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Role of DDE Exposure in Type 2 Diabetes Mellitus: Association with Biochemical Markers and Diabetes Prevalence

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By

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Type 2 diabetes mellitus (T2D) is a metabolic disease characterized by hyperglycemia, insulin resistance, dyslipidemia, and beta cell dysfunction. T2D prevalence has been increasing with not all clear causes identified, while the use of synthetic chemicals has risen. Because genetics can only explain a small percentage of disease development, more attention is being given to associations of environmental chemical exposure and disease. Epidemiological evidence suggests environmental exposure to organochlorine compounds (OC) including dichlorodiphenyltrichloroethane (DDT) and its bioaccumulative metabolite dichlorodiphenyldichloroethylene (DDE) is associated with T2D prevalence and is hypothesized to play a role in contributing to T2D.

The purpose of this research was to perform an *in vitro* study of DDE exposure and its effect on liver hepatocyte and pancreatic beta cell functions that regulate biochemical markers implicated in T2D, and determine an association of DDE exposure with T2D from a population exhibiting a high prevalence of T2D living in an area once highly exposed to OC. Human blood samples from diabetics and non-diabetics were analyzed for any significant association of DDE levels with biochemical markers of T2D

and T2D presence. The *in vitro* effect of DDE exposure on the regulation of hepatocyte lipid metabolism and secretion with respect to triglyceride (TG), apolipoprotein B (ApoB), sortilin-1 (Sort-1), and microsomal triglyceride transfer protein (MTP) levels implicated in dyslipidemia was investigated. Finally, the *in vitro* effect of DDE exposure on the regulation of beta cell insulin secretion with respect to insulin, oxidative stress (ROS), prohormone convertase (PC), and pancreatic-duodenal homeobox-1(PDX-1) levels implicated in beta cell dysfunction was investigated.

Based on our results, DDE levels were not associated with an increased risk of T2D prevalence from this study population, although DDE levels were correlated with some biochemical markers of T2D. ApoB secretion, Sort-1 and MTP levels were increased after DDE exposure, while TG accumulation was decreased in hepatocytes. Insulin secretion and PC levels were increased after DDE exposure, while ROS and PDX-1 levels were increased but not significantly. Although no causative association of DDE exposure with T2D prevalence was found, a potential mechanism of DDE's effect on regulating biochemical markers of T2D was identified.

DEDICATION

I would like to dedicate this work first to my wife Tiffany Ward for supporting me throughout this academic journey and always being a shining light during difficult times. I also would like to dedicate this work to my unborn daughter, who has given me a renewed perspective and greater purpose in life. Finally, I would like to dedicate this work to my parents, Lola and the late Willie Ward Jr., for believing in me always and supplying me with the tools to succeed under any circumstances. Thank you and I love you all.

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First and foremost I would like to thank Dr. Janice Chambers for giving me the opportunity to pursue a doctoral degree in research. Her mentorship and guidance has allowed me to succeed towards the next step in my career. I am very grateful for this life changing opportunity. I would also like to thank all of my committee members for their professional contributions to me as a graduate student. Lastly, I would like to thank Mississippi State University for giving me one of the best educational experiences I could imagine.

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CHAPTER I

INTRODUCTION

Environmental Toxicology investigates the adverse effects of any biological, chemical, and physical agents on living organisms and their environment. Thus, this field of study explores how potentially toxic agents can directly or indirectly affect the physiology of organisms and assess the risk associated with these substances that may be hazardous to organisms and their environment. Indeed, the risks associated with these toxic agents may outweigh the benefits of their usage. The overall significant impact of this field of study is vital in increasing awareness for the better protection of human health and the environment. Therefore, this project addresses these main ideas and emphasizes the need for caution of future usage of potential environmental contaminants by investigating the effects of known environmental contaminant exposure on public health outcomes.

Historical Significance of DDE Exposure

Organochlorine compounds (OC) were synthetic chemicals used abundantly for agricultural purposes throughout the world until banned for their adverse effects on human health and the environment (Longnecker et al., 1997; EPA, 1999). Dichlorodiphenyltrichloroethane (DDT) was developed as one of the first OC in the 1940s with worldwide potential for multiple uses as an insecticide (Morgan and Roan, 1971). DDT was cheaply manufactured and used in agriculture and industry for the

benefits of insect control in institutions, homes, and crops, and in pest control because of its neurotoxic effects (CDC, 2012). DDT's mechanism of action is to prevent closure of the sodium channels in nerve cells causing them to fire rapidly producing hyper-excitability in an organism usually resulting in paralysis and death (Henderson and Woolley, 1970; Davies et al., 2007). DDT was a very effective insecticide that was administered for many years, until recognized for its adverse effects in the environment (Sharpe and Irvine, 2004; Turusov et al., 2002). Although DDT was banned for use in the United States by the 1970s, its bioaccumulative metabolite dichlorodiphenyldichloroethylene (DDE) persists for years in the environment (EPA, 2011). A study on OC exposure in the general U.S. population revealed that serum DDT levels were detected in the range of 2-58 ppb, while serum DDE levels were detected in the range of 1-378 ppb (Murphy and Harvey, 1985). DDT accumulates in soil and is slowly degraded by microorganisms over time to produce DDE (Fig. 1.). DDE, an environmental contaminant, is highly lipophilic, slowly excreted from the body, and levels in the body have the potential to increase throughout one's lifetime based on its biological half-life values (EPA, 1999). Exposure to residues of DDE is still possible and can be detected in air, water, soil, and food samples (Fisher et al., 2003). In organisms exposure to DDE residues has been detected in fat, urine, breast milk, and blood (EPA, 1999). Human exposure to DDE is mostly due to its existing presence in pre-exposure high agriculture areas for past insecticide/pesticide use and from its biomagnification throughout the food chain mainly from aquatic life and produce consumption (CDC, 2012). DDT's half-life in soil is around 22 days to 15-30 years depending on conditions, while DDT's and DDE's half-life is between 5-10 years in the body (ATSDR, 2002).

DDE exposure levels may represent a lifetime of exposure as individuals are potentially exposed to DDE residues on multiple occasions. Exposure to these organic pollutants is still possible and a worldwide threat due to their usage in underdeveloped countries including Africa and India as insecticides for malaria control and on food products that are imported to the United States (Fisher et al., 2003). Because genetics can only explain a small percentage of disease development, more attention is being given to associations of environmental chemical exposure and disease (Hunter, 2005). Data from the 1999-2002 National Health and Nutrition Examination Survey (NHANES) conducted by the Centers for Disease Control and Prevention (CDC) identified an association of OC with type 2 diabetes mellitus (T2D) (Lee et al., 2006; Lee et al., 2007). Based on data from the CDC NHANES study, DDE was detected in 99.7% of human plasma samples, and the highest concentration of DDE levels detected in human serum was around 16 μM (which was similar to concentrations used in this study) after adjusting for adipose tissue body burden of OC (CDC, 2012; Howell and Mangum, 2011). Research by others has also shown that DDE exposure is associated with an increased risk for T2D prevalence from a population once highly exposed to OC (Ukropec et al., 2010; Son et al., 2010). *In vivo* animal studies by others has shown that DDT/DDE exposure as well as similar OC are associated with an increased risk for obesity, insulin resistance, and other metabolic disorders (La Merrill et al., 2014; Ruzzin et al., 2010). For example, *in vivo* OC exposure has been implicated in altering liver function in mice such as affecting lipid metabolism (Sato et al., 2008). Studies from Howell's lab have shown that OC exposure affects adipocyte function *in vitro* (Howell and Mangum, 2011). Research by others has shown that OC exposure affects cell signaling mechanisms of liver cell function *in vitro* such as

regulating protein kinase C levels (Moser and Smart, 1989). Previous research has shown an epidemiological association between individuals with or at risk for T2D and the presence of higher levels of DDE in their blood (Lee et. al, 2006). Vietnam War Veterans exhibiting exposure to the OC 2,3,7,8-tetrachlorodibenzodioxin (TCDD) have a higher prevalence of insulin resistance and T2D (Kern et al., 2004). Research by others has provided epidemiological evidence of an association between high exposure levels of OC contaminants such as DDE, transnonachlor, and oxychlordane in plasma samples of people with metabolic disorders and T2D (Lee at el., 2006). Furthermore, long-term DDE exposure has been associated with an increased risk for biochemical markers of dyslipidemia in diabetics and non-diabetics (Lee et. al, 2011). Long-term DDE exposure has been implicated to predict abnormal lipid profiles in diabetics and non-diabetics over time (Lee et. al, 2011). Long-term exposure to OC might affect how beta cells in the pancreas synthesize and secrete insulin. Research by others has shown that rats exposed to the OC endosulfan exhibited a dramatic increase in blood glucose levels compared to non-exposed control groups (Kalender et al., 2004). Pancreatic beta cells of rats exposed to endosulfan *in vivo* showed structural changes such as swelling of the mitochondria implicating oxidative stress and mitochondrial dysfunction, possibly through the induction of NADPH oxidase isoforms that regulate oxidative stress levels and by the decreased production of adenosine triphosphate (ATP) in the mitochondria. Furthermore, mice exposed to the OC TCDD exhibited reduced glucokinase gene expression and glucose transporter transcript levels, causing a rise in blood glucose levels similar to that of T2D (Sato et al., 2008), and impaired glucose-stimulated secretion of insulin in rat treated islets (Novelli et al., 2005). TCDD is capable of decreasing glucose sensing by

the pancreas and impairs glucose stimulated insulin secretion (Hectors et al., 2011). Although these results were observed from different OC, DDE exposure may exhibit similar effects *in vivo* and *in vitro*. Scientific data suggest environmental exposure to some of these OC is associated with T2D and may affect physiological functions in the body that contribute to T2D (Lee et al., 2011; Lee et al., 2007). These epidemiological and experimental data suggest a somewhat strong association between environmental contaminant exposure and T2D, and implicate that there are more factors involved in the development of T2D than over-nutrition and a lack of exercise, possibly environmental factors (Lee et al., 2011). The epidemiological association of environmental contaminant exposure with T2D remains inconclusive even though the rate of T2D has increased (CDC, 2009).

Type 2 Diabetes Prevalence

T2D is a chronic metabolic disease characterized by hyperglycemia, insulin resistance, dyslipidemia, and beta cell dysfunction (Keane et al., 2011). The major complications that are associated with T2D are heart disease, stroke, obesity, high blood pressure, blindness, kidney disease, neuropathy, amputation, and death (Amos et al., 1997). T2D is the most common form of diabetes and affects between 90%-95% of the millions of people living with all forms of diabetes in the United States (CDC, 2011). The prevalence of T2D has increased dramatically over recent years with millions of people being diagnosed in the United States each year (MSDH, 2012). Epidemiological data indicates that 25.8 million children and adults in the United States, which accounts for about 8.3% of the population, are living with T2D. Statistical analysis of this population concludes that about 18.8 million people are diagnosed with diabetes, with 7

million people being undiagnosed (CDC, 2011). Unfortunately, 79 million people are reported to be living with pre-diabetes, and new cases of T2D are being diagnosed in people age 20 and older by the millions every year (Shaw et al., 2010). It is estimated that the total cost of managing T2D reached 218 billion dollars in the United States in 2007 (CDC, 2011). Many factors are hypothesized as contributing to T2D (Kahn and Flier, 2000). One factor such as exposure to common environmental contaminants could play a role in the pathogenesis of T2D (Lee et al., 2006; Carpenter, 2008). Genetic factors only account for about 15-25% of this prevalence, suggesting that other factors including environmental factors may play a role in its pathogenesis (Hunter, 2005). The prevalence of T2D has been increasing concurrent with the increasing use of synthetic chemicals (Neel and Sargis, 2011).

The prevalence of T2D is higher in the southern region of the United States, specifically the state of Mississippi (Fig. 2.). Mississippi's Delta region has an extremely high prevalence of T2D compared to the rest of the United States (CDC, 2008). The Mississippi Delta region has higher exposure levels to OC compared to other regions of Mississippi based on unpublished data from our lab investigating soil samples for OC contamination throughout the entire state of Mississippi. The majority of residents in the Delta region are African American, and African Americans have a greater risk of developing T2D (CDC, 2011). These individuals are living in a region once highly exposed to OC contaminants and exhibit high levels of biochemical markers associated with T2D. Some of these biochemical markers include altered levels of insulin, free fatty acids, very low density lipoproteins (VLDL), low density lipoproteins (LDL), high

density lipoproteins (HDL), and changes in the levels of proteins associated with or that regulate these biochemical markers.

The state of Mississippi is ranked in the top five of the unhealthiest states in the United States. Mississippi is ranked second in the entire United States for overall diabetes prevalence based on a 2010 study (MSDH, 2012). The Mississippi Delta region has the highest prevalence of T2D in Mississippi (Mendy et al., 2015). Belzoni, MS is an agricultural community that was once highly exposed to the synthetic chemical DDT. DDT was declared illegal because of its hazardous effects to human health and the environment and banned in 1972 for use in the United States (EPA, 2011). Although many decades have passed since DDT was banned many countries still use DDT for the benefit of agricultural and health reasons (Cohn et al., 2015). Because of this, high levels of its metabolite, DDE, are still being identified in populations all over the world (Taylor et al., 2013). This Mississippi Delta population is significant for the potential to exhibit higher than normal levels of exposure to the OC DDE. Thus, individuals living in Belzoni, MS may be a highly suitable population to investigate a potential association between high exposure levels of DDE and T2D. This Delta population has never been studied for the association of DDE exposure and T2D although the prevalence of T2D has increased in this area.

Type 2 Diabetes and Liver Function

Individuals with T2D exhibit abnormal liver cell function. Insulin resistance precedes T2D and occurs when cells in the body become insensitive to the action of insulin (Martin et al., 1992). The liver is responsible for glucose production which is regulated by insulin and glucagon levels. In the liver insulin regulates lipid and glucose

metabolism to maintain a healthy balance of lipid and glucose levels (Rader, 2007). When the liver loses its ability to be regulated by insulin, lipid and glucose metabolism are altered resulting in insulin resistance and hyperglycemia (DeFronzo and Ferrannini, 1991). During the progression of hyperglycemia cells in the body do not properly uptake and utilize glucose for cellular functions such as for glucose storage, which eventually results in excess glucose production by the liver and higher glucose levels in the blood. Hyperglycemia further affects liver function such as contributing to dyslipidemia by altering lipid metabolism (Michael et al., 2000). The effect of glucotoxicity in the liver causes oxidative stress which alters cell signaling mechanisms that regulate lipid metabolism and secretion such as fatty acid synthesis. Dyslipidemia, a state of altered lipid levels, is characterized by an increased production of lipids including adipose derived free fatty acids, lipid associated apolipoprotein B (ApoB), and triglycerides (TG) into the circulatory system (Benoist and Grand-Perret, 1996).

Apolipoproteins are proteins that exist on the surface of lipoproteins, which are composed internally of TG and cholesteryl esters. ApoB is a protein that binds to other lipids to form lipoprotein molecules. Two forms of ApoB exist in the body and are significantly associated with dyslipidemia and T2D (Ginsberg et al., 2005). ApoB-100 is synthesized in the liver and is essential for the formation of VLDL. ApoB-48 synthesized primarily in the small intestine, is a truncated form of ApoB containing the amino-terminal portion of ApoB-100, and is essential for the formation of chylomicrons (Veniant et al., 1998). ApoB-48 is synthesized as a result of ApoB mRNA splicing (Liu et al., 2010). ApoB-100 is the main protein component of lipoproteins including VLDL. One VLDL particle consists of one ApoB molecule and numerous attached TG and

cholesterol molecules. ApoB is an atherogenic related protein whose levels are increased by the presence of free fatty acids and decreased by the action of insulin (Jin et al., 2008). Free fatty acids stimulate ApoB secretion by providing an abundance of TG to nascent ApoB molecules therefore increasing ApoB translocation across the ER membrane (Macri and Adeli, 1997). ApoB plays an important role in lipid transport and clearance in the body. For example, ApoB is important in transporting lipids such as TG to other tissues in the body to be used for energy by facilitating the binding of lipoprotein molecules to their cell receptors (Fisher and Ginsberg, 2002). It is necessary for the synthesis and secretion of TG containing lipoproteins such as VLDL (Boren et al., 1994). The increased secretion of ApoB associated lipids thus increases circulatory levels of TG, cholesterol, and VLDL particles thereby promoting dyslipidemia (Julius, 2003). ApoB levels are significantly higher in individuals exhibiting dyslipidemia and T2D (Qiu et. al, 2006).

TG are synthesized as a product of free fatty acid hydrolysis by liver enzymes including triacylglycerol hydrolases/carboxylesterases (TGH) (Lehner and Verger, 1997) and serve as secondary energy source after carbohydrates for cellular functions (Howard, 1987). TG are a biochemical marker implicated in T2D and levels are usually higher in individuals with diabetes compared to non-diabetics (Albrink and Man, 1958). The increased levels of TG in the liver are a result of free fatty acid efflux from lipolysis, uptake of VLDL and chylomicron remnants, re-esterification by TGH, and hepatic lipogenesis during insulin resistance (Ginsberg et al., 2005). TG are a companion biochemical marker for ApoB molecules. TG are composed of a glycerol molecule attached to three fatty acids. Sufficient triglyceride levels are important in the formation

of nascent ApoB to form a completed ApoB molecule, which is secreted by the liver or small intestine into the bloodstream attached to other lipids (Sparks et al., 2012). Otherwise, in the absence of sufficient TG levels, ApoB remains in its nascent form and is degraded by intracellular proteasomes resulting in a decrease of ApoB secretion. Excess TG levels or hypertriglyceridemia is associated with T2D (Julius, 2003).

The ability of TG molecules to attach to nascent ApoB molecules is regulated by microsomal triglyceride transfer protein (MTP) and the enzyme TGH (Manchekar et al., 2004). The rate-limiting step for ApoB synthesis, assembly, and secretion involves the joint action of MTP and TGH (Lehner and Verger, 1997). MTP is an endoplasmic reticulum related protein that is important in the transfer of lipids between vesicles (Hussain et al., 2003). MTP is a lipid transporter protein that regulates lipidation of lipoprotein molecules for secretion. MTP is important in the transport of neutral lipids including cholesterol and TG to nascent ApoB in order to form complete ApoB lipoproteins (Taghibiglou et. al, 2000). Research by others has shown that MTP levels are associated with T2D *in vivo*. Increased MTP levels will result in the increased synthesis and secretion of TG containing ApoB lipids that contribute to T2D development (Bartels et al., 2002). The interaction of MTP with ApoB preserves ApoB from intracellular degradation and promotes lipidation (lipid assembly) and lipid secretion (Higuchi et al., 2011).

In the absence of increased MTP levels, ApoB is degraded by intracellular proteasomes and secretion is decreased due to the increased protein levels of sortilin-1 (Sort-1) (Cardozo et al., 2002). Sort-1 is an intracellular protein that regulates ApoB degradation (Strong and Rader, 2012). During normal liver cell function, Sort-1 binds to

ApoB and transports it to the proteasome for degradation to maintain a homeostasis of lipid secretion. Although ApoB is degraded, basal levels of ApoB are still secreted from the liver and small intestine at a homeostatic rate (Chamberlain et al., 2013). A disruption of this homeostatic rate regulated by Sort-1 could affect how lipids are secreted from the liver thereby contributing to complications associated with dyslipidemia and T2D. Insulin levels positively regulate Sort-1 protein levels. For example, Sort-1 levels were found to be decreased in *in vivo* and *in vitro* models of insulin resistance and diabetes along with an increase in ApoB and MTP levels from the liver (Ai et al., 2012). Therefore, Sort-1 plays a very important role in regulating ApoB levels in the body.

T2D can result in altered lipid levels as well as the altered levels of proteins attached to these lipids. Paraoxonase-1 (PON) is an endogenous serum esterase attached to HDL molecules found in the blood (Aviram et al., 1999). PON is synthesized and secreted by the liver in high amounts and lower amounts in other tissues (Abbott et al., 1995). The main functions of PON are protection against oxidative stress by preventing lipid oxidation, hydrolysis of esters, and increasing antioxidant levels (Costa et al., 2005). PON protects LDL from forming oxidized lipid products induced by oxidative stress (Furlong et al., 2010). PON is non-specific and has the ability to hydrolyze organophosphate compounds such as paraoxon, the metabolite of parathion, therefore making it protective against organophosphate pesticide poisoning (Boesch-Saadatmandi et al., 2010). Previous data concluded that PON levels are decreased in cases of T2D (Manning et al., 2012). It is well established that African Americans are at a greater risk for metabolic diseases and exhibit differences in PON activity compared to Caucasians

(Davis et al., 2009). Furthermore, exposure to OC is associated with changes in lipid profiles *in vivo* (Shamir et al., 2005). OC exposure may play a role in the alteration of PON levels identified in individuals with altered lipid profiles exhibiting T2D (Saha et al., 1991). For example research from our laboratories show that the OC dieldrin affects PON levels *in vitro* by the induction of nuclear receptors that regulate PON synthesis (Dail et al., 2014). The potential association of OC exposure with PON levels may complement the association of OC exposure with abnormal lipid profiles and T2D.

Type 2 Diabetes and Pancreatic Function

The function of the pancreas to regulate insulin is impaired in T2D (Lim et al., 2011). Insulin is produced by the beta cells of the pancreas in response to elevated extracellular glucose concentrations in the body. This extracellular glucose enters pancreatic beta cells through glucose transporters (GLUT2) by facilitated diffusion across the cell membrane (Skelin et al., 2010). Once inside the cell, glucose is metabolized by the enzyme glucokinase to glucose-6-phosphate and then to pyruvate which enters the tricarboxylic acid cycle in the mitochondria to increase the production of adenosine triphosphate (ATP). ATP binds to and closes the ATP-dependent-potassium channel in the plasma membrane resulting in depolarization of the plasma membrane. This membrane depolarization results in the opening of the voltage-gated calcium channels and an increase in influx of extracellular calcium. High levels of calcium influx raises the cell's electrical activity and cause insulin secretory granules to travel to the plasma membrane to eventually release insulin from beta cells (Ashcroft and Rorsman, 1989). The pancreas is responsible for insulin production which is regulated by glucose levels. In the pancreas beta cells uptake excess glucose from the blood and secrete insulin to

further regulate glucose levels in other cells of the body (Schuit et al., 2001). Hyperglycemia is detrimental to pancreatic beta cell function. Excess glucose levels or glucotoxicity alter the function of beta cells to synthesize and secrete insulin. Glucotoxicity causes oxidative stress in beta cells which blunt cell signaling mechanisms responsible for insulin regulation such as the production of sufficient ATP levels. Long-term hyperglycemia causes beta cells to secrete high amounts of insulin in a short amount of time (Leahy et al., 1992). Beta cells continue to secrete high amounts of insulin resulting in hyperinsulinemia until these cells lose their ability to secrete sufficient levels of insulin over time resulting in beta cell burnout and beta cell dysfunction (Porte and Kahn, 2001).

Beta cell dysfunction is a state of altered cellular function in pancreatic beta cells including changes in intracellular oxidative stress levels, insulin synthesis, and insulin secretion (Makaji et al., 2011). During beta cell dysfunction the regulation of insulin synthesis is disrupted at the site of transcription, post-translational modification, and secretion in beta cells. Individuals with T2D exhibit a decline in the ability of their pancreatic beta cells to properly regulate or produce sufficient amounts of insulin as result of insulin resistance, which leads to beta cell dysfunction and depletion of beta cell mass (Dixon et al., 2004). Hyperglycemia in T2D results from defective insulin secretion and impaired insulin action on target tissues or insulin resistance, which produces a rise in circulating blood glucose levels with T2D progression (McClenaghan, 2007). The decline in pancreatic beta cell function in T2D has been linked to glucose toxicity. Hyperglycemia adversely affects beta cells by causing defective insulin secretion (Robertson et al., 2003). Individuals that exhibit less beta cell mass or less effective beta

cell function are at a higher risk for developing T2D (Tushuizen et al., 2007). Insulin resistance that is accompanied by the dysfunction of pancreatic islet beta cells results in failure to control blood glucose levels (Kahn, 2003). Abnormalities in beta cell function are critical in defining the risk and development of T2D (Kahn et al., 2006). Therefore, biochemical markers of beta cell dysfunction ranging from insulin synthesis, secretion, and insulin regulatory proteins are potential contributors to the development of T2D (Weir, 2004).

The main function of insulin is to increase cell permeability to monosaccharides, amino acids, and fatty acids (Itoh et al., 2003). Insulin blunts the use of fat as an energy source by inhibiting the release of glucagon. Insulin is provided within the body in a constant proportion to remove excess glucose from the blood (Duckworth et al., 1998). Insulin can be regulated by endogenous lipids and proteins, but glucose is the primary regulator of insulin. Insulin is secreted at basal levels from beta cells in the absence of glucose and secretion is increased in response to stimulatory glucose levels (Schuit et al., 1988). The body utilizes insulin to decrease circulatory blood glucose levels by inducing the liver to decrease glucose production and the adipose and muscle tissue to uptake and metabolize glucose for energy use or storage in order to maintain normal blood glucose homeostasis (Abel et al., 2001).

The insulin molecule is regulated enzymatically in phases during synthesis. Insulin is the metabolite of preproinsulin, which is a precursor molecule formed from insulin gene transcription (Bollheimer et al., 1998). During post-translational modification, preproinsulin is cleaved by the serine protease, prohormone convertase (PC), to proinsulin. PC is an enzyme whose main function is to regulate the proteolytic

cleavage of incomplete peptides to their complete forms. The proinsulin molecule is further cleaved by PC to yield two products: mature insulin and connecting (C)-peptide (Fu et al., 2013). PC levels are important in the diagnosis of T2D as changes in these levels have been associated with biochemical markers of the metabolic syndrome. PC regulates the synthesis of insulin by multiple steps: transcription from the insulin gene, protein translation, and post-translational modification of protein conformation (Weiss, 2009). Disruption of this enzymatic pathway contributes to altered insulin homeostasis and beta cell dysfunction (Rhodes and Alarcon, 1994).

Pancreatic-Duodenal Homeobox 1 (PDX-1) is a transcription factor present in beta cells. It plays a vital role in regulating insulin gene transcription. The function of PDX-1 depends on the cellular metabolism of glucose and the production of oxidative stress. PDX-1 exists as an inactive protein in the cytoplasm of beta cells and translocates to the nucleus of beta cells as an active protein via glucose induced phosphorylation (Macfarlane et al., 1999). PDX-1 has been shown to regulate the expression of a number of proteins involved in glucose sensing, insulin secretion, and genes involved in pancreatic islet growth and development including insulin, glucokinase, and islet amyloid polypeptide (McKinnon and Docherty, 2001). PDX-1 has been shown to induce insulin and glucokinase gene expression (Watada et al., 1996). Research by others has shown that the reduced expression of PDX-1 is associated with beta cell dysfunction and hyperglycemia. For example, research by others has shown that mutations in the PDX-1 gene could lead to abnormalities in islet function that has the potential to result in T2D in humans and mice (Brissova et al., 2005).

Increased oxidative stress has been associated with cases of T2D (Kahn, 2003). Oxidative stress occurs when the levels of reactive oxygen species (ROS) are higher than the levels of antioxidant molecules in a cell. Oxidative stress occurs naturally in cells through normal cell function and metabolism. Oxidative stress has a major influence on beta cell function and is a product of glucose induced insulin secretion (Evans et al., 2003). Pancreatic beta cells express lower levels of antioxidant enzymes and are highly vulnerable to damage by oxidative stress markers induced by endogenous factors such as excess glucose or lipids including free fatty acids. Oxidative stress can result in damage to DNA, lipids, and proteins in cells (Klaunig et al., 1998). ROS are biochemical markers of oxidative stress. Common ROS include superoxide (O_2^-), hydroxyl radical ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2). DDE exposure has been known to play a role in the induction of these oxidants *in vitro*. ROS generation is dependent upon the activity of membrane bound NADPH oxidase and its isoforms. DDE exposure has been shown to increase the levels of some of these NADPH oxidase isoforms *in vitro* (Song et al., 2014). ROS target many signal transduction molecules in pancreatic beta cells that regulate insulin secretion and beta cell function. DDE exposure results in an elevation of ROS levels *in vitro*, and an association between increased ROS levels and alterations in the insulin signaling pathway has been demonstrated (Pi et al., 2007).

Scientific Rationale and Study Approach

Environmental exposure to OC is associated with T2D and may be a potential risk factor that contributes to T2D prevalence by its hypothesized effects on regulating biochemical markers of T2D *in vitro*. The epidemiological association of T2D with OC exposure has yet to be solved. The purpose of this study was to investigate an association

of DDE exposure with biochemical markers of T2D or with T2D presence from an at-risk population for metabolic diseases as well as investigate an association of DDE exposure with biochemical markers of T2D *in vitro* to investigate DDE exposure as a risk factor for T2D. The overall hypothesis of this study is that potentially exhibiting high DDE levels is a risk factor associated with biochemical markers of T2D and the increased prevalence of T2D. Following thorough investigation, this study will answer whether DDE exposure plays a role in T2D prevalence reported by others by observing DDE's epidemiological association with biochemical markers of T2D and by investigating DDE's *in vitro* effect on mechanisms of liver hepatocytes and pancreatic beta cells that regulate these biochemical markers of T2D. Therefore, this study demonstrated the need for an *in vitro* scientific investigation into the means behind this association. Liver cell hepatocyte function was studied for the effects of DDE that could lead to changes in biochemistry that are reflective of T2D. Pancreatic beta cell function was studied for the effects of DDE that could lead to changes in biochemistry that are reflective of T2D. Finally, the significance of OC exposure with T2D was investigated through an epidemiological study that identified biochemical markers of metabolic disease between diabetic and non-diabetic individuals exhibiting high DDE exposure. These findings could contribute to the overall understanding of environmental exposure and metabolic disease development.

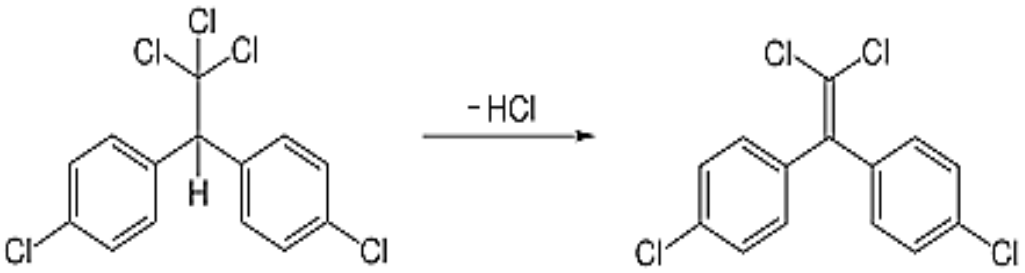

Figure 1 Organochlorine compound structure of dichlorodiphenyltrichloroethane (DDT) and its metabolite dichlorodiphenyldichloroethylene (DDE).

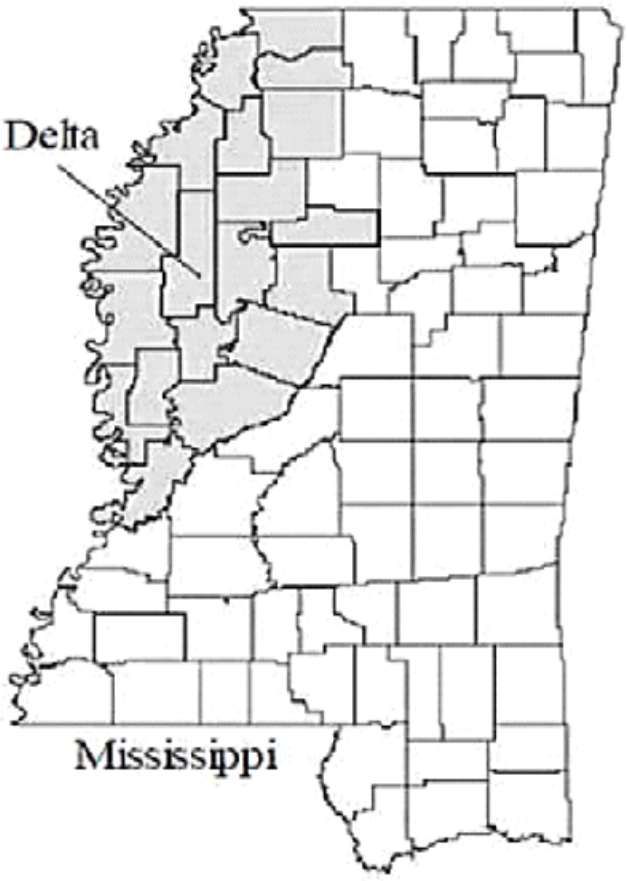

Figure 2 Map of the Mississippi Delta region.

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CHAPTER II

IN VITRO EFFECT OF DDE EXPOSURE ON THE REGULATION OF LIPID METABOLISM AND SECRETION IN MCA-RH7777 HEPATOCYTES: A POTENTIAL ROLE IN DYSLIPIDEMIA ASSOCIATED WITH TYPE 2 DIABETES MELLITUS

Introduction

Environmental exposure to the organochlorine compound (OC) contaminant dichlorodiphenyldichloroethylene (DDE) is associated with type 2 diabetes mellitus (T2D) according to epidemiological studies and may play a role in risk factors that contribute to T2D such as dyslipidemia (Lee et al, 2011). Dyslipidemia, a state of altered lipid levels, plays a role in the progression of insulin resistance and T2D (Rader, 2007). Exposure to environmental contaminants is a possible factor that could play a role in the pathogenesis of T2D (Lee et al., 2006; Carpenter, 2008). DDE exposure has been associated with biochemical markers of dyslipidemia (Lee et al., 2011). The liver is a potential target for DDE exposure since it plays a role in regulating lipid metabolism and secretion (Fig. 3.) and a disruption of this process by DDE exposure may cause dyslipidemia to occur (Mulvihill et al., 2009).

The purpose of this study was to investigate any changes that may occur in free fatty acid induced lipid metabolism and secretion of McArdle-RH7777 (McA) hepatocytes exposed to DDE *in vitro* with respect to biochemical markers identified in

dyslipidemia including apolipoprotein B (ApoB), microsomal triglyceride transfer protein (MTP), sortilin-1 (Sort-1), and triglycerides (TG). ApoB is a lipoprotein molecule synthesized in the liver, important in lipid transport and clearance, and is needed for the synthesis and secretion of TG containing lipids such as very low-density lipoproteins (VLDL) from the liver (Boren et al., 1994). ApoB levels are significantly higher in individuals exhibiting dyslipidemia and T2D (Qiu et. al, 2006). The increased secretion of ApoB molecules thus increases circulatory levels of TG, cholesterol, and VLDL particles thereby promoting dyslipidemia (Julius, 2003). TG are synthesized from free fatty acids that are metabolized in the liver and serve as a secondary energy source after carbohydrates for cellular functions (Howard, 1987). TG are important in the formation of nascent ApoB to form a completed ApoB molecule, which is then secreted by the liver or small intestine into the bloodstream (Sparks et al., 2012). TG are a biochemical marker implicated in T2D and levels are usually higher in individuals with diabetes compared to non-diabetics (Albrink and Man, 1958). The rate-limiting step for ApoB synthesis, assembly, and secretion involves the action of MTP and TGH (Hussain et al., 2003; Lehner and Verger, 1997). MTP is important in the transport of neutral lipids including cholesterol and TG to nascent ApoB in order to form complete ApoB lipoproteins (Taghibiglou et. al, 2000). The interaction of MTP with ApoB preserves ApoB from intracellular degradation and promotes lipidation (lipid assembly) and lipid secretion (Cardozo et al., 2002). Sort-1 is an intracellular protein that regulates ApoB degradation (Strong and Rader, 2012). Sort-1 binds to ApoB in the liver and transports it to the proteasome for degradation to maintain a homeostatic level of lipid secretion

(Chamberlain et al., 2013). Sort-1 protein levels were found to be decreased in *in vivo/in vitro* models of insulin resistance and T2D (Ai et al., 2012).

The hypothesis of this study is that exposure to DDE will alter lipid synthesis, accumulation, transport, degradation, and/or secretion in McA cells. The goal was to identify *in vitro* effects of DDE on McA cell mechanisms involved in lipid metabolism and secretion. The rodent hepatoma McA cell line was used as a mechanistic model for liver cell function. McA cells secrete lipids in response to fatty acids and are a reliable cell line for studying lipid metabolism and secretion (Boren et al., 1994). McA liver cells are significant because they secrete both ApoB-100 and its truncated form, ApoB-48, and *in vivo* studies by others using rodent models indicate that both forms of ApoB are secreted from the liver (Veniant et al., 1998). McA cells are beneficial for studying lipid metabolism and secretion as they express biochemical pathways similar to lipid signaling pathways identified in primary hepatocytes. McA cells express the transcription factor Srebp-1c needed for lipid synthesis, but lack expression of the TGH needed for lipid assembly while relying on only MTP expression for lipid assembly. However, McA cells express both forms of ApoB while human primary hepatocytes do not, therefore limiting this cell line as the best model for studying lipid metabolism and secretion. McA cells also show differences in glucose and fatty acid uptake when compared to primary hepatocytes (Hansson et al., 2004). McA cells are derived from a cancerous cell line (rat hepatomas) and do not completely represent normal hepatocyte function as compared to using primary cells of human origin.

Materials and Methods

The McArdle-RH7777 (McA) rodent cell line was purchased from American Tissue Culture Collection (Manassas, VA). 10% Neutral Buffered Formalin, Super Signal West Pico Chemiluminescence kit, 0.05% trypsin in 0.53mM ethylenediaminetetraacetic acid (EDTA) solution, and Sort-1 antibody were purchased from Thermo Fisher Scientific (Pittsburgh, PA). Certified fetal bovine serum (FBS), Dulbecco's Modified Eagles Medium (DMEM) complete growth medium, and penicillin-streptomycin were purchased from Invitrogen (Grand Island, NY). p,p'-DDE was purchased from Chem Service (West Chester, PA). Phosphate buffered saline (PBS) was purchased from Mediatech Inc. (Manassas, VA). Non-fat dry milk and Bradford protein assay kit were purchased from Bio-Rad (Hercules, CA). ApoB antibody, goat anti-rabbit IgG-HRP, donkey anti-goat IgG-HRP, beta-actin antibody, radio-immunoprecipitation assay (RIPA) lysis buffer system, and Western Blotting Luminol Reagent were purchased from Santa Cruz (Dallas, TX). MTP antibody was purchased from Aviva Systems Biology (San Diego, CA) and Santa Cruz (Dallas, TX). Bovine serum albumin-fatty acid free (BSA), oleic acid (OA) conjugated to BSA, Oil-red-O dye, 2X-sodium dodecyl sulfate (SDS) sample loading buffer, and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO). Triglyceride (TG) assay kit was purchased from Cayman Chemicals (Ann Arbor, MI). Crystal violet dye solution was purchased from Active Motif (Carlsbad, CA).

McA cells were grown in supplemented DMEM containing 20% FBS and a 1% solution of 100 IU/ml penicillin with 0.1 mg/ml streptomycin in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C in a T-75 cell culture flask until 70%-90%

confluent. Confluent cells were washed with PBS and detached from the culture flask with a solution of 0.05% trypsin in 0.53mM EDTA. Cells were pelleted by centrifugation and re-suspended in supplemented DMEM to be seeded overnight in a 12-well cell culture plate to adhere. The next day supplemented DMEM was removed and adherent cells were washed with PBS and treated with serum-free DMEM to starve cells of serum during an overnight incubation. Following overnight incubation, serum-free DMEM was removed and cells were washed with PBS and subjected to the following experimental protocols.

Experimental Design

Experiment 1. The effect of free fatty acid exposure on McA liver cell lipid secretion was investigated with respect to ApoB protein levels. OA, a free fatty acid, was used to induce lipid metabolism and secretion in McA liver cells based on studies by others. Following standard cell culture protocols, serum starved cells were treated with either a final concentration of 400 µM OA conjugated to 1.2% BSA, 100 µM OA conjugated to 0.3% BSA, or 1.2 % BSA only in DMEM (OA is conjugated to BSA and the concentration of BSA varies based on the concentration of OA) for 24 hours for a total of 3 treatment groups repeated in triplicate in each of 3 individual experimental trials. Cell media was assayed for the detection of secreted ApoB protein levels using SDS-PAGE with Western Blot Analysis.

Experiment 2. The concentration-response effect of DDE exposure on free fatty acid induced lipid secretion of ApoB was investigated. Serum starved cells were exposed to final concentrations of 1, 10, or 100 µM DDE dissolved in 0.1% DMSO simultaneously with 400 µM OA conjugated to 1.2% BSA or 400 µM OA conjugated to

1.2% BSA only in DMEM for 24 hours for a total of 4 treatment groups repeated in triplicate in each of 3 individual experimental trials. Cell media was assayed for the detection of secreted ApoB protein levels using SDS-PAGE with Western Blot Analysis. Cell lysates were assayed for intracellular ApoB, MTP, and Sort-1 protein levels using SDS-PAGE with Western Blot Analysis.

Experiment 3. Following the concentration-response analysis, the effect of DDE exposure on free fatty acid induced lipid secretion was investigated using a single concentration (10 μM) DDE. To investigate the effect of DDE exposure on free fatty acid induced lipid secretion, serum starved cells were exposed to 10 μM DDE simultaneously with 100 or 400 μM OA conjugated to 0.3 or 1.2% BSA, 100 or 400 μM OA conjugated to 0.3 or 1.2% BSA, 10 μM DDE, or 0.3 or 1.2% BSA only in DMEM for 24 hours for a total of 4 treatment groups repeated in triplicate in each of 3 individual experimental trials. Cell media was assayed for the detection of secreted ApoB protein levels using SDS-PAGE with Western Blot Analysis. Cell lysates were assayed for intracellular ApoB, MTP, and Sort-1 protein levels using SDS-PAGE with Western Blot Analysis.

Experiment 4. The effect of DDE exposure on free fatty acid induced TG accumulation in McA cells was investigated. Serum starved cells were exposed to 10 μM DDE simultaneously with 100 μM OA conjugated to 0.3% BSA, 100 μM OA conjugated to 0.3% BSA, 10 μM DDE, or 0.3% BSA only in DMEM for 24 hours for a total of 4 treatment groups repeated in triplicate in each of 3 individual experimental trials. Cell lysates were subjected to a colorimetric assay for the detection of TG accumulation in liver cells (Cayman Chemicals).

Experiment 5. The effect of DDE exposure on free fatty acid induced TG secretion was investigated. Serum starved cells were exposed to 10 μM DDE simultaneously with 100 μM OA conjugated to 0.3% BSA, 100 μM OA conjugated to 0.3% BSA, 10 μM DDE, or 0.3% BSA only in DMEM for 24 hours for a total of 4 treatment groups repeated in triplicate in each of 3 individual experimental trials. Cell media was subjected to lipid extraction techniques (Folch et al., 1957) for the detection of secreted lipid molecules and then analyzed using a colorimetric assay for the detection of secreted TG molecules from liver cells (Cayman Chemicals).

Experiment 6. The effect of DDE exposure on free fatty acid induced neutral lipid accumulation (triglycerides and cholesteryl esters) in McA liver cells was investigated. Serum starved cells were exposed to 10 μM DDE or simultaneously with 100 μM OA conjugated to 0.3% BSA, 100 μM OA conjugated to 0.3% BSA, or 0.3% BSA only in DMEM for 24 hours for a total of 4 treatment groups repeated in triplicate in each of 3 individual experimental trials. Following experimentation, adherent cells were analyzed for the detection of lipid accumulation using lipid staining techniques.

Assay Development

Assay 1. Bradford Protein Analysis. Following all experimental protocols, the cell media was collected and stored at -80°C and cells were washed with PBS and lysed in RIPA buffer with or without protease and phosphatase inhibitors and stored at -80°C for future analysis. The protein concentrations of whole cell lysates were separated from cell debris after centrifugation at 16.1 relative centrifugal force for 4°C using an Allegra X-14R Centrifuge (Beckman Coulter). The diluted protein concentrations of cell media and lysates from each treatment group were quantified by the Bradford protein assay. All

treatment groups were normalized to the protein concentrations of their respective cell lysates for Western Blot Analysis.

Assay 2. SDS-PAGE with Western Blot Analysis. Protein concentrations were normalized for all treatment samples using 50 mM Tris-hydrochloride buffer (pH 7.4 at 4°C). Equal amounts of 2X-SDS sample loading buffer were added to each treatment sample at a 1:1 dilution. Diluted protein samples (25 µg) were heated at 95°C and allowed to cool. After cooling, samples were pipetted at equal volumes per well on a 10% polyacrylamide gel and subjected to electrophoresis for the separation of protein bands by molecular weight at 100 volts for 15 minutes and 120 volts for 1 hour using a Mini-PROTEAN 3 system. Following electrophoresis, the gel containing the protein bands was transferred to a 0.45 µm polyvinylidene fluoride (PVDF) membrane with electrophoresis at 20 volts for 30 minutes using a Trans-Blot SD semi-dry electrophoretic transfer cell system (Bio-Rad). After a successful transfer, ApoB, MTP, or Sort-1 protein levels were detected on a 0.45 µm PVDF membrane using the following antibodies: ApoB-primary antibody, MTP-primary antibody, Sortilin-1-primary antibody, beta-actin-primary antibody, goat anti-rabbit IgG-HRP-secondary antibody, and donkey anti-goat IgG-HRP-secondary antibody. Membranes were incubated with a blocking solution (5% milk) to prevent non-specific binding of antibodies. Following successful blocking, membranes were incubated with primary antibodies diluted 1:500 at 4°C overnight. After overnight incubation, membranes were washed with a solution of Tris-buffered saline containing 0.05% Tween-20 for 15 minutes. After washing, membranes were incubated with secondary antibodies diluted 1:2000 for 1 hour at room temperature to detect protein bands. After washing again for 15 minutes, membranes containing the

appropriate antibody conjugated proteins were subjected to a chemical reaction of a 1:1 combination of super signal peroxide and luminol enhancer solution for 1 minute. The horseradish peroxidase conjugated to the secondary antibody mediated the oxidation of luminol in the presence of hydrogen peroxide resulting in light emission or chemiluminescence. Following this reaction, protein bands were visualized using the Chemi-Doc XRS System with Image Lab Software (Bio-Rad). Protein band density was interpreted using ImageJ Software supplied by the National Institutes of Health (NIH).

Assay 3. Lipid Extraction Analysis. One milliliter of a chloroform:methanol (2:1) solution was added to the cell media of each sample. Samples were vortexed and incubated for 1 hour on ice. Following incubation, 200 µl of water was added to each sample and samples were vortexed and centrifuged at 3,000 x g for 5 minutes. After centrifugation, the supernatant was discarded and the lower lipid phase of each solution was collected and allowed to dry at room temperature under nitrogen gas. The remaining pellet of each sample was redissolved in a solution of 60 µl tert-butanol combined with a 40 µl solution of Triton-X-100:methanol (2:1) and vortexed. Redissolved lipid molecules were collected and subjected to a colorimetric assay for the detection of TG efflux from liver cells (Cayman Chemicals).

Assay 4. TG Analysis. Ten µl of lipid extracted cell media or cell lysate samples per treatment were pipetted into a 96-well microplate. One hundred fifty µl of a diluted enzyme mixture was added to each sample well to initiate the hydrolysis of TG to free fatty acid and glycerol. Glycerol is further metabolized by the enzyme mixture to hydrogen peroxide. Hydrogen peroxide then reacts with the enzyme mixture to produce a purple colored product. Samples were incubated at room temperature for 15 minutes to

allow the enzymatic reaction to take place. Following incubation, TG levels were detected by measuring the absorbance of this purple product from each sample well at 540 nm using a SpectraMax M5 spectrophotometer (Molecular Devices). TG levels were quantified using a TG standard curve from a dilution of purified TG standard.

Assay 5. Oil-red-O Lipid Analysis. McA cells were washed in PBS and fixed to tissue culture treated plates using a 10% formalin solution for 1 hour. After fixation, cells were washed with 60% isopropanol and incubated with an Oil-red-O dye solution (0.7 grams Oil-red-O powder dissolved in 200 ml 100% isopropanol) for 10 minutes before the detection of lipid accumulation. After incubation, cells were washed with distilled water and allowed to dry at room temperature until all liquid was removed. An aliquot of 100% isopropanol was administered to each cell treatment to elute all stained lipids of dye. Lipid accumulation was measured from the eluted dye of stained lipids based on their absorbance at 520 nm in a 96-well microplate using a SpectraMax M5 spectrophotometer (Molecular Devices). Following lipid analysis, cells were washed with PBS until all dye was removed and stained with a crystal violet dye solution to detect the number of intact cells per treatment based on their absorbance at 595 nm. Stained lipid values per treatment were normalized to the absorbance values of cells per treatment.

Statistical Analysis

The effect of treatments on biochemical markers was assessed by mixed model analysis using PROC MIXED in SAS for windows 9.4 (SAS Institute, Inc., Cary, NC). Treatment was included as a fixed effect in separate models for each biochemical marker. Experimental trials were included as a random effect. If treatment was significant,

Tukey's adjustment for multiple comparisons was used to determine differences in least square means. A p-value of ≤ 0.05 was considered statistically significant based on least square means differences for each treatment group. Graphical data were expressed as the mean ± the standard error of the mean (SEM) for each treatment group repeated in triplicate for each of 3 individual experimental trials.

Results

OA, an endogenous free fatty acid, was administered to induce lipid metabolism and secretion from McA liver cells to verify that the assay was performed correctly. After a 24 hour exposure with or without OA, McA liver cell ApoB secretion was significantly increased in OA exposed cells compared to non-exposed basal lipid secretion. Immunoblotting of the cell media detected that OA is a significant inducer of ApoB secretion from liver cells as expected (Fig. 4.).

DDE exposure significantly increased ApoB secretion with increasing concentrations compared to the controls. After a 24 hour exposure of cells to different concentrations of DDE with co-exposure to OA, cell media was collected and subjected to immunoblotting for the detection of ApoB proteins secreted into the media. DDE exposure at 10 and 100 μM exhibited a positive concentration-response effect on ApoB secretion (Fig. 5.).

DDE exposure increased ApoB secretion as concluded from the previous protocol. Therefore the effect of DDE on lipid synthesis of ApoB in McA cells was investigated. The analysis of intracellular ApoB levels from cell lysates based on DDE exposure was determined using the previous concentration-response protocol. This protocol identified that DDE at 10 μM significantly increased ApoB secretion. For all

future experiments, McA cells were exposed to this concentration of DDE for 24 hours with or without co-exposure to OA. Immunoblotting of the cell lysates indicated no significant increase in intracellular ApoB protein levels for DDE exposed cells in the presence or absence of OA compared to controls (data not shown).

The idea of whether DDE exposure may play a role in lipid transport by affecting the intracellular assembly and formation of complete lipid products by way of neutral lipid preservation and transport in McA liver cells was investigated. A 24 hour exposure of McA cells to DDE with or without co-exposure to OA was administered to test this hypothesis. Immunoblotting of cell lysates indicated an increase in intracellular MTP protein levels for DDE exposed cells with co-exposure to OA, but not for DDE exposed cells without OA compared to controls (Fig. 6.).

The idea of whether DDE exposure may affect ApoB secretion by altering intracellular lipid degradation was investigated. Cell lysates from DDE exposure with or without co-exposure to OA were subjected to immunoblotting. DDE exposure significantly decreased the intracellular protein levels of Sort-1 with or without co-exposure to OA after 24 hours (Fig. 7.).

The effect of DDE exposure on TG synthesis and accumulation was investigated to identify any significant changes in TG accumulation levels in McA liver cells. After a 24 hour exposure to DDE with or without co-exposure to OA, cell lysates were subjected to a colorimetric assay for the detection of intracellular TG levels. DDE exposure in the presence of OA appears to decrease TG accumulation in liver cells, while the opposite effect was observed in OA exposed cells (Fig. 8.). Cell media from these same studies were extracted for lipid molecules and subjected to a colorimetric assay for the detection

of TG secretion levels. DDE exposure with or without co-exposure to OA did not have a significant effect on TG secretion levels (data not shown).

The idea of whether DDE exposure may play a role in the regulation of neutral lipid accumulation was investigated. McA cells were exposed to DDE with or without co-exposure to OA for 24 hours. Lipid accumulation was allowed to take place in liver cells during this 24 hour period. OA induced neutral lipid accumulation was decreased in the presence of DDE compared to controls. Based on these data, DDE exposure appears to affect the accumulation of lipids by decreasing neutral lipid accumulation in liver cells (Fig. 9.).

Discussion

Dichlorodiphenyltrichloroethane (DDT) was developed as one of the first synthetic OC insecticides (CDC, 2012). OC exposure was once a prevalent event and is still a threat to human health and the environment based on evidence of some OC contaminants, DDE, being detected in soil, air, and water samples (EPA, 2011). Individuals chronically exposed to OC may be at a greater risk of developing T2D or exhibiting biochemical markers of T2D (Lee et al., 2007). Some of these biochemical markers are regulated by the functions of the liver: lipid metabolism and secretion. DDE exposure may cause adverse effects to the functions of the liver such as dyslipidemia and was investigated *in vitro*.

Serum free fatty acid levels are elevated in T2D. Free fatty acids undergo decreased beta-oxidation and increased esterification to produce more TG during insulin resistance and dyslipidemia (Julius, 2003). Insulin resistance increases free fatty acid lipid levels in the body, and ApoB levels are significantly higher in individuals with

dyslipidemia and T2D (Qiu et. al, 2006). An increase in ApoB levels will potentially result in adverse health effects because of the increased presence of TG molecules available for metabolism that will further increase free fatty acid levels in the body, thereby promoting dyslipidemia (Ginsberg et al., 2005). Although humans produce two different forms of ApoB (ApoB-48/ApoB-100), measuring total ApoB levels may serve as a more reliable biochemical marker for the prediction of metabolic dysfunction-related diseases such as T2D. However, the livers of mice and rats express ApoB mRNA editing activity and synthesize both forms of ApoB (Veniant et al., 1998). Therefore, this study investigated total ApoB levels and their association with dyslipidemia *in vitro*.

Dyslipidemia is a characteristic of the metabolic syndrome and T2D. Exhibiting biochemical markers of dyslipidemia including abnormal lipid levels is used in the diagnosis of T2D (Benoist and Grand-Perret, 1996). There is an epidemiological association between lipid biochemical markers and the prevalence of T2D (Kannel, 1985). OC exposure may further contribute to this epidemiological association (Ukropec et al., 2010). For example, DDE exposure was a predictor of higher lipid levels over a time course of 20 years post exposure in non-diabetics (Lee et al., 2011). One of the liver's functions is the synthesis and secretion of lipids and the disruption of that process could have detrimental effects. The liver is susceptible to OC exposure as *in vitro* research has shown adverse effects on liver cell function after OC exposure (Moser and Smart, 1989). Therefore, the effect of exposure to the OC contaminant DDE on McA liver cell function with respect to lipid metabolism and secretion was investigated and identified a potential *in vitro* association with biochemical markers of dyslipidemia.

These experimental data support the hypothesis that DDE exposure alters lipid secretion levels *in vitro* and may serve as an important risk factor for predicting dyslipidemia and T2D *in vivo* (La Merrill et al., 2014). Free fatty acid induced lipid secretion yielded significantly higher levels of secreted ApoB lipids for DDE exposed cells versus controls. Our immunoblotting techniques yielded detectable protein levels of a lower molecular weight amino-terminal fragment of ApoB between 40-70 kDa as observed in other studies (Macri and Adeli, 1997). DDE exposure appears to affect the secretion of ApoB from free fatty acid stimulated McA liver cells, therefore suggesting a role for DDE exposure in contributing to dyslipidemia.

Intracellular ApoB protein levels in cell lysates were not significantly different for DDE exposed cells or OA exposed cells compared to controls, therefore diminishing a role of DDE exposure in the regulation of ApoB synthesis. MTP protein levels in cell lysates were increased in free fatty acid stimulated cells exposed to DDE, suggesting a role of DDE in regulating the transport and assembly of ApoB containing lipids as observed in other studies (Bartels et al., 2002). DDE exposure appears to affect mechanisms that regulate ApoB degradation and may explain the increased secretion of ApoB containing lipids observed in liver cells exposed to DDE. Sort-1 protein levels were decreased in DDE exposed and OA exposed cells compared to controls in this study indicating ApoB is rescued from intracellular degradation as observed in other studies (Strong and Rader, 2012). DDE exposure decreased free fatty acid induced TG accumulation, although no effect was observed with respect to TG secretion. DDE exposure could decrease the rate of TG accumulation by regulating lipid transport mechanisms or through induction of beta oxidation mechanisms.

Neutral lipid transport is a vital mechanism in the lipidation or degradation of ApoB molecules and their secretion. Neutral lipids such as TG and cholesterol are synthesized from free fatty acids. Lipid accumulation depends on the regulation of lipid secretion. In the liver, lipid accumulation will decrease as lipid secretion increases, and will increase because of a decrease in lipid secretion (Ginsberg et al., 2005). Free fatty acid induced intracellular TG and neutral lipid accumulation levels were decreased with DDE exposure compared to controls. However, TG secretion levels did not differ between DDE exposed cells compared to controls possibly due to lipid reuptake by liver cells or poor recovery of lipid extracted molecules. Furthermore, previous *in vitro* studies have shown that free fatty acid stimulation of ApoB secretion does not necessarily increase levels of TG secretion (Chamberlain et al., 2013).

DDE exposure appears to play a role in dyslipidemia by affecting mechanisms associated with lipid metabolism and secretion. DDE exposure seems to have a significant effect on free fatty acid induced lipid accumulation and secretion and may regulate intracellular proteins involved in lipid synthesis, transport, and degradation in liver cells. One could further hypothesize that DDE exposure seems to rescue ApoB from intracellular degradation by some unknown mechanism that may affect free fatty acid beta oxidation, esterification, and/or intracellular protease activity.

These data support the hypothesis that DDE exposure is associated with McA liver cell dyslipidemia by its effect on lipid synthesis, transport, accumulation, degradation, and/or secretion (Fig. 10.). DDE exposure may exacerbate the liver's secretion of lipids thereby contributing to biochemical markers of dyslipidemia observed in T2D. This *in vitro* mechanistic association may explain the epidemiological

association of DDE exposure with biochemical markers of dyslipidemia and T2D (Lee et al., 2011). These *in vitro* findings support data by others that DDE exposure is associated with biochemical markers of dyslipidemia and T2D *in vivo* (Ruzzin et al., 2010).

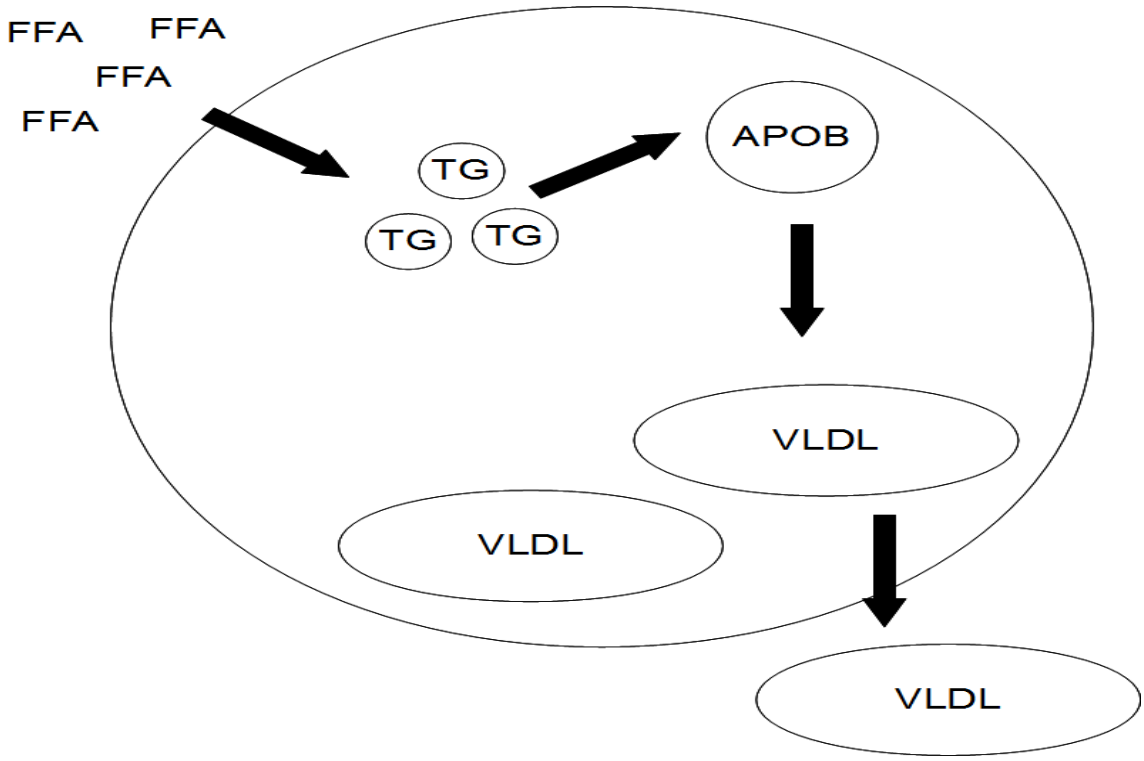


Figure 3 Mechanism of Liver Cell Lipid Secretion.

Lipid secretion is induced by the metabolism of free fatty acids (FFA) to triglycerides (TG). TG are transported to apolipoprotein B (ApoB) molecules to form lipidated ApoB. Lipidated ApoB is then attached to very low density lipoproteins (VLDL) and secreted from liver cells.

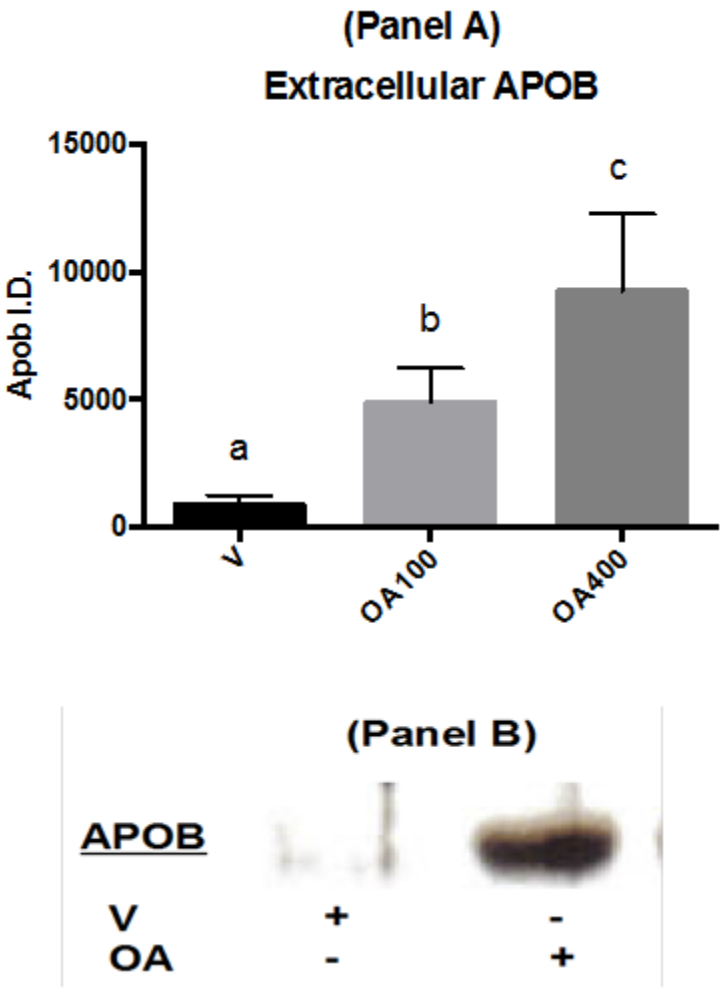


Figure 4 (Panel A) Treatment of McA cells stimulated with 100 µM oleic acid (OA) conjugated to 0.3% BSA, 400 µM OA conjugated to 1.2% BSA, or 1.2% BSA only (vehicle) (V) for 24 hours. (Panel B) Western blot density of ApoB secretion.

Measurement of ApoB secretion for treatments (OA) and vehicle control (V) in 3 experimental trials. Means that do not share the same letter are significantly different ($p \leq 0.05$), as determined by a mixed model analysis with Tukey's adjustment for multiple comparison of least square means. Error bars represent the mean ± the standard error of the mean (SEM) for each treatment group. ApoB secretion levels were increased after OA exposure.

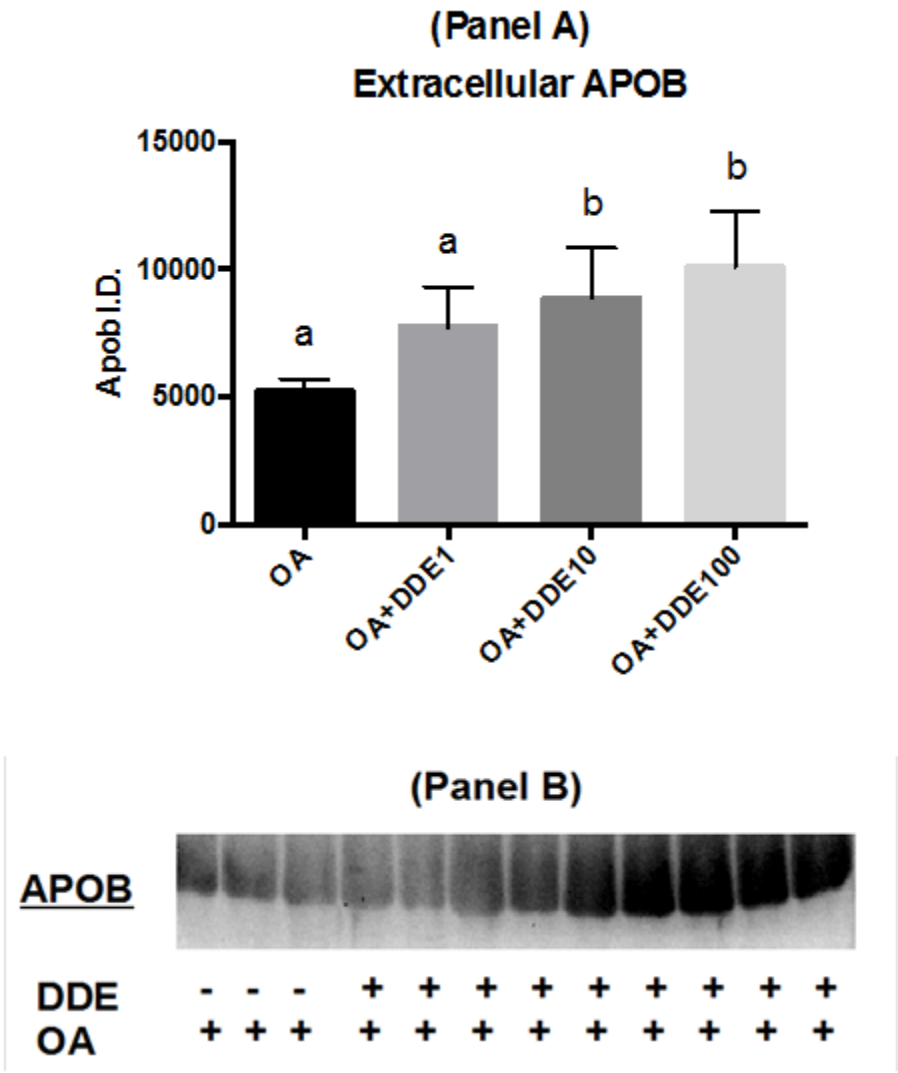
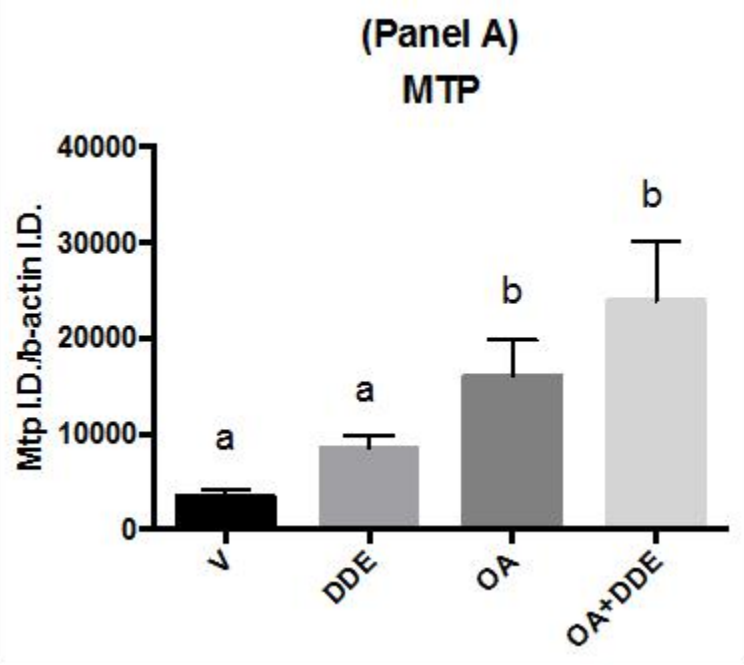


Figure 5 (Panel A) Treatment of McA cells exposed to 1, 10, or 100 µM DDE simultaneously with 400 µM oleic acid (OA) or 400 µM OA only for 24 hours. (Panel B) Western blot density of ApoB secretion.

Measurement of ApoB secretion for treatments (OA+DDE) and control (OA) in 3 experimental trials. Means that do not share the same letter are significantly different ($p \leq 0.05$), as determined by a mixed model analysis with Tukey's adjustment for multiple comparison of least square means. Error bars represent the mean ± the standard error of the mean (SEM) for each treatment group. ApoB secretion levels were increased after DDE exposure at 10 and 100 µM. 1 µM DDE exposure did not have a significant effect on ApoB secretion compared to control.



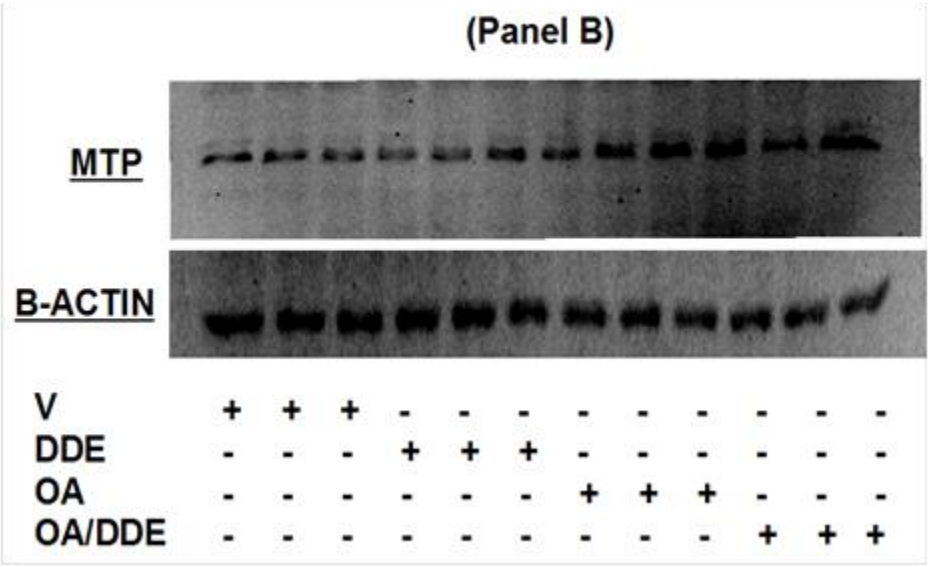
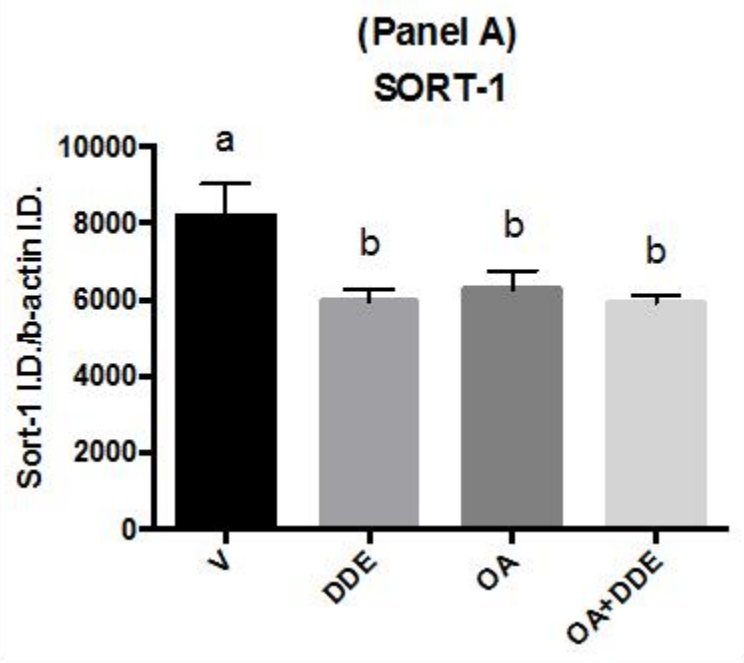


Figure 6 (Panel A) Treatment of McA cells exposed to 10 µM DDE only or simultaneously with 400 µM oleic acid (OA), 400 µM OA only, or 1.2% BSA (vehicle) (V) for 24 hours. (Panel B) Western blot density of MTP.

Measurement of MTP protein levels of cell lysates for treatments (DDE, OA, OA+DDE) and control (V) in 3 experimental trials. Means that do not share the same letter are significantly different ($p \leq 0.05$), as determined by a mixed model analysis with Tukey's adjustment for multiple comparison of least square means. Error bars represent the mean ± the standard error of the mean (SEM) for each treatment group. Intracellular MTP protein levels were increased after DDE exposure with co-exposure to OA.



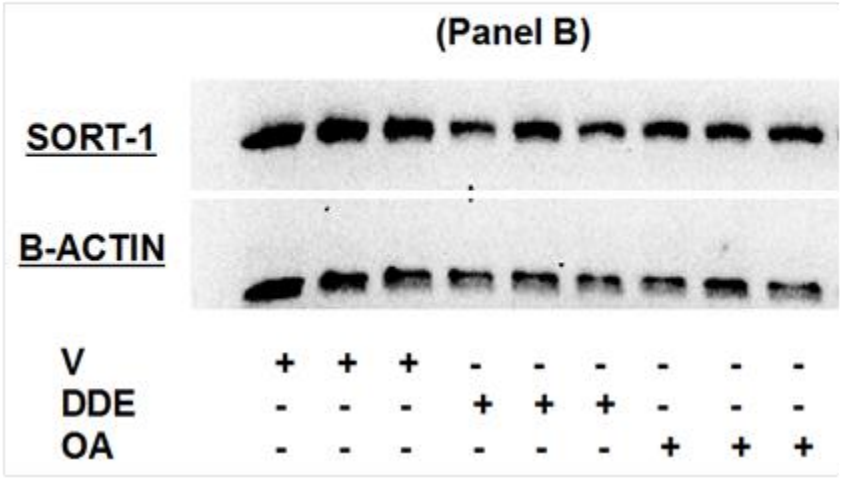


Figure 7 (Panel A) Treatment of McA cells exposed to 10 µM DDE only or simultaneously with 100 µM oleic acid (OA), 100 µM OA only, or 0.3% BSA (vehicle) (V) for 24 hours. (Panel B) Western blot density of Sort-1.

Measurement of Sort-1 protein levels of cell lysates for treatments (DDE, OA, OA+DDE) and control (V) in 3 experimental trials. Means that do not share the same letter are significantly different ($p \leq 0.05$), as determined by a mixed model analysis with Tukey's adjustment for multiple comparison of least square means. Error bars represent the mean ± the standard error of the mean (SEM) for each treatment group. Intracellular Sort-1 protein levels were decreased after DDE exposure with or without co-exposure to OA.

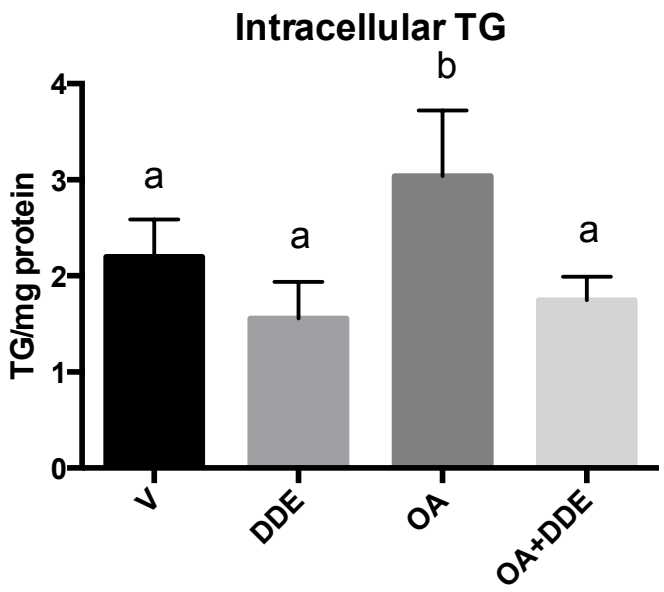


Figure 8 Treatment of McA cells exposed to 10 µM DDE only or simultaneously with 100 µM oleic acid (OA), 10 µM DDE only, 100 µM OA only, or 0.3% BSA (vehicle) (V) for 24 hours.

Measurement of triglyceride accumulation in cell lysates of treatments (DDE, OA, OA+DDE) and control (V) in 3 experimental trials. Means that do not share the same letter are significantly different ($p \leq 0.05$), as determined by a mixed model analysis with Tukey's adjustment for multiple comparison of least square means. Error bars represent the mean ± the standard error of the mean (SEM) for each treatment group. OA stimulated intracellular TG accumulation was decreased after DDE exposure.

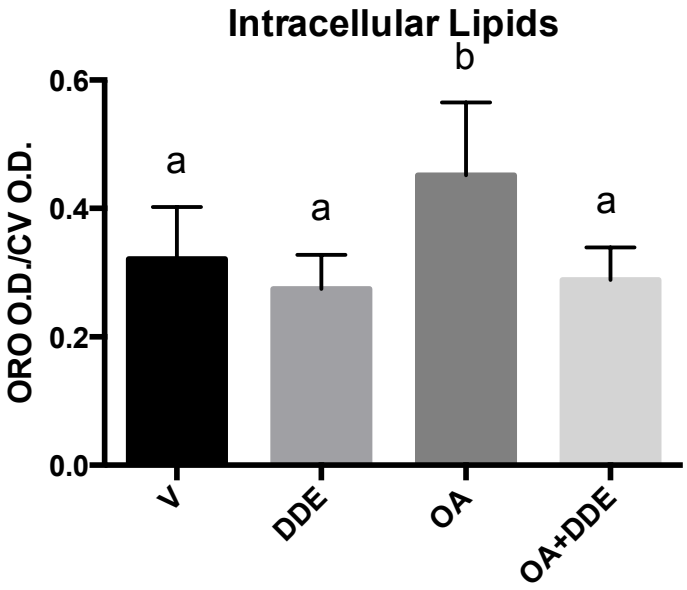


Figure 9 Treatment of McA cells exposed to 10 µM DDE simultaneously with 100 µM oleic acid (OA), 10 µM DDE only, 100 µM OA only, or 0.3% BSA (vehicle) (V) for 24 hours.

Measurement of total neutral lipid accumulation in intact cells of treatments (DDE, OA, OA+DDE) and control (V) in 3 experimental trials. Means that do not share the same letter are significantly different ($p \leq 0.05$), as determined by a mixed model analysis with Tukey's adjustment for multiple comparison of least square means. Error bars represent the mean ± the standard error of the mean (SEM) for each treatment group. Free fatty acid induced neutral lipid accumulation was decreased after DDE exposure.

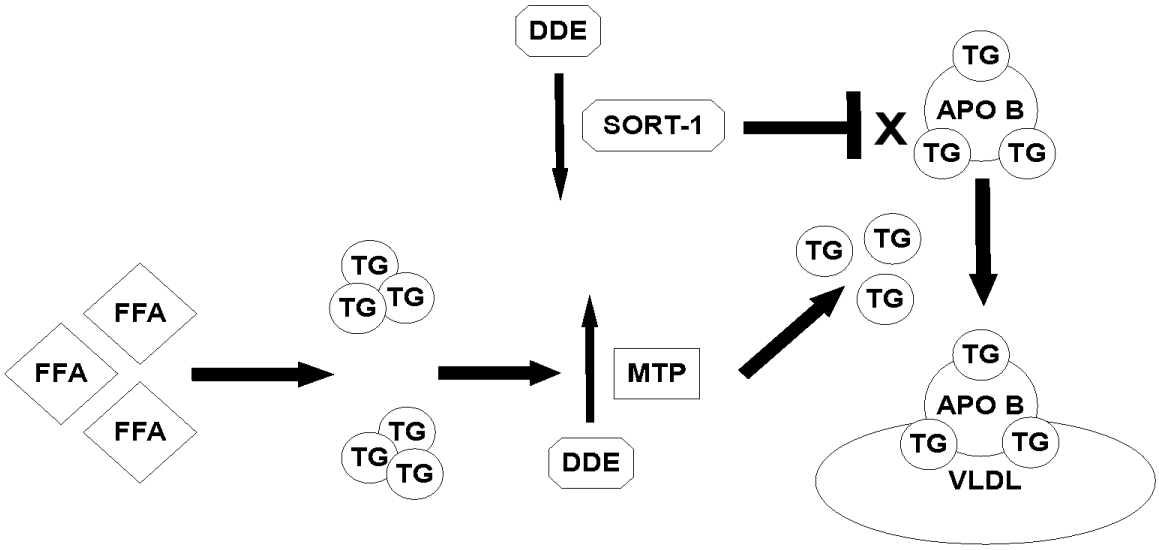


Figure 10 Proposed Mechanism of Dichlorodiphenyldichloroethylene (DDE) Induced Lipid Metabolism and Secretion.

DDE exposure may exert its effect on free fatty acid (FFA) lipid metabolism and lipid secretion by altering the function of biochemical markers involved in lipid synthesis and degradation. DDE exposure decreases the protein levels of Sortilin-1 (Sort-1), reducing apolipoprotein B (ApoB) degradation, while simultaneously DDE exposure increases the protein levels of microsomal triglyceride transfer protein (MTP) which increases lipid transport of neutral lipids such as triglyceride (TG) molecules in liver cells. This event may preserve the overall protein levels of ApoB available for lipidation to form completed lipid molecules such as very low-density lipoproteins (VLDL). The more lipid molecules that are available in liver cells, the more lipids will be secreted from liver cells. DDE exposure is an environmental factor that may alter lipid function in liver cells that could promote dyslipidemia.

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CHAPTER III

IN VITRO EFFECT OF DDE EXPOSURE ON THE REGULATION OF B-TC-6 PANCREATIC BETA CELL INSULIN SECRETION AND ITS ASSOCIATION WITH BETA CELL DYSFUNCTION AND TYPE 2 DIABETES MELLITUS

Introduction

Epidemiological evidence in humans suggests that exhibiting higher levels of some organochlorine compounds (OC), including dichlorodiphenyltrichloroethane (DDT), and its metabolite, dichlorodiphenyldichloroethylene (DDE), is associated with type 2 diabetes mellitus (T2D) (Lee et al., 2006). Based on recent literature, individuals chronically exposed to DDE may be at a greater risk of developing T2D or exhibiting biochemical markers of T2D (Lee et al., 2011). Individuals with T2D exhibit a decline in the ability of their pancreatic beta cells to secrete insulin, which leads to beta cell dysfunction, depletion of beta cell mass, and apoptosis (Dixon et al., 2004). The pancreas is an organ that plays a role in regulating glucose homeostasis by the function of its beta cells to produce sufficient amounts of the hormone insulin (Lim et al., 2011). During T2D development, beta cells of the pancreas undergo physiological changes that lead to beta cell dysfunction (Makaji et al., 2011). The pancreas could be a potential target of OC exposure. Pancreatic beta cells of rats exposed to the OC endosulfan exhibited swelling of the mitochondria resulting in mitochondrial dysfunction and

yielding an increased risk of hyperglycemia (Kalendar et al., 2004). The OC DDE may affect mechanisms in pancreatic beta cells that regulate biochemical markers of beta cell dysfunction and T2D. Long-term exposure to DDE may affect how beta cells in the pancreas synthesize and secrete insulin. Therefore, by using Beta-Tumor Cell-6 (B-TC-6) murine pancreatic beta cells, the effect of DDE exposure on beta cell function was investigated. B-TC-6 cells are a reliable cell line for insulin secretion studies because they secrete sufficient amounts of insulin in response to glucose stimulation. B-TC-6 cells are derived from transgenic mice pancreatic beta cells expressing the pseudogene construct composed of the simian virus-40 t-antigen (SV40) early region controlled by the rat insulin II gene promotor responsible for insulin synthesis and secretion. However, B-TC-6 cells do not respond to different concentrations of glucose in the same manner as primary beta cells (Hohmeier and Newgard, 2004). B-TC-6 cells are derive from a cancerous cell line (mouse insulinoma) and are not the best model for studying insulin secretion compared to using primary cells of human origin.

The purpose of this study was to investigate the *in vitro* effect of DDE exposure on the regulation of pancreatic beta cell function with respect to biochemical markers implicated in beta cell dysfunction: insulin, pancreatic and duodenal homeobox factor-1 (PDX-1), prohormone convertase (PC), and reactive oxygen species (ROS). Insulin is a secreted peptide hormone central to regulating carbohydrate and fat metabolism. Insulin is synthesized and secreted by pancreatic beta cells in response to elevated extracellular blood glucose concentrations (Itoh et al., 2003) (Fig. 11.). When control of insulin regulation is altered, T2D will result (CDC, 2011). PDX-1 is a transcription factor present in beta cells that regulates insulin gene transcription (McKinnon and Docherty,

2001). Research by others has shown that the reduced expression of PDX-1 is associated with beta cell dysfunction, hyperglycemia, and T2D (Brissova et al., 2005). PC is a proteolytic enzyme responsible for the enzymatic cleavage of insulin to its mature form for secretion from beta cells (Fu et al., 2013). Disruption of this enzymatic pathway contributes to altered insulin secretion and beta cell dysfunction (Rhodes and Alarcon, 1994). ROS are a biochemical marker of oxidative stress and play a role in beta cell function (Evans et al., 2003). Pancreatic beta cells express low levels of antioxidants and oxidative stress has been associated with T2D development (Kahn, 2003). These findings will potentially support the epidemiological association of DDE exposure with T2D prevalence by testing our hypothesis: DDE exposure will adversely affect beta cell function resulting in the altered synthesis and secretion of insulin from pancreatic beta cells *in vitro*.

Materials and Methods

The B-TC-6 murine cell line and 0.05% trypsin in 0.53mM ethylenediaminetetraacetic acid (EDTA) solution were purchased from American Tissue Culture Collection (Manassas, VA). Certified fetal bovine serum (FBS), Dulbecco's Modified Eagles Medium (DMEM) with or without glucose, and penicillin-streptomycin were purchased from Invitrogen (Grand Island, NY). p,p'-DDE was purchased from Chem Service (West Chester, PA). Phosphate buffered saline (PBS) was purchased from Mediatech Inc. (Manassas, VA). Radio-immunoprecipitation assay (RIPA) lysis buffer system, PDX-1 antibody, insulin antibody, goat anti-rabbit IgG-HRP, beta-actin antibody, and Western Blotting Luminol Reagent were purchased from Santa Cruz (Dallas, TX). Dimethyl sulfoxide (DMSO), 2X-sodium dodecyl sulfate (SDS) sample loading buffer,

bovine serum albumin-fatty acid free (BSA), hydrogen peroxide (H_2O_2), D-glucose, pGlu-Arg-Thr-Lys-Arg-7-amido-4-methylcoumarin trifluoroacetate salt (p-Glu), and 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) were purchased from Sigma Aldrich (St. Louis, MO). Non-fat dry milk and Bradford protein assay kit were purchased from Bio-Rad (Hercules, CA). Super Signal West Pico Chemiluminescence kit was purchased from Thermo Fisher Scientific (Pittsburgh, PA). High-throughput Time Resolved Fluorescence (HTRF) Insulin Assay was purchased from Cisbio (Bedford, MA). Mercodia Insulin Enzyme-Linked Immunosorbent Assay (ELISA) was purchased from Mercodia (Winston Salem, NC).

B-TC-6 cells were grown in supplemented DMEM containing 15% heat inactivated FBS and a 1% penicillin/streptomycin solution in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C until 70% confluent. Confluent cells were washed with PBS and detached from the culture flask with a 0.05% trypsin in 0.53mM EDTA solution. Cells were pelleted by centrifugation and re-suspended in supplemented DMEM to be seeded overnight in a 12-well cell culture plate to adhere. The next day supplemented DMEM was removed and adherent cells were washed with PBS and treated with serum-free/glucose-free DMEM to starve cells of serum and glucose during an overnight incubation. Following overnight incubation, serum-free/glucose-free DMEM was removed and cells were washed with PBS and subjected to the following experimental protocols.

Experimental Design

Experiment 1. The effect of glucose exposure on pancreatic beta cell function was investigated with respect to insulin secretion levels. D-glucose was used to induce

insulin secretion in B-TC-6 cells. Following standard cell culture protocols, serum and glucose starved cells were treated with 0, 5, 10, or 15 mM glucose in DMEM for 2 hours for a total of 4 treatment groups repeated in triplicate in each of 3 individual experimental trials. Cell media was assayed for the detection of secreted insulin protein levels using an immunoassay specific for insulin (Cisbio).

Experiment 2. The effect of DDE exposure on the regulation of insulin secretion was investigated. Serum and glucose starved cells were treated with 10 μM DDE dissolved in 0.1% DMSO, 1 μM H_2O_2, or 0.1% DMSO for 24 hours with or without co-exposure to 5 mM glucose in DMEM for a total of 3 treatment groups repeated in triplicate in each of 3 individual experimental trials. H_2O_2 was used as a positive control to decrease insulin secretion. Cell media was assayed for the detection of secreted insulin protein levels using an immunoassay specific for insulin (Cisbio).

Experiment 3. The effect of glucose exposure on intracellular ROS levels in B-TC-6 cells was investigated. Serum and glucose starved cells were exposed to 0, 5, or 10 mM glucose in DMEM for 3 hours for a total of 3 treatment groups repeated in triplicate in each of 3 individual experimental trials. Cells were assayed for the detection of intracellular ROS levels using spectrophotometry.

Experiment 4. The effect of DDE exposure on ROS levels in B-TC-6 cells was investigated. Serum and glucose starved cells were exposed to 10 μM DDE, 1 μM H_2O_2, or 0.1% DMSO only with or without co-exposure to 5 mM glucose in DMEM for 3 hours for a total of 3 treatment groups repeated in triplicate in each of 3 individual experimental trials. Cells were assayed for the detection of intracellular ROS levels using spectrofluorometry.

Experiment 5. The effect of DDE exposure on the regulation of insulin transcription with respect to PDX-1 and insulin protein levels was investigated. Serum and glucose starved cells were exposed to 1, 10, or 100 μM DDE or 0.1% DMSO in DMEM for 24 hours for a total of 4 treatment groups repeated in triplicate, or exposed to 10 μM DDE, 1 μM H_2O_2, or 0.1% DMSO for 24 hours with an additional exposure to 5 mM glucose in DMEM for 2 hours for a total of 3 treatment groups repeated in triplicate in each of 3 individual experimental trials. Cell lysates were assayed for intracellular PDX-1 and insulin protein levels using SDS-PAGE with Western Blot Analysis.

Experiment 6. The effect of DDE exposure on the regulation of post-translational modification of insulin with respect to PC enzymatic activity was investigated. Serum and glucose starved cells were exposed to 1, 10, or 100 μM DDE or 0.1% DMSO in DMEM for 24 hours for a total of 4 treatment groups repeated in triplicate, or exposed to 10 μM DDE, 1 μM H_2O_2, or 0.1% DMSO for 24 hours with an additional exposure to 5 mM glucose in DMEM for 2 hours for a total of 3 treatment groups repeated in triplicate in each of 3 individual experimental trials. Cell lysates were assayed for the detection of PC enzyme activity using spectrophotometry.

Assay Development

Assay 1. Bradford Protein Analysis. Following all experimental protocols, the cell media was collected and stored at -80°C and adherent cells were washed with PBS and lysed in RIPA buffer with or without protease/phosphatase inhibitors and stored at -80°C for future analysis. The protein concentrations of whole cell lysates were separated from cell debris after centrifugation at 16.1 RCF for 4°C using an Allegra X-14R Centrifuge (Beckman Coulter). The protein concentrations of cell media and lysates

from each treatment group were quantified by the Bradford protein assay. All treatment groups were normalized to the protein concentrations of their respective cell lysates for Western Blot Analysis.

Assay 2. SDS-PAGE with Western Blot Analysis. Protein concentrations were normalized for all treatment samples using 50 mM Tris-hydrochloride buffer (pH 7.4 at 4°C). Equal amounts of 2X-SDS sample loading buffer were added to each treatment sample at a 1:1 dilution. Diluted protein samples were heated at 95°C and allowed to cool. After cooling, samples were pipetted at equal volumes per well on a 10% polyacrylamide gel and subjected to electrophoresis for the separation of protein bands by molecular weight at 100 volts for 15 minutes and 120 volts for 1 hour using a Mini-PROTEAN 3 system. Following electrophoresis, the gel containing the protein bands was transferred to a 0.45 µm polyvinylidene fluoride (PVDF) membrane with electrophoresis at 20 volts for 30 minutes using a Trans-Blot SD semi-dry electrophoretic transfer cell system (Bio-Rad). After a successful transfer, PDX-1 and insulin protein levels were detected on a 0.45 µm PVDF membrane using the following antibodies: PDX-1-primary antibody, insulin-primary antibody, beta-actin-primary antibody, and goat anti-rabbit IgG-HRP-secondary antibody. Membranes were incubated with a blocking solution (5% milk) to prevent non-specific binding of antibodies. Following successful blocking, membranes were incubated with primary antibodies diluted 1:500 at 4°C overnight. After overnight incubation, membranes were washed with a solution of Tris-buffered saline containing 0.05% Tween-20 for 15 minutes. After washing, membranes were incubated with secondary antibodies diluted 1:2000 for 1 hour at room temperature to detect protein bands. After washing again for 15 minutes, membranes

containing the appropriate antibody conjugated proteins were subjected to a chemical reaction of a 1:1 combination of super signal peroxide and luminol enhancer solution for 1 minute. The horseradish peroxidase conjugated to the secondary antibody mediated the oxidation of luminol in the presence of hydrogen peroxide resulting in light emission or chemiluminescence. Following this reaction, protein bands were visualized using the Chemi-Doc XRS System with Image Lab Software (Bio-Rad). Protein band density was quantified using ImageJ Software supplied by the National Institutes of Health (NIH).

Assay 3. Insulin Secretion Analysis. Cell media was collected and assayed for insulin secretion levels. Fifty µl of cell media per treatment was pipetted into a 96-well microplate. Twenty five µl each of anti-insulin Ab-cryptate and anti-insulin-Ab-XL665 antibodies were added to each sample well for the detection of insulin protein levels. These two antibodies recognize insulin molecules simultaneously. When they are in close proximity with each other, fluorescence resonance energy transfer (FRET) occurs between them. This FRET increases as insulin concentration increases. Samples were incubated for 2 hours at room temperature to increase antibody detection of insulin. After incubation, insulin secretion levels were detected by measuring the FRET values from each sample well at 620/665 nm using a SpectraMax M5 spectrophotometer (Molecular Devices). Insulin levels were quantified using an insulin standard curve from diluted solutions of purified insulin protein.

Assay 4. Intracellular ROS Analysis. After glucose or DDE exposure, cells were washed with PBS and treated with a fluorescently labeled dye solution (25 µM DCFDA). Cells were incubated for 1 hour with DCFDA to identify markers of oxidative stress (ROS). DCFDA, a cell permeable fluorescent probe, is oxidized by intracellular ROS

into a fluorescent product (2′,7′-dichlorofluorescein). Intracellular ROS levels in cells were representative of oxidative stress levels and were measured fluorescently at 485/535 nm from intact cells or cell lysates in a 96-well microplate using a SpectraMax M5 spectrophotometer (Molecular Devices).

Assay 5. PC Enzymatic Analysis. Cell lysates were diluted with a solution (100 mM sodium acetate buffer, pH 6.0, 10 mM $CaCl_2$) and incubated for 15 minutes. Following incubation, cell lysates were treated with 25 µM p-Glu and incubated for 1 hour at 37°C in a 96-well microplate. p-Glu is a fluorescently labeled substrate for the enzyme PC. After incubation, samples were measured for the hydrolysis of p-Glu to its metabolite (amino-4-methylcoumarin) by intracellular protein levels of PC. PC hydrolysis activity was measured from the fluorescent emission of the metabolite at 380/460 nm in a 96-well microplate using a SpectraMax M5 spectrophotometer (Molecular Devices).

Statistical Analysis

The effect of treatments on biochemical markers was assessed by mixed model analysis using PROC MIXED in SAS for windows 9.4 (SAS Institute, Inc., Cary, NC). Treatment was included as a fixed effect in separate models for each biochemical marker. Experimental trials were included as a random effect. If treatment was significant, Tukey's adjustment for multiple comparisons was used to determine differences in least square means. A p-value of ≤ 0.05 was considered statistically significant based on least square means differences for each treatment group. Graphical data were expressed as the mean ± the standard error of the mean (SEM) for each treatment group repeated in triplicate for each of 3 individual experimental trials.

Results

Glucose was administered to induce insulin secretion from B-TC-6 cells to verify that the cells were responding as expected. Cells were exposed to different concentrations of glucose to investigate a concentration-response effect on insulin secretion. Insulin secretion levels measured from immunoassaying of cell media were quantified for the detection of any significant difference between glucose-exposed and non-exposed cells. Exposure to glucose significantly increased insulin secretion in a concentration-dependent manner (Fig. 12.).

The effect of DDE exposure plus glucose exposure on beta cell insulin secretion was investigated. After a 24 hour exposure to DDE with or without exposure to glucose for 2 additional hours, cell media was collected and subjected to immunoassay for the detection of insulin secretion levels. Exposure of B-TC-6 cells to DDE caused a significant increase in basal and glucose stimulated insulin secretion levels (Fig. 13.) (Fig. 14.).

The idea that glucose metabolism induces changes in oxidative stress levels was investigated. Therefore, a concentration-response effect of glucose exposure on beta cell insulin secretion was measured. Exposure of B-TC-6 cells to increasing concentrations of glucose increased ROS levels in conjunction with increasing insulin secretion, as expected (Fig. 15.).

The idea that DDE exposure may regulate biochemical markers of oxidative stress in B-TC-6 cells was investigated. Cells were exposed to DDE with or without glucose for 3 hours to test this hypothesis. DDE exposure with or without glucose exposure

yielded a trend towards increased ROS levels compared to controls, although not significant (data not shown).

The effect of DDE exposure on the regulation of insulin synthesis by the transcription factor PDX-1 was investigated. Immunoblotting of cell lysates for the intracellular detection of PDX-1 revealed that DDE exposure alone yielded a trend for increased PDX-1 protein levels compared to controls, although not significant (data not shown). However, the opposite effect was observed from DDE exposed cells after glucose stimulated insulin secretion, although not significant (data not shown).

The effect of DDE exposure on the regulation of post-translational modification of insulin was investigated. The enzyme activity of endogenous PC from cell lysates exposed to DDE with or without glucose stimulated insulin secretion was measured. PC enzyme activity levels were increased in DDE exposed cells (Fig. 16.), but decreased in DDE and glucose exposed cells (Fig. 17.).

Discussion

Multiple cell signaling pathways can be activated to affect the production of insulin in beta cells (Fu et al., 2013). The abnormal regulation of pathways involved in the synthesis and secretion of insulin can be detrimental to overall beta cell function (Rhodes and Alarcon, 1994). Many factors can contribute to the altered regulation of insulin signaling in beta cells. One factor such as the effect of DDE exposure on insulin signaling has not been explored. Investigating this factor is significant because OC including DDE are lipophilic compounds that persist for years in the environment and bioaccumulate in organisms (CDC, 2012; EPA, 1999). Therefore, we investigated the potential *in vitro* effect of DDE exposure on beta cell function because of the

epidemiological association of DDE with T2D (Lee et al., 2007). Environmental exposure to DDE could play a role in affecting biochemical markers of beta cell function, potentially resulting in beta cell dysfunction and T2D development (Fig. 18.).

Overall, this study may explain the *in vitro* mechanisms behind the recent epidemiological data that DDE exposure is a risk factor for the potential development of T2D (Son et al., 2010). The purpose of the present study was to investigate whether *in vitro* exposure to the environmental contaminant DDE leads to changes in beta cell biochemical markers that are consistent with the development of T2D. Therefore, these data support our hypothesis that DDE exposure alters beta cell function by the changes observed in biochemical markers that regulate insulin secretion.

Glucose exposure had a significant effect on insulin secretion in this study similar to the observations of others (Skelin et al., 2010). DDE exposure significantly increased insulin secretion from B-TC-6 cells with or without co-exposure to glucose suggesting a role in regulating insulin synthesis. ROS levels were not significantly different in DDE exposed cells in the presence or absence of glucose diminishing oxidative stress a possible mechanistic pathway for the regulation of insulin secretion. H_2O_2 exposure did not increase ROS levels and decrease insulin secretion from beta cells, which was not expected (Pi et al., 2007). DDE exposure may increase basal insulin secretion by inducing cell mechanisms that regulate post-translational modification and insulin secretion. PDX-1 protein levels in cell lysates were not increased in DDE exposed cells compared to non-exposed cells diminishing a role of DDE in regulating insulin transcription. DDE exposure may play a role in regulating insulin translational modification by its effect on PC enzyme activity that is responsible for the synthesis of

insulin *in vitro*. During non-glucose stimulation, DDE exposure increased overall insulin secretion and PC levels in B-TC-6 cells. However, glucose stimulation after DDE exposure only increased insulin secretion while decreasing PC levels. This could suggest that DDE exposure initially increases insulin secretion but long-term may actually decrease insulin secretion by blunting glucose induced regulation of insulin synthesis and secretion. DDE exposure may cause similar effects observed in beta cell burnout (hyperinsulinemia, increased cellular stress, and late-stage decreased insulin secretion) of beta cells identified in the progression of T2D (Dixon et al., 2004). DDE exposure could have an effect on the regulation of beta cell function by its adverse effect on insulin synthesis and secretion, which over time could contribute to the progression of beta cell dysfunction and T2D (Weir, 2004). Based on this study, one could hypothesize that individuals exposed to high levels of the OC contaminant DDE may be at a greater risk of exhibiting biochemical markers of beta cell dysfunction associated with T2D. The goal of this research was to understand the potential adverse effects associated with the exposure of environmental contaminants on human health and the environment and associate environmental exposure as a possible factor in the development of chronic diseases such as T2D.

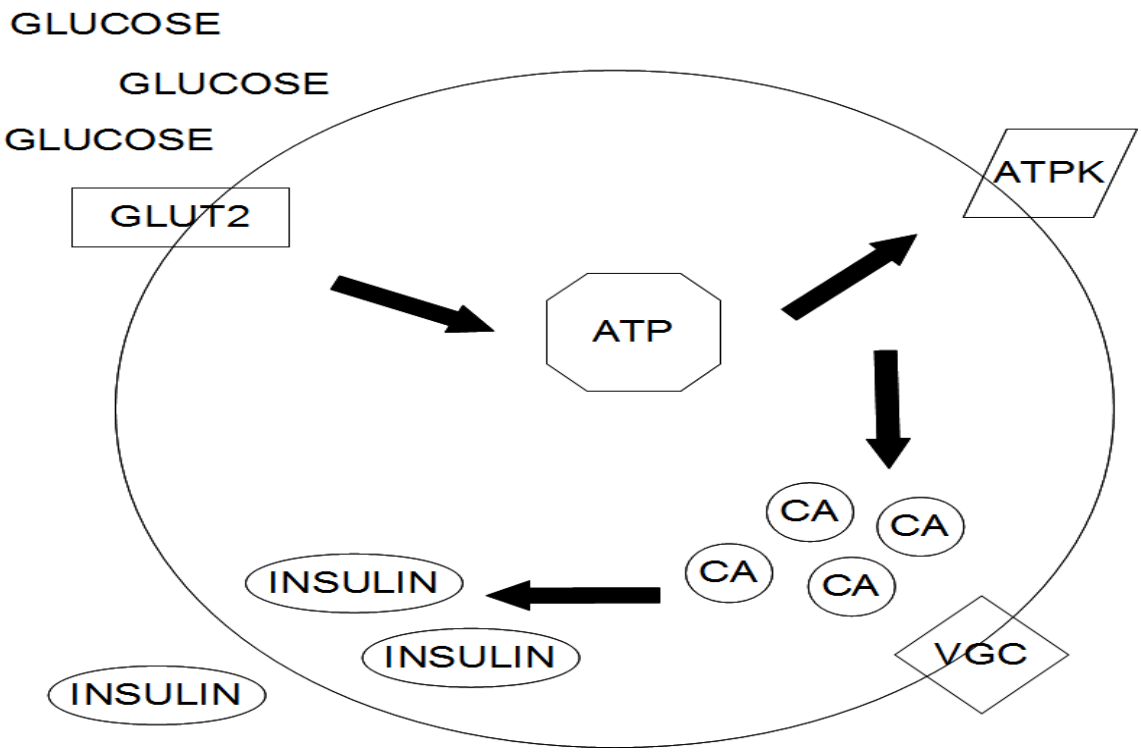

Figure 11 Mechanism of Pancreatic Beta Cell Insulin Secretion.

Glucose enters the beta cell through glucose transporter-2 (GLUT2). Once inside the cell glucose metabolism increases the levels of adenosine triphosphate (ATP). ATP binds to and closes the ATP-dependent-potassium channel (ATPK). This causes the cell membrane to depolarize and opens the voltage-gated calcium channel (VGC). The increased influx of calcium ions causes insulin that is stored in intracellular vesicles to be released out of the cell resulting in insulin secretion.

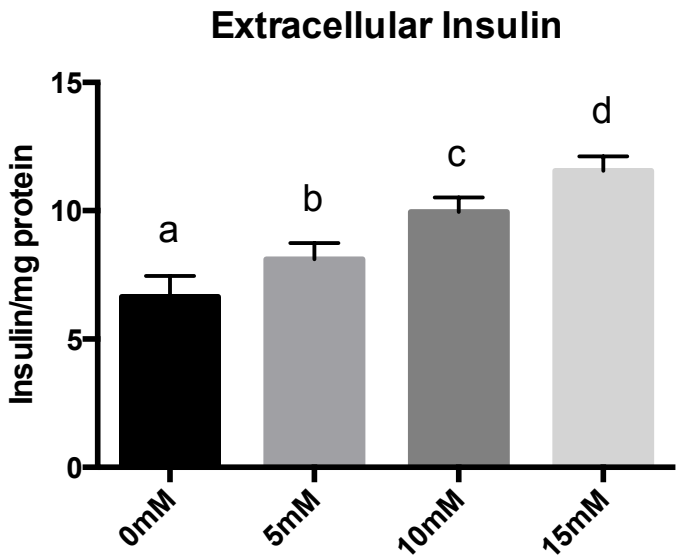

Figure 12 Treatment of B-TC-6 cells exposed to 0, 5, 10, or 15 mM glucose for 2 hours.

Measurement of insulin secretion for treatments (5, 10, 15 mM) and control (0 mM) in 3 experimental trials. Means that do not share the same letter are significantly different ($p \leq 0.05$), as determined by a mixed model analysis with Tukey's adjustment for multiple comparison of least square means. Error bars represent the mean ± the standard error of the mean (SEM) for each treatment group. Insulin secretion was increased with increasing glucose concentrations.

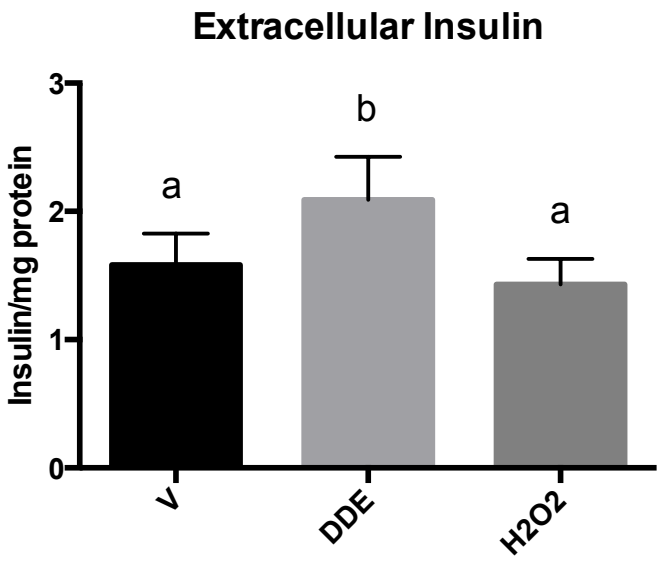

Figure 13 Treatment of B-TC-6 cells exposed to 10 µM DDE, 1 µM H_2O_2, or 0.1% DMSO (vehicle) (V) without glucose for 24 hours.

Measurement of insulin secretion for treatments (DDE, H_2O_2) and control (V) in 3 experimental trials. Means that do not share the same letter are significantly different ($p \leq 0.05$), as determined by a mixed model analysis with Tukey's adjustment for multiple comparison of least square means. Error bars represent the mean ± the standard error of the mean (SEM) for each treatment group. Basal insulin secretion increased after DDE exposure compared to control.

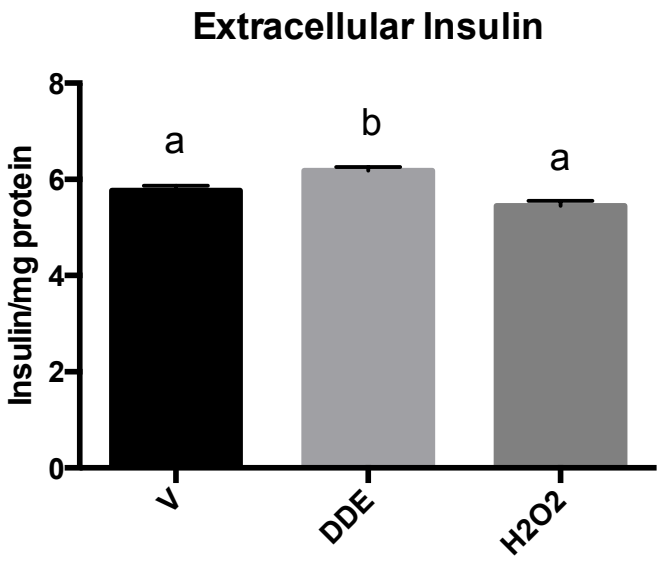

Figure 14 Treatment of B-TC-6 cells exposed to 10 µM DDE, 1 µM H_2O_2, or 0.1% DMSO (vehicle) (V) for 24 hours and stimulated an additional 2 hours with 5 mM glucose.

Measurement of insulin secretion for treatments (DDE, H_2O_2) and control (V) in 3 experimental trials. Means that do not share the same letter are significantly different ($p \leq 0.05$), as determined by a mixed model analysis with Tukey's adjustment for multiple comparison of least square means. Error bars represent the mean ± the standard error of the mean (SEM) for each treatment group. Glucose stimulated insulin secretion increased after DDE exposure compared to control.

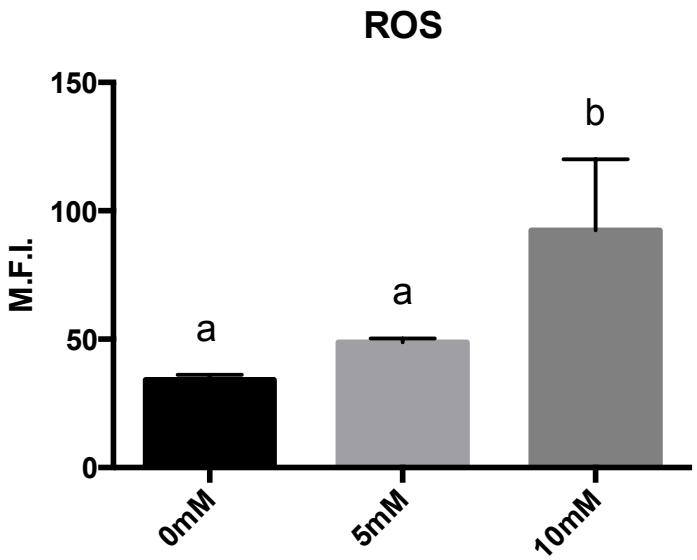

Figure 15 Treatment of B-TC-6 cells exposed to 0, 5, or 10 mM glucose for 3 hours.

Measurement of intracellular ROS levels for treatments (5, 10 mM) and control (0 mM) in 3 experimental trials. Means that do not share the same letter are significantly different ($p \leq 0.05$), as determined by a mixed model analysis with Tukey's adjustment for multiple comparison of least square means. Error bars represent the mean ± the standard error of the mean (SEM) for each treatment group. ROS levels were only increased with 10 mM glucose exposure.

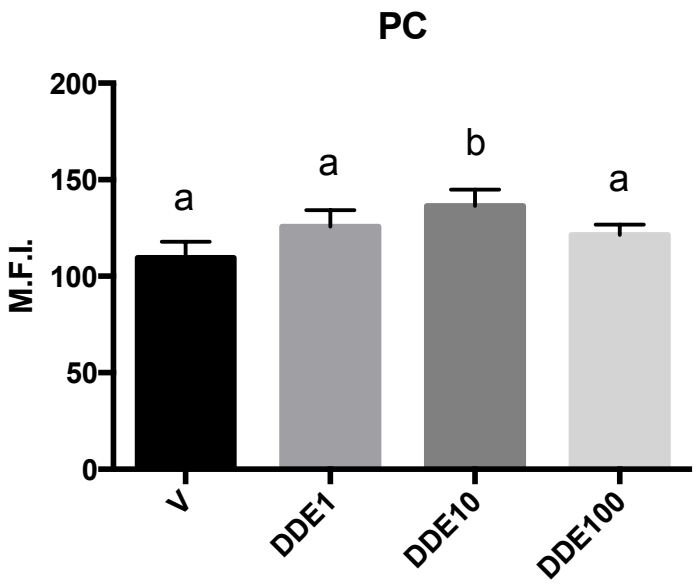

Figure 16 Treatment of B-TC-6 cells exposed to 1, 10, or 100 µM DDE or 0.1% DMSO (vehicle) (V) for 24 hours.

Measurement of PC enzyme activity of cell lysates for treatments (DDE) and control (V) in 3 experimental trials. Means that do not share the same letter are significantly different ($p \leq 0.05$), as determined by a mixed model analysis with Tukey's adjustment for multiple comparison of least square means. Error bars represent the mean ± the standard error of the mean (SEM) for each treatment group. Although no concentration-response effect was observed with increasing DDE concentrations, basal PC enzyme activity was increased after 10 µM DDE exposure compared to control.

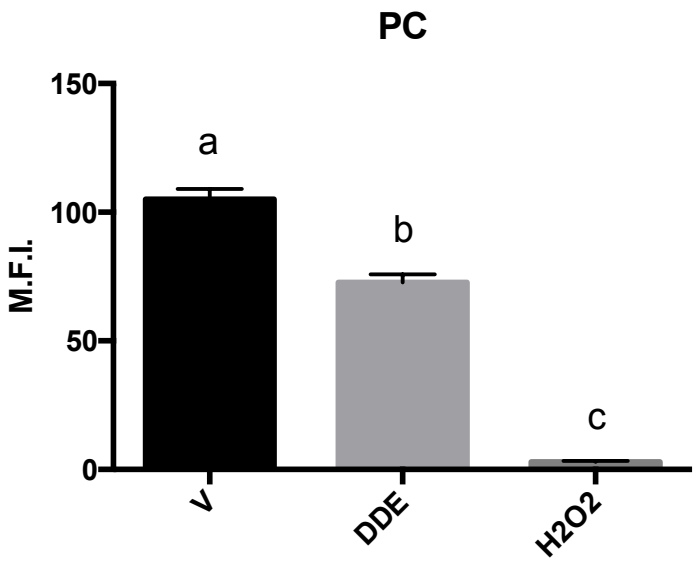

Figure 17 Treatment of B-TC-6 cells exposed to 10 µM DDE, 1 µM H_2O_2, or 0.1% DMSO (vehicle) (V) for 24 hours and stimulated with 5 mM glucose for 2 hours.

Measurement of PC enzyme activity levels of cell lysates for treatments (DDE, H_2O_2) and control (V) in 3 experimental trials. Means that do not share the same letter are significantly different ($p \leq 0.05$), as determined by a mixed model analysis with Tukey's adjustment for multiple comparison of least square means. Error bars represent the mean ± the standard error of the mean (SEM) for each treatment group. Although DDE exposure alone increased basal PC levels, PC levels were decreased after glucose exposure compared to control.

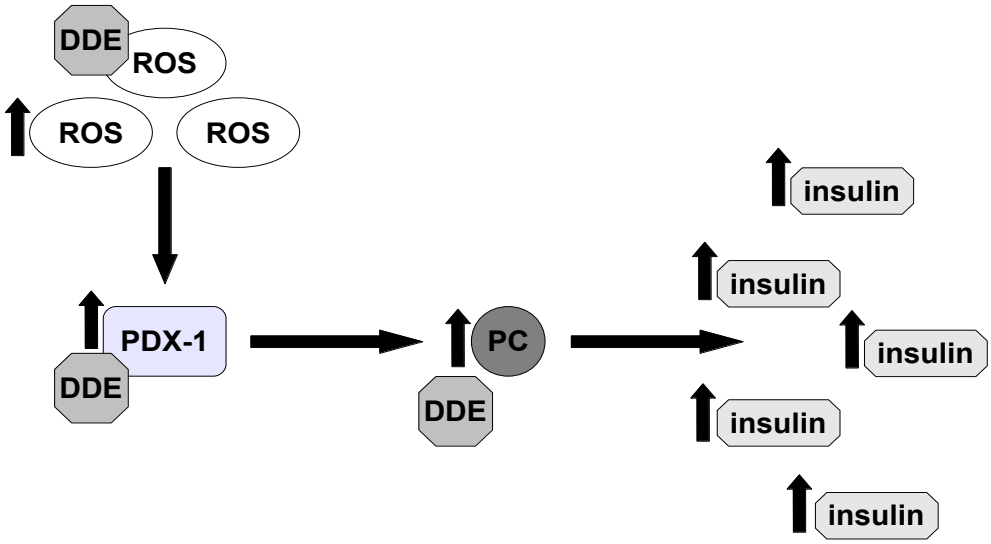

Figure 18 Proposed Mechanism of Dichlorodiphenyldichloroethylene (DDE) Induced Beta Cell Dysfunction.

DDE exposure may induce reactive oxygen species (ROS) levels, increase the expression of the insulin transcription factor pancreatic and duodenal homeobox factor-1 (PDX-1), or increase the enzyme activity of prohormone convertase (PC). Alteration in any one of these processes increases the synthesis and/or secretion of insulin from beta cells, representative of beta cell dysfunction. However, glucose exposure after DDE exposure may further induce ROS levels while having no significant effect on PDX-1 and PC protein levels. This produces the opposite effect of DDE exposure alone resulting in a decrease of insulin secretion, which is also representative of beta cell dysfunction.

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CHAPTER IV

DDE EXPOSURE IN A POPULATION EXHIBITING HIGH TYPE 2 DIABETES PREVALENCE: AN EPIDEMIOLOGICAL ASSOCIATION OF GEOGRAPHICAL EXPOSURE WITH BIOCHEMICAL MARKERS OF TYPE 2 DIABETES MELLITUS

Introduction

The prevalence of type 2 diabetes mellitus (T2D) is increasing by the millions every year worldwide (Amos et al., 1997). Because genetics can only explain a small percentage of disease development, more attention is being given to environmental exposure and disease association (Hunter, 2005). The prevalence of T2D has been increasing with not all clear causes identified, while the use of synthetic chemicals has risen (Neel and Sargis, 2011). Exposure to environmental contaminants is a factor that could play a role in the pathogenesis of T2D (Lee et al., 2006; Carpenter, 2008). Belzoni, MS, is an agricultural community in the Mississippi Delta region that was once highly exposed to the synthetic organochlorine compound (OC) dichlorodiphenyltrichloroethane (DDT) and is still polluted with levels of the environmental contaminant dichlorodiphenyldichloroethylene (DDE) based on soil sample studies from our lab (340 ng DDE/g soil). The Mississippi Delta region also has a high prevalence of T2D, so the associations of chemical contamination with disease prevalence should be investigated.

Due to the increased epidemiological evidence of OC contaminants being associated with T2D, the goal of this study was to investigate this association in a population exhibiting a high prevalence of T2D which also has a high risk of OC exposure. The working hypothesis for this study was that individuals currently living in high exposure regions for OC who exhibit T2D or biochemical markers of T2D could have higher than average levels of the OC contaminant DDE in their blood. Although DDE levels might be found in all individuals, having higher levels detected in blood samples from diabetics versus non-diabetics would support research by others of an association with T2D (Lee et al., 2011). Therefore, the purpose of this study was to investigate DDE exposure as a possible environmental risk factor for the association of biochemical markers implicated in T2D: Apolipoprotein B (ApoB), triglycerides (TG), and paraoxonase-1 (PON). ApoB plays an important role in lipid transport and clearance. ApoB levels are significantly higher in individuals exhibiting dyslipidemia and T2D (Qiu et. al, 2006). TG are synthesized from free fatty acids in the liver. TG are a biochemical marker implicated in T2D and levels are usually higher in individuals with diabetes compared to non-diabetics (Albrink and Man, 1958). PON functions as a protective antioxidant against the oxidation of lipids. Previous epidemiological data concludes that PON levels are decreased in T2D (Manning et al., 2012).

Materials and Methods

Human blood samples were collected from the Gorton Rural Health Clinic (Belzoni, MS). Apolipoprotein B (ApoB) Enzyme-Linked Immunosorbent Assay (ELISA) kit was purchased from Mabtech Inc. (Cincinnati, OH). Paraoxon (ultra pure) was a generous gift from Dr. Howard Chambers from Mississippi State University

(Mississippi State, MS). Phenyl acetate, acetonitrile, ethyl acetate, hexane, deionized water, ethylenediaminetetraacetic acid (EDTA), calcium chloride ($CaCl_2$), and bovine serum albumin-fatty acid free (BSA) were purchased from Sigma Aldrich (St. Louis, MO). Disposable pipette extraction (DPX) columns were purchased from DPX Labs (Columbia, SC). Carbon-13-p,p'-DDT and carbon-13-trans-nonachlor internal standard solutions were purchased from Cambridge Isotopes (Tewksbury, MA). Phosphate buffered saline (PBS) was purchased from Mediatech Inc. (Manassas, VA).

Human blood samples were obtained from a population in which our laboratory has obtained access through the Gorton Rural Health Clinic (Belzoni, MS) following Institutional Review Board (IRB) approval by Mississippi State University (MSU). Individuals within this population were patients of the Gorton Rural Health Clinic and represent a small subset of the Mississippi Delta region. This study population consisted of 77 subjects between the ages of 25 and 65 who were all females of African American descent and living in the Mississippi Delta region. The demographic and clinical data of each individual were collected by the study doctors and nurses from the Gorton Clinic (height, weight, blood pressure, waist size, history of high blood pressure and smoking, family history of heart disease, occupation, job history, use of medication, exposure to pesticides, diabetes presence, heart disease, and lipid levels). Serum, plasma, red blood cell, and urine samples were obtained from all individuals. Individuals were compensated with a $10 gift card to a local department store for their participation. All samples were collected with informed consent and public health records were kept confidential. Samples were de-identified prior to any scientific and/or statistical analysis by MSU researchers.

Experimental Design/Assay Development

Assay 1. OC Extraction Analysis. Human plasma samples were tested for levels of DDE with a solid phase extraction method using DPX columns with quantitation via gas chromatography with mass spectrometry (GC/MS) detection. After obtaining human plasma samples, aliquots of 1 ml were used for OC extractions. Samples were thawed and 100 µl of an analytical internal standard mix of carbon-13-p,p'-DDT and carbon-13-trans-nonachlor solution were added to 1 ml of each sample. The samples were vortexed for 1 minute and allowed to incubate for 5 minutes at room temperature. Two ml of acetonitrile were added to each sample and vortexed for 1 minute and incubated at room temperature for 5 minutes. The samples were centrifuged at 1,200 x g for 10 minutes and the supernatant was collected into clean test tubes. Two ml of deionized water was added to the supernatant and the extract was aspirated using a DPX column for 30 seconds. The liquid layer was extracted out of the column using a syringe and discarded. Following the extraction phase, 0.5 ml of wash solution (33% acetonitrile in water) was aspirated into the column for 10 seconds and discarded. One ml of elution solvent (1:1 ratio of ethyl acetate/hexane) was aspirated into the column for 10 seconds and the eluent was collected in a clean conical tube for a total of 2 times. The eluents were combined and dried under a stream of nitrogen gas. The total eluent was resuspended with 100 µl elution solvent and dried under nitrogen gas again. After drying, the eluent was resuspended in a final solution of 100 µl elution solvent and placed in a GC vial with a glass insert. The DDE levels in the final elution solvent were detected via GC/MS detection.

Assay 2. GC/MS Analysis. The analysis of DDE levels from the extracted samples were quantified by isotope dilution via GC/MS. A targeted analysis using isotope labeled internal standards were administered using the single ion monitoring (SIM) mode of the 6890N Network GC System (Agilent Technologies). This analysis was used to quantify levels of the DDE analytes detected. The limit of detection (LOD) is 100 pg/ml for DDE, and the mean percent recovery detected around 90%. DDE levels from samples were detected using a 30m x 0.25mm i.d. DB-5MS column supplied by J&W Scientific (Folsom, CA). Helium was administered as the carrier gas at a flow rate of 1 ml per minute with an injector temperature of 275°C and a transfer line temperature of 270°C. Each sample was analyzed with an average run time of 27.24 minutes. An untargeted analysis was also performed using the full-scan mode of the GC/MS on a random selection of samples in order to identify any additional OC pesticide analytes detectable in the samples. All analytes detected were identified via Agilent Technologies' deconvolution reporting software and summated to the targeted analysis in the SIM mode if detected in at least 50% of samples for analysis. OC levels expressed as nanogram of OC analyte per milliliter of plasma (ng/ml) from each sample were adjusted for the total plasma lipid levels for each subject, which is representative of total OC body burden, since OC are lipophilic and are expected to partition in lipid molecules/adipose tissue in the body (Schisterman et al., 2005). Thus, OC levels normalized on total blood lipids (cholesterol esters, free cholesterol, phospholipids, and triglycerides) are a proxy for adipose tissue OC levels. Lipid levels for each subject were calculated from their clinical lipid data using the simplified equation, [total lipids (mg/dl) = 2.27 × total cholesterol (mg/dl) + triglycerides (mg/dl) + 62.3)] which is derived from a broader

equation described by Phillips et al. (1989), assuming a cholesterol ester percentage of 73% and the regression analysis between total cholesterol and phospholipid concentrations. Total calculated lipid levels were expressed as gram of lipid per liter of plasma (g/L), and lipid adjusted OC levels were standardized (plasma OC concentrations divided by plasma lipids concentrations) and expressed as ng of OC analyte per gram of lipid (ng/g) (Bergonzi et al., 2009).

Assay 3. ApoB Analysis. Human serum samples were analyzed for the detection of ApoB levels using an immunoassay specific for human ApoB (Mabtech, Inc.). Clear microplates (96-well) were coated with 100 µl per well of a monoclonal antibody (LDL 20/17) solution diluted to 2 µg/ml, and incubated overnight at 4°C. Following incubation, plates were washed with PBS and blocked for non-specific binding using 200 µl of PBS with 0.05% Tween-20 containing 1% BSA at room temperature for 1 hour. After blocking, plates were washed with PBS and 100 µl of serum samples and standards were added in triplicate wells and incubated at room temperature for 1 hour. Plates were washed with PBS and ApoB antigen levels were targeted using 100 µl per well of a biotinylated monoclonal antibody (LDL 11) solution diluted to 1 µg/ml with incubation for 1 hour at room temperature. Plates were washed with PBS and incubated with 100 µl per well of a detection antibody solution, streptavidin-horseradish peroxidase diluted 1:1000, for 1 hour at room temperature. After incubation, plates were washed with PBS and incubated in 100 µl of tetramethylbenzidine (TMB) substrate solution (T-0440) for 15 minutes in the dark. A stop solution of 50 µl 1N sulfuric acid (H_2SO_4) was added to the TMB solution to produce a colored product representative of ApoB protein levels. This colored product was measured at 450 nm using a ThermoMax spectrophotometer

(Molecular Devices). ApoB levels were quantified using a calibrated human recombinant ApoB standard that yielded a standard curve representative of ApoB levels.

Assay 4. PON Enzymatic Analysis with Paraoxon. Human serum samples were investigated for the quantitation of PON enzyme activities based on the hydrolysis of paraoxon (Fig. 19., A). Human serum samples were thawed at 37°C and stored on ice until further analysis. One hundred seventy five µl of calcium buffer solution (0.2 M Tris-hydrochloride (HCl), 2 mM $CaCl_2$, pH 8.0,) per sample was added in triplicate amounts to the first 3 wells on the first two rows of a 96-well clear microplate. One hundred seventy five µl of EDTA buffer solution (0.1 M Tris-HCl, 1 mM EDTA, pH 8.0) per sample was added in triplicate amounts to the first 3 wells on the third row of a 96-well clear microplate for a total of 9 wells per sample. Sixty µl of calcium buffer solution was added to 3 microcentrifuge tubes per sample along with 15 µl of serum in the same tubes. Forty µl of EDTA buffer solution was added to 3 microcentrifuge tubes per sample along with 10 µl of serum in the same tubes. All samples were vortexed and remained on ice until further analysis. Twenty five µl of the calcium dilution was added to the first 3 wells on the first two rows per sample and 25 µl of the EDTA dilution was added to the first 3 wells on the third row per sample. The samples were incubated on a plate shaker for 5 minutes at 37°C. After incubation, 2 µl of a solution of 1.2 mM paraoxon dissolved in ethanol was added to all sample wells. Samples were further incubated at 37°C on a plate shaker for an additional 20 minutes. Following final incubation, 50 µl of enzyme stop solution (20 mM EDTA with 2% Tris-base dissolved in H_2O) was added to inhibit further enzyme activity. Enzyme esterase activity was measured from the production of the paraoxon metabolite, *p*-nitrophenol and quantified at

405 nm using a Sunrise spectrophotometer (Tecan). EDTA inhibited PON activity levels were subtracted from calcium activated PON activity levels per sample to yield the total mean levels of PON activity.

Assay 5. PON Enzymatic Analysis with Phenyl Acetate. Human serum samples were investigated for the quantitation of PON enzyme activities based on the hydrolysis of phenyl acetate (Fig. 19., B). Human serum samples were thawed at 37°C and stored on ice until further analysis. Nine hundred ninety µl of calcium buffer solution was added to duplicate microcentrifuge tubes and the same amount of EDTA buffer solution was added to a third microcentrifuge tube for a total of 3 tubes per sample. All sample tubes were incubated at 37°C in a water bath with gentle shaking for 3 minute intervals per sample. Ten µl of serum was added to all three tubes per sample, vortexed, and incubated at 37°C for 10 minutes. After incubation, 10 µl of 50 mM phenyl acetate dissolved in ethanol was added to each triplicate sample tube and vortexed for 5 seconds. The sample solution from each tube was aliquoted into ultraviolet cuvettes and PON hydrolysis activity was measured at 270 nm using a BioMate 3 spectrophotometer (Thermo Scientific). Arylesterase activity of phenyl acetate hydrolysis via PON to its metabolite, phenol, was detected over a 2 minute interval. Enzyme activity was quantified as the yield of calcium activated enzyme activity minus EDTA inhibited enzyme activity.

Statistical Analysis

All data analyses were performed using Statistical Analysis Software (SAS) for Windows version 9.4 (SAS Institute, Inc., Cary, NC). DDE, APOB, TG, PON and other biochemical markers (parameter levels): high density lipoproteins (HDL), low density

lipoproteins (LDL), cholesterol (CHOL), body mass index (BMI), fasting blood glucose (FBG), hemoglobin A1C (HGAC), use of statins (STAT), use of fibrates (FIB), use of niacin (NIA) use of tobacco (TOB), and age (AGE), were collected from each individual to test for any significant association with T2D presence. Descriptive statistics of parameter levels for continuous variables of diabetics and non-diabetics were analyzed using PROC UNIVARIATE to identify means ± standard deviation (SD) values, while PROC FREQ was used to compare categorical variables of diabetics and non-diabetics to identify proportional values. Basic linear regression analysis was used to test for any significant correlation between DDE levels and other parameter levels using PROC GLM. Univariable logistic regression analysis was used to test for any significant association of individual parameter levels with increased T2D risk by measuring odds ratios (OR) and 90% confidence interval (CI) for each parameter using PROC LOGISTIC. Individual parameters associated ($p \leq 0.25$) with T2D risk were used as candidates for development of a multivariable logistic regression model using PROC LOGISTIC. Because of the small sample size, a p-value of ≤ 0.1 was considered statistically significant and a CI including the numeral 1 as a value was considered not statistically significant.

Results

Following the collection of all clinical and demographic data for each individual, descriptive statistics of parameter levels for diabetics and non-diabetics were analyzed (Table 1.). Based on our analysis, DDE, ApoB, PON, BMI, and AGE levels were similar for diabetics and nondiabetics. FBG levels, which is used to diagnosis diabetes, were not

similar for diabetics and non-diabetics as expected. However, HGAC levels, which is also used to diagnosis diabetes, were similar for diabetics and non-diabetics.

All parameters were analyzed for their potential individual association with T2D presence using logistic regression analysis. Based on the statistical analysis, HDL levels (OR=0.955, p-value=0.0436), FBG levels (OR=1.037, p-value=0.0029), PHAC levels (OR=1, p-value=0.1312), and STAT levels (OR=0.387, p-value=0.0620) were significantly associated with T2D, p-value $\leq$ 0.25 (Table 2.).

Parameters significant for T2D risk from the univariable logistic regression model: HDL, PHAC, and STAT levels (except for FBG which is representative of diabetes diagnosis), were subjected to multivariable logistic regression analysis to test for any significant combined association with T2D risk. After identifying the best model, parameter levels for DDE were added to the final model to observe a potential association for increased T2D risk. Based on our analysis, there was not a significant increase in association for any parameter with T2D risk, even though HDL and STAT levels both yielded a p-value $\leq$ 0.1 (Table 3.). DDE levels did not increase the risk of association for these parameters with T2D.

Because no association was found for DDE and other parameters with T2D using logistic regression analysis, DDE levels were investigated for any significant association with other biochemical markers/parameters of T2D from this study using linear regression analysis. Based on the results, only AGE and HGAC levels were found to be associated with DDE levels, p-value $\leq$ 0.1 (Table 4.). DDE levels were not associated with any significant trend among lipid levels for diabetics and non-diabetics.

Discussion

Genetic factors contribute to only a small portion of the pathology of disease development in organisms. A majority of non-genetic factors including environmental factors may play a large role in the progression of metabolic diseases (Neel and Sargis, 2011). Past epidemiological data has linked many adverse health effects in humans to exposure to environmental chemicals including DDE (Lee et al., 2006). Research by others has shown an epidemiological association between individuals with or at risk for T2D and the presence of higher levels of DDE in their blood (Lee et al., 2007). In a study by Ukropec et al. (2010), OC exposure was associated with an increased risk of diabetes prevalence in a population living in a highly polluted area in Eastern Slovakia for persistent organic pollutants based on OC levels found in their serum: (DDE:OR=1.86, CI=1.17-2.95) (DDT:OR=2.48, CI=1.77-3.48). Individuals in the upper quintiles of this study exhibited average DDE levels ≥ 1,500 ng/g, similar to the DDE levels observed from our study population. Data from the NHANES study showed that serum levels of OC from a population exhibiting diabetes were associated with diabetes prevalence: (DDE: OR=2.3, CI=1.0-5.5) (Lee et al., 2006). Also, DDE levels from the NHANES study were detected in over 90% of samples, which was similar to DDE levels detected in our study. OC levels from a population in South Korea with low level background exposure were associated with diabetes risk: (DDT: OR=10.6, CI=1.3-84.9) (DDE: OR=12.7, CI=1.9-83.7) (Son et al., 2010). Diabetic individuals from this study exhibited serum DDE levels around 650 ng/g, which was similar to the lower quintiles plasma DDE levels of diabetic and non-diabetics from our current study and a previous study by Eden et al. (2014). DDE levels from this South Korean population exhibited an

even higher risk of association with diabetes prevalence when they were adjusted to the covariates BMI and AGE. Thus, higher BMI and AGE levels were both correlated with DDE levels from this study, but only AGE levels (p-value ≤ 0.1) were correlated with DDE levels from our study. However, Eden et al. (2014) showed that DDE levels were associated with diabetes prevalence with increasing AGE levels and decreasing BMI levels, although our study showed no statistically significant difference for average BMI and AGE levels between diabetics and non-diabetics. Also, BMI levels were not a significant risk factor for diabetes prevalence in African Americans from our study, similar to studies by Eden et al. (2014). A study by Lee et al. (2011) showed that low dose DDE levels predicted higher BMI and triglyceride levels in non-diabetics over a 20 year time period, while studies by Turyk et al. (2009) showed that high DDE levels were associated with diabetes prevalence over a 20 year time period.

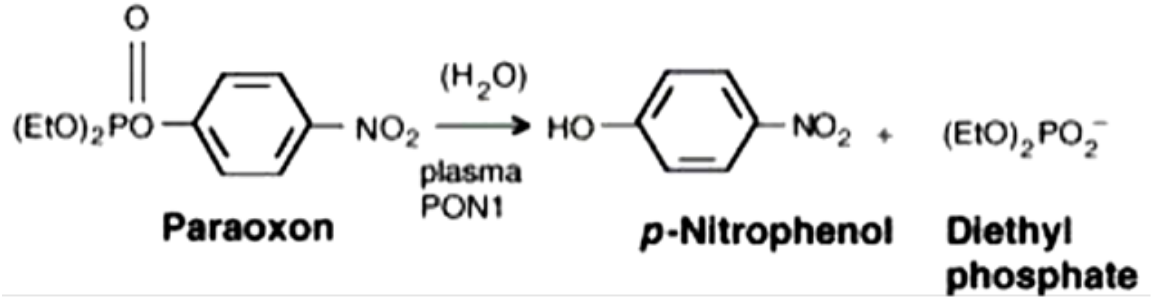
Long-term DDE exposure has been associated with an increase of lipid levels based on epidemiological studies (Lee et al., 2011). Animal studies have also shown a positive association between OC levels and biochemical markers of dyslipidemia (La Merrill et al., 2014). ApoB is a biochemical marker of dyslipidemia implicated in T2D and was also a parameter investigated in this study (Qiu et al., 2006). DDE exposure is associated with abnormal lipid profiles and with T2D, while lower levels of PON activity have been observed in T2D (Ruzzin et al., 2010). PON is attached to HDL lipids and HDL lipids are decreased while LDL lipids are increased in T2D (Manning et al., 2012). Therefore, PON levels could vary in the body based on certain pathophysiological conditions such as T2D. Diabetics are known to have decreased PON levels compared to non-diabetics (Ikeda et al., 1998). Variations in PON activity levels are due to changes in

lipid levels, which could be affected by DDE exposure. PON levels are directly associated with lipid levels of HDL (Aviram et al., 1999; Abbott et al., 1995). However, lower HDL and higher LDL levels implicated in dyslipidemia contribute to lower PON levels (Saha et al., 1991). Diabetics exhibit higher LDL and lower HDL levels as well as lower PON levels (Shamir et al., 2005).

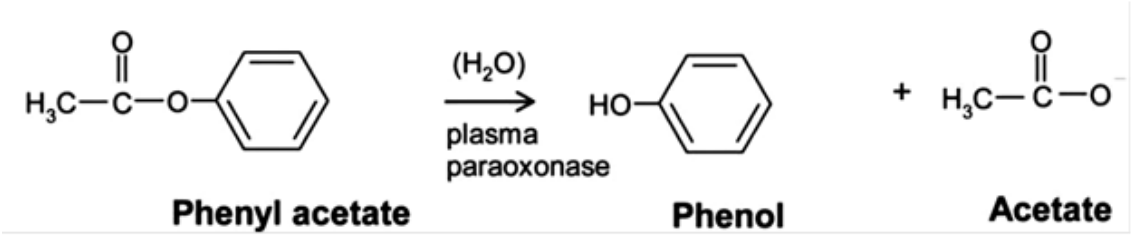
This Mississippi Delta population is significant because it has a high prevalence of T2D. Our study hypothesis was that individuals living in a high risk exposure area for OC could have higher than average exposure levels of DDE in their blood and exhibit biochemical markers associated with T2D. This epidemiological study from our lab identifying DDE levels in blood samples from diabetics and non-diabetics could potentially support research by others of DDE levels being associated with T2D (Taylor et al., 2013). Based on the results of this study, DDE levels were not a significant risk factor for directly predicting T2D presence: (DDE: OR=1, CI=1.00-1.00) compared to another study from our lab observing OC levels in a United States Air Force study (DDE: OR=1.56, CI=1.22-1.99) (Eden et al., 2014). Therefore, it was important to identify if DDE levels may be indirectly associated with biochemical markers of T2D. DDE levels were only associated with HGAC and AGE levels. However, this study does not support our overall hypothesis of DDE levels being associated with T2D presence.

The Mississippi Delta region is a high exposure area for DDE contamination. This could explain why plasma DDE levels were not significantly different between diabetics (2,169 ± 4,041 ng/g lipid) and non-diabetics (1,890 ± 2,236 ng/g lipid). Higher DDE levels in individuals may reach a certain level of saturation until there is no real relationship observed with T2D. Serum ApoB levels were not greater in diabetics (260 ±

118) compared to non-diabetics (257 ± 113). Therefore, these data do not support previous research by others finding an association of OC exposure with lipid biochemical markers of T2D. Serum PON levels were not significantly different between diabetics (147 ± 44) and non-diabetics (140 ± 56), which was not expected. DDE levels were not directly associated with T2D presence based on our epidemiological studies probably due to the small sample size and all samples being selected from a high exposure area for OC. Therefore, obtaining results from a larger study sample is needed before any conclusions can be derived. Studies by others observing a relationship between DDE and T2D in individuals with high body burdens of DDE levels is interesting compared to our study which found no association with high DDE levels and T2D. This study does not support previous studies that identified DDE levels with T2D association. Regardless of scientific outcome, understanding the epidemiological and physiological association of OC compounds with chronic diseases such as T2D is vital in determining their overall impact on human health and the environment.



(A)



(B)

Figure 19 Metabolism of paraoxon (A) and phenyl acetate (B) by paraoxonase.

Table 1 Descriptive Statistics of Parameter Means for Diabetics (D) and Non-diabetics (ND).

Analysis of Parameter Means, n=77		
Parameter	**Diabetic (D), n=30**	**Non-diabetic (ND), n=47**
DDE (ng/g)	2168.72 ± 4040.74	1890.44 ± 2235.85
APOB (ng/ml)	260.46 ± 118.22	257.34 ± 113.10
PON (µmol/min/L)	147.09 ± 44.73	140.38 ± 55.64
PHAC (µmol/min/L)	27869.83 ± 2866.14	26430.11 ± 4502.30
TG (mg/dl)	124.17 ± 73.52	111.13 ± 53.87
BMI (kg/m^2)	35.12 ± 8.82	35.75 ± 8.09
AGE (years)	46.77 ± 10.11	45.51 ± 10.31
HDL (mg/dl)	45.57 ± 10.22	51.21 ± 12.15
LDL (mg/dl)	116.63 ± 44.12	120.04 ± 30.51
CHOL (mg/dl)	187.00 ± 47.40	193.30 ± 33.48
HGAC (%)	7.90 ± 1.95	6.57 ± 1.06
FBG (mg/dl)	170.14 ± 117.43	97.40 ± 15.33
STAT (yes/no)	(13/16)	(11/35)
TOB (yes/no)	(11/18)	(17/29)
FIB (yes/no)	(0/29)	(0/46)
NIA (yes/no)	(0/29)	(1/45)

Measurement of parameter levels between groups for n=77 samples, mean ± the standard error of the mean (SEM). Parameters measured were dichlorodiphenyldichloroethylene (DDE), apolipoprotein B (APOB), paraoxon hydrolysis (PON), phenyl acetate hydrolysis (PHAC), triglyceride (TG), body mass index (BMI), age (AGE), high density lipoprotein (HDL), low density lipoprotein (LDL), cholesterol (CHOL), hemoglobin A1C (HGAC), fasting blood glucose (FBG), statin (STAT), tobacco (TOB), fibrate (FIB), and niacin (NIA). All Parameter levels excluding FBG levels were not significantly different between groups based on mean values.

Table 2 Univariable Logistic Regression Analysis.

Univariable Data Analysis, n=77			
Parameter	Odds ratio	95% CI	p-value
DDE (ng/g)	1	1.000-1.000	0.7111
APOB (ng/ml)	1	.996-1.004	0.9064
PON (μmol/min/L)	1.003	.994-1.012	0.5749
PHAC (μmol/min/L)	1	1.000-1.000	0.1312
LDL (mg/dl)	0.997	.985-1.010	0.6853
HDL (mg/dl)	0.955	.913-.999	0.0436
TG (mg/dl)	1.003	.996-1.011	0.3746
BMI (kg/m^2)	0.991	.937-1.048	0.7471
AGE (years)	1.012	.967-1.060	0.5958
HGAC (%)	2.532	.500-12.822	0.2617
STAT (yes/no)	0.387	.143-1.049	0.062
TOB (yes/no)	1.043	.399-2.722	0.9322
CHOL (mg/dl)	0.996	.984-1.008	0.4921
FBG (mg/dl)	1.037	1.012-1.061	0.0029

Statistical association of each parameter with T2D prevalence. Parameters measured were dichlorodiphenyldichloroethylene (DDE), apolipoprotein B (APOB), paraoxonase-1 (paraoxon (PON)/phenyl acetate (PHAC), low density lipoprotein (LDL), high density lipoprotein (HDL), triglyceride (TG), body mass index (BMI), age (AGE), hemoglobin A1C (HGAC), fasting blood glucose (FBG), cholesterol (CHOL), statin (STAT), and tobacco (TOB). Measurement of an individual association with T2D prevalence based on odds ratios (OR), 95% confidence interval (CI), and p-values for each parameter for n=77 samples. A p-value of ≤ 0.25 was considered statistically significant. HDL, PHAC, FBG, and STAT levels were found to be associated with increased T2D risk.

Table 3 Multivariable Logistic Regression Analysis.

Multivariable Data Analysis, n=77			
Parameter	Odds ratio	95% CI	p-value
HDL (mg/dl)	1.046	0.995-1.099	0.0708
STAT (yes/no)	0.362	0.124-1.056	0.0628
DDE (ng/g)	1.000	1.000-1.000	0.6008

Statistical association of all significant parameters for T2D risk. Parameters measured were dichlorodiphenyldichloroethylene (DDE), high density lipoprotein (HDL), and statin (STAT). Measurement of a combined risk for T2D presence using odds ratios (OR), 95% confidence intervals (CI), and p-values for each parameter for n=77 samples. A p-value of ≤ 0.1 was considered statistically significant. There was no significant increase in risk for T2D among combined parameters.

Table 4 Linear Regression Analysis.

Linear Regression Analysis, n=77		
Parameter	R-square	p-value
APOB (ng/ml)	0.0025	0.6821
PON (µmol/min/L)	0.0102	0.4094
PHAC (µmol/min/L)	0.0201	0.245
LDL (mg/dl)	0.0027	0.6698
HDL (mg/dl)	0.0061	0.5223
TG (mg/dl)	0.0256	0.1892
BMI (kg/m^2)	0.0016	0.7476
AGE (years)	0.3265	0.0001
HGAC (%)	0.1699	0.0506
FBG (mg/dl)	0.0022	0.7013
CHOL (mg/dl)	0.0005	0.849

Statistical association of dichlorodiphenyldichloroethylene (DDE) levels with predicting other parameters of T2D. Parameters measured were apolipoprotein B (APOB), paraoxon hydrolysis (PON), phenyl acetate hydrolysis (PHAC), low density lipoprotein (LDL), high density lipoprotein (HDL), triglyceride (TG), body mass index (BMI), age (AGE), hemoglobin A1C (HGAC), fasting blood glucose (FBG), and cholesterol (CHOL). Measurement of an individual association of DDE with other parameters of T2D based on R-square and p-values for n=77 samples. A p-value of ≤ 0.1 was considered statistically significant. DDE levels were found to be associated with AGE and HGAC levels.

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CHAPTER V

CONCLUSIONS

The threat of dichlorodiphenyldichloroethylene (DDE) exposure is still relevant based on evidence of all living organisms exhibiting some level of DDE contamination. Some adverse effects of DDE exposure have been identified while other effects have yet to be defined. One of those adverse effects is the association with biochemical markers of metabolic diseases. Type 2 diabetes mellitus (T2D) is a metabolic disease associated with many biochemical markers. These biochemical markers are significant in revealing adverse effects to an organism. Individuals may be at a greater risk for exhibiting these adverse effects in areas where contamination from dichlorodiphenyltrichloroethane (DDT) exposure is ubiquitous in the environment. Thus, DDE exposure qualifies as an environmental factor.

T2D prevalence has risen dramatically, especially in countries where DDT is still be used and areas with historical exposure to high levels of DDE residues. Thus, DDE exposure may be an environmental factor that is associated with the increasing prevalence of T2D. DDE exposure may have a mechanistic association in T2D development, although most scientific evidence claims only an observational association of DDE exposure with T2D. Based on results from this study, DDE exposure appears to be associated with dyslipidemia and beta cell dysfunction by its effect on liver hepatocyte and pancreatic beta cell function *in vitro*, although no direct association of DDE exposure

with T2D was found in epidemiological studies. The regulation of lipid metabolism and secretion in McA liver cells was significantly altered as a result of DDE exposure. DDE exposure altered the levels of proteins involved in regulating lipid metabolism and secretion implicated in dyslipidemia. DDE exposure significantly altered insulin synthesis and secretion levels in B-TC-6 cells. The levels of proteins involved in regulating insulin synthesis and secretion implicated in beta cell dysfunction were also altered. DDE exposure may change how liver hepatocytes and pancreatic beta cells function in maintaining protective mechanisms in order to prevent T2D development. Long-term DDE exposure could favor the progression of dyslipidemia, beta cell dysfunction, and potential T2D development. Dyslipidemia and beta cell dysfunction are both contributors to T2D progression and any biological, chemical, or physical agent that promotes liver dyslipidemia and/or beta cell dysfunction is therefore also contributing to T2D progression. DDE contamination could be that chemical agent. This research supported the findings of others who identified either an epidemiological and/or *in vitro/in vivo* association of DDT/DDE exposure with T2D or biochemical markers of T2D. An epidemiological association between biochemical markers and the prevalence of T2D already exists, but DDE exposure may further contribute to this epidemiological association.

Based on epidemiological studies, no significant association between DDE levels and T2D was observed from this study population. However, DDE exposure was associated with some biochemical/clinical markers (AGE, HGAC) implicated in various metabolic diseases such as T2D. DDE exposure may have an indirect association with T2D by its association with biochemical markers, which was also observed in some

previous studies by others. Because of the small sample size of this study population, the statistical power of tested parameters was probably diminished. All samples were collected from African Americans, who are known to exhibit a higher prevalence of T2D, and could play a role in explaining the lack of association of biochemical markers with T2D prevalence in this population. It was concluded from this study that diabetics and non-diabetics living in high exposure areas for DDE contamination exhibited no significant difference among biochemical markers for T2D. Because all samples were from high exposure areas for DDE contamination, this could explain the similarities among biochemical markers between diabetics and non-diabetics because DDE exposure could potentially play a role in regulating these biochemical markers based on experimental studies by others. To continue, non-diabetics usually do not exhibit similar biochemical markers clinical data when compared to diabetics. Research by others supports the idea that DDE exposure has an individual association with some of these biochemical markers and this could explain why levels were similar between both groups. Randomly selecting samples from diabetics and non-diabetics from all regions of Mississippi to observe DDE exposure would have been more significant. This study does not support research by others of DDE exposure being associated with T2D. Overall, the increasing prevalence of T2D may one day be explained by the increase in exposure to environmental chemicals such as DDE, although more research is needed on this subject matter.