INVESTIGATING THE EFFECTS OF GESTATIONAL EXPOSURE TO THE FLAME RETARDANT, TRIPHENYL PHOSPHATE, ON MATERNAL LIVER IN C57BL/6 MICE

by

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Abstract

Due to the recent banning of polybrominated diphenyl ethers (PBDE), the use of other flame retardants has increased, including triphenyl phosphate (TPP), an organophosphate ester flame retardant, which is contained in many consumer and industry products. As such, there is widespread exposure to TPP globally, including pregnant women. At present, data and knowledge surrounding the toxicological effects of TPP is limited, but some studies suggest endocrine and metabolic disrupting effects. In this study we examined the effects of gestational exposure to TPP (0, 5, 25, and 50 mg/kg on gestational day 8, 10, 12, and 14 via intraperitoneal injection) on the maternal liver of C57BL/6 mice through analysis of mRNA expression of genes involved in the insulin-like growth factor (Igf) 1 signaling pathway, including Igf1, its receptor, Igf1r, and downstream signaling proteins: insulin receptor substrates (Irs) 1 and 2 along with expression of peroxisome proliferator-activated receptor alpha (Ppar-α), a gene important in glucose and lipid metabolism, and carboxylesterase (CE) activity, an enzyme important in lipolysis and lipid metabolism. Using quantitative real-time PCR we determined that exposure to TPP resulted in no significant changes in the expression of Igf-1r, Irs-1, and Ppar-α. However, there was a significant decrease in the mRNA expression of Igf-1 and Irs-2. These findings suggest that gestational exposure to TPP may cause toxic endocrine and metabolic disrupting effects. Based on these findings, further studies are warranted to examine the associations between widespread TPP exposure and levels of Igf-1, Igf-1r, Irs-1, Irs-2, and Ppar-α in the potential development of maternal and fetal toxicity. Additionally, a CE activity assay was conducted with no significant changes observed between TPP treatment groups. Taken together, these results suggest that TPP may alter mRNA expression of genes and further research is required into the effect of TPP on both mRNA expression and CE activity.
Co-Authorship

The research conducted in this thesis was performed by the candidate, Victoria E. Restivo, under the supervision of Dr. Louise M. Winn and a postdoctoral fellow, Dr. Nicola A. Philbrook.
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List of Common Abbreviations

°C  Degrees Celsius
µg  Microgram
µL  Microlitre
Akt  Protein kinase B
AU  Activity Units
BHT  Butylated hydroxytoluene
BPA  Bisphenol A
BSA  Bovine serum albumin
cDNA  Complementary deoxyribose nucleic acid
CE  Carboxylesterase
CPF  Chlorpyrifos
Ct  Cycle threshold
CYP  Cytochrome P450
DAG  Diacylglycerol
DDT  Dichlorodiphenyltrichloroethane
DNA  Deoxyribose nucleic acid
EDTA  Ethylenediamine tetra acetic acid
g  Gravity
GH  Growth hormone
Hprt  Hypoxanthine guanine phosphoribosyl transferase
i.p.  Intraperitoneal
Igf-1  Insulin-like growth factor 1
Igf-1r  Insulin-like growth factor 1 receptor
Irs-1  Insulin receptor substrate 1
Irs-2  Insulin receptor substrate 2
LC50  Lethal concentration for 50% of the population
LEC  Lowest effective concentration
mRNA  Messenger RNA
mg/kg  Milligram/kilogram
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>PBDE</td>
<td>Polybrominated diphenyl ethers</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated biphenyl</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>$Ppar-\alpha$</td>
<td>Peroxisome proliferator activated receptor alpha</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>TBP</td>
<td>Tributyl phosphate</td>
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<tr>
<td>TPP</td>
<td>Triphenyl phosphate</td>
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Introduction and Literature Review

Flame retardants are a group of chemicals that are added to manufactured materials, finishes, and coatings for the purpose of preventing the spread of fire. Flame retardants are typically included in products to meet flammability standards and can be found in both industry and consumer products, specifically plasticizers, textiles, electrical/electronics, foam seating, bedding, furniture, furnishings, lubricants, greases, building materials, hydraulic fuels, and insolation (van der Veen & de Boer, 2012). Flame retardants adhere to the surface of the object they are protecting thus reducing flammability by interfering with the material’s combustion; when exposed to heat the flame retardant decomposes before the polymer it is protecting, preventing flammable gasses from forming (Rahman et al., 2001).

There are several classes of flame retardants including mineral, inorganic, organohalogen compounds, and organophosphorus compounds. Inorganic brominated flame retardants, specifically polybrominated diphenyl ethers (PBDE), were the cheapest flame retardants available in the past and as such, PBDEs were used extensively to decrease the flammability of objects. PBDE’s were eventually discontinued in Canada and globally as they were found to have effects of toxicity, persistence, and bioaccumulation resulting in health and environmental risks (Rahman et al., 2001 & Morris, 2014). Prior to their discontinuation, PBDEs were used extensively around the world due to their reasonable price and thermal stability relative to other flame retardants (Rahman et al. 2001). PBDE’s are resistant towards acids and bases, heat and light, along with reducing and oxidizing compounds (Allchin et al., 1998) and as a result of their resistant nature, are not readily decomposed and are persistent in the environment. In humans, PBDE’s do not move through the biotransformation phase and as a result are not easily excreted, thus PBDE’s are found to be persistent in the body and play a role in interfering with hormone
regulation (De Boer et al., 1998). Previous to the heavy use of PBDEs, the use of polychlorinated biphenyl (PCB) and dichlorodiphenyltrichloroethane (DDT) were relied upon. When these compounds were discontinued due to their detrimental environmental and health effects, the use of PBDEs increased and as a result human exposure to PBDEs also increased. In fact, it has been determined that between 1981 and 1992 the amount of PBDE levels found in human breast milk increased from 0.21 to 16.24 ng/g milk lipid (Renner, 2000). In a study by Rahman et al. (2001), it was found that human resistance to metabolizing PBDEs resulted in accumulation and encouraged malignant cell division and DNA replication. Finally, in 2004, PBDEs were found to be a major concern for human health in Europe and the United States, and for this reason PBDEs are no longer manufactured (Environment Canada, 2013).

Similar to the increased use of PBDEs following the discontinued production of PCBs and DDT, the banning of PBDEs has led to the increased use of many alternative flame retardants, specifically aromatic organophosphate esters like triphenyl phosphate (TPP). TPP, the compound of interest in this research, is a molecule containing both a phosphorous group and aromatic group that has become more frequently produced. Although PBDEs are not the focus of this study, the background information surrounding PBDEs is helpful in understanding the potential risks associated with the current widespread use of TPP as its alternative. In a comparative study by Behl et al. (2016) it was found that both PBDE and TPP exposure in Caenorhabditis elegans inhibited major biological endpoints; TPP was observed to have comparable potency to the PBDEs with a lowest effective concentration (LEC) of 0.16 µM. Furthermore, Behl et al. (2016) also showed that TPP inhibited both larval development and reproduction, suggesting that TPP may have similar levels of toxicity to PBDEs. As such, there are many concerns surrounding TPP’s potential toxicity including neurotoxic, teratogenic, and
metabolic effects.

**Exposure:**

TPP is dissolved into material or coating, but is not chemically bound to the structure it is protecting, leaving it free to diffuse through the material to the surface allowing some to escape. Since TPP does not adhere permanently to the structure it is added to, but instead is slowly released from the surface and replaced by more TPP diffusing from the material, it can escape into air, surface water, drinking water, sediment, wildlife, and human tissue (Sundkvist et al., 2010). Data on TPP toxicity is limited, but some studies have been conducted in animals including *Daphnia magna*, fish, zebrafish embryos, mice, and rats, in addition to other studies assessing the effect of environmental exposure to TPP in humans (Lin, 2009, Betts, 2013, Morris et al, 2014, Liu et al., 2013 & Patisaul, et al., 2013).

It has been found that adults and children may be exposed to 5.8 and 57 µg/ kg/day, respectfully, to total organophosphates; the increase in children may be a result of increased hand to mouth activity along with proximity to and ingestion of dust in the home (Marklund et al., 2005). In locations that are held to strict fire safety standards, like public infrastructure, the exposure levels to TPP were elevated by up to four fold (Marklund et al., 2005).

The broad application of TPP results in its widespread environmental exposure in the population, including pregnant women, but TPP has not been found to be overtly carcinogenic, neurotoxic, or teratogenic to humans (Betts, 2013). Human exposure can be examined by measuring metabolite levels in urine (Meeker, et al., 2013); organophosphates and their metabolites are detectable in 96% of human urine samples tested (Morris, 2014). Furthermore, a study by Hoffman, et al. (2013), studied urinary TPP metabolites throughout pregnancy and results showed that exposure to TPP was widespread.
**Effects:**

Although the human exposure to TPP is low in the environment, the toxicity of TPP may be greater than other chemicals. In a study in *D. magna*, by Lin (2009) on the comparative effect of TPP and another flame retardant, tributyl phosphate (TBP), both highly produced chemicals in the European Union, it was confirmed that although TPP exposure is lower it can have extremely toxic effects. TBP is also an organophosphorous flame retardant and is mainly used as a solvent in the manufacturing of plastics, textiles, and clothing (Thomas & Maccaskie, 1996). Lin (2009) determined that TPP toxicity, measured by lethal concentration (LC50), was ten times higher than TBP (0.51 and 5.48 mg/L respectively), indicating that although TPP is found in lower concentrations in the environment it has comparable toxicity with chemicals found in high concentrations like TBP.

It is clear that humans are widely exposed to TPP, thus during gestation pregnant mothers are equally exposed. Teratogenic effects following exposure to TPP during gestation are largely unknown due to the complicated relationship between the maternal-fetal unit, however it is well known that compounds that the mother is exposed to can be transferred across the placenta into the fetus. This is concerning for both mother and child, especially since this transfer may occur during a time of critical fetal growth and development resulting in body system damage, including but not limited to the central nervous system, digestive system, skeletal system, and endocrine systems. A cohort study conducted by Roze et al. (2009) tested pregnant women for their blood level concentrations of organohalogen flame retardants in the thirty-fifth week of pregnancy, and later followed up with the children at 5-6 years old. Their findings determined that blood brominated flame retardant levels were correlated with neurotoxic effects manifested in lower fine manipulative abilities, and reduced attention (Roze et al., 2009). This supports the
idea that there is placental transfer of flame retardants and highlights the concerns associated with TPP exposure during gestation and effects on child development. Furthermore, transplacental transfer of flame retardants, specifically Firemaster 550 - a flame retardant that is made up of a mixture of components including TPP, has been observed through the detection of compounds in both the dam and offspring hepatic tissue following gestational exposure in rats (Patisaul et al., 2013). Furthermore, studies exploring gestational exposure are complex due to the pregnancy-related changes in metabolism. In a study exploring exposure to bisphenol A (BPA), a compound found in flame retardants, it was found that urine concentrations of BPA were 26% higher in pregnant women than non-pregnant women and furthermore, concentrations of BPA in urine samples of the pregnant women studied increased by 33% while pregnant compared to pre-pregnancy (Mahalingaiah et al., 2008). These findings highlight the complicated changes that occur during pregnancy may affect distribution, metabolism and clearance, for example increased glomerular filtration, creatinine clearance and urine output along with changes in toxicant metabolism (Mahalingaiah et al., 2008).

TPP has also been thought to cause toxic effects in humans as a result of TPP’s high lipophilicity and resistance to excretion from the body (Rahman et al., 2001). Given these chemical properties, TPP can accumulate in the body and has been detected in human adipose tissue and blood (Jensen, 1987). Recent studies suggest that TPP has metabolic and endocrine disrupting effects following exposure, for example altered hormone levels and reduced semen quality in men (Meeker & Stapleton, 2010). More specifically, Morris et al. (2014), found that exposure to TPP inhibits carboxylesterase (CE) enzyme activity in mouse liver tissue and alters aspects of lipid metabolism causing hypertriglyceridemia. CEs found in the liver are important in phase 1 metabolism of toxicants (eventually leading to excretion of the substance), and have a
role in lipid metabolism. Morris et al. (2014) found that when mice were exposed to TPP, changes in the liver lipidome were observed, including increased diacylglycerols (DAG), along with increased levels of phospholipids, lysophospholipids, and neutral lipids.

Preliminary unpublished data from the Winn lab suggests that there is evidence of metabolic and endocrine disrupting effects after exposure to TPP, specifically increased gene expression of insulin-like growth factor-1 (Igf-1), insulin-like growth factor-1 receptor (Igf-1r), insulin receptor substrate-1 (Irs-1), insulin receptor substrate-2 (Irs-2) and peroxisome proliferator-activated receptor alpha (Ppar-a). Additionally, in a study by Liu et al. (2013), it was found that exposure to TPP in zebrafish embryos/larvae resulted in up-regulated mRNA expression of Ppar-a. Furthermore, it is well known that Igf-1, Igf-1r, Irs-1, Irs-2, and Ppar-a are important genes involved in lipid metabolism, leading to many endocrine and metabolic effects.

Mechanisms of Interest:

The proposed mechanism of TPP metabolic toxicity being examined in this study is through the insulin/Igf signaling pathway, as shown in Figure 1. Insulin like growth factor protein, Igf-1, is synthesized in the liver and acts as an endocrine hormone leading to growth and development; Igf-1 is very important for metabolism. Growth hormone (GH) acts as a trophic regulating hormone that stimulates the secretion of Igf-1 from the liver. Metabolically, GH and Igf-1 are anabolic and promote protein synthesis, which is essential for tissue growth. Igf-1 signaling occurs through its receptor Igf-1r, which is comprised of two extracellular alpha subunits linked by disulfide bonds with hormone binding sites and two beta subunits (Laviola et al., 2007). Igf-1r has a high affinity for Igf-1 and when bound initiates phosphorylation of the beta subunit, which acts as a site for Irs-1 and Irs-2. Irs-1 and Irs-2 proteins are homologous and
are both able to sit in the active site of Igf-1r (Gallagher et al., 2010). Once Irs-1 and Irs-2 are bound they undergo tyrosine phosphorylation by the insulin receptor, and act as binding sites for other proteins leading to the activation of downstream signaling of the phosphatidylinositol-3 kinase (PI3K) pathway and activation of protein kinase B (Akt) (Bondy, Cheng, Zhong, & Lee, 2006). Akt induces glycogen and protein synthesis and is involved in lipid synthesis and insulin-stimulated glucose uptake (Wong, & Sul, 2010). Furthermore, this pathway is controlled by negative feedback and when Irs proteins are phosphorylated they leave the active site of Igf-1r, stopping all downstream effects through the PI3K pathway (Paz, et al., 1997). As such, it is well characterized that altered insulin and Igf signaling is related to metabolic dysregulation including impaired glucose metabolism, increased gluconeogenesis, and insulin resistance. There are several metabolic disorders associated with the insulin/Igf signaling pathway including type 2 diabetes and metabolic syndrome (Glund & Zierath, 2005 & Kahn, 1994).

Additionally, Ppar-α is a nuclear receptor responsible for glucose and lipid metabolism, playing an important role in the regulation of genes involved in lipid metabolism and is linked to diseases such as hyperlipidemia, diabetes, and obesity (Contreras, Torres, & Tovar, 2013). Lipid and lipid derived molecules like polyunsaturated fatty acids and xenophobic drugs, bind to Ppars, which are then transported into the nucleus, bind to an element on the promoter region and activate the transcription of genes required for glucose and lipid metabolism, along with cytochrome P450 (CYP) in the liver. Ppar-α is highly expressed in the liver where it acts as a lipid sensor responding to fatty acids by stimulating the transcription of specific genes including genes involved in beta-oxidation, fatty acid uptake and binding, and lipoprotein assembly and transport (Reddy & Rao, 2006). Furthermore, in a study by Yakar et al. (2004), a link between the insulin/Igf-1 signaling pathway was explored; it was found that when GH, an activator of Igf-
1, was increased a decrease in Ppar-α occurs, which results in decreased acyl-CoA oxidase and fatty acid transporters, thus impacting lipid metabolism. In a study by Liu (2012), after exposure to TPP in zebrafish there was up-regulation of Ppar-α mRNA, suggesting that the Ppar-α pathway may be the primary pathway affected by TPP. There have also been studies that show that Ppar-α is associated with the progression of type 2 diabetes (Conteras, Torres, & Tovar, 2013).

CE enzymes are also found in the liver and are responsible for the phase 1 metabolism of toxicants. Additionally, CEs play a role in lipid metabolism converting triacylglycerides to glycerol and fatty acids. Ross and Borazjani (2007) determined that CEs control the lipolysis of triglycerides in mouse adipose tissues and if left unregulated can result in high levels of fatty acids in the circulation along with lipotoxicity. Furthermore, TPP has been found to cause changes in the lipidome of the liver, manifesting in increased diacylglycerols (DAGs), increased levels of phospholipids, lysophospholipids, and neutral lipids (Morris, 2014). Adverse metabolic outcomes may occur as a result of TPP interference in metabolic pathways such as lipotoxicity, which can lead to obesity and type 2 diabetes, impaired insulin production and resistance, and glucose intolerance.
Figure 1: The insulin/Igf-1 pathway.

Igf-1 (synthesized in and secreted from the liver) binds to and phosphorylates the receptor Igf-1r, which binds Irs-1 and Irs-2. Irs-1 and Irs-2 act as binding sites for other proteins leading to the downstream signaling of the PI3K pathway and activation of Akt, inducing glycogen and protein synthesis, lipid synthesis, and insulin stimulated glucose uptake. When Irs proteins are
phosphorylated they leave the binding site stopping all downstream effects through the PI3K pathway.

**Research Question:**

Although TPP has not been found to be overtly toxic, while limited there are some studies demonstrating possible effects of TPP following gestational exposure in animal models. To further investigate the possible effects of gestational exposure to TPP, the Winn Laboratory exposed pregnant mice during gestation to TPP and collected both maternal and fetal tissue. My role in this larger study was to investigate a portion of the metabolic disrupting effects of TPP on the maternal liver tissue. Metabolic toxicity was studied by examining the effects on lipid metabolism through the insulin/IGF signaling pathway and CE enzyme activity.

The overall goal of this thesis research was to test the following hypothesis:

1. As a result of exposure to TPP, CE activity will be inhibited and mRNA expression of *Igf-1, Igf-1r, Irs-1, Irs-2, and Ppar-a* will be increased in maternal liver.

The objectives of this thesis research were:

1. To isolate maternal liver RNA from TPP exposed and non-exposed pregnant mice and use qRT-PCR to determine *Igf-1, Igf-1r, Irs-1, Irs-2, and Ppar-a* mRNA expression.

2. To determine the effects of TPP on CE activity in maternal liver by preparing microsomal fractions and performing a CE activity assay.
**Materials and Methods**

C57BL/6 female mice (n=51) were impregnated (denoted gestational day 1) and exposed on gestational days 8, 10, 12, and 14 to varying doses of TPP purchased from Sigma-Aldrich (Oakville, Ontario), via intraperitoneal injection in corn oil. TPP exposure groups included: oil/0 mg/kg (n=21), 5 mg/kg (n=10), 25 mg/kg (n=10), and 50 mg/kg (n=10). On gestational day 19 dams and fetuses were euthanized, pups were removed and organs were retained and stored in a -80 ºC freezer for further analysis into the effect of TPP exposure. All methods pertaining to mouse care and control were carried out in accordance to the guidelines of the Queen’s University Animal Care Committee and the Canadian Council on Animal Care with the welfare of the mice in mind throughout. Although the focus of this research is based on TPP exposure and effects on maternal liver further research is being conducted utilizing the dam and fetus by the Winn lab in the Department of Biomedical and Molecular Sciences at Queen’s University.

**RNA Isolation:**

First, RNA isolation was conducted to determine gene expression in dams. The maternal liver was excised from the right lobe using a razor blade and RNA was subsequently isolated. With the use of the Qiagen RNeasy Mini Kit (Qiagen, Toronto, Ontario), RNA was isolated, the concentration was measured using a Biochrom NanoVue Plus Spectrometer to determine isolation accuracy, and the isolated RNA was run on agarose gel to determine purity. Following isolation, complementary DNA (cDNA) was synthesized by reverse transcription using the Applied Biosystems High Capacity cDNA Reverse Transcription master mix kit (Thermo Fisher, Waltham, Massachusetts) and subsequently used in quantitative real time PCR (qRT-PCR) as the template for amplification for Qiagen RT2 primers (Qiagen, Toronto, Ontario) for the genes *Igf-*1, *Igf-1r*, *Irs-*1, *Irs-*2, *Ppar-*α, and the control *Hprt*. Primers were mixed with SYBR Green PCR
Master Mix (Thermo Fisher, Waltham, Massachusetts) and RNAse free H$_2$O, along with 10 nM diluted cDNA for each sample (n=6) in each treatment group (0, 5, 25, and 50 mg/kg). Efficiency curves were created to show the reaction progress along with accuracy during experimentation (80 ≤ percent efficiency ≤ 120), and then qRT-PCR was conducted and mean cycle threshold (Ct) values were recorded and used to quantify mRNA using the delta delta Ct method for later statistical analysis. Gene expression (mRNA transcript levels) was normalized and graphed to the geometric mean of the control gene, Hprt, using the delta delta Ct method. This strategy is used to eliminate variation and allow comparison between the exposed and non-exposed samples.

**Carboxylesterase (CE) Activity Assay:**

Finally, the effect of TPP on CE activity was tested using a CE Spectrophotometric Activity Assay. Before beginning the assay liver microsome fractions were prepared at 0-4°C, using the protocol indicated in the Principles and Methods of Toxicology, 5th edition (Hayes, 2001). Livers were weighed and placed in four times their weight of buffer A (0.10 M Tris-acetate buffer (pH 7.4), 0.10 M KCl, 1.0 mM ethylenediamine tetraacetic acid (EDTA), and 20 µM butylated hydroxytoluene (BHT)), then homogenized with the use of a glass rod. The homogenate was centrifuged at $10^4 \times g$ for 20 minutes and the supernatant was saved. The supernatant was then centrifuged at $10^5 \times g$ for 60 minutes to give a microsomal pellet (the supernatant was discarded). A volume of buffer B (0.10 M potassium pyrophosphate buffer (pH 7.4), 1.0 mM EDTA, and 20 µM BHT) equal to the discarded supernatant was added to the pellet. Microsomes were then removed from the glycogen pellet by homogenization with a rod and re-centrifuged at $10^5 \times g$ for 60 minutes. The final supernatant was discarded and the resulting pellet was homogenized with a rod in buffer C (10 mM of Tris-acetate buffer (pH 7.4), 1.0 mM EDTA, and 20% glycerol) and
stored in a -80 °C freezer.

Following the microsomal preparations, the protein concentration was determined by conducting a Bradford Assay. First, seven standards were prepared using dilutions of bovine serum albumin (BSA) and phosphate-buffered saline (PBS) to make 800 µL and combined with 200 µL of Bio-Rad dye (Bio-Rad, Mississauga, Ontario). The mixture was then vortexed briefly and pipetted into 1.5 mL cuvettes and read by the Bio-Rad spectrophotometer at 595 nm. Once the standards were determined, microsomes were diluted to 1:2500 with PBS and combined with 200 µL of Bio-Rad dye, and protein concentration was measured.

Following the microsomal preparations, the activity assay was conducted using the continuous spectrophotometric assay detailed by Ross and Borazjani (2007). First, a 1 mM solution of p-nitrophenyl valerate in 50 mM Tris-HCl buffer at a pH 7.4 was prepared. 150 µL of this solution was pipetted into the wells of a 96 well plate. The plate was then incubated for 5 minutes at 37ºC in an absorbance plate reader, SpectraMax iD3. The liver fractions were then assayed and diluted with a 50 mM Tris-HCl buffer at a pH of 7.4 to make 5, 10, and 15 mg protein dilutions. Three protein concentrations were made to determine the concentration that had the most linear slope for later use in specific activity calculations. Following incubation, 150 µL of the diluted liver fractions are added to the wells. The formation of p-nitrophenolate is monitored at 450 nm for 5 minutes. Then the non-enzymatic rate is measured by adding 50 mM Tris-HCl buffer at a pH of 7.4 into the wells, and the slopes of each activity curve is converted to specific activities using the following equation: specific activity = (absorbance units, AU/min – [slope of activity curve])(mmol cm/13 AU x L - [extinction coefficient of p-nitrophenolate at pH 7.4] (0.0003 litres – [reaction volume in the well])) (1/χ mg protein – [total amount of protein in each well]) (1/1.0 cm – [light pathlength]) (10^3 µmol/mmol –[unit conversion factor]). Therefore,
CE activity was assessed using the rate of product formation of yellow coloured p-nitrophenolate from p-nitrophenyl.

**Statistical Analysis:**

ANOVAs were conducted for all analyses with the variable TPP exposures (0, 5, 25, and 50 mg/kg). Statistical analyses and graphs were produced using Prism 7 (Graph Pad, La Jolla, California). A sample size of six was used to determine mRNA expression, and a sample size of three was used to determine the CE activity. Statistical significance was stated for tests with $p \leq 0.05$. 

Results

There were no significant differences found in *Igf-1r* (p=0.4316), *Irs-1* (p=0.8594), and *Ppar-α* (0.0751) mRNA expression, while exposure to TPP resulted in significant decreases in mRNA expression in *Igf-1* (p=0.0034) and *Irs-2* (p=0.0134) as seen in Figure 2. Multiple comparisons were conducted and significant decreases between the control, 5 mg/kg (p=0.0049), 25 mg/kg (p=0.0049), and 50 mg/kg (p=0.0027) treatment groups were observed in *Igf-1* mRNA expression. Multiple comparisons were also conducted for *Irs-2* mRNA expression and significant decreases between the control, 5 mg/kg (p=0.0237), 25 mg/kg (p=0.0168), and 50 mg/kg (0.0146) treatment groups were observed. Furthermore, although non-significant, it was found that decreases in mRNA expression also occurred in progressive treatments for *Igf-1r* and *Irs-1*. *Ppar-α*’s mRNA expression showed a non-significant increase for the 5 mg/kg exposure, and a non-significant decreased in expression for the 25 and 50 mg/kg treatment groups when compared to the control. Additionally, there were no significant differences observed in CE activity after exposure to 0, 5, 25, or 50 mg/kg of TPP when normalized to the control (p=0.7880) evidenced in Figure 3.
Figure 2: The effect of TPP on C57BL/6 maternal mouse liver transcript levels.

The transcript levels of genes found in maternal mouse livers following exposure to 0 (n=6), 5 (n=6), 25 (n=6), and 50 (n=6) mg/kg of TPP in corn oil via intraperitoneal injection on gestational days 8, 10, 12, and 14. A) The relative Igf-1 transcript levels normalized to the control B) The relative Igf-Ir transcript levels normalized to the control C) The relative Irs-1 transcript levels normalized to the control D) The relative Irs-2 transcript levels normalized to the control E) The relative Ppar-α transcript levels normalized to the control. Asterisks signify a significant difference compared to the controls (p ≤ 0.05).

Figure 3: The effect of TPP on carboxylesterase activity.

The relative carboxylesterase activity of maternal mouse livers when exposed to 0 (n=3), 5 (n=3), 25 (n=3), and 50 (n=3) mg/kg of TPP in corn oil via intraperitoneal injection normalized to the control.
Discussion

Information surrounding the effects of TPP is limited, especially with respect to teratogenic effects. There have been some studies conducted in animal models following gestational exposure that suggest that TPP has metabolic and endocrine disrupting effects. In this study, C57/BL6 were impregnated and exposed during the gestational period to 0, 5, 25, and 50 mg/kg of TPP via intraperitoneal injection. Maternal liver was used to determine mRNA expression of \( \text{Igf-1, Igf-1r, Irs-1, Irs-2, and Ppar-\alpha} \) through qRT-PCR, along with CE activity activity through a CE activity assay. It was hypothesized that as a result of exposure to TPP, CE activity would be inhibited and mRNA expression of \( \text{Igf-1, Igf-1r, Irs-1, Irs-2, and Ppar-\alpha} \) would be increased in maternal liver. The expression of the aforementioned mRNA and the activity of CE is integral in maintaining the insulin/IGF pathway, necessary for metabolism, lipid regulation, and growth.

mRNA Expression:

In this study the hypothesis that TPP exposure in maternal C57BL/6 mice would increase expression of \( \text{Igf-1, Igf-1r, Irs-1, Irs-2, and Ppar-\alpha} \) was investigated. The mechanism of TPPs effect in humans is largely unknown, but based on a study conducted using zebrafish embryos (Liu et al., 2013), it was hypothesized that following TPP exposure up-regulated expression of mRNA genes associated with phase 1 and 2 metabolism, lipid regulation, and growth would be observed.

However, the results of this study showed decreased mRNA expression of hepatic insulin/IGF signaling genes: \( \text{Igf-1, Igf-1r, Irs-1, Irs-2, and Ppar-\alpha} \) after exposure to TPP in the maternal liver. It is well-characterized that chemicals can directly bind and block hormone receptors, blocking receptor activated gene transcription as a result (Cooper & Kavlock, 1997).
As such, TPP may work to block gene transcription resulting in overall decreases in mRNA. This type of mechanism is seen with other chemicals including ethanol, which has been shown to decrease maternal Igf-1 mRNA expression in maternal rats (Shankar et al., 2007). This was determined to be caused by ethanol inhibiting the autophosphorylation of Igf-1r, thus preventing all downstream signaling (Resnicoff et al., 1993). Furthermore, mice deficient in Igf-1 were found to have reduced Igf-1 in circulation with increased GH, which leads to insulin insensitivity and impaired Irs signaling (Cho, et al., 2001 & Yakar et al., 2001). With respect to our study, the observed decrease in mRNA expression of the genes under study after exposure to TPP suggests that TPP inhibits transcription in the signaling pathway causing deficiencies in downstream signaling. Therefore, it is possible that TPP causes inhibition of the insulin/Igf-1 signaling pathways in a similar manner to ethanol toxicity, however more research into its effects on the signaling pathway are required.

As shown in Figure 2, Irs-1 and Irs-2 mRNA expression also decreased in maternal liver following TPP exposure. Irs-1 deficiencies are also observed in male patients with type 2 diabetes (Krook, et al., 2000). Furthermore, while studies involving Irs-2 knockouts demonstrate impaired glucose homeostasis, insulin resistance in the liver and skeletal muscle, and decreased pancreatic beta-cell mass, resulting in the development of type 2 diabetes (Krook et al., 2000 & Withers et al., 1998). These studies highlight the importance of decreased expression of Irs-1 and Irs-2 in metabolism through the insulin/Igf-1 pathway and the development of type 2 diabetes.

These findings highlight the complex role of the insulin/IGF signaling pathway contributing to metabolic and endocrine dysfunction. The observed results may indicate that TPP impairs the insulin/IGF signaling pathway in the body and may be associated with the development of metabolic disorders. Future studies that focus on the mechanism of TPP-induced
toxicity within the insulin/IGF signaling pathway by examining the expression of phosphorylated proteins, Igf-1, Igf-1r, Iris-1, Iris-2, and Ppar-α are necessary in order to determine the full effects of TPP toxicity.

**CE Activity:**

With respect to the CE activity assay, it was hypothesized in this study that there would be an observed decrease in CE activity with exposure to increasing doses of TPP relative to the no treatment group. In this study, the results of the CE activity assay were insignificant, however, in a study by Morris et al. (2014) it was discovered that exposure to TPP in mice resulted in significant inhibition of CE enzyme activity.

CE enzymes are expressed in high concentrations in the liver and within adipose tissue and are known to be involved in lipolysis where stored triglycerides are hydrolyzed into fatty acids and used as energy (Wei, Gao & Lehner, 2007). In patients living with type 2 diabetes or insulin resistance, lipolysis is typically increased to increase the number of fatty acids in the tissue and glycerol in the liver. The reason for this is to enhance glucose production in order to provide sufficient energy to cells and tissues. This is consistent with findings that suggest that increased lipolysis is a central feature of insulin resistance and type 2 diabetes (Samuel & Shulman, 2012 & Cusi, 2010). As such, it has been observed that CE activity is further increased in subjects with increased white fat – type 2 diabetics and obese individuals (Dominiguez et al., 2014). Dominiguez et al. (2014) determined that increased CE activity resulted in a greater release of fatty acids into the circulation, which may be a response to insulin resistance in an attempt to raise glucose production in the body. Additionally, it was determined that CE enzymes act as a target for bioactive compounds that promote lipid storage in adipocytes and when treated with a CE inhibitor, insulin sensitivity and metabolic syndrome ensued
Therefore, it may be suggested that TPP, a bioactive compound, may result in the inhibition of CE activity and interfere with lipolysis; this interference may result in decreased energy provided to tissues and cells thus disrupting the metabolic pathway.

In this study, CE activity was investigated to determine whether there was a link to lipid metabolism, specifically to examine if CE activity would be inhibited after exposure to TPP. In a study by Quiroga et al. (2012) on mice, it was determined that CE plays a regulatory role in hepatic fat metabolism; increased lipogenesis and lipoprotein secretion was observed in knockout CE models, leading to hyperlipidemia, increased fat deposition, obesity, fatty liver, hyperinsulinemia, insulin insensitivity, and decreased energy expenditure. The knockout CE mice were observed to gain more weight in addition to white adipose tissue weight, larger adipocytes, along with increased lipid in the plasma compared with the wildtype mice (Quiroga et al., 2012). Furthermore, the knockout mice were discovered to have an increase of alanine aminotransferase (ALT), a well-known marker of liver damage (Quiroga et al., 2012). Mice deficient in CE also showed insulin insensitivity due to increased activation via phosphorylation of AKT, whose mechanism is shown in Figure 1. The increased lipid availability as a result of CE inhibition is likely the foremost contributor to insulin sensitivity manifesting in enhanced insulin signaling in the liver and compromised insulin signaling in peripheral tissue (Quiroga et al., 2012). Thus, it may be suggested that dysregulation in hepatic lipid metabolism is largely effected by CE inhibition.

In addition to research that utilized CE knockout models previously mentioned, there have been studies conducted that show the effects of flame retardants on carboxylesterase activity. In a study by Medina-Cleghorn et al. (2014), exposure to organophosphate chlorpyrifos (CPF) in 6 week old mice was found to inhibit CEs causing a pathophysiological response with

(Dominiguez et al., 2014).
respect to lipid metabolism. Specifically, changes in the liver lipidome, including several different classes of lipids, of mice were observed (Medina-Cleghorn, 2014). Furthermore, other flame retardants have been identified in the environment and found to have similar effects on CE activity. As evidenced in a study by Patisaul et al. (2013), Firemaster 550 – a common flame retardant also used as a replacement for PBDEs, was found to be a potential obesogen, contributor to metabolic syndrome, and cause endocrine disrupting effects in rats exposed to 100 or 1000 µg/day during gestation and lactation, including inhibition of CE activity in dams. CEs are directly implicated in the metabolism of TPP thus, when CEs are inhibited as a result of TPP exposure it may contribute to increased availability of TPP in the body leading to greater metabolic effects (Patisaul et al., 2013). Finally, the findings previously mentioned echo the research contributions of Morris et al. (2014), who found that TPP, inhibits specific liver CE enzymes irreversible phosphorylation off TPP to the CE active site resulting in alterations in hepatic lipid metabolism. Furthermore, Morris et al. (2014) suggests that TPP exposure causes hypertriglyceridemia, a condition of elevated triglycerides often caused by diabetes, and obesity, which further interrupts lipid metabolism, and that prolonged TPP exposures have the same result as CE deficient mice.

As mentioned previously, the study conducted by Morris et al. (2014) observed inhibition of CE activity while research conducted in this study did not observe a significant change in CE activity. This unexpected result may have been due to several factors. For one, Morris et al. (2014) studied mice who were not pregnant, whereas this research involved studying pregnant mice and TPP exposure during gestation. Exposure during gestation may potentially result in complex metabolic interactions that could have changed the outcome of our activity assay when compared to the study conducted by Morris et al. (2014). Changes is metabolism during
pregnancy can be further highlighted in a study by Villeneuve et al. (1971) where rabbits were bred and exposed to Aroclor (a potent PCB) daily for 28 days; upon conducting an activity assay using o-nitrophenylbutyrate researchers observed an increase in CE activity in maternal liver, a result that also contrasts the results presented by Morris et al (2014). Furthermore, the research conducted by Morris et al. (2014) was based on an exposure of 200 mg/kg of TPP administered via intraperitoneal injection, while this study exposed mice at 5, 25, and 50 mg/kg of TPP in order to assess the effects of chronic exposure to mice. Therefore, it is possible that the exposure used was too small to elicit an effect on the maternal liver. Additionally, CE activity has been observed to be reduced at certain time points during pregnancy with some subtypes being active at early time points and others later in pregnancy (Fortin, Aleksunes & Richardson, 2013). Since our assay examined all CE subtypes at once, it is possible that ones that did not have a change in activity masked ones that did, thus it is possible that a reduction in a specific CE activity occurred at different time point during gestation that went undetected during the final assay as a result of our liver collection period.

Further research is necessary to determine the effects of chronic low-dose exposure to TPP on subtype specific CE enzyme activity in both maternal and fetal mice to determine whether there are changes in effect during pregnancy. As such, the results of the CE activity assay conducted on fetal livers will be necessary to provide insight into the placental transfer of TPP and to determine whether placental transfer between the maternal-fetal unit plays a protective role with respect to the fetus during TPP exposure. Moving forward, it would be beneficial to manipulate the dose of TPP delivered to determine if CE inhibition is observed at higher exposures similar to that shown in the research conducted by Morris et al. (2014), and to collect liver at different time points during pregnancy to account for potential changes in CE
activity during pregnancy.

**Conclusions:**

In summary, the results of this study suggest that exposure to TPP during gestation may affect the mRNA expression of genes important in the insulin/Igf signaling pathway, while further investigation is required to determine the effect on CE activity. Taken together, this research points toward metabolic effects including insulin resistance, lipid metabolism, and the development of metabolic disorders as a result of TPP exposure. This study will add to current limited research in gestational exposure to TPP. Future studies should focus on the molecular mechanisms associated with impairment of the insulin/Igf pathway through up regulation of genes involved in these pathways and the effect of dosage on specific CE activity, along with the potential complexities associated with metabolic pathways during pregnancy.
References


Summary

Due to the recent banning of PBDE flame retardants, the use of other flame retardants has increased, specifically the use of organophosphate TPP in product manufacturing has significantly increased. There is very limited research surrounding the potential toxicity of TPP following exposure, but an increasing number of preliminary animal studies, including this study, suggest that TPP may have metabolic and endocrine disrupting properties. This may be particularly important following gestational exposure to TPP. It is known that pregnant women are exposed equally to TPP as the general population, but have more complicated metabolic enzyme expression and activity. Furthermore, the developing embryo will likely be at increased risk for toxicity given the lack of detoxifying capacity and unique signaling pathways that occur during this period of life. Our research into metabolic enzyme activity and lipid metabolism following maternal exposure in mice has indicated that after exposure to TPP there is:

1. A significant decrease in mRNA expression in *Igf-1* and *Irs-2*, and non-significant decreases in *Igf-1r, Irs-1, and Ppar-a*, which may be a result of inhibition within the insulin/IGF pathway

2. Results of the CE activity assay were insignificant and should be studied further

Our findings will add to the limited data associated with gestational exposure to this widespread chemical, however further research into the mechanisms of TPP toxicity is necessary in determining the full effects on metabolic and endocrine toxicity.