ballard and Hanson 1969; Hanson and Reshef 1997). In animals with predominant mitochondrial isoforms of PEPCK, oxaloacetate (OAA) that is generated in the mitochondria via pyruvate carboxylase can be converted to phosphoenolpyruvate (PEP). However, due to the poor permeability of OAA in the mitochondria, animals that have the dominant form of PEPCK located in the cytosol, must first convert OAA to malate and transport malate into the cytosol. Once in the cytosol, malate is oxidized to OAA by malate dehydrogenase (MDH) where the

cytosolic form of PEPCK can then convert OAA to PEP (Hanson and Reshef 1997). The cytosolic form of PEPCK in the rat liver consists of 621 amino acids, has a molecular weight of 69 kD (Beale et al. 1985) and a half-life of 6-8 hours (Ballard et al. 1974).

Other Roles of PEPCK

PEPCK is also present in nongluconeogenic tissues and, in the case of adipose tissue and small intestine, is proposed to aid in providing glycerol backbone for triglyceride synthesis via the process of glyceroneogenesis. (Hanson and Reshef 1997; Reshef et al. 1970) Another role of PEPCK is cataplerosis or removal of citric acid cycle anions. Hakimi et al. (2005) evaluated the metabolic consequences of deleting the cytosolic form of PEPCK (PEPCK-C) in the liver and kidney of mice in utero. [Fetal livers do not contain PEPCK, as glucose is provided by the mother, but it appears at birth corresponding to the needs of glucose production (Hanson and Reshef 1997)]. Mice were killed two or three days after birth. The PEPCK knockout mice did not gain weight one day after birth. Blood glucose levels in PEPCK knockout mice were 60% lower than controls. Lipid accumulated in the liver of the PEPCK knockout mice and the triglyceride level in the liver was twice the amount of controls. The same elevated level of triglycerides was also seen in the blood of PEPCK knockout mice. The blood concentration of β-hydroxybutyrate, a ketone body, was three times higher than control, and the levels of malate and lactate were 10 and 2.5 times higher, respectively, than control (Hakimi et al. 2005). These results show the importance of PEPCK, not only in the process of GNG, but also in maintaining homeostasis of energy metabolism.

Regulation of PEPCK

The gene for PEPCK-C is regulated at the transcriptional level via induction and repression. During times of starvation, PEPCK-C is induced and consumption of a high carbohydrate diet will reduce PEPCK synthesis. Glucagon, a hormone released during times of low blood sugar, and thyroid hormone both increase PEPCK-C synthesis while insulin, released during times of high blood sugar, inhibits PEPCK-C synthesis (Ballard and Hanson 1967; Gurney et al. 1994; Hanson and Reshef 1997).

The mechanism of glucagon induction involves glucagon binding to its surface receptor, which stimulates G_s-protein. Stimulated G-protein will then stimulate adenylyl cyclase causing an increase in cAMP levels. The increase in cAMP levels causes a phosphorylation of protein kinase A (PKA). Activated PKA then phosphorylates cAMP regulatory element binding protein (CREB), which binds to cAMP response element-1 (CRE-1). CRE-1 is located in Region 1 of the PEPCK-C gene promoter and is in close proximity to the start location of transcription (Roesler et al. 1988, 1989; Yabaluri and Bashyam 2010). If PEPCK mRNA is not translated, existing PEPCK mRNA will be degraded with a half-life of about 40 minutes (Cimbala et al. 1982). However, the presence of cAMP stabilizes PEPCK mRNA and extends the half-life to 140-250 minutes (Hod and Hanson 1988). In contrast, glucocorticoids and thyroid hormone diffuse through the plasma membrane and bind to receptors located in the cytosol. The occupied receptors translocate into the nucleus and bind to glucocorticoid response elements (GRE1 or -2) and a thyroid response element (TRE) on the PEPCK gene promoter, respectively (Yabaluri and Bashyam 2010), to promote PEPCK transcription.

Insulin acts in an opposing mechanism to glucagon. The presence of insulin decreases the levels of cAMP causing PKA to remain inactive and does not phosphorylate CREB (Harvey and Ferrier 2011). Insulin can also phosphorylate a forkhead transcription factor (FOXO1) that normally is responsible for activating the PEPCK gene. Phosphorylation of FOXO1 renders it inactive by decreasing FOXO1's binding affinity to the insulin response sequence (IRS) (O'Brien et al. 1995; Zhang et al. 2006), removing PEPCK gene activation.

Xenobiotic Effects on GNG and PEPCK

The effect of PBDE exposure *in vivo* and *in vitro* on GNG and PEPCK is currently unknown. However, studies using other xenobiotic compounds have examined effects on GNG and PEPCK. Male Wistar rats were administered an oral dose of 100 mg/kg/day of Phenobarbital, a pharmaceutical drug used to treat epilepsy and diabetes, for 15 days. Hepatocytes were isolated and exposed to gluconeogenesis precursors lactate/pyruvate, alanine, glycerol and glutamine. Glucose production was inhibited by 50% and PEPCK activity was also inhibited by 50% (Argaud et al. 1991). Chauvin et al. (1996) found hepatic PEPCK enzyme kinetics from Phenobarbital-treated rats, 100mg/kg/day for 14 days was altered: Vmax and Km was reduced by about 50% and 30%, respectively. The researchers also determined the effect of Phenobarbital treatment on PEPCK activity was post-translational as PEPCK protein and mRNA did not decrease (Chauvin et al. 1996).

Viluksela et al. (1999) administered oral dosages of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a compound present in Agent Orange, ranging from 50 to 9600 μg/kg/day to male and female Han/Wistar rats for 10 days. PEPCK activity decreased in

a dose-dependent manner with the highest dose of TCDD reducing maximal activity by about 50% in males and 25% in females. Plasma glucose levels were also slightly decreased compared to controls (Viluksela et al. 1999), suggesting that TCDD represses PEPCK activity and, as a result, alters glucose homeostasis.

Vijayan et al. (2006) used Anadromous arctic char, a fish that resides in Arctic bodies of water, and administered 0 or 100 mg/kg/day of Aroclor 1254, a commercial polychlorinated biphenyl (PCB) mixture, for 4 months. The researchers treated two groups of fish but fasted one group to mimic the natural fasting pattern seen during the winter months and continuously fed another group for comparison. Results showed a 55% reduction in plasma glucose and a 66% reduction of PEPCK activity in the 100 mg/kg fasted group compared to both the 0 mg/kg fasted group and the 100 mg/kg fed group (Vijayan et al. 2006). These data suggest that, in fish, PCB not only reduces PEPCK activity but also inhibits glucose production in the fasted, but not fed, state.

Summary

PBDE treatment has been shown to increase hepatic CYP RNA, protein and activity. PBDEs also act as a ligand and activator of the nuclear receptor, PXR. Animal models with activated PXR have shown a decrease in hepatic PEPCK mRNA. It has been established that exposure of animals to certain xenobiotics reduces activity of PEPCK, the key enzyme found in the gluconeogenesis pathway. The effect of PBDE exposure of rats on hepatic PEPCK activity is unknown.

Hypothesis

The purpose of this study was to determine the effect of *in vivo* PBDE treatment of rats on hepatic PEPCK activity and *in vivo* glucose homeostasis: it tested the hypothesis that hepatic PEPCK activity is reduced by *in vivo* PBDE treatment of rats.

CHAPTER II

MATERIALS AND METHODS

Animals

Forty-eight male Wistar rats, (Charles River Laboratory, Wilmington, MA) were used for this study. All rats weighed between 75-100 grams upon arrival. The rats were housed under controlled atmosphere and a 12- hour light/dark cycle. They were fed standard chow and water *ad libitum*. The University of New Hampshire's Animal Care and Use Committee approved all procedures, #110406.

Treatment

The rats were allowed to adjust to their new habitat for five days prior to the start of treatment. The rats were randomly assigned to two different gavage treatments: 14 mg PBDE / kg body weight / day (n = 24) or corn oil control (n = 24). Rats were gavaged daily, generally between 8 and 9 am. Initial body weights were obtained at the onset of treatment and body weight was recorded three times per week. Rats were euthanized after a 16 – hour fast at 3, 14 and 28 days of treatment (n = 8), using carbon dioxide gas. (Gas was allowed to fill the chamber for 90 seconds, followed by 90 seconds of no gas.)

Solutions

PBDE Gavaging Solution

- Two grams of DE – 71, a commercialized mixture of penta – PBDEs (Great Lakes Chemical Corporation, West Lafayette, IN through a generous gift from Dr. David Szabo) was removed from the original glass vial via a small metal spatula, dissolved into 2400 µl of hexane in a secondary glass vial and vigorously mixed. The mixture was placed in a 37°C water bath and stirred with a glass rod until PBDEs were completely dissolved. The mixture was poured into an amber glass bottle containing 60 ml of corn oil. An additional 51 ml of corn oil were added and the bottle was vortexed for 30-60 seconds to mix contents thoroughly. The hexane was evaporated under the hood using compressed nitrogen for 5 – 10 hours. The solution was stored in an amber bottle at room temperature.

Corn Oil Gavaging Solution

- Hexane, 2400 µl, was added to 111 ml of corn oil in an amber bottle. The vial was vortexed for 30-60 seconds to mix contents thoroughly. The hexane was then evaporated using compressed nitrogen under the hood for 5 – 10 hours. The solution was stored in an amber bottle at room temperature.

Preparation of Blood and Liver Samples

Blood

Rats were removed from the euthanasia chamber, the abdominal cavity was spread and a lateral incision was made to expose the internal organs. The diaphragm was then cut to expose the heart. Approximately 5 ml of blood was obtained via cardiac

puncture using a 0.2% EDTA-rinsed 18G-1.5" needle attached to a 5 ml syringe. Blood was transferred to a 6 ml EDTA – treated tube (Thermo Fisher Scientific, Waltham, MA) and kept on ice until it was transported back to the lab. Once in the lab, the collected blood was centrifuged at 3000 rpm for 15 minutes at 4°C in an IEC – Centra8R centrifuge. Approximately 1-2.5 ml of plasma was removed, aliquoted into cryovials in the amount of 250 – 400 µl, frozen and stored at -80°C.

Liver

The liver was removed, weighed, cut into approximately two-gram portions, flash frozen in liquid nitrogen, wrapped in aluminum foil and stored at -80°C.

Preparation of Liver Cytosol Fraction

Liver cytosol fraction was prepared, with modifications, as described by Chauvin et al. (1996). Liver was thawed, weighed (approx. 2 g), minced with scissors and placed in nine volumes of working homogenizing solution (refer to Solutions). Tissue was then homogenized for 15 seconds using a Powerstat® polytron (Thermo Fisher Scientific, Waltham, MA) at a setting of 60.

Homogenized liver was centrifuged at 3116.425 x g (5000 rpm) for 15 minutes at 4°C in a Sorvall Evolution RC centrifuge. The supernatant was removed and centrifuged at 59466 x g (30000 rpm) for 60 minutes at 4°C in a Beckman L8-80 ultracentrifuge. Supernatant was removed, placed on ice and used for PEPCK enzyme-coupled assay analysis. Total protein concentration of the supernatant was measured via a commercial DC protein assay (BioRad, Hercules, CA).

Solutions

Stock Solution of 0.5 M Potassium Phosphate, pH 7.0

- The solution was made by placing 13.609 g of potassium phosphate (FW:136.09) into 150 ml ddH_2O. The pH was adjusted to 7 with NaOH and the final volume was brought to 200 ml. Solution was stirred and stored at 4°C.

Stock Solution of 50 mM PMSF

- The solution was made by placing 0.1742 g of PMSF (FW: 174.19) in 100% ethanol with the final volume being brought to 20 ml. Solution was vortexed until particles dissolved and stored at 4°C.

Stock Solution of 50 mM DTT

- The solution was made by placing 0.154 g of DTT (FW: 154.25) in ddH_2O with the final volume being brought to 20 ml. Solution was stirred and stored at 4°C.

Stock Solution of 20 mM EDTA

- The solution was prepared by placed 0.7368 g of EDTA (FW: 368.4) into ddH_2O with the final volume being brought to 100 ml. The solution was stirred and stored at 4°C.

Stock Solution of 0.5 mM Leupeptin

- The solution was prepared by placing 0.0021 g of leupeptin (FW: 426.6) into ddH_2O with the final volume being brought to 10 ml. The solution was stirred, aliquoted into cryovials (100 µl) and frozen at -20°C.

Working Homogenizing Solution

- Twelve milliliters of 0.5 M Potassium Phosphate stock solution, 1.2 ml of 50 mM PMSF stock solution, 1.2 ml of 50 mM DTT stock solution, 3 ml of 20 mM

EDTA stock solution and 120 μl of 0.5 mM leupeptin stock solution were mixed with the final volume being brought to 60 ml with ddH_2O. The final concentration of the working homogenizing solution consisted of 0.1 M potassium phosphate, pH 7; 1 mM PMSF; 1 mM DTT; 1 mM EDTA; and 1 μM leupeptin.

Analysis of Blood and Liver

Blood Analysis

Blood was analyzed for fasting glucose, insulin and C-peptide. Fasting glucose was measured using a commercial glucose oxidase kit (Sigma-Aldrich, St. Louis, MO). Insulin and C-peptide were measured using commercial ELISA and RIA kits, respectively (Millipore, Billerica, MA).

Liver Cytosol Fraction

The liver cytosol was used for a Phosphoenolpyruvate Carboxykinase (PEPCK) enzyme-coupled assay and was performed, with modifications, as described by Chauvin et al. (1996). The enzyme-coupled assay utilizes malate dehydrogenase (MDH) to convert malate to oxaloacetate (OAA). PEPCK then converts OAA to phosphoenolpyruvate (PEP). MDH requires nicotinamide adenine dinucleotide (NAD^+) as a coenzyme resulting in the generation of the reduced form of nicotinamide adenine dinucleotide (NADH). (Figure 4) The presence of NADH can then be measured spectrophotometrically at 340 nm.

A 3-ml acrylic cuvette contained the following: 12.5 mM Tris, pH 8.0; 0.75 mM $MnCl_2$; 1 mM NAD^+; 6 U of malate dehydrogenase and varying amounts of malate (0, 0.125, 0.25, 0.375, 0.5, 1, 2, 4 and 8 mM), as shown in Table 1. The mixture was allowed to equilibrate in a 37°C water bath for three minutes.

Figure 4: PEPCK enzyme-coupled assay

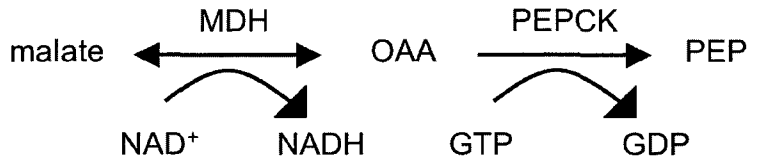


Figure 4: The PEPCK enzyme-coupled assay. The generation of OAA from malate via MDH provides PEPCK with the necessary substrate to synthesize PEP.

One hundred and twenty µl of liver cytosol fraction was added and allowed to equilibrate in a 37°C water bath for an additional one minute. To start the reaction, 1 mM GTP was added with a final volume of 2 ml. NADH formation was measured spectrophotometrically at 340 nm. The assay was performed in duplicate for each amount of malate. The assay was programmed to run for 15 seconds / cycle for an average of 4 minutes (~16 cycles).

Beer's law was used to determine the activity (velocity, v) of PEPCK (A= Ebc). 'A' is the change in OD, 'E' is the extinction coefficient of NADH (6200 L/ (mol x cm), 'b' is the path length of the cuvette (1 cm) and 'c' is the concentration (mol/L). The following equations were used to normalize for the amount of protein (µmol / min / mg protein) and the amount of liver (µmol / min / g wet weight liver).

Protein:

$$\frac{\left[\left(\frac{\text{(Average A/min)}}{\text{6200 L/(mol x cm)}}\right) \times 10^6 \text{ umol/mol} \times \text{0.002 L}\right]}{\left[\left(\frac{\text{Average protein conc. mg/ml}}{\text{1000 ul/ml}}\right) \times \text{120 ul}\right]}$$

Liver:

$$\frac{\left[\left(\frac{\text{(Average A/min)}}{\text{6200 L/(mol x cm)}}\right) \times 10^6 \text{ umol/mol} \times 0.002 \text{ L}\right]}{\left[\left(\frac{\text{[liver (g) / buffer (ml)]}}{\text{1000 ul/ml}}\right) \times 120 \text{ ul}\right]}$$

Solutions

Stock Solution of 100 mM Tris, pH 8.0

- The solution was prepared by placing 1.211 g of Tris (FW: 121.1) in 80 ml of ddH_2O. pH was adjusted to 8.0 with HCl and the final volume was brought to 100 ml. The solution was stored at 4°C.

Stock Solution of 5 mM $MnCl_2$

- The solution was prepared by placing 0.0989 g of $MnCl_2$ (FW: 197.9) in ddH_2O with the final volume being brought to 100 ml. The solution was stored at 4°C.

Stock Solution of 10 mM NAD^+

- The solution was prepared by placing 0.1326 g of NAD^+ (FW: 663.4) in 20 ml of ddH_2O. The solution was mixed thoroughly and then aliquoted into cryovials in the amount of 500 µl and stored at -20°C.

Stock Solution of 1:500 dilution of MDH (Sigma Aldrich, 2.2 mg/ml)

- Ten µl of MDH was placed into 490 µl of 0.9% NaCl. Mixture was made fresh for each assay and kept on ice for the duration of the experiment.

0.9% NaCl:

- The solution was prepared by dissolving 0.9 grams of NaCl in ddH_2O and the volume was brought to 100 ml. The solution was stored at 4°C.

Stock Solution of 10 mM Malate

- The solution was prepared by placing 0.0356 g of malate (FW: 178.05) in 20 ml of ddH_2O. The solution was stored at 4°C.

Stock Solution of 20 mM Malate

- The solution was prepared by placing 0.0712 g of malate (FW: 178.05) in 20 ml of ddH_2O. The solution was stored at 4°C.

Stock Solution of 10 mM GTP

- The solution was prepared by placing 0.1046 g of GTP (FW: 523.2) in 20 ml of ddH_2O. The solution was mixed thoroughly and then aliquoted into cryovials in the amount of 500 µl and stored at -20°C.

Table 1: Assay Cuvette

0 mM malate	
12.5 mM Tris	250 µl
0.75 mM $MnCl_2$	300 µl
1 mM NAD^+	200 µl
1:500 MDH	50 µl
1 mM GTP	200 µl
10 mM malate	0 µl
ddH_2O	880 µl
liver cytosol	120 µl

4 mM malate	
100 mM Tris	250 µl
5 mM $MnCl_2$	300 µl
10 mM NAD^+	200 µl
1:500 MDH	50 µl
10 mM GTP	200 µl
10 mM malate	800 µl
ddH_2O	80 µl
liver cytosol	120 µl

8 mM malate	
100 mM Tris	250 µl
5 mM $MnCl_2$	300 µl
10 mM NAD^+	200 µl
1:500 MDH	50 µl
10 mM GTP	200 µl
20 mM malate *	800 µl
ddH_2O	80 µl
liver cytosol	120 µl

Table 1: Examples of solution amounts added to assay cuvette. Water amount changed with corresponding malate amount. A 20 mM concentration of malate was used for 8 mM cuvette in order to maintain a final volume of 2 ml.

Statistics

Statistical analysis was performed on JMP 8.0 software. A two-way ANOVA – general linear model analysis was performed using PBDE treatment and time as main effects and PBDE treatment x time as a covariate. Significance was set as $p < 0.05$. Outliers were excluded using a Q-test (Fritz and Schenk 1969).

CHAPTER III

THE EFFECT OF IN VIVO PBDE TREATMENT ON HEPATIC PHOSPHOENOLPYRUVATE CARBOXYKINASE ENZYME KINETICS IN MALE WISTAR RATS

INTRODUCTION

Phosphoenolpyruvate carboxykinase (PEPCK) is a key regulatory enzyme in gluconeogenesis (GNG) that converts oxaloacetate to phosphoenolpyruvate and is dependent on guanosine triphosphate (GTP)- or inosine triphosphate (ITP)-. PEPCK is present in the liver, kidney, lung, heart, small intestine and adipose tissue and resides in both the mitochondria and cytosol of most mammals, although its distribution varies. For example, in humans, 60% of PEPCK is found in the mitochondria with 40% in the cytosol. In rats, only 10% is found in the mitochondria while 90% is in the cytosol (Hanson and Garber 1972; Hanson and Reshef 1997)

Cytosolic PEPCK (PEPCK-C) is highly regulated by diet and hormones including thyroid, glucagon, glucocorticoids and insulin. (Ballard and Hanson 1969; Hanson and Reshef 1997) The gene for PEPCK-C is regulated at the transcriptional level via induction and repression. During times of starvation, PEPCK-C is induced and consumption of a high carbohydrate diet will reduce synthesis. Glucagon, a hormone released during times of low blood sugar, and thyroid hormone both increase PEPCK-C

synthesis while insulin, released during times of high blood sugar, inhibits gene synthesis (Ballard and Hanson 1967; Gurney et al. 1994; Hanson and Reshef 1997).

Studies have been conducted examining effects of xenobiotics on hepatic GNG and PEPCK. Phenobarbital (PB) exposure of rats inhibited hepatocyte glucose production and PEPCK activity by 50% (Argaud et al. 1991). Chauvin et al. (1996) found PEPCK enzyme kinetics from PB-treated rats decreased Vmax and Km by about 50% and 30%, respectively (Chauvin et al. 1996). Following exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), male rats had a 50% decrease of PEPCK activity at the highest dose and female rats had a 25% decrease. Plasma glucose levels were also slightly decreased compared to controls (Viluksela et al. 1999). A 55% reduction of plasma glucose and a 66% reduction of PEPCK activity were also observed in fish following exposure to a polychlorinated biphenyl mixture (Vijayan et al. 2006).

Another class of xenobiotic is polybrominated diphenyl ethers (PBDEs). PBDEs are a family of lipophilic, flame retardant chemicals introduced in the 1970's. PBDEs are used in consumer products including plastics, electronics, furniture and carpeting to slow the rate at which products burn (Darnerud et al. 2001). PBDEs are only mixed with the polymer resin. Therefore, as products break down, PBDEs have the ability to leach into the environment because they are not chemically bound to the product (Darnerud et al. 2001; Kim et al. 2006; McDonald 2002). Since PBDEs are resistant to chemical and physical breakdown, they can bioaccumulate in the environment and even biomagnify within the food chain (Alaee and Wenning 2002; Costa et al. 2008; Hale et al. 2003). PBDEs have been found in soil sediment, marine animals, fish-eating birds, dairy products, and human breast milk, serum and adipose tissue (Andersson and Blomkviet

1981; Dunn et al. 2010; Inoue et al. 2006; Jansson et al. 1987; Lorber 2008; Petreas et al. 2003; Schecter et al. 2010; Schecter et al. 2003; Stafford 1983; Watanabe et al. 1987). Moreover, PBDEs are classified as an endocrine disrupting chemical as they have been shown to alter thyroid and estrogen family hormone homeostasis (Hallgren and Darnerud 2002; Zhou et al. 2001; Zhou et al. 2002). A disruption in insulin stimulated glucose metabolism was also observed in adipocytes isolated from *in vivo* PBDE treatment of rats (Hoppe and Carey 2007). The effect of PBDE treatment on PEPCK activity is unknown.

The purpose of this study was to determine the effect of *in vivo* PBDE treatment of rats on hepatic PEPCK activity and fasting plasma glucose and insulin. Our hypothesis was that hepatic PEPCK activity is reduced by *in vivo* PBDE treatment of rats.

MATERIALS AND METHODS

Animals

Forty-eight male Wistar rats, (Charles River Laboratory, Wilmington, MA) were used for this study. All rats weighed between 75-100 grams upon arrival. The rats were housed under controlled pressure and a 12- hour light/dark cycle. They were fed standard chow and water *ad libitum*. The University of New Hampshire's Animal Care and Use Committee approved all procedures, #110406.

Treatment

The rats were allowed to adjust to their new habitat for five days prior to the start of treatment. The rats were randomly assigned to two different gavage treatments: 14 mg PBDE / kg body weight / day (n = 24) or corn oil control (n = 24). Rats were gavaged daily, generally between 8 and 9 am. Initial body weights were obtained at the onset of treatment and body weight was recorded three times per week. Rats were euthanized after a 16 – hour fast at 3, 14 and 28 days of treatment (n = 8), using carbon dioxide gas.

Preparation of Blood and Liver Samples

Blood

Approximately 5 ml of blood was obtained via cardiac puncture using a 0.2% EDTA rinsed 18G-1.5" needle and a 5 ml syringe. Blood was transferred to a 6 ml EDTA – treated tube (Thermo Fisher Scientific, Waltham, MA) and kept on ice until it was transported back to the lab. Once in the lab, the collected blood was centrifuged at 3000

rpm for 15 minutes at 4°C in an IEC – Centra8R centrifuge. Approximately 1-2.5 ml of plasma was removed, aliquoted into cryovials in the amount of 250 – 400 µl, frozen and stored at -80°C.

Liver

The liver was removed, weighed, cut into approximately two-gram portions, flash frozen in liquid nitrogen, wrapped in aluminum foil and stored at -80°C.

Preparation of Liver Cytosol Fraction

Liver cytosol fraction was prepared as described by Chauvin et al. (1996) with the following additional details. A liver portion was thawed, weighed (approx. 2 g), minced with scissors and placed in nine volumes of working homogenizing solution. Tissue was then homogenized for 15 seconds using a Powerstat® polytron (Thermo Fisher Scientific, Waltham, MA) at a setting of 60.

Analysis of Blood and Liver

Blood Analysis

Blood was analyzed for fasting glucose (glucose oxidase kit, Sigma-Aldrich); insulin and C-peptide (ELISA and RIA kits, respectively, Millipore), according to manufacturer's instructions.

Liver Cytosol Fraction

The liver cytosol was used for a phosphoenolpyruvate carboxykinase (PEPCK) enzyme-coupled assay, which was performed with modifications, as described by Chauvin et al. (1996). The enzyme-coupled assay utilizes malate dehydrogenase (MDH) to convert malate to oxaloacetate (OAA). PEPCK then converts OAA to

phosphoenolpyruvate (PEP). MDH requires nicotinamide adenine dinucleotide (NAD^+) as a coenzyme resulting in the generation of the reduced form of nicotinamide adenine dinucleotide (NADH), which can be measured spectrophotometrically at 340 nm.

A 3-ml acrylic cuvette contained the following with a final volume of 2 ml: 12.5 mM Tris, pH 8.0; 0.75 mM $MnCl_2$; 1 mM NAD^+; 6 U of malate dehydrogenase and varying concentrations of malate (0 – 8 mM). The mixture was allowed to equilibrate in a 37°C water bath for three minutes. One hundred and twenty µl of liver cytosol fraction was added and allowed to equilibrate in a 37°C water bath for an additional one minute. To start the reaction, 1 mM GTP was added. NADH formation was measured spectrophotometrically at 340 nm at 23°C. The assay was performed in duplicate for each concentration of malate.

In order to normalize Vmax for mg of protein, total protein concentration of the cytosol was measured via a commercial DC protein assay (BioRad).

Statistics

Statistical analysis was performed on JMP 8.0 software. A two-way ANOVA – general linear model analysis was performed using PBDE treatment and time as main effects and PBDE treatment x time as a covariate. Significance was set as $p < 0.05$. Outliers were excluded using a Q-test (Fritz and Schenk 1969).

RESULTS

Animal Measurements

Final body weight, weight gain over the treatment period, final liver weight and liver as a percent of body weight are represented as mean ± SEM at days 3, 14 and 28 (Table 2). There was a significant effect of time ($p < 0.05$) on final body weight and the effect of PBDE treatment on final body weight was approaching significance ($p = 0.07$). There was a significant effect of time ($p < 0.05$), but not treatment, on weight gain over the treatment period. Final liver weight was not different between control and treatment but liver weight as a percent of body weight was significantly higher in PBDE-treated rats compared to control ($p < 0.05$).

In order to compare the effect of treatment on liver weight as a function of time, data for liver weight and liver weight as a percent of body weight are displayed in Figure 5 and 6. There was a significant effect of time ($p < 0.05$) on final liver weight (Figure 5). Liver weight as a percent of body weight was higher in the PBDE group with an effect of treatment ($p < 0.05$) and time ($p < 0.05$) (Figure 6). The slopes of the control and treatment trendlines are similar ($m = -0.023$ and $m = -0.022$, respectively) indicating the effect of treatment was independent of time.

PEPCK Enzyme Kinetics

Velocity versus substrate concentration plots for PEPCK (Figures 7 - 9) are expressed as the mean of 8 experiments ± SEM; plots follow standard Michaelis-Menton

kinetics. Each individual velocity vs. substrate curve was fit via SigmaPlot 10 to generate Km and Vmax values; data for 8 replicates at each time point and treatment are represented as mean ± SEM in Table 3. There was no effect of treatment or time on Km values. Vmax values, normalized for protein, were 26%, 44% and 43% lower on average in the treatment group, compared to control at 3, 14 and 28 days, respectively, with an effect of treatment ($p < 0.05$) and time ($p < 0.05$) (Figure 10). The effect of treatment was independent of time as the slopes of trendlines for control and treatment are similar ($m = -0.0003$ and $m = -0.0004$, respectively). Similar results were seen when Vmax values were normalized for liver weight (Figure 11). The effect of treatment was independent of time, however, the three-fold difference of control and treatment trendline slopes ($m = -0.0077$ and $m = -0.0229$, respectively) suggested a treatment x time interaction ($p = 0.11$). Vmax values, expressed as total liver PEPCK activity, were 18%, 34% and 41% lower on average in the treatment group compared to control at 3, 14 and 28 days, respectively, with an effect of treatment ($p < 0.05$), time ($p < 0.05$) and an interaction effect ($p < 0.05$) (Figure 12).

Blood Measurements

Fasting glucose, insulin and C-peptide are represented as mean ± SEM in Table 4. There was no effect of treatment on any of the values. In order to compare the effect of treatment on glucose, insulin and C-peptide as a function of time, data for each value displayed in Figure 13, 14 and 15. There was a significant effect of time ($p < 0.05$) for fasting glucose but no significant effect of treatment (Figure 13) and fasting insulin (Figure 14). By 28 days, insulin values of the PBDE treatment group were trending to lower values compared to control but this apparent interaction was not significant ($p =$

0.17) (Figure 15). This response was mimicked by the C-peptide values; by 28 days, C-peptide values of the treatment group were trending towards lower values compared to control (Figure 15), with an effect of time ($p < 0.05$) but the interaction effect was not significant ($p = 0.13$).

Table 2: Body Weight, Weight Gain and Liver Weight at 3, 14 and 28 days of treatment

Time (Days)	Final body weight (g)		Weight gain (g)		Final liver weight (g)		Liver weight as % of BW (%)	
	Control [C]	Treatment [T]	C	T	C	T	C	T
3	142.9 ± 4.2	141.3 ± 1.7	20.0 ± 0.8	17.3 ± 0.7	6.38 ± 0.21	6.73 ± 0.2	4.47 ± 0.11	4.77 ± 0.16
14	221.4 ± 4.4	213.0 ± 5.6	81.9 ± 3.1	78.1 ± 4.5	9.71 ± 0.31	10.08 ± 0.29	4.4 ± 0.15	4.73 ± 0.05
28	316.0 ± 6.5	305.6 ± 3.6	171.3 ± 5.5	168.5 ± 3.4	12.33 ± 0.38	12.93 ± 0.42	3.9 ± 0.1	4.22 ± 0.1

Table 2: Animal measurements represented as mean ± SEM (n = 8 per group). Final weight and weight gain was similar among PBDE-treated group and control, both with an effect of time ($p < 0.05$). Liver weights were not different between control and PBDE-treated group, but there was a significant effect of time ($p < 0.05$). Final liver weight expressed as a percent of body weight was higher in PBDE-treated group with an effect of treatment ($p < 0.05$) and time ($p < 0.05$).

Figure 5: Final liver weight at 3, 14 and 28 days of treatment

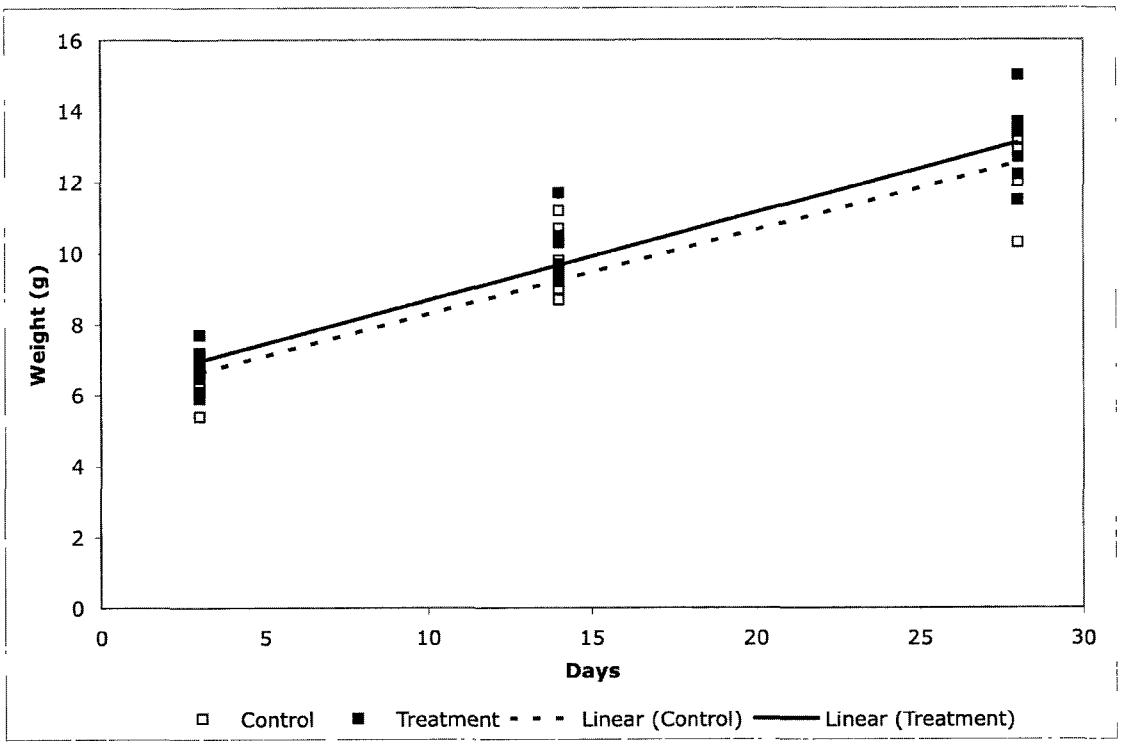


Figure 5: Final liver weight measured at 3, 14 and 28 days. There was an effect of time ($p < 0.05$).

Figure 6: Liver Weight as Percent of Body Weight at 3, 14 and 28 days of treatment

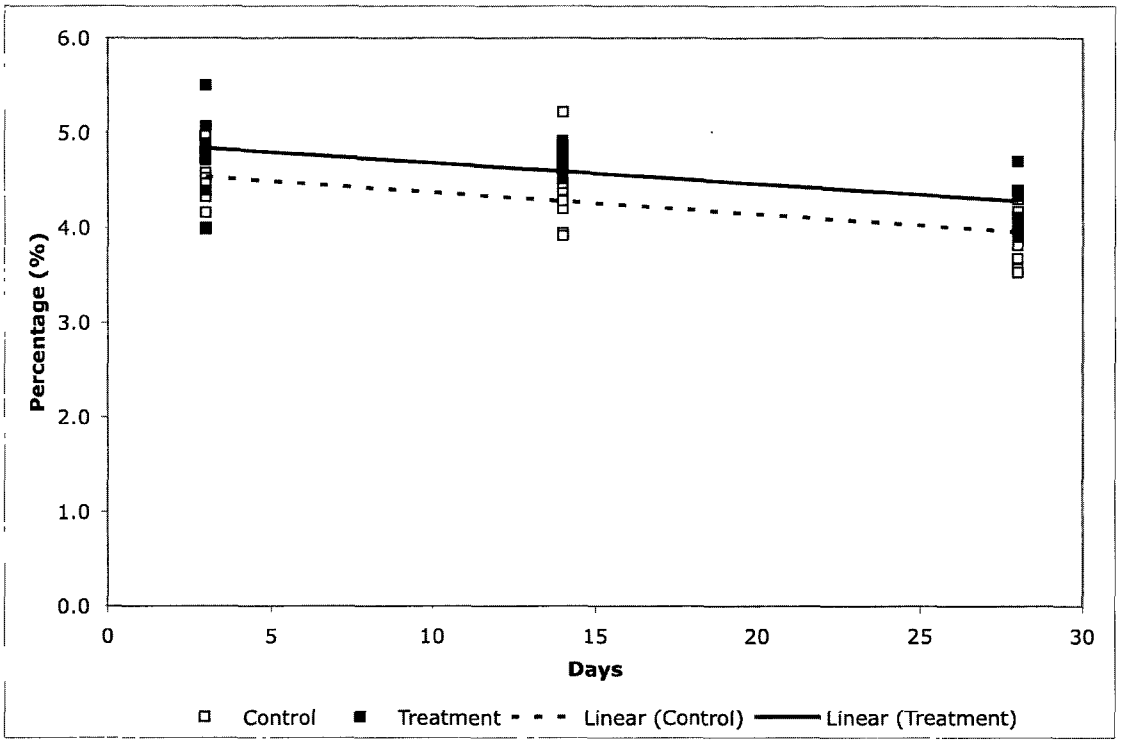
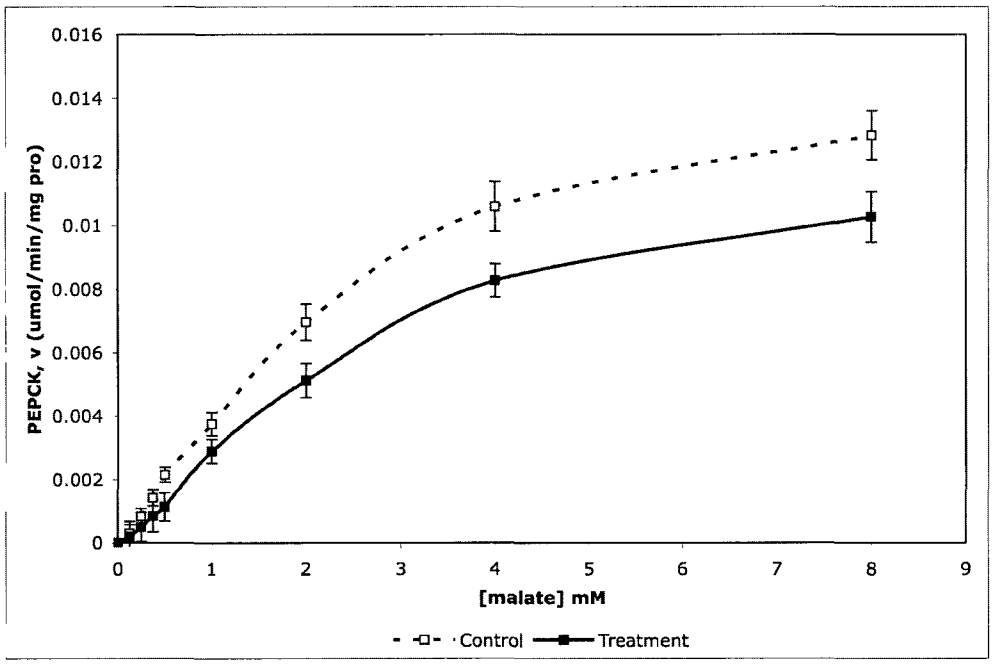


Figure 6: Liver weight expressed as a percent of body weight. Liver weight as a percent of body weight was higher in the PBDE group with an effect of treatment ($p < 0.05$) and time ($p < 0.05$).

Figure 7: 3 day PEPCK, velocity vs. substrate curves, expressed per mg protein and per gram liver

A:



B:

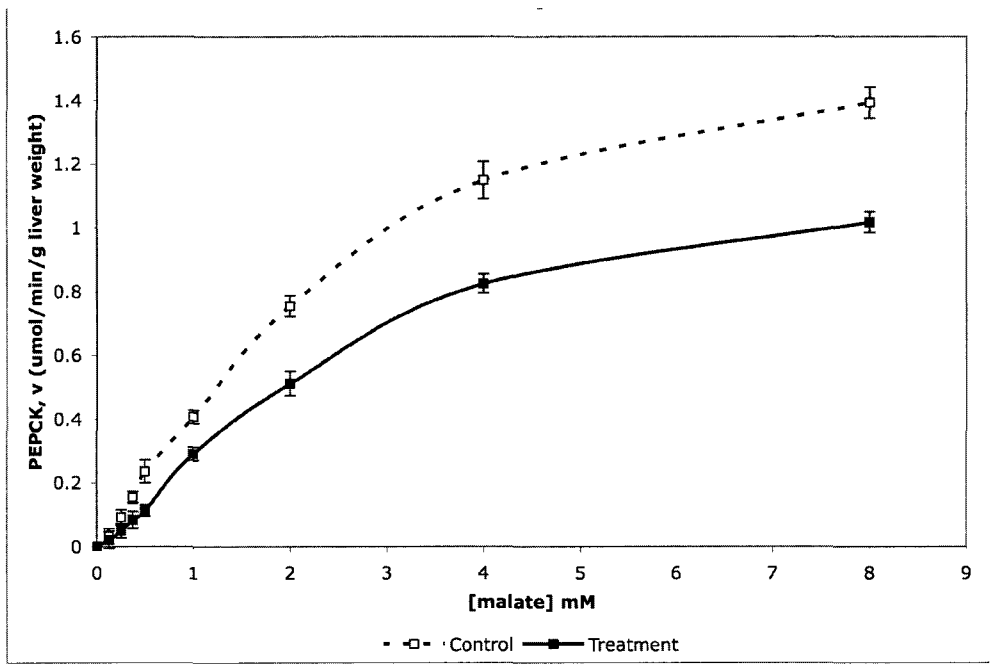
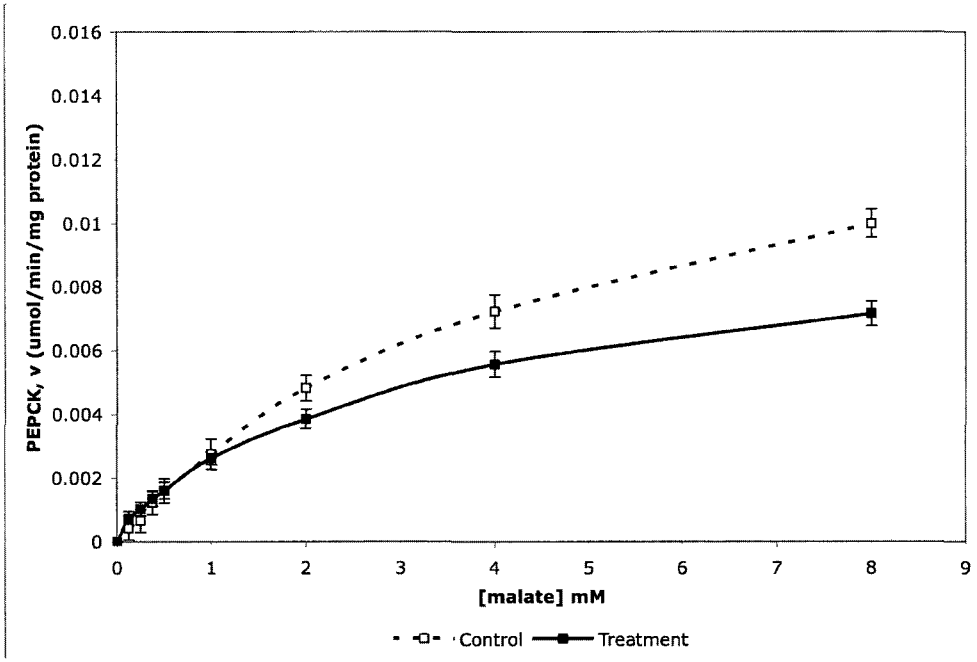


Figure 7: A: PEPCK activity at 3 days, normalized for mg protein for control (❑) animals vs. treatment (■) animals (n = 8, mean ± SEM). **B:** PEPCK activity at 3 days normalized for gram of liver for control (❑) animals vs. treatment (■) animals (n = 8, mean ± SEM).

Figure 8: 14 day PEPCK, velocity vs. substrate curves, expressed per mg protein and per gram liver

A:



B:

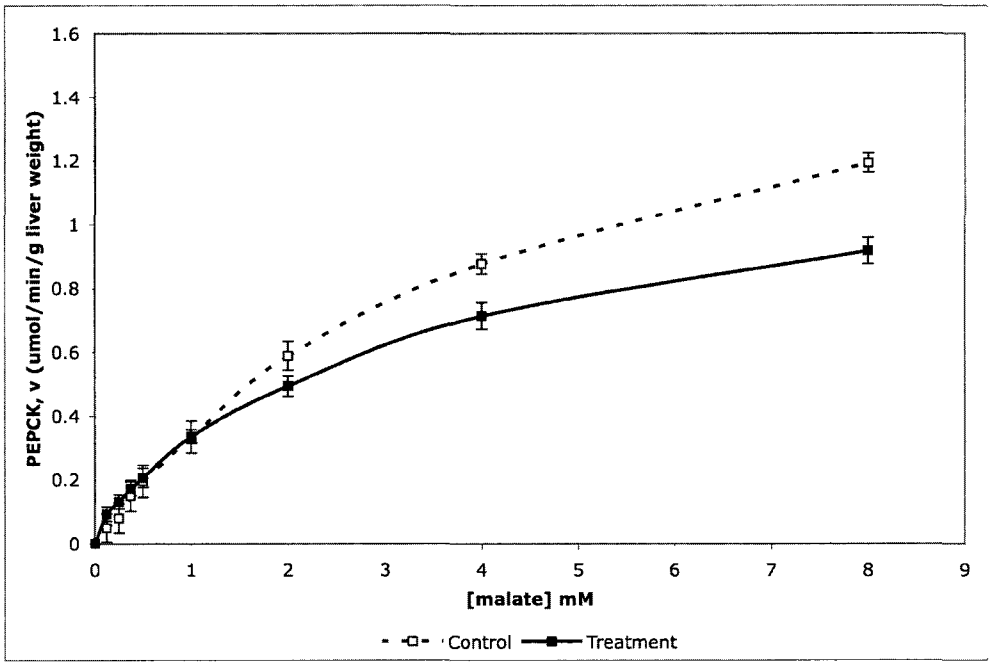
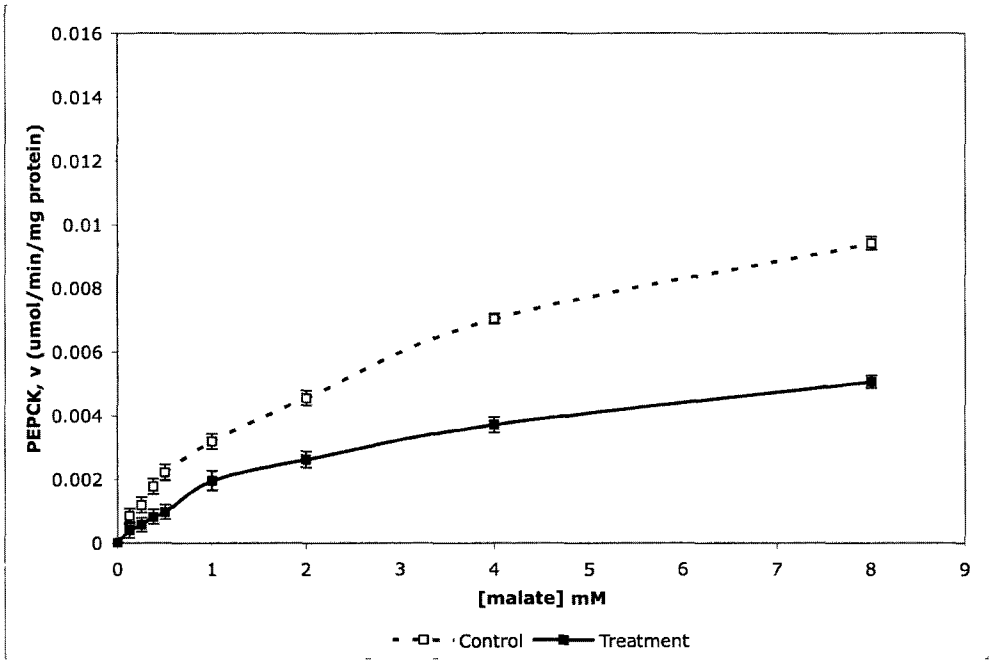


Figure 8: A: PEPCK activity at 14 days, normalized for mg protein for control (❑) animals vs. treatment (■) animals (n = 8, mean ± SEM). **B:** PEPCK activity at 14 days normalized for gram of liver for control (❑) animals vs. treatment (■) animals (n = 8, mean ± SEM).

Figure 9: 28 day PEPCK, velocity vs. substrate curves, expressed per mg protein and per gram liver

A:



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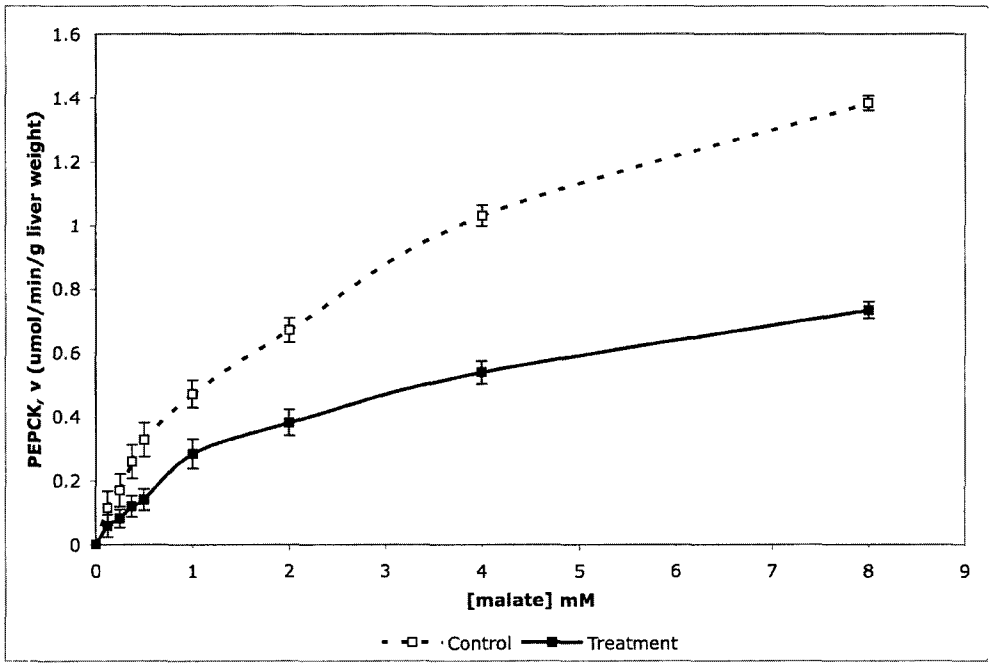


Figure 9: A: PEPCK activity at 28 days, normalized for mg protein for control (❑) animals vs. treatment (■) animals (n = 8, mean ± SEM). **B:** PEPCK activity at 28 days normalized for gram of liver for control (❑) animals vs. treatment (■) animals (n = 8, mean ± SEM).

Table 3: PEPCK Enzyme Kinetics at 3, 14 and 28 days of treatment

Time (Days)	Km [malate] (mM)		Vmax (μmol/min/mg protein)		Vmax (μmol/min/g liver weight)		Total PEPCK Activity (μmol/min/liver)	
	Control [C]	Treatment [T]	C	T	C	T	C	T
3	3.89 ± 0.77	4.67 ± 0.98	0.023 ± 0.003	0.017 ± 0.002	2.2 ± 0.18	1.71 ± 0.12	14.1 ± 1.32	11.4 ± 0.67
14	4.63 ± 0.42	3.8 ± 0.24	0.016 ± 0.001	0.009 ± 0.001	1.92 ± 0.11	1.23 ± 0.05	18.7 ± 1.3	12.4 ± 0.5
28	4.17 ± 0.5	5.72 ± 0.88	0.014 ± 0.001	0.008 ± 0.001	1.99 ± 0.09	1.12 ± 0.09	24.6 ± 1.28	14.6 ± 1.46

Table 3: Curve fitting and calculation of PEPCK enzyme kinetics, Km and Vmax, was performed on SigmaPlot 10 shown as mean ± SEM at days 3, 14 and 28 (n = 8 per group). Km values were similar between the two groups. Vmax values, regardless of units expressed, all had an effect of time ($p < 0.05$) and an effect of treatment ($p < 0.05$). Vmax, normalized for g liver weight, had an interaction effect approaching significance ($p = 0.11$). Total PEPCK activity had a significant interaction effect ($p < 0.05$).

Figure 10: PEPCK Vmax (μmol/min/mg protein) at 3, 14 and 28 days of treatment

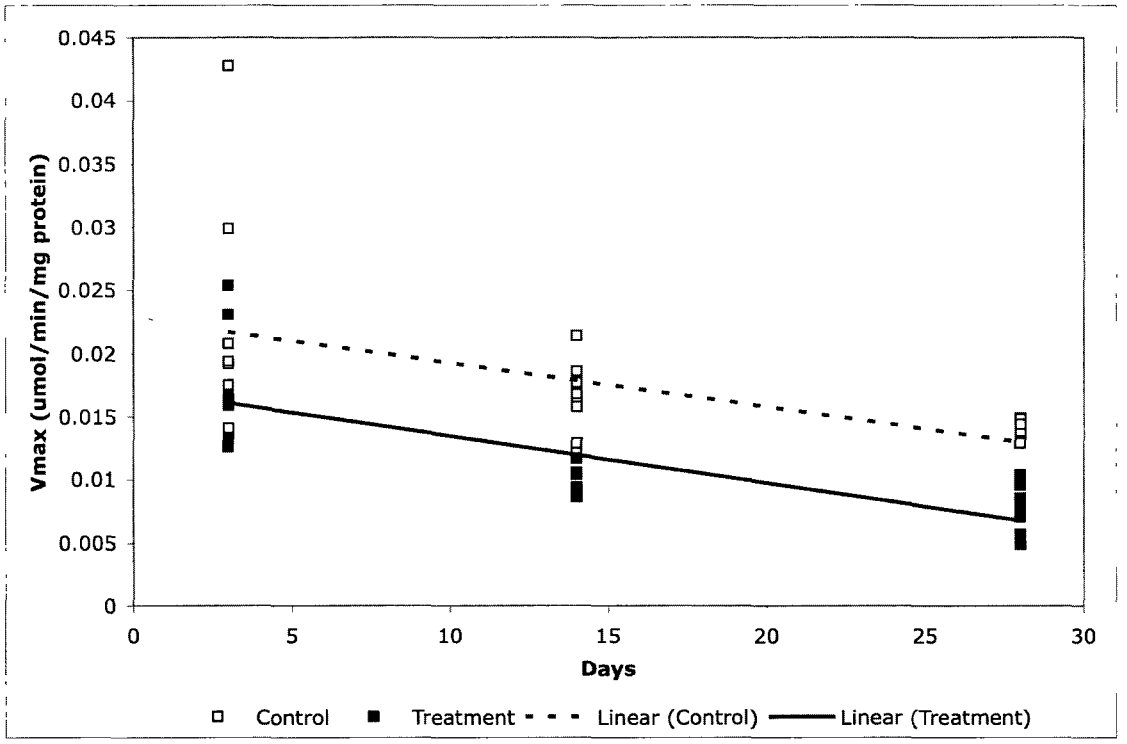


Figure 10: Vmax values, normalized for protein, were lower in the treatment group with an effect of treatment ($p < 0.05$) and time ($p < 0.05$). The effect of treatment was independent of time as the slopes of trendlines for control ($m = -0.0003$) and treatment ($m = -0.0004$) are similar.

Figure 11: PEPCK Vmax (μmol/min/g liver weight) at 3, 14 and 28 days of treatment

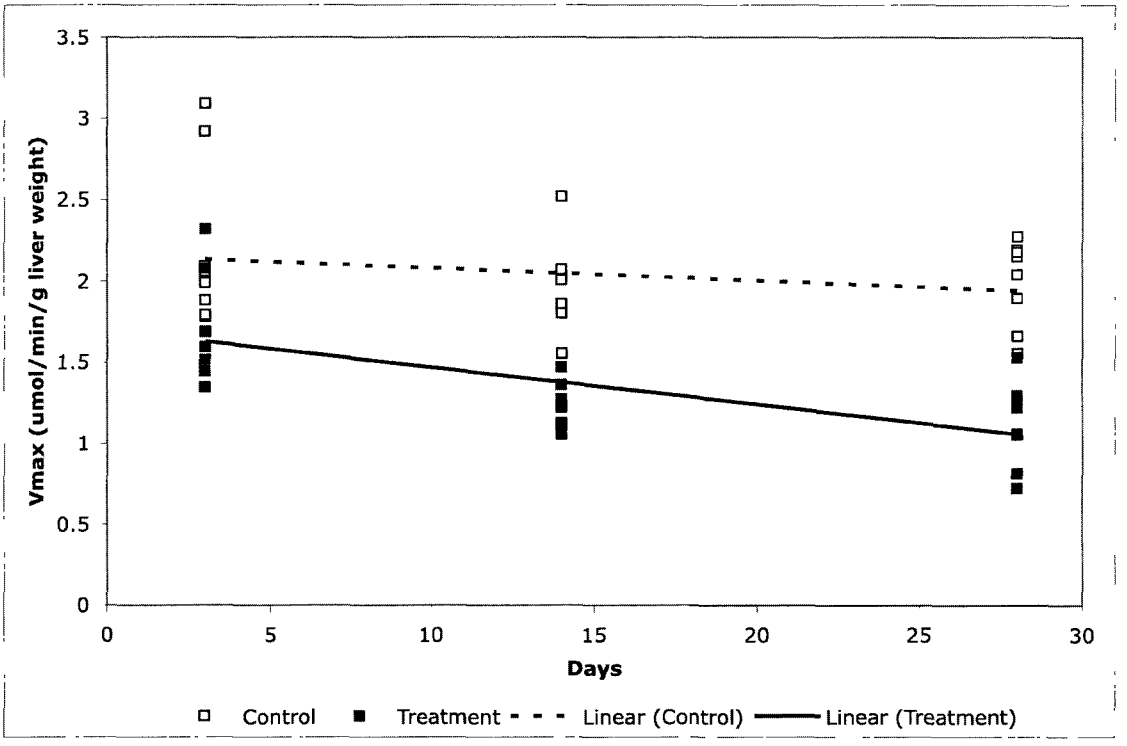


Figure 11: Vmax values, normalized for liver weight, were lower in the treatment group with an effect of treatment ($p < 0.05$) and time ($p < 0.05$). The effect of treatment was independent of time. The three-fold difference of trendline slopes, control m = -0.0077 and treatment m = -0.0229, suggests a treatment x time interaction ($p = 0.11$).

Figure 12: Total PEPCK Activity at 3, 14 and 28 days of treatment

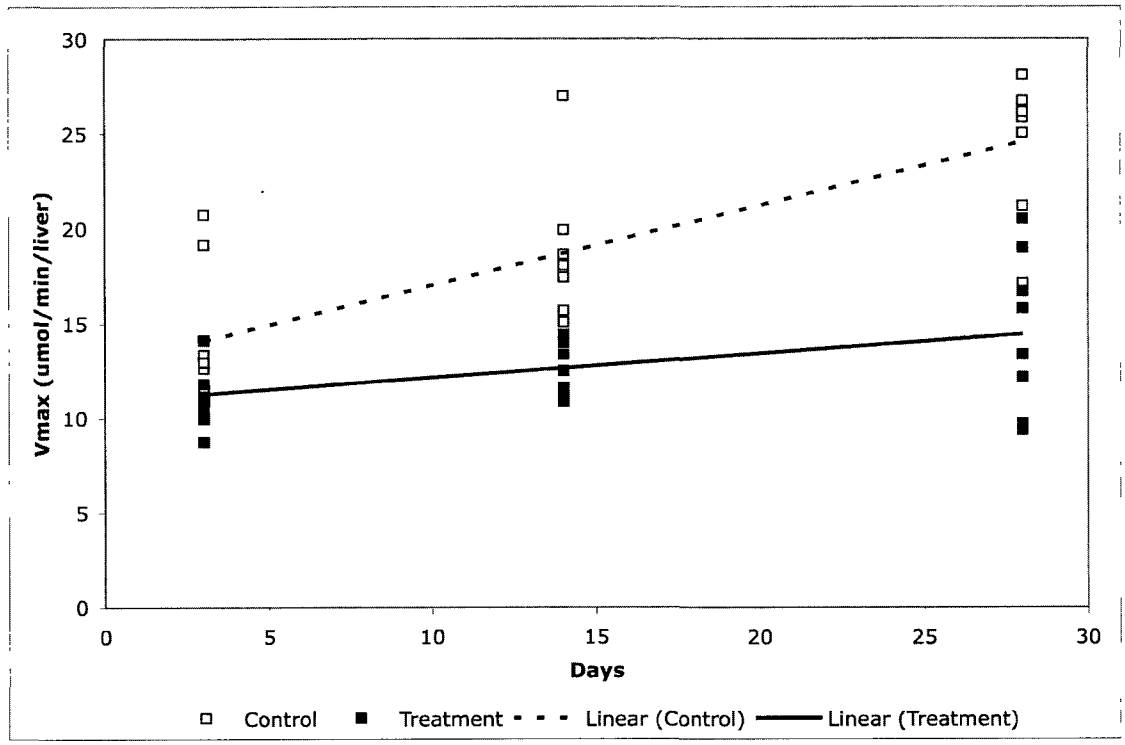


Figure 12: Velocity values, normalized for total PEPCK activity, were lower in the treatment group with an effect of treatment ($p < 0.05$), time ($p < 0.05$) and an interaction effect ($p < 0.05$).

Table 4: Fasting plasma glucose, insulin and C-peptide at 3, 14 and 28 days of treatment

Time (Days)	Fasting Glucose (mg/dl)		Insulin (ng/ml)		C-peptide (pM/0.1 ml)	
	C	T	C	T	C	T
3	89.33 ± 4.23	93.43 ± 6.73	0.226 ± 0.019	0.208 ± 0.029	232.4 ± 33.19	231.9 ± 34.89
14	148.64 ± 12.59	153.81 ± 10.06	0.237 ± 0.016	0.274 ± 0.032	369.9 ± 49.58	402.6 ± 43.01
28	184.36 ± 7.88	200.47 ± 8.17	0.572 ± 0.043	0.434 ± 0.075	698.3 ± 47.32	555.3 ± 74.06

Table 4: Fasting plasma glucose, insulin and C-peptide are represented as mean ± SEM (N = 8 per group). There was no significant effect of treatment on glucose, insulin or C-peptide levels.

Figure 13: Fasting plasma glucose at 3, 14 and 28 days of treatment

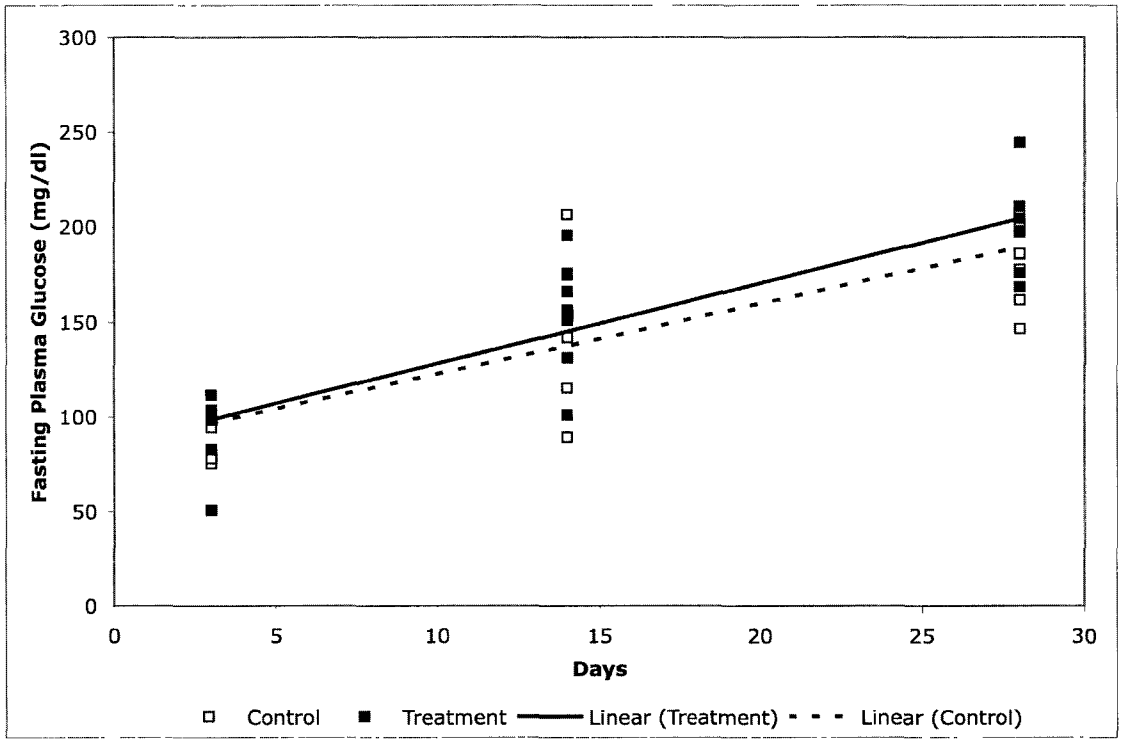


Figure 13: Plasma glucose following 16-h fast. There was a significant effect of time ($p < 0.05$) for fasting glucose but no significant effect of treatment.

Figure 14: Plasma Insulin at 3, 14 and 28 days of treatment

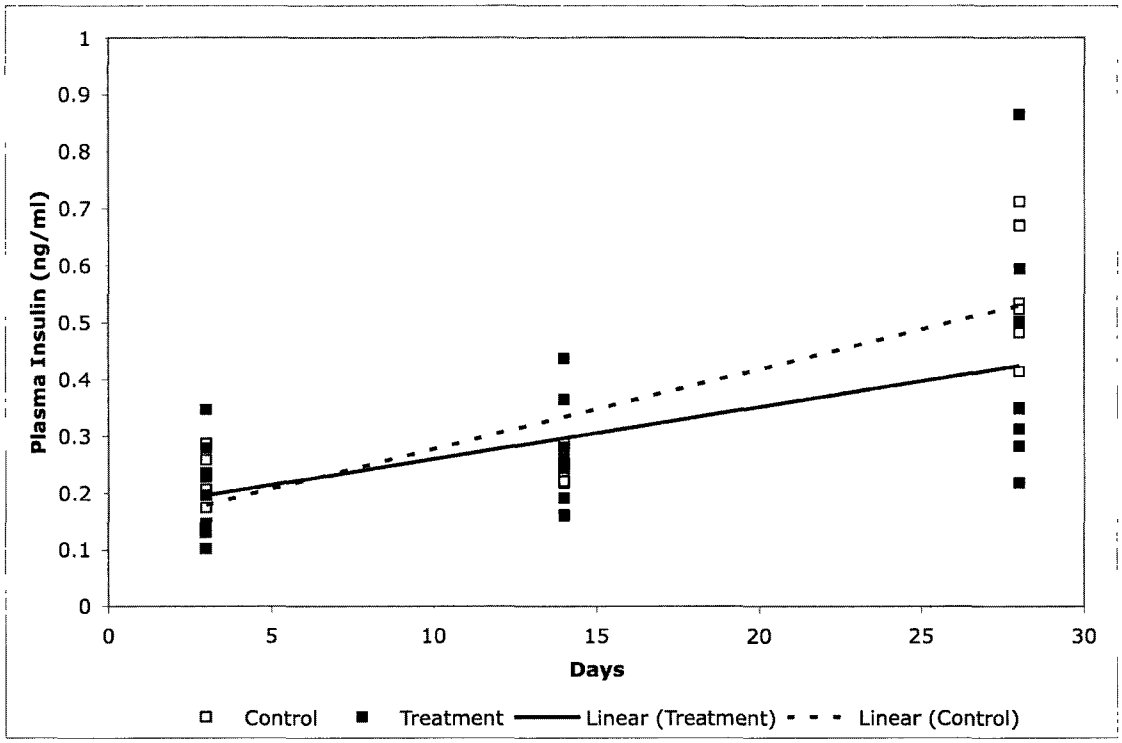


Figure 14: There was an effect of time ($p < 0.05$) for insulin levels. The interaction effect was not significant ($p = 0.17$) despite dissimilar slopes (control $m = 0.014$, treatment $m = 0.0091$).

Figure 15: Plasma C-peptide at 3, 14 and 28 days

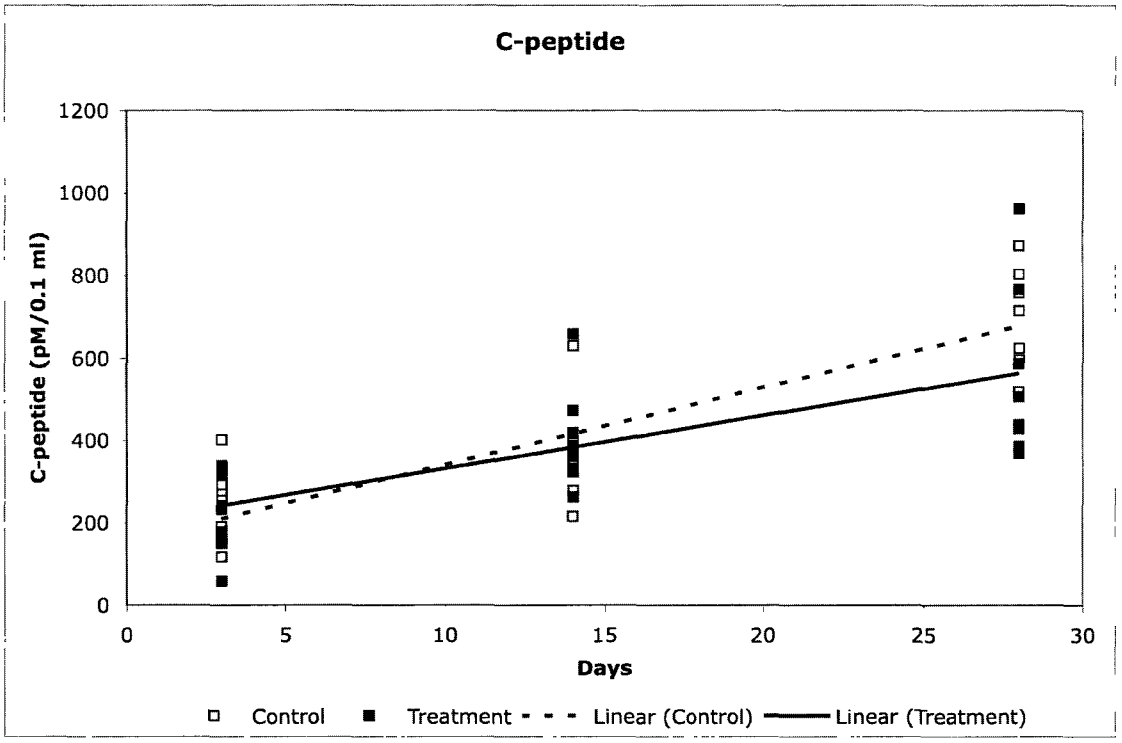


Figure 15: There was an effect of time ($p < 0.05$) for C-peptide. The interaction effect was not significant ($p = 0.13$) despite dissimilar slopes (control $m = 18.73$, treatment $m = 12.848$).

DISCUSSION

This study is the first to investigate the effects of *in vivo* PBDE treatment on hepatic phosphoenolpyruvate carboxykinase (PEPCK) enzyme kinetics. We hypothesized that hepatic PEPCK activity would be reduced by *in vivo* PBDE treatment of rats. Our findings support this hypothesis and demonstrate that PBDEs disrupt PEPCK enzyme kinetics.

First, we provide evidence of a reduction in PEPCK Vmax after 3 days of PBDE treatment that persisted through 28 days. To our knowledge, four papers exist that have investigated the effect of xenobiotic exposure on hepatic PEPCK. Our findings are similar to work by Argaud et al. (1991) and Chauvin et al. (1996) who exposed rats to 100 mg/kg/day of Phenobarbital for two weeks and found a 50% decrease of PEPCK Vmax, expressed as μmol/min/g liver wet weight. We found a 40% reduction in Vmax (μmol/min/g liver wet weight) in the treatment group compared to control at 14 days. The researchers also found a 30% decrease in Km in treatment rats compared to control (Argaud et al. 1991; Chauvin et al. 1996) In contrast to their findings, we found there was no disruption of PEPCK Km. This observation in combination with the reduction in Vmax, suggests a disruption in transcription and/or translation of the PEPCK gene or protein, respectively. This would need to be confirmed by determining protein levels of PEPCK following PBDE treatment. Our findings are also consistent with work by

Viluksela et al. (1999) who exposed rats to a range of 50-9600 μg/kg of TCDD for 10 days and found a maximum Vmax reduction of 50% with the highest dose in male rats compared to control (Viluksela et al. 1999). Vijayan et al. (2006) also found a 66% reduction of PEPCK activity in the treatment group compared to control Anadromous arctic char following exposure to 100 mg/kg of Aroclor 1254 (Vijayan et al. 2006).

Second, we examined the physiological effects of PBDE treatment on glucose metabolism by analyzing fasting glucose, insulin and C-peptide values. Despite the decrease in PEPCK Vmax, we found fasting glucose was not impacted by PBDE treatment. This is consistent with the work of She et al. (2003) using liver PEPCK-knockout mice. The liver PEPCK-knockout mice maintained fasting blood glucose levels that were similar or even higher than control. She et al. (2003) suggest that this maintenance of blood glucose levels could be attributed to an up-regulation of gluconeogenesis in extrahepatic tissues such as the kidney (She et al. 2003). Work by Hakimi et al. (2005) confirms the importance of PEPCK in the kidney. Hepatic and kidney PEPCK knockout mice did not gain weight one day after birth, had 60% lower glucose levels, twice the level of triglycerides in the blood and liver and three times the level of ketone bodies compared to control (Hakimi et al. 2005).

Our findings are in contrast to Viluksela et al. (1999) who saw a decrease in plasma glucose of treatment rats exposed to TCDD compared to control and Vijayan et al. (2006) who, in addition to a reduction of PEPCK Vmax, also found a 55% reduction of plasma glucose in fish following exposure to PCB. These discrepancies are most likely due to using different species models (fish vs. rat), different chemicals (TCDD or PCB

vs. PBDE), different dosages (9600 μg/kg TCDD or 100 mg/kg PCB vs. 14 mg/kg DE-71) and different time periods (10 days or 4 months vs. 28 days).

Unpublished work done in our lab found insulin was 30% lower after 28 days of PBDE treatment compared to control, although this was not statistically significant ($p = 0.075$) (Allgood 2009). Allgood used the same dose of DE-71 for the same length of time and the same species of Wistar rat. Although the treatment effect was also not significant in our work, we found a 24% decrease in insulin at 28 days. It is possible that this decrease could be exacerbated by treatment time.

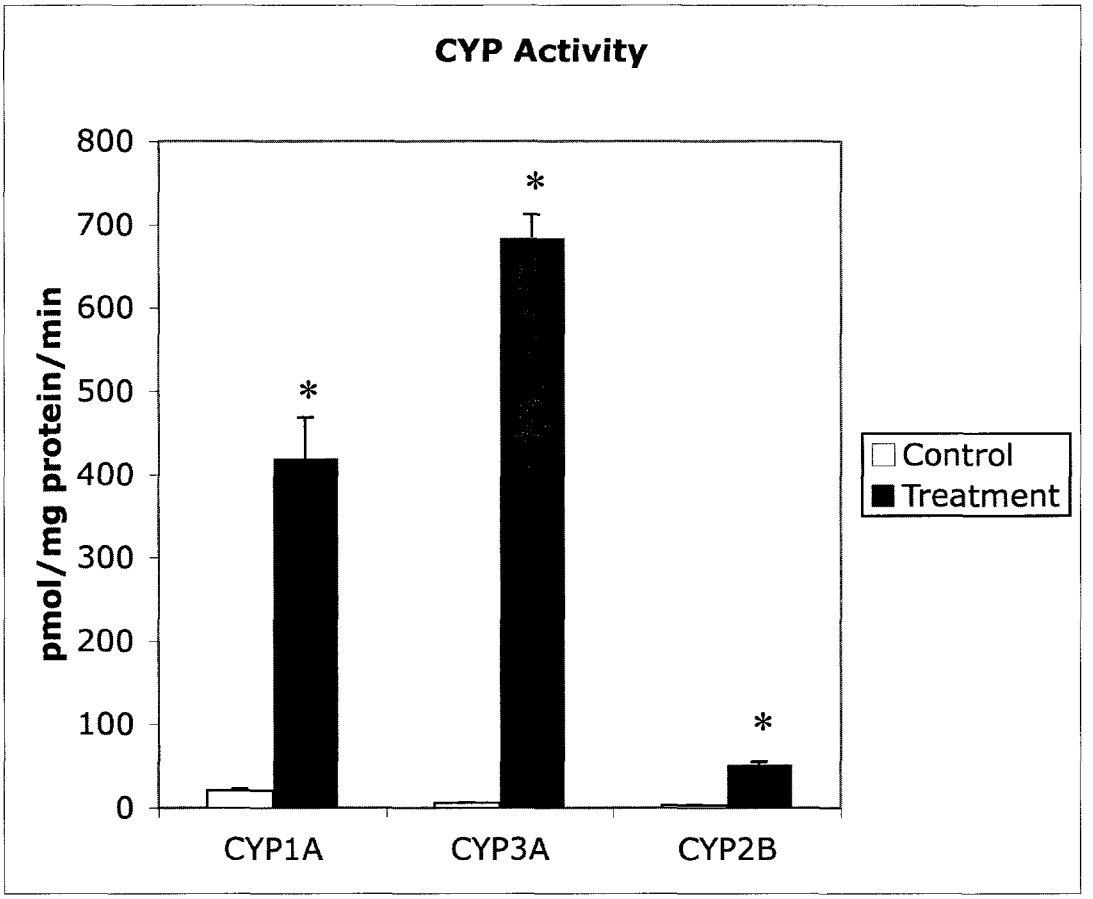
There are several possible mechanisms by which PBDE exposure could affect PEPCK activity. First, it is known that PBDE exposure decreases thyroid hormone, T_4, in rats (Hallgren and Darnerud 2002; Zhou et al. 2001; Zhou et al. 2002). Thyroid hormone normally diffuses through the nucleus and binds to its nuclear receptor (NR). The NR-thyroid hormone complex will then bind to the thyroid response element on the PEPCK gene promoter (Yabaluri and Bashyam 2010). Therefore, PEPCK gene promotion could be reduced due to low levels of thyroid hormone. Another possible mechanism is via another nuclear receptor, pregnane X receptor (PXR). It is known that PBDEs are a ligand to PXR (Fery et al. 2009; Pacyniak et al. 2007). It is also known that activated PXR results in a reduced gene expression of PEPCK (Bhalla et al. 2004; Zhou et al. 2006) via direct interaction with the forkhead transcription factor, FOXO1, a PEPCK gene activator (Kodama et al. 2004). Therefore, a plausible mechanism of action is that PBDE exposure activates PXR, inhibiting FOXO1 from binding to the response element on the PEPCK gene promoter, causing a reduction in PEPCK gene and, ultimately, a reduction in PEPCK activity. This mechanism, however, requires validation.

The decreased activity of PEPCK combined with unaffected levels of glucose and a tendency toward lower levels of insulin following exposure to PBDEs suggests mild glucose metabolism disruption in rats. There is very limited evidence that this disruption occurs in humans. In 2008, a cross-sectional study using NHANES data examined the correlation between diabetes and serum levels of brominated flame retardant. There was a significant positive correlation between polybrominated biphenyl-153 (PBB-153) and diabetes (defined as fasting glucose levels of >126 mg/dL and non-fasting glucose levels of >200 mg/dL) and a monotonic association between PBDE-153 and diabetes (Lim et al. 2008). Correlation does not mean causation, however, and further investigation is warranted to determine if environmental chemicals are contributing to the diabetes epidemic occurring in the United States.

Our findings, in combination with existing literature, confirm that exposure to xenobiotics such as PB, PCB and PBDEs affect glucose metabolism by decreasing PEPCK activity and, in some cases, altering plasma glucose and insulin levels. Future work should focus on the mechanism of this disruption by examining protein and mRNA expression of PEPCK following PBDE exposure to determine if the disruption occurs at the transcriptional and/or translational level.

APPENDIX A

Figure 1: Effect of PBDE treatment on cytochrome P450 enzymes: CYP1A, -3A and -2B.



This series of experiments examined the effect of *in vivo* PBDE exposure of rats on cytochrome P450 (CYP) enzyme activity. In summer of 2010, sixteen male Wistar rats were gavaged with two types of treatment: corn oil or corn oil with 14 mg/kg PBDE for 28 days. They has access to food from 8 AM to 5 PM and water *ad libitum*. Approximately 1.5 g of liver from animals were sent to North Carolina for CYP activity analysis. CYP activity was measured via a fluorometric assay as described previously (Szabo et al. 2009). CYP activity was significantly induced by treatment compared to control (* $p < 0.05$ compared to control). CYP1A was induced in the treatment group by 95%, CYP3A by 99% and CYP2B by 94% on average compared to control.

These data confirm that PBDE exposure induces hepatic CYP activity in rats.

APPENDIX B

University *of* New Hampshire

Research Integrity Services, Service Building
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Fax: 603-862-3564

23-May-2011

Carey, Gale B
Molecular, Cellular & Biomedical Sciences, Kendall Hall
Durham, NH 03824

IACUC #: 110406
Project: Polybrominated Diphenyl Ethers and Diabetes
Category: C
Approval Date: 18-May-2011

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category C on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the research potentially involves minor short-term pain, discomfort or distress which will be treated with appropriate anesthetics/analgesics or other assessments.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jessica A. Bolker, Ph.D.
Chair

cc: File

REFERENCES

Alaee M, Wenning RJ. 2002. The significance of brominated flame retardants in the environment: current understanding, issues and challenges. Chemosphere 46(5): 579-582.

Allgood EL. 2009. The effect of diet on polybrominated diphenyl ether (PBDE) exposure on adipocyte and whole body metabolism in male Wistar rats. M.S. Thesis, Durham: University of New Hampshire.

Andersson O, Blomkviet G. 1981. Polybrominated aromatic pollutants found in fish in Sweden. Chemosphere 10(9): 1051-1060.

Argaud D, Halimi S, Catelloni F, Leverve XM. 1991. Inhibition of gluconeogenesis in isolated rat hepatocytes after chronic treatment with phenobarbital. Biochem J 280 (Pt 3): 663-669.

Ballard FJ, Hanson RW. 1967. Phosphoenolpyruvate carboxykinase and pyruvate carboxylase in developing rat liver. Biochem J 104(3): 866-871.

Ballard FJ, Hanson RW. 1969. Purification of phosphoenolpyruvate carboxykinase from the cytosol fraction of rat liver and the immunochemical demonstration of differences between this enzyme and the mitochondrial phosphoenolpyruvate carboxykinase. J Biol Chem 244(20): 5625-5630.

Ballard FJ, Hopgood MF, Reshef L, Hanson RW. 1974. Degradation of phosphoenolpyruvate carboxykinase (guanosine triphosphate) in vivo and in vitro. Biochem J 140(3): 531-538.

Beale EG, Chrapkiewicz NB, Scoble HA, Metz RJ, Quick DP, Noble RL, et al. 1985. Rat hepatic cytosolic phosphoenolpyruvate carboxykinase (GTP). Structures of the protein, messenger RNA, and gene. J Biol Chem 260(19): 10748-10760.

Bhalla S, Ozalp C, Fang S, Xiang L, Kemper JK. 2004. Ligand-activated pregnane X receptor interferes with HNF-4 signaling by targeting a common coactivator PGC-1alpha. Functional implications in hepatic cholesterol and glucose metabolism. J Biol Chem 279(43): 45139-45147.

Chauvin C, Brilloit-Petit C, Argaud D, Catelloni F, Velours J, Leverve XM. 1996. The inhibition of phosphoenolpyruvate carboxykinase following in vivo chronic phenobarbital treatment in the rat is due to a post-translational event. Eur J Biochem 238(1): 207-213.

Cimbala MA, Lamers WH, Nelson K, Monahan JE, Yoo-Warren H, Hanson RW. 1982. Rapid changes in the concentration of phosphoenolpyruvate carboxykinase mRNA in rat liver and kidney. Effects of insulin and cyclic AMP. J Biol Chem 257(13): 7629-7636.

Costa LG, Giordano G, Tagliaferri S, Caglieri A, Mutti A. 2008. Polybrominated diphenyl ether (PBDE) flame retardants: environmental contamination, human body burden and potential adverse health effects. Acta Biomed 79(3): 172-183.

Darnerud PO, Eriksen GS, Johannesson T, Larsen PB, Viluksela M. 2001. Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology. Environ Health Perspect 109 Suppl 1: 49-68.

Dunn RL, Huwe JK, Carey GB. 2010. Biomonitoring polybrominated diphenyl ethers in human milk as a function of environment, dietary intake, and demographics in New Hampshire. Chemosphere 80(10): 1175-1182.

Dunnick JK, Nyska A. 2009. Characterization of liver toxicity in F344/N rats and B6C3F1 mice after exposure to a flame retardant containing lower molecular weight polybrominated diphenyl ethers. Exp Toxicol Pathol 61(1): 1-12.

Fery Y, Buschauer I, Salzig C, Lang P, Schrenk D. 2009. Technical pentabromodiphenyl ether and hexabromocyclododecane as activators of the pregnane-X-receptor (PXR). Toxicology 264(1-2): 45-51.

Fischer D, Hooper K, Athanasiadou M, Athanasiadou I, Bergman A. 2006. Children show highest levels of polybrominated diphenyl ethers in a California family of four: a case study. Environ Health Perspect 114(10): 1581-1584.

Fritz JS, Schenk GH. 1969. Quantitative analytical chemistry. 2d ed. Boston,: Allyn and Bacon.

Gurney AL, Park EA, Liu J, Giralt M, McGrane MM, Patel YM, et al. 1994. Metabolic regulation of gene transcription. J Nutr 124(8 Suppl): 1533S-1539S.

Hakimi P, Johnson MT, Yang J, Lepage DF, Conlon RA, Kalhan SC, et al. 2005. Phosphoenolpyruvate carboxykinase and the critical role of cataplerosis in the control of hepatic metabolism. Nutr Metab (Lond) 2: 33.

Hakk H, Letcher RJ. 2003. Metabolism in the toxicokinetics and fate of brominated flame retardants--a review. Environ Int 29(6): 801-828.

Hale RC, Alaee M, Manchester-Neesvig JB, Stapleton HM, Ikonomou MG. 2003. Polybrominated diphenyl ether flame retardants in the North American environment. Environ Int 29(6): 771-779.

Hallgren S, Darnerud PO. 2002. Polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and chlorinated paraffins (CPs) in rats-testing interactions and mechanisms for thyroid hormone effects. Toxicology 177(2-3): 227-243.

Hanson RW, Garber AJ. 1972. Phosphoenolpyruvate carboxykinase. I. Its role in gluconeogenesis. Am J Clin Nutr 25(10): 1010-1021.

Hanson RW, Reshef L. 1997. Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. Annu Rev Biochem 66: 581-611.

Harrad S, Ibarra C, Diamond M, Melymuk L, Robson M, Douwes J, et al. 2008. Polybrominated diphenyl ethers in domestic indoor dust from Canada, New Zealand, United Kingdom and United States. Environment International 34: 232-238.

Harvey RA, Ferrier DR. 2011. Lippincott's illustrated reviews: Biochemistry. 5th ed. Philadelphia: Wolters Kluwer Health.

Hewitt NJ, Lecluyse EL, Ferguson SS. 2007. Induction of hepatic cytochrome P450 enzymes: methods, mechanisms, recommendations, and in vitro-in vivo correlations. Xenobiotica 37(10-11): 1196-1224.

Hod Y, Hanson RW. 1988. Cyclic AMP stabilizes the mRNA for phosphoenolpyruvate carboxykinase (GTP) against degradation. J Biol Chem 263(16): 7747-7752.

Hoppe AA, Carey GB. 2007. Polybrominated diphenyl ethers as endocrine disruptors of adipocyte metabolism. Obesity 15: 2942-2950.

Huwe J. 2005. Bioaccumulation of decabromodiphenyl ether (BDE-209) from the diet into Sprague-Dawley rats. . Organohalogen Compounds 67: 633-635.

Ihunnah CA, Jiang M, Xie W. 2011. Nuclear receptor PXR, transcriptional circuits and metabolic relevance. Biochim Biophys Acta 1812(8): 956-963.

Inoue K, Harada K, Takenaka K, Uehara S, Kono M, Shimizu T, et al. 2006. Levels and concentration ratios of polychlorinated biphenyls and polybrominated diphenyl ethers in serum and breast milk in Japanese mothers. Environ Health Perspect 114(8): 1179-1185.

Jansson B, Asplund L, Olsson M. 1987. Brominated flame retardants - ubiquitous environmental pollutants? Chemosphere 16: 2343-2349.

Kim YJ, Osako M, Sakai S. 2006. Leaching characteristics of polybrominated diphenyl ethers (PBDEs) from flame-retardant plastics. Chemosphere 65(3): 506-513.

King MW. 2011. Gluconeogenesis. Available: http://themedicalbiochemistrypage.org/gluconeogenesis.html [accessed 04/28/2011 2011].

Kodama S, Koike C, Negishi M, Yamamoto Y. 2004. Nuclear receptors CAR and PXR cross talk with FOXO1 to regulate genes that encode drug-metabolizing and gluconeogenic enzymes. Mol Cell Biol 24(18): 7931-7940.

Lee E, Kim TH, Choi JS, Nabanata P, Kim NY, Ahn MY, et al. 2010. Evaluation of liver and thyroid toxicity in Sprague-Dawley rats after exposure to polybrominated diphenyl ether BDE-209. J Toxicol Sci 35(4): 535-545.

Lim JS, Lee DH, Jacobs DR, Jr. 2008. Association of brominated flame retardants with diabetes and metabolic syndrome in the U.S. population, 2003-2004. Diabetes Care 31(9): 1802-1807.

Lorber M. 2008. Exposure of Americans to polybrominated diphenyl ethers. J Expo Sci Environ Epidemiol 18(1): 2-19.

McDonald TA. 2002. A perspective on the potential health risks of PBDEs. Chemosphere 46(5): 745-755.

Mercado-Feliciano M, Bigsby RM. 2008. The polybrominated diphenyl ether mixture DE-71 is mildly estrogenic. Environ Health Perspect 116(5): 605-611.

Nedelcheva V, Gut I. 1994. P450 in the rat and man: methods of investigation, substrate specificities and relevance to cancer. Xenobiotica 24(12): 1151-1175.

Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, et al. 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 6(1): 1-42.

Norris JM, Kociba RJ, Schwetz BA, Rose JQ, Humiston CG, Jewett GL, et al. 1975. Toxicology of octabromobiphenyl and decabromodiphenyl oxide. Environ Health Perspect 11: 153-161.

O'Brien RM, Noisin EL, Suwanichkul A, Yamasaki T, Lucas PC, Wang JC, et al. 1995. Hepatic nuclear factor 3- and hormone-regulated expression of the phosphoenolpyruvate carboxykinase and insulin-like growth factor-binding protein 1 genes. Mol Cell Biol 15(3): 1747-1758.

Orn U, Klasson-Wehler E. 1998. Metabolism of 2,2',4,4'-tetrabromodiphenyl ether in rat and mouse. Xenobiotica 28(2): 199-211.

Pacyniak EK, Cheng X, Cunningham ML, Crofton K, Klaassen CD, Guo GL. 2007. The flame retardants, polybrominated diphenyl ethers, are pregnane X receptor activators. Toxicol Sci 97(1): 94-102.

Petreas M, She J, Brown FR, Winkler J, Windham G, Rogers E, et al. 2003. High body burdens of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) in California women. Environ Health Perspect 111(9): 1175-1179.

Poulos TL. 1988. Cytochrome P450: molecular architecture, mechanism, and prospects for rational inhibitor design. Pharm Res 5(2): 67-75.

Poulos TL. 2005. Structural and functional diversity in heme monooxygenases. Drug Metab Dispos 33(1): 10-18.

Reshef L, Hanson RW, Ballard FJ. 1970. A possible physiological role for glyceroneogenesis in rat adipose tissue. J Biol Chem 245(22): 5979-5984.

Roesler WJ, Vandenbark GR, Hanson RW. 1988. Cyclic AMP and the induction of eukaryotic gene transcription. J Biol Chem 263(19): 9063-9066.

Roesler WJ, Vandenbark GR, Hanson RW. 1989. Identification of multiple protein binding domains in the promoter-regulatory region of the phosphoenolpyruvate carboxykinase (GTP) gene. J Biol Chem 264(16): 9657-9664.

Roper CS, Simpson AG, Madden S, Serex TL, Biesemeier JA. 2006. Absorption of [14C]-tetrabromodiphenyl ether (TeBDE) through human and rat skin in vitro. Drug Chem Toxicol 29(3): 289-301.

Sakai H, Kim EY, Petrov EA, Tanabe S, Iwata H. 2009. Transactivation potencies of Baikal seal constitutive active/androstane receptor by persistent organic pollutants and brominated flame retardants. Environ Sci Technol 43(16): 6391-6397.

Sanders JM, Burka LT, Smith CS, Black W, James R, Cunningham ML. 2005. Differential expression of CYP1A, 2B, and 3A genes in the F344 rat following exposure to a polybrominated diphenyl ether mixture or individual components. Toxicol Sci 88(1): 127-133.

Schecter A, Haffner D, Colacino J, Patel K, Papke O, Opel M, et al. 2010. Polybrominated diphenyl ethers (PBDEs) and hexabromocyclodecane (HBCD) in composite U.S. food samples. Environ Health Perspect 118(3): 357-362.

Schecter A, Pavuk M, Papke O, Ryan JJ, Birnbaum L, Rosen R. 2003. Polybrominated diphenyl ethers (PBDEs) in U.S. mothers' milk. Environ Health Perspect 111(14): 1723-1729.

She P, Burgess SC, Shiota M, Flakoll P, Donahue EP, Malloy CR, et al. 2003. Mechanisms by which liver-specific PEPCK knockout mice preserve euglycemia during starvation. Diabetes 52: 1649-1654.

Sjodin A, Hagmar L, Klasson-Wehler E, Kronholm-Diab K, Jakobsson E, Bergman A. 1999. Flame retardant exposure: polybrominated diphenyl ethers in blood from Swedish workers. Environ Health Perspect 107(8): 643-648.

Stafford CJ. 1983. Halogenated diphenyl ethers identified in avian tissues and eggs by GC/MS. Chemosphere 12: 1487-1495.

Stapleton HM, Kelly SM, Pei R, Letcher RJ, Gunsch C. 2009. Metabolism of polybrominated diphenyl ethers (PBDEs) by human hepatocytes *in vitro*. Environ Health Perspect 117(2): 197-202.

Szabo DT, Richardson VM, Ross DG, Diliberto JJ, Kodavanti PR, Birnbaum LS. 2009. Effects of perinatal PBDE exposure on hepatic phase I, phase II, phase III, and deiodinase 1 gene expression involved in thyroid hormone metabolism in male rat pups. Toxicol Sci 107(1): 27-39.

Talsness CE, Kuriyama SN, Sterner-Kock A, Schnitker P, Grande SW, Shakibaei M, et al. 2008. In utero and lactational exposures to low doses of polybrominated diphenyl ether-47 alter the reproductive system and thyroid gland of female rat offspring. Environ Health Perspect 116(3): 308-314.

Tompkins LM, Wallace AD. 2007. Mechanisms of cytochrome P450 induction. J Biochem Mol Toxicol 21(4): 176-181.

Vijayan MM, Aluru N, Maule AG, Jorgensen EH. 2006. Fasting augments PCB impact on liver metabolism in anadromous arctic char. Toxicol Sci 91(2): 431-439.

Viluksela M, Unkila M, Pohjanvirta R, Tuomisto JT, Stahl BU, Rozman KK, et al. 1999. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on liver phosphoenolpyruvate carboxykinase (PEPCK) activity, glucose homeostasis and plasma amino acid concentrations in the most TCDD-susceptible and the most TCDD-resistant rat strains. Arch Toxicol 73(6): 323-336.

von Meyerinck L, Hufnagel B, Schmoldt A, Benthe HF. 1990. Induction of rat liver microsomal cytochrome P-450 by the pentabromo diphenyl ether Bromkal 70 and half-lives of its components in the adipose tissue. Toxicology 61: 259-274.

Wahl M, Guenther R, Yang L, Bergman A, Straehle U, Strack S, et al. 2010. Polybrominated diphenyl ethers and arylhydrocarbon receptor agonists: Different toxicity and target gene expression. Toxicol Lett 198(2): 119-126.

Watanabe O, Kashimoto T, Tatsukawa R. 1987. Polybrominated biphenyl ethers in marine fish, shellfish and river and marine sediments in Japan. Chemosphere 16: 2389-2396.

Wu N, Herrmann T, Paepke O, Tickner J, Hale RC, Harvey E, et al. 2007. Human exposure to PBDEs: associations of PBDE body burdens with food consumption and house dust concentrations. Environmental Science & Technology 41(5): 1584-1589.

Yabaluri N, Bashyam MD. 2010. Hormonal regulation of gluconeogenic gene transcription in the liver. J Biosci 35(3): 473-484.

Zhang W, Patil S, Chauhan B, Guo S, Powell DR, Le J, et al. 2006. FoxO1 regulates multiple metabolic pathways in the liver. J Biol Chem 281(15): 10105-10117.

Zhou J, Zhai Y, Mu Y, Gong H, Uppal H, Toma D, et al. 2006. A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. J Biol Chem 281(21): 15013-15020.

Zhou T, Ross DG, DeVito MJ, Crofton KM. 2001. Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. Toxicol Sci 61(1): 76-82.

Zhou T, Taylor MM, DeVito MJ, Crofton KM. 2002. Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. Toxicol Sci 66(1): 105-116.