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The Investigation of Chlorpyrifos Exposure on Hypertension and Metabolic Syndrome Prevalence and its Dysregulatory Effects within Hypothalamic Blood Pressure and Metabolism Pathways

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A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Environmental Health Sciences 2022

Abstract

The Investigation of Chlorpyrifos Exposure on Hypertension and Metabolic Syndrome Prevalence and its Dysregulatory Effects within Hypothalamic Blood Pressure and Metabolism Pathways

By Frank E. Glover III

Hypertension (HTN) is the leading cause of morbidity and mortality associated with cardiovascular diseases, and the global prevalence of HTN is predicted to increase over the ensuing decades. Hypertension is also a component of the metabolic syndrome (MetS), a cluster of conditions that significantly increases the risk of heart disease, stroke and Type II diabetes. While strong risk factors for HTN and MetS have been identified including age, smoking, and high-salt diets, the complete etiology remains to be fully understood. Recently, the contribution of environmental toxicants such as OP insecticides in the etiology of HTN and MetS has come to light.

Organophosphate (OP) insecticides are a group of environmentally ubiquitous chemicals that have been in use for decades to increase crop yield and lower transmissions of vector-borne diseases. They function primarily to disrupt synaptic function in insects via acetylcholinesterase inhibition, resulting in paralysis and death. Despite their utility, OPs have been strongly associated with several adverse health outcomes, most notably central nervous system (CNS) dysregulation linked with cognitive delay in children, and neurodegenerative diseases in adults. Organophosphates have also been demonstrated to have off-target consequences in not only the CNS, but also peripheral organs including the heart, liver, and kidneys. These adverse outcomes have been observed at concentrations below the threshold to induce acute toxicity, and may reflect concentration levels representing everyday exposures.

While several *in vitro* and *in vivo* studies have found associations between OP exposure, HTN, and MetS, the generalizability of these studies is lacking, and their results remain conflicting and inconclusive. Additionally, the biological mechanisms underlying the association between OPs, HTN, and MetS remain to be elucidated. Our group hypothesized that exposure to OP insecticides at environmentally relevant concentrations are associated with the risk of HTN and MetS, and that these associations are related to effects of OPs on hypothalamic pathway targets.

Using a nationally representative cohort of U.S. adults from the NHANES 2013-2016 survey cycles, we quantified the association between general population exposure of OP insecticides, HTN, and MetS. Our findings revealed significant associations between OP exposure and systolic blood pressure, diastolic blood pressure, odds of abnormal pulse pressure, and the odds of MetS and MetS components, specifically low high-density lipoprotein (HDL) and hypertriglyceridemia. Using a murine hypothalamic cell line, we performed a targeted *in vitro* investigation of the effects by chlorpyrifos, the most used OP insecticide, on the mRNA and protein expression of well-established blood pressure and metabolism-regulating pathways components. Hypothalamic cells were exposed to a range of environmentally relevant

concentrations of chlorpyrifos and chlorpyrifos-oxon, at either 24 hour or 4-day timepoints. We observed both concentration and time-dependent changes in the mRNA and protein expression of our hypothalamic targets. Computational benchmark dose (BMD) modeling was used to estimate points of departure for fold changes of mRNA and protein expression of pathway targets at various concentrations, and a value of 10% change in expression relative to the control was set as the predetermined BMD response. We were able to successfully calculate BMD and benchmark lower limit (BMDL) values for monotonic dose-response curves, and these values can ultimately be to calculate daily reference doses and to inform public health policies.

Our findings support previous studies implicating OP insecticides in the pathogenesis of HTN and MetS. This study contributes important novel information to the toxicological profile of OP insecticides like chlorpyrifos using a BMD model, and provides unique insights into the pathogenesis of OP-related HTN and metabolic dysfunction, specifically related to the hypothalamus. Furthermore, we have demonstrated that OP insecticides are associated with blood pressure changes and MetS at everyday levels of exposure, findings which support initiatives to reduce exposure to OPs and to develop safer alternatives. Future studies are warranted to corroborate our results, test these associations in whole organisms, and to expand on the proposed biological mechanisms underlying these associations.

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Chapter 1: Introduction

Hypertension Background

Public Health Burden/Epidemiology

Hypertension (HTN) is the leading cause of morbidity and mortality associated with cardiovascular diseases, and remains an increasingly serious public health challenge globally(1-3). Hypertension accounts for roughly 8 million deaths per annum worldwide (approximately 13-15% of the total), and significantly increases one's risk of stroke (220% increase in risk compared to normotensive individuals), chronic kidney disease, and dementia(4-6). Studies show that mortality from both ischemic heart disease and stroke doubles with each 20 mmHg increment of systolic blood pressure and/or 10 mmHg increment of diastolic blood pressure(7, 8). From 1999 to 2016, the age-standardized prevalence of HTN in some areas the U.S. has decreased, and the proportion of control among those treated for HTN slightly improved(3). However, the absolute HTN burden has increased overall, and among those being treated, the control rate has not consistently improved in all subgroups. The most recent CDC data shows that the age-adjusted prevalence of HTN among U.S. adults is 45%, and within certain subgroups and regions of the country, prevalence estimates can exceed 50%(9, 10). For example, men generally tend to have higher rates of HTN (51%) compared to women (39%), non-Hispanic blacks have the highest rates of HTN (57%) compared to other racial/ethnic groups, and regionally the south-east U.S. (>40%) has the highest rates(9).

Economics of HTN

In addition to posing a significant public health burden, HTN has a substantial impact on the macro and microeconomics of countries worldwide(11). In the U.S., HTN is estimated to

cost between $131 to $198 billion annually. These estimated totals include the cost of direct health care services for individuals, medications to treat high blood pressure, and loss of productivity from premature death(12). A study pooling data between 2003-2014 from the Medical Expenditure Panel Survey found that individuals with HTN face nearly $2,000 higher annual healthcare costs compared to their normotensive peers(13). Thus, by improving our understanding of the etiology of HTN and ultimately our ability to prevent and manage HTN, we can significantly cut healthcare costs for individuals and global economies.

Clinical Characteristics

Traditionally, HTN has been clinically defined as a systolic pressure greater than 140mmHg and/or a diastolic blood pressure greater than 90 mmHg. However, according to the most recent guidelines for HTN by the American College of Cardiology (ACC), HTN is now defined as blood pressure exceeding 130/80 mmHg(14). The rationale for this reclassification is based on evidence of increased risk of cardiovascular events seen in blood pressure values between the 120-139 mmHg systolic and/or 80-89 mmHg diastolic ranges(15). The classifications of HTN diagnosis can be further stratified into prehypertension (120-139/80-89 mmHg), stage I hypertension (140-159/ 90-99 mmHg), and stage II hypertension (≥160/≥100 mmHg). For a reliable diagnosis of HTN, at least 2–3 office visits over 1–4-week intervals (depending on the blood pressure level) are required to confirm the diagnosis of HTN(14). The diagnosis can be made on a single visit, if blood pressure is ≥180/110 mm Hg and there is evidence of cardiovascular disease(16). Most individuals with HTN do not display any apparent symptoms, which has led to HTN being referred to as the “silent killer.” When symptoms are present, they are usually indicative of long-standing HTN and include involvement of multiple

organ systems. Symptoms of uncontrolled HTN can include early morning headaches, nosebleeds, irregular heart rhythms, vision changes, and ringing in the ears (tinnitus) (17). Individuals with severe HTN can experience fatigue, nausea, vomiting, confusion, anxiety, chest pain, and muscle tremors.

Types of Hypertension (Primary vs. Secondary)

Hypertension can be classified as primary ("essential") or secondary(18). Primary HTN represents over 80% of all HTN cases, in which the cause for elevated blood pressure is unknown. In secondary HTN, the pathogenesis of HTN is clear, such as inheritable genetic conditions (e.g. primary hyperaldosteronism, Liddle's syndrome, paragangliomas), medication use (e.g. contraceptives, estrogen supplementation, steroids, stimulants), or toxic exposures (e.g. illicit drugs, alcohol, heavy metals, pesticides)(19-21). Regardless of the specific type of HTN, the risks associated with adverse cardiovascular events is similar. As our understanding of biological, psychosocial, and environmental factors related to HTN improves, we can hopefully shift more of the primary HTN cases into the secondary classification. Improved understanding of specific causes of HTN can likely improve our development of therapies and the prevention and management of HTN.

Treatments and Treatment Gaps

There are several medication classes and lifestyle programs that have been developed in the efforts to control HTN, and public health guidelines recommend a combined regiment of medication use and lifestyle modifications for optimal blood pressure reduction(22, 23). Most notably, drugs that directly target blood pressure-regulating pathways within the cardiac, renal,

and neurological systems have been the most commonly prescribed (e.g. diuretics, beta-blockers, angiotensin II receptor blockers, calcium channel blockers, alpha blockers, etc.). A total of over seventy drugs in fifteen different classes, many of which are also available in single pill combinations, have been approved for the treatment of HTN in the U.S.(24). In addition to current therapies, newer classes of vasopeptidase inhibitors, aldosterone synthase, and soluble epoxide hydrolase, as well as agonists of natriuretic peptide A and vasoactive intestinal peptide receptor 2 have been approved or are in phases II/III of development(24). Non-pharmacological treatments such as lifestyle modifications have been shown to lower HTN risk and improve management of HTN. These modifications include weekly aerobic exercise, lowering salt intake, and cessation of alcohol use and smoking(23). Despite the numerous treatment options available and advancements in HTN management, less than 30% of adults with HTN have their blood pressure under control(7). Additionally, studies have shown that increasing the number of medications for a multi-drug therapy yields diminishing returns beyond a certain number, but comes with a wide variety of side effects that can negatively affect quality of life(25). Proper management of HTN is likely hampered by several reasons, including inadequacies in the treatment of HTN and mitigation of risk factors, inadequacies which may be related to an incomplete understanding of factors related to HTN.

Blood Pressure Control by Organ Systems

Definition of Blood Pressure

Blood pressure is defined as the force applied by circulating blood against the walls of vessels, usually within the large arteries(26). A healthy blood pressure is crucial to maintaining

the perfusion of organ systems to allow for oxygenation of tissues, transportation of important substrates and enzymes, and removal of metabolic waste and toxicants(27, 28). Blood pressure is tightly regulated primarily through communications between the cardiac, renal, and nervous systems. The equation for blood pressure can be written as follows:

$$BP = CO\ x\ SVR \quad \text{and} \quad CO = HR\ X\ SV$$

Where BP stands for blood pressure, CO cardiac output, SVR systemic vascular resistance, HR heart rate, and SV stroke volume. Mechanical properties of organs and their production of effector hormones can have indirect and/or direct effects on each of these variables that defines blood pressure. The following organs discussed are crucial to maintaining a healthy blood pressure at baseline, and perturbations within a number of pathways belonging to these organ systems have been implicated in the pathogenesis of HTN. ‘

Systolic Blood Pressure vs. Diastolic Blood Pressure

Systolic blood pressure is the top number in a blood pressure reading and is defined as the pressure within the arteries when the heart is contracting to pump blood (systole). The diastolic blood pressure is the bottom number and is defined as the pressure within the arteries when the heart is relaxing to fill with blood (diastole). Biological systems can either concurrently or independently modify systolic and diastolic blood pressure, and isolated systolic or diastolic HTN have both been shown to significantly increase morbidity and mortality(29). The sympathetic nervous system acts to increase systolic blood pressure by primarily influencing heart rate and stroke volume(30). The sympathetic nervous system also influences diastolic blood pressure by modulating systemic peripheral resistance via vasoconstriction.

Factors that affect diastolic blood pressure are generally vasoactive substances, which modulate systemic vascular resistance through vasoconstricting or vasodilating arteries. In contrast to the sympathetic nervous system, parasympathetic activity acts to slow the heart rate, reduce contractility, and increase vasodilation, actions that tend to lower blood pressure.

Another difference seen between systolic and diastolic blood pressure is observed in aging. The Framingham Heart Study found that systolic blood pressure tends to increase continuously from age 30-84 and greater(31). Generally, systolic, and diastolic blood pressure tend to rise concomitantly until age 50, after which diastolic blood pressure begins to fall, and isolated rises in systolic blood pressure continue.

Control of Blood Pressure by the Heart

Traditionally, the heart is recognized as one of the main effectors of blood pressure regulation. The heart is a large organ comprised of muscle and contractile tissue, that functions to pump blood oxygenated blood it receives from the pulmonary circulation into the systemic circulation. This is achieved through a cyclical phase of contraction and relaxation phases, where the heart is either contracting during systole to eject blood through the aorta into the systemic vasculature, or relaxing during diastole to fill with blood from the pulmonary system(32). The heart receives numerous parasympathetic and sympathetic inputs from the brainstem (specifically the medulla), inputs that regulate the heart rate (how quickly or slowly the heart is pumping blood), stroke volume (how much blood is pumped into the systemic circulation each cycle), and the contractile force (how strongly the heart can pump blood)(33). The heart is also able to directly detect changes in blood pressure through its baroreceptors,

and in turn can send afferent signals to the brain, where the activity of the parasympathetic and sympathetic nervous systems can be modified(34). Additionally, the heart contains several types of α-adrenergic and β-adrenergic receptors that are activated by catecholamines(35). Binding of catecholamines to α-adrenergic receptors generally leads to vasoconstriction, while binding to β-adrenergic receptors leads to increased heart rate and contractility of the heart. Both actions can ultimately lead to increases in blood pressure.

Control of Blood Pressure by the Kidneys

The kidneys are two retroperitoneal organs that act together to filter the blood and excrete water soluble waste products and play a crucial role in maintaining a healthy blood pressure through involvement with the renin-angiotensin-aldosterone system (RAAS). Renal artery perfusion directly influences sodium absorption and excretion, a process known as pressure natriuresis(36). When the kidneys receive less than normal sodium, chloride, blood flow, or receive increased sympathetic stimulation, renin is produced in the juxtaglomerular cells in the proximal tubule. The actions of renin and the ensuing cascade of RAAS pathways maintain a healthy blood pressure by stimulating the kidney to resorb sodium and fluids into the body, which ultimately increases blood pressure(37). These functions are primarily regulated by angiotensin II, a hormone with powerful vasoconstrictive activity. Angiotensin II has direct actions on the proximal tubule of the kidney to increase sodium resorption, the vasculature to increase vasoconstriction, and functions as a transcription factor of vasoactive hormones such as antidiuretic hormone (ADH)(38).

Control of Blood Pressure by the Adrenals

The adrenal glands are hormone-producing organs that sit atop each kidney, and help to regulate metabolism, immune system function, and blood pressure. These endocrine glands serve as key sites that coordinates the stress response via the hypothalamic-pituitary-adrenal axis and the sympathetic-adrenal system(39). The adrenals are divided into an outer region (cortex) and an inner region (medulla), and each region is responsible for producing specific hormones. The cortex produces steroid hormones such as glucocorticoids (e.g. cortisol) and mineralocorticoids (e.g. aldosterone), and the medulla produces catecholamines such as epinephrine and norepinephrine(40). Cortisol, aldosterone, and the catecholamines regulate blood pressure through several mechanisms. Aldosterone and cortisol can act directly on the kidneys to increase sodium reabsorption, which ultimate acts to increase effective circulating volume and blood pressure(41). Catecholamines can bind to α-adrenergic receptors located on blood vessels, resulting in vasoconstriction and increases in blood pressure(42). Cortisol can also increase β-receptor sensitivity of the Na/K+ ATPase pumps in the heart to catecholamines, which ultimately leads to increased heart rate, increased contractility, and increased blood pressure(43).

Control of Blood Pressure by the Lungs and Liver

The lungs and liver play minor but important roles in the maintenance of blood pressure. The liver produces a number of important enzymes and hormones that are used as mediators of blood pressure control throughout the body (e.g. cytochrome P450 enzymes, steroids, angiotensinogen)(44). Angiotensinogen is produced by the liver, where it can be

cleaved into angiotensin I by renin in the plasma. The lungs contain angiotensin converting enzyme (ACE) which cleaves angiotensin I into angiotensin II, the primary form of angiotensin that acts to increase blood pressure(45).

Control of Blood Pressure by the Pituitary Gland

The pituitary gland is an endocrine organ located at the base of the brain that functions to regulate several physiological processes such as energy, appetite, growth, and metabolism. It forms direct vascular and neuronal attachments with the hypothalamus, and receives direct inputs from the hypothalamus that modulates the release of its hormones(46). The pituitary is divided into an anterior zone (adenohypophysis) and a posterior zone (neurohypophysis), each zone responsible for the production and storage of specific hormones (e.g. FSH, LH, ACTH, TRH, GnRH, ADH)(47). In circumstance of stress and increased simulation from the hypothalamus, the pituitary glands can release adrenocorticotropic releasing hormone (ACTH). Adrenocorticotropic releasing hormone stimulates the adrenal glands to produce cortisol, which in turn can activate a cascade of events to increase blood pressure. Additionally, anti-diuretic hormone (ADH) produced in the hypothalamus can be stored in the posterior zone, and then released under states of low blood pressure and stress.

Control of Blood Pressure by the Vasculature

The vascular endothelium plays a crucial role in the basal and dynamic regulation of circulation and blood pressure. The arteries serve as the conduits for blood transportation, and their mechanical properties allow them to constrict and relax in response to endogenous and exogenous stimuli(48). Alpha-adrenergic receptors located on the endothelium of blood vessels

are activated by catecholamines, which leads to vasoconstriction and ultimately increases in blood pressure. Additionally, a variety of vasoactive substances is synthesized in the endothelium (e.g. nitric oxide and endothelin). Nitric oxide acts to vasodilate the arteries, while endothelin acts to constrict the arteries(49). Endothelial dysfunction refers to a condition comprising attenuated endothelium-dependent vasodilatation and endothelial inflammatory activation, which can result in perturbations of pathways regulating vascular tone(50). Perturbations in the expression of vasoactive substances as well as pathways directly targeting the vasculature such as the RAAS and sympathetic nervous systems can lead to HTN(48).

Control of Blood Pressure by the Hypothalamus

Historically, the contribution of the CNS to the initiation and maintenance of HTN has been well-known, and early treatments of high blood pressure included procedures such as sympathetic nerve denervation to restore the balance of the autonomic nervous system(51, 52). In the modern era, much of the attention given to therapies and management of HTN have been focused on targeting components of the cardiac and renal systems. However, studies estimate that at least 50% or more of the etiology of HTN may be related to CNS aberrations, especially within the hypothalamus(30, 53).

The hypothalamus is a neuroendocrine organ that lies along the third ventricle in the brain, and serves as the master regulator of blood pressure through modulating the parasympathetic and sympathetic nervous system. The hypothalamus is divided into several distinct nuclei, each containing the cell bodies of specific neurons that regulate a number of biological processes(54). These nuclei include the preoptic, paraventricular (PVN), supraoptic,

anterior, dorsomedial, ventromedial, lateral, arcuate (ARC), mamillary, and posterior nuclei. Most notably, the PVN is the central site for sympathetic nervous system modulation regarding blood pressure regulation(55, 56). Peripheral organs can sense changes to blood pressure and send afferent signals to the PVN, which then initiates activation of pre-sympathetic neurons. These neurons project directly to the brainstem, where they can increase sympathetic drive to peripheral organs. In addition to the PVN, the ARC plays a key role in cardiovascular and sympathetic regulation(57). The ARC communicates primarily with the PVN, SFO, and lateral hypothalamus, and can also send direct projections to the sympathetic preganglionic neurons at the level of intermediolateral cell column of the thoracic spinal cord.

In addition to the peripheral RAAS of the kidneys and adrenals, central RAAS modulation within the CNS influences the major vasomotor center in the brainstem, the rostral ventrolateral medulla (RVLM), which contains sympathetic premotor neurons that are responsible for generating and maintaining vasomotor tone and resting levels of arterial blood pressure(58). The central RAAS components converge within the hypothalamus, where not only do peripheral RAAS components localize, but also central RAAS components are synthesized by glial cells within the hypothalamus(59). The key effector molecule of central RAAS is angiotensin II, which is responsible for regulating fluid balance, sodium intake, thirst, and ultimately the maintenance of BP(60). Angiotensin II primarily functions within the supraoptic nucleus and PVN, and binding to the angiotensin II type 1A receptor can result in water and sodium intake, modulation of the baroreceptor reflex, and increased vasopressin secretion(58). In addition to RAAS effects, angiotensin II has also been demonstrated to increase the activity of the sympathetic nervous system within the hypothalamus, which ultimately leads to

increased heart rate and catecholamine release from the adrenals(61). Angiotensin II also increases the synthesis and secretion of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and glucocorticoids(62, 63). Several studies in spontaneously hypertensive rats that serve as animal models for primary HTN, indicate that brain RAAS is more active in these animals than in the normotensive controls, suggesting the importance of central RAAS in the pathogenesis of HTN(64).

The hypothalamus also controls the central circadian clock system, which in turn regulates a number of physiological processes such as metabolism, immune-response, renal function, sleep-wake cycles, and blood pressure(65). The central clock is located in the suprachiasmatic nucleus of the hypothalamus and is composed of a core group of transcription factors that act in a Transcription Translation Oscillating (TTO) loop (e.g. CLOCK, BMAL1, Period (Per), and Cryptochrome (Cry)(66). The *BMAL1* gene has been demonstrated to be integral to the maintenance of normal blood pressure, where knockout *BMAL1* mice have increased endothelial dysfunction, blood pressure dysregulation, and increased arteriosclerotic disease(67). Other knock-out studies in mice lacking *CLOCK*, *Per*, and *Cry* have also demonstrated blood pressure dysregulation, metabolic dysregulation, and effects on the aldosterone-dependent resorption of sodium in the kidneys(68, 69). In addition to knock-out studies, many genetic rodent models of HTN have perturbations within the hypothalamus, regardless of the etiology (e.g. DOCA salt mouse, spontaneously hypertensive rat, schlager mouse)(70). Intervention studies where centrally acting HTN therapies are administered have been able to restore the blood pressure to normal in many cases, even when aberrations in the

peripheral organs such as the kidneys persist(53). These studies further demonstrate the importance of the hypothalamus in regard to blood pressure regulation.

Metabolic Syndrome

Public Health Burden and Epidemiology

Metabolic syndrome (MetS) is a cluster of cardiometabolic risk factors that significantly increases one's risk for a number of chronic illnesses, including chronic kidney disease, arthritis, various cancers, and cardiovascular disease(71). Metabolic syndrome can be present in up to 30% of HTN patients, and in these cases the presence of MetS significantly increases the morbidity and mortality of cardiovascular disease(72). Among U.S. adults aged 18 years and older, the prevalence of metabolic syndrome rose by more than 35% from 1988–1994 to 2007–2012, increasing from 25.3% to 34.2%(73). As seen with HTN, MetS prevalence can vary significantly between subpopulations within the U.S., where non-Hispanic Black and Mexican American citizens generally have the highest rates of MetS. Prevalence of MetS increases rapidly with age, and given the demographic trend in the US population of increasing age, further increases in metabolic syndrome prevalence are to be expected, with concomitant increases in related chronic diseases and conditions(74).

Economics of Metabolic Syndrome

Metabolic syndrome imposes a substantial economic burden globally, and the presence of specific combinations of metabolic syndrome risk factors in an individual are markers for high utilization and costs among patients receiving medical care(75). The largest components of costs included the management of prevalent Type II diabetes and incident cardiovascular

events. In addition to diabetes, the combination of other risk clusters are major drivers of utilization and costs, and estimates show these factors can increase a person's healthcare costs nearly 1.6-fold, or about $2,000 per year(76). Mean annual costs per hypertensive patient were around three-fold higher in subjects with MetS compared to those without, and rose incrementally with the additional number of MetS components present(72).

Clinical Characteristics

The clinical characteristics of MetS include abdominal obesity, dyslipidemia (reduced high-density lipoprotein (HDL) cholesterol, elevated plasma triglycerides (TG), increased low-density lipoprotein (LDL) cholesterol), HTN, elevated fasting glucose, and insulin resistance(77). A diagnosis of MetS requires the presence of at least three of the following: a waistline of 40 inches or more for men and 35 inches or more for women; a blood pressure exceeding 135/85 mmHg or taking antihypertensive medications; a serum triglyceride level exceeding 150 mg/dL; a fasting serum glucose exceed 100 mg/dL or taking glucose-lowering medications; an HDL level lower than 40 mg/dL for men or lower than 50 mg/dL for women(78). A common feature of MetS is insulin resistance, which can lead to impaired regulation of glucose homeostasis. Metabolic syndrome patients may present with or without Type II diabetes, and studies have shown that those individuals with Type II diabetes have significantly higher morbidity and mortality(79).

Treatments for Metabolic Syndrome

The treatments for MetS are designed to target each of the five possible metabolic abnormalities, and usually include a combination of pharmacological and non-pharmacological

treatments. Pharmacological options include medications that lower LDL cholesterol and triglycerides, and raise HDL cholesterol (e.g. statins, fibrates, DPP-4 inhibitors, GLP-1 agonists)(77). Non-pharmacological options include weight loss, a consistent and manageable exercise routine (30-60 minutes a day), diet modification (usually lowering sodium and sugar), limiting alcohol, and smoking cessation(80). The treatment of MetS becomes complex in the clinical setting of insulin resistance, where special attention should be paid to the metabolic side effects of antihypertensive drugs(81). In these cases, preference should be given to renin-angiotensin system inhibitors and calcium channel blockers rather than to β-blockers and diuretics.

Metabolism Regulation by Organ Systems

Metabolic Regulation by the Liver

The liver is an essential metabolic organ that governs the body's energy utilization processes and maintains appropriate levels of various sugars, lipids, and proteins. Metabolic activity in the liver is tightly controlled by various hormones like insulin, glucagon, a number of transcription factors and coactivators, and by the sympathetic and parasympathetic nervous systems(82). The liver regulates blood glucose levels through the processes of glycogenolysis (breaking down stored glycogen into glucose) and gluconeogenesis (*de novo* glucose production). In general, increased sympathetic system activity stimulates hepatic gluconeogenesis, whereas the parasympathetic system suppresses gluconeogenesis(83).

The liver also functions to regulate the production, use, and storage of triglycerides and fatty acids(84). In the fed state, glycolytic products are used to synthesize fatty acids through *de*

novo lipogenesis. Long-chain fatty acids are incorporated into triacylglycerol, phospholipids, and cholesterol esters in hepatocytes, and these complex lipids are stored in lipid droplets and membrane structures, or secreted into the circulation as VLDL particles(85). Fatty acids are packed into VLDL particles and delivered to adipose tissue and other extrahepatic tissues through the bloodstream. Numerous transcription factors and coactivators, including CREB, FOXO1, ChREBP, SREBP, PGC-1α, and CRTC2, control the expression of the enzymes which catalyze the rate-limiting steps of liver metabolic processes, thus controlling liver energy metabolism. Ultimately, aberrant energy metabolism in the liver can promote insulin resistance, diabetes, and contribute to metabolic syndrome risk.

Metabolic Regulation by the Pancreas

The pancreas is an important endocrine organ that produces a number of key metabolic hormones and is primarily responsible for maintaining healthy blood sugar levels of glucose. Insulin and glucagon are two key hormones involved in glucose homeostasis and are produced in pancreatic beta and alpha cells, respectively(86). When blood sugar levels are too high, insulin is secreted to stimulate glucose storage in the liver, muscles, and adipose tissue, which can lead to weight gain in excess. The pancreas releases glucagon in response to low blood glucose levels, which in turn stimulates the liver to undergo glycogenolysis to convert stored glycogen into glucose(87). Pancreatic dysfunction has been strongly linked to metabolic diseases, most notably hypertriglyceridemia, hypercalcemia, and Type I and II diabetes(88).

Metabolic Regulation by Adipose Tissue

The adipose tissue functions to store excess fatty acids, which can be utilized for energy in states of prolonged fasting and starvation(89). Under these conditions, adipose tissue also produces and releases non-esterified fatty acids (NEFAs) and glycerol via lipolysis. Adipose tissue is classified as white or brown, where white adipose tissue functions as a key energy reservoir for other organs, and the brown adipose tissue accumulates lipids for cold-induced adaptive thermogenesis(90). In response to increases in energy demand, the adipose tissue can undergo remodeling, a process that has been implicated in obesity and metabolic syndrome(89).

Metabolic regulation by the Gastrointestinal Tract

The gastrointestinal (GI) tract plays an important role in the absorption of nutrients from our food and the maintenance of normal blood glucose levels, and aberrations in GI function have been implicated in the development of diabetes and MetS(91). The GI tract functions to deliver glucose into the circulation, and secretes a number of glucoregulatory hormones to ensure maintenance of normal glucose levels in fasting and postprandial states. Afferent neurohormonal signals from the GI tract convey information regarding incoming nutrients to the brain, initiating changes in eating behavior and energy expenditure, to maintain energy balance(92). The GI tract also contains numerous microorganisms that contribute to its dynamic ecosystem. Combinations of these microorganisms influence host immune responses, modulation of metabolism, and the breakdown of nutrients. For example, gram-negative bacteria produce lipopolysaccharide (LPS) in their cell walls, which can act as a pro-

inflammatory cytokine to induce low-grade inflammation(93). Disruption of the gut flora by a number of factors has been implicated in the pathogenesis of HTN and MetS(94).

Metabolic Regulation by the Hypothalamus

Within the CNS, the hypothalamus helps to exert central control over metabolic homeostasis(95). The hypothalamus is able to directly sense changes in metabolite concentrations such as fatty acids within blood circulation(96). Peripheral organs can also sense changes in metabolites and send afferent signals to the hypothalamus. There are two ways for the hypothalamus to signal to the peripheral organs: by stimulating the autonomic nerves of the sympathetic and parasympathetic systems, and/or by releasing hormones from the pituitary gland(97). After receiving information from afferent nerves, the hypothalamus sends signals to peripheral organs, including the liver, to induce functional changes to maintain homeostasis.

Hypothalamic nuclei, particularly the arcuate nucleus, regulate several pathways to maintain metabolic homeostasis. Of note, leptin signaling within the hypothalamus is especially important for regulating hunger and satiety, and dysregulation of hypothalamic leptin signaling has been implicated in obesity and metabolic syndrome(98, 99). Additionally, brain-derived neurotropic factor (BDNF) signaling in the hypothalamus has been shown to play an important role in regulation of energy balance. In twelve-month old mice, BDNF gene transfer prevented the development of aging-associated metabolic declines such as aging-associated weight gain, reducing adiposity, improving glucose tolerance, increasing energy expenditure, and suppressing inflammatory genes in the hypothalamus and adipose tissues(100, 101). BDNF also

stimulates glucose transport, mitochondrial biogenesis, and cellular bioenergetics to protect neurons against injury and disease(102). In the CNS and peripherally, BDNF can insulin sensitivity and parasympathetic tone, and impaired BDNF signaling is implicated in MetS etiology.

Many of the central pathways within the hypothalamus that regulate blood pressure also regulate energy metabolism, and these pathways have biological enzymes and receptors in common(103, 104). As seen with HTN, studies from previous data suggest that MetS has a central neuroendocrine origin in the form of enhanced engagement of the hypothalamic-pituitary-adrenal (HPA) axis(105, 106). Thus, it is likely that exogenous stimuli that increase sympathetic drive within the hypothalamus also increase the activity of energy regulating pathways, and these changes may contribute to the pathogenesis of HTN and MetS.

The Immune System, Blood Pressure, and Metabolism

The immune system plays an integral role in the pathogenesis of HTN and MetS, and efforts to understand factors related to HTN and MetS etiology would be incomplete without its consideration(107, 108). Many individuals with HTN and MetS exist in proinflammatory states, which further emphasizes the role of the immune system in these conditions(109). Within the hypothalamus, there is a well-established inflammatory basis in the etiology of HTN, where pro-inflammatory molecules like NFkB are implicated in the initiation and/or progression of HTN(104). *In vivo* rodent models have demonstrated the contribution of the immune system to HTN. For example, in a study of pre-hypertensive rats given a selective toll-like receptor 4(TLR4) blocker, blockade of TLR4 in the brain significantly reduced the salt-induced prehypertensive

response, likely through downregulation of reactive oxygen species and the restorage of neurotransmitter balance in the PVN(55). Transforming growth factor Beta (TGF-β) is an anti-inflammatory cytokine important for blood pressure regulation, and blockade of TGF-β signaling prior to HTN induction accelerated HTN progression. Conversely, supplementation of TGF-β1 substantially suppressed neuroinflammation, kidney norepinephrine levels, and blood pressure(110). In addition to its direct cholinergic and non-cholinergic effects, chlorpyrifos has been shown to modulate the activity of the immune system directly, and this may play into the pathogenesis of HTN and MetS, through either a direct causal pathway and/or interacting with other toxicities of chlorpyrifos(111, 112).

Angiotensin II has been demonstrated to not only have vasoconstrictive actions to increase blood pressure, but also functions as a pro-inflammatory molecule(113). Increased levels of circulating angiotensin II can induce the development of HTN, which is mediated by increased production of reactive oxygen species (ROS) in the subfornical organ of the CNS(114). Thus, it is it possible that the stimulation of the sympathetic nervous system and RAAS by chlorpyrifos, both which utilize angiotensin II as a primary effector molecule, increases the activation of the immune system while increasing blood pressure and metabolic activity.

Figure 1.

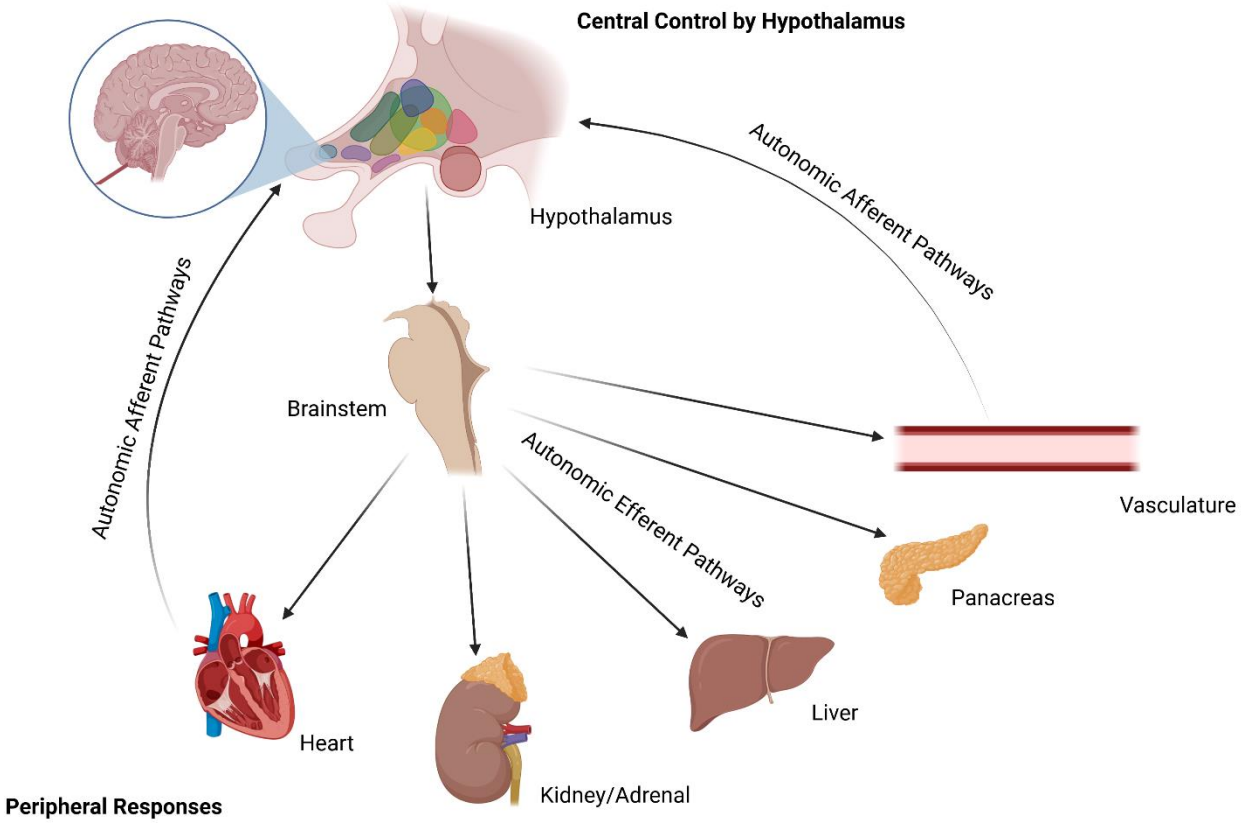


Figure 1: Hypothalamic control of blood pressure regulation and metabolic function requires extensive crosstalk with peripheral organs. Hypothalamic nuclei can directly sense changes in levels of circulating metabolites and hormones and receives inform via afferent pathways from peripheral organs. In response, the hypothalamus can modulate sympathetic and parasympathetic inputs to the brainstem (medulla) via efferent pathways. Those inputs reach peripheral target organs and ultimately modulate their activity to maintain homeostasis of blood pressure and metabolism.

Risk Factors for HTN and Metabolic Syndrome

The strongest identified risk factors for HTN are sedentary lifestyle, aging, high-salt diet, smoking, alcohol use(115). Most of these risk factors are considered “modifiable”, whereby if an individual is able to adjust the frequency of these activities, blood pressure control can

improve(116). Studies have shown that lifestyle modifications including cessation of smoking, drinking, and increasing physical activity can lead to marked reductions in blood pressure(117, 118). Risk factors for MetS share the previously mentioned risk factors in common with HTN, and include postmenopausal status in women, high BMI, and high carbohydrate and sugar intake(119). As seen with modifiable HTN risk factors, taking actions to reduce smoking, drinking, and increasing physical activity has been shown to lower weight, improve lipid profiles, and lower blood glucose levels(120). Education status was also shown to be independently associated with metabolic syndrome in the NHANES 2007-2012 survey cycles(121). Currently, these factors alone do not fully explain the incidence and prevalence of HTN and MetS. In addition to these known risk factors, emerging evidence has begun to investigate the role of the environment in the pathogenesis of HTN and MetS, specifically exposure to pesticides.

Hypertension, Metabolic Syndrome, and Environmental Exposures

History of Environmental Exposures and Hypertension/Metabolic syndrome

Pesticides are a group of various chemical compounds (natural or synthetic) extensively used in modern agriculture to enhance the quality and quantity of crop yields. Over the last several decades, their use has been integral for meeting the nutritional needs of an ever-growing population globally, as they significantly lower the overall loss of crops to insects and weeds(122). Additionally, the implementation of pesticides has significantly reduced the transmission of vector-borne diseases such as malaria, dengue, and filariasis(123). Over 800 pesticides are registered for use in the U.S., and pesticide classes include herbicides,

nematicides, fungicides, rodenticides, and insecticides(124, 125). Each of these classes of pesticides has specific mechanisms of actions to kill weeds or insects by targeting various biological pathways necessary for growth (e.g. enzyme activity, macromolecule production, gas exchange etc.)(126). In the U.S., insecticides are the second most used pesticide class following herbicides.

Insecticides have several physicochemical properties that allow them to persist and accumulate within environmental and biological compartments such as the soil, water, air, blood/plasma, and body fat. These properties include various combinations of lipophilicity and lipophobicity, low vapor pressures, and resistance to microbial degradation and photodegradation(127). Not only do these properties allow them to accumulate in their intended target areas, but also confer the stability necessary to be transported across far distances. Specifically, the phenomenon of pesticide drift occurs when chemical droplets and residues are transported through the air to unintended sites, as well as through soil and run-off water(128). These unintended sites can include playgrounds, residential homes, and many public places where people can easily come into contact. As a result, insecticides contribute significantly to global pollution of soil and water, damage microflora and microfauna, and pose serious health risks within the general population(124).

Despite their benefits, the increased use of insecticides has paralleled the increase in incidence and prevalence of chronic diseases such as HTN and MetS, and their use has been implicated in the etiology and pathogenesis of these diseases(129). Insecticides have been demonstrated to mimic or antagonize natural hormones in human body which disturbs hormonal balance, affect immune function, impair cognitive function, and lead to fertility and

cancers(130). Organophosphate insecticides are the largest class of insecticides used in the U.S. They have been associated with HTN and MetS in several *in vivo*, *in vitro*, and cohort studies, but the results remain conflicting and inconclusive. Chlorpyrifos, the most used OP, has been the subject of a number of studies.

Chlorpyrifos Background

Origin of Chlorpyrifos

Organophosphate insecticides are acetylcholinesterase inhibitors that were first developed as human nerve gas agents during World War II(131). They were adapted as insecticides when it was discovered that they effectively killed insects via the same mechanism as in humans, but at much lower concentrations.(132). Organophosphate insecticides in general were also marketed as replacements for the previously more toxic and biopersistent organochlorine insecticides, most notably DDT(133). Some of the earlier OPs include diazinon, parathion, and fenthion, the uses of which have been banned in the U.S. since 2004 due to strong evidence of neurotoxicity in humans. Chlorpyrifos, an OP insecticide still widely used, was first registered for use by Dow Chemical in 1965(134). The introduction of chlorpyrifos to the market helped reduce weeds and foliage in agricultural settings, lower mosquito density, as well as reduce the loss of crops to insect damage. Chlorpyrifos was also heavily used in residential settings as a structural termite control agent in the early 2000s(135). Between 2009 and 2013, roughly 6 million pounds of chlorpyrifos were applied to over 10 million acres of land annually.

Restrictions on Manufacturing and Use

In response to a wealth of studies implicating chlorpyrifos and other OP insecticides in the pathogenesis of neurocognitive diseases, endocrine disruption, and cardiovascular anomalies, there has been a significant push to reduce the production and use of chlorpyrifos in the U.S. and the EU(136-138). Residential use of chlorpyrifos was restricted in 2001, and was limited to agricultural use and mosquito control(139). However, in 2016 the EPA deemed the current epidemiological risk assessment data insufficient to warrant an overall ban(132). In 2020, the European Union banned all sales of chlorpyrifos and restricted its use in all areas. As of 2021, the EPA has deemed the data sufficient to ban chlorpyrifos in both residential and agricultural sectors, and phaseouts of its production and use began in February 2022. While this is an important step forward, these restrictions do not include chlorpyrifos use on crops solely intended for export, nor crops imported from countries where chlorpyrifos is still legal. Global use of chlorpyrifos is still permitted in both agricultural and/or residential settings in dozens of countries in Africa and Asia, meaning the public health burden of chlorpyrifos will be relevant for the foreseeable future. Furthermore, specific regulations on the disposal of the currently produced chlorpyrifos have not been imposed. These reservoirs of chlorpyrifos may serve as a significant exposure risk depending on where and how they are disposed of.

Trends in Chlorpyrifos and Metabolites in the Environment

Since restrictions initiated in the early 2000s, there have been several cohort and population level studies that have measured trends in OP insecticides and their metabolites in the environment overtime. Work by Barr et. al. has shown that the mean concentrations of

chlorpyrifos metabolites has significantly decreased overtime, and another study comparing the concentrations of OP metabolites in 2009 to NHANES 2002 show that mean concentrations of urinary OP metabolites have decreased since the implementation of initial phaseouts in 2000(140, 141). In contrast, a Swedish study collected data on OP metabolites between 2000-2017 found increasing temporal trends of TCPy (1.7%/year)(142). Other studies have shown that even decades following the initiation bans and restrictions, appreciable amounts of OP insecticides and their metabolites are quantified in biological matrices and the environment(143, 144). It remains to be seen what effect the current exposure amounts of chlorpyrifos have on various health outcomes.

Chemical Characteristics and Exposure Routes

Chlorpyrifos is a hydrophobic, colorless to white crystalline solid, with a mild mercaptan odor. Chlorpyrifos initially enters the environment via direct application to target sites (*e.g.*, soil, foliage, seed treatments, urban surfaces). Chlorpyrifos is reported to have short to moderate persistence in the environment because of several dissipation pathways that might occur concurrently, such as volatilization, photolysis, abiotic hydrolysis, and microbial degradation(145, 146). However, the half-life varies significantly depending on the properties of the environmental compartment (e.g. half-lives for hydrolysis of chlorpyrifos in water are inversely dependent on pH), and reported half-lives range from fifteen days to several months(147). As it accumulates in the environment, chlorpyrifos may move off-site via spray drift, volatilization, and runoff. Indeed, pesticides have been quantified in remote environments such as wildlife in the arctic, which may be a function of its bioaccumulation within organisms and/or its long-distance transportation through water or air.(148). Human exposure to

chlorpyrifos occurs primarily through residues in food, skin contact, and air dispersion. Within homes, children are easily exposed from inhalation, toys, and carpets(135).

In addition to chemical properties of chlorpyrifos and environmental media, the timing of chlorpyrifos spray is an important variable determining the biopersistence of chlorpyrifos. The timing of spray can vary based on the season, and typically coincides with the highest densities of pests and optimal times for crop growth(149). This timing of application of in relation to local climatic conditions, rainfall, and patterns of weather may have significant effects on the degradation, potential for movement, and exposures of non-target organisms(150).

Mechanisms of Action

Chlorpyrifos provides broad-spectrum coverage to kill several pests, including weeds, ants, mosquitos, and roundworms. The primary mechanism of action of chlorpyrifos is irreversible inhibition of acetylcholinesterase, located primarily at neuromuscular junctions and cholinergic synapses in the CNS and peripheral nervous systems(151). Acetylcholinesterase is an enzyme responsible for the degradation of acetylcholine, a potent neurotransmitter. Acetylcholinesterase inhibition is achieved by the chlorpyrifos-oxon form, which is produced through an enzymatic desulfuration process in the liver(152). When acetylcholinesterase is inhibited, excess acetylcholine accumulates within the synaptic cleft between neurons, leading to overactivation of acetylcholine-dependent (“cholinergic”) pathways (Figure 2). The ensuing cascade of cholinergic pathways can lead to organism paralysis, cell growth inhibition, and eventual death.

Figure 2.

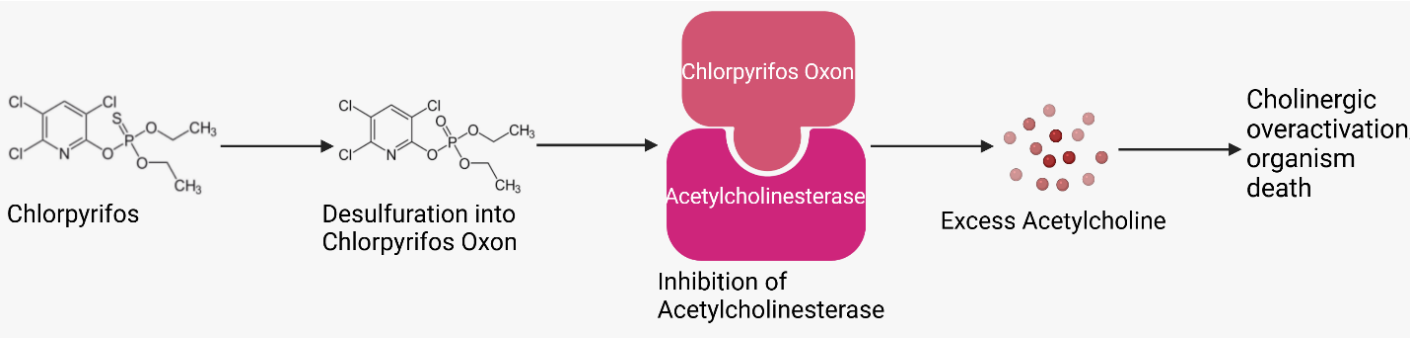


Figure 2: Transformation of chlorpyrifos into chlorpyrifos-oxon is achieved by an activating desulfuration reaction catalyzed by cytochrome P450 (CYP2B6) enzymes in the liver. Chlorpyrifos-oxon is then able to irreversibly bind to acetylcholinesterase and phosphorylate the serine hydroxyl group located in the active site. With acetylcholinesterase inhibited, acetylcholine binding increases cholinergic neurotransmission, and this can ultimately lead to paralysis and death in target organisms.

Chlorpyrifos Inactivation and Degradation

The toxicity of chlorpyrifos is primarily determined by its biotransformation within the body, which is governed primarily by the cytochrome P450 system. Metabolic activation of chlorpyrifos into chlorpyrifos-oxon by CYP2B6 occurs in the liver. After activation, chlorpyrifos is further metabolized by CYP2C19 and CYP3A4 enzymes in the liver and plasma through a dearylation process into 3,5,6-trichloro-2-pyridinol (TCPy) and diethylthiophosphate(152). Additional detoxification occurs by A-esterases and paraoxonases, that hydrolyze the phosphate ester bonds of chlorpyrifos and chlorpyrifos-oxon to form TCPy, diethylthiophosphate (from chlorpyrifos), or diethylphosphate (from chlorpyrifos-oxon)(153). Virtually all chlorpyrifos and chlorpyrifos-oxon is metabolized into water-soluble metabolites that are excreted through the urine, and only trace amounts of parent compounds are

detectable in the urine(154). Variations in the pharmacokinetics and pharmacodynamics of chlorpyrifos can lead to varying sensitivities to the insecticide. In addition to the biochemical properties of chlorpyrifos, metabolism kinetics also depend on individual capacities for organ systems such as the liver, plasma, and kidneys to metabolize, transport, and excrete chlorpyrifos. Activities of these organ systems can vary significantly from person to person, and this heterogeneity may contribute to case-by-case variations in chlorpyrifos toxicity. For example, population-level polymorphisms seen in cytochrome P450 and paroxonase expression have been implicated in varying sensitivities of individuals to chlorpyrifos, where studies have shown that individuals with greater CYP2B6 content and lower CYP2C19 content were predicted to be most sensitive to both OPs(155). Additionally, lifestyle practices such as smoking and alcohol consumption can affect the expression and activity of a number of P450 enzymes(156, 157).

Central Nervous System Toxicity

Chlorpyrifos was developed as a potent neurotoxicant, where its intended action is to overstimulate cholinergic pathways leading to prolonged nerve transmissions and death. In addition to cholinergic dysfunction in the CNS, chlorpyrifos effects have been linked to risk of neurodegenerative diseases in adults including Parkinson's and Alzheimer's, though the exact mechanisms aren't fully understood(158-160). Currently proposed mechanisms of chlorpyrifos-related Parkinson's include impaired dopaminergic signaling(161, 162). In rats, chlorpyrifos has been demonstrated reduces the density of dopaminergic neurons, which play a crucial role in motor function and cognition. The loss of dopaminergic neurons can manifest as Parkinsonian symptoms including impaired motor coordination accompanied by bone loss and osteoporosis

(163). Chlorpyrifos has also been shown to increase levels of Amyloid β precursor proteins in various brain regions, which may be a mechanism through which chlorpyrifos increases Alzheimer's risk(164, 165).

Through actions unrelated to cholinergic drive, numerous studies have determined strong associations between development exposure to chlorpyrifos and various forms of nervous system impairment, most notably cognitive delay in children(166, 167). One mechanism underlying this pathology is believed to be *in utero* exposure to chlorpyrifos during crucial stages of glial proliferation and differentiation of cells that integrate complex tasks such as memory and cognition(168). Exposure to chlorpyrifos *in utero* is believed to induce lasting changes on gene pathways related to cognition, which can manifest as deficits in working memory and IQ among children(169). At the cellular level, chlorpyrifos has the ability to inhibit DNA synthesis, interfere with adenylate cyclase signaling, obstruct transcription factor binding to DNA, and enhance reactive oxygen species formation. These effects are observed at concentrations much lower than what is necessary to elicit cholinergic toxicity. It is likely that the long-lasting effects myelination, synaptic plasticity, and architectural modeling of the CNS has overlap with the pathology of other health conditions.

In addition to the primary enzymatic activity of acetylcholinesterase, acetylcholinesterase also functions in the developing nervous system as a morphogenic factor to increase axonal growth(170). Axogenesis is required for a healthy establishment and remodeling of neural connections that regulate the autonomic nervous system, and impairment of acetylcholinesterase activity may be implicated in dysregulations of axogenesis. It has been hypothesized that this mechanism is responsible for the functional deficits observed in children

and animals following developmental exposure to OPs, and the increased vulnerability of the developing nervous system to OPs. Exposure to low-dose and acute levels of chlorpyrifos can also induce an organophosphorus ester-induced chronic neurotoxicity (OPICN) syndrome(171). This syndrome is marked by a delayed onset of ataxia and upper motor neuron spasticity, accompanied by a Wallerian-type degeneration of axons and myelin in the most distal portion of tracts in both the central and peripheral nervous systems(172). These effects have been observed in rats, where their difficulty to perform cognitive tasks and mazes persist months after the initial exposure to chlorpyrifos.

Peripheral Organ Effects of Chlorpyrifos

In addition to the central nervous system, cholinergic pathway components exist in various peripheral nervous organ systems, and this can lead to the unintended effects of chlorpyrifos throughout the body (Figure 3). For example, there several muscarinic and nicotinic cholinergic receptors within the heart that use acetylcholine as primary ligands. As a result, acetylcholinesterase inhibition peripherally within the cardiac system increases cholinergic activity, leading to reduced heart rate, contractility, and conduction velocity in the sinoatrial and atrioventricular nodes(173). Within the somatic nervous system found in skeletal muscle, overactivity of cholinergic pathways at the neuromuscular junction can lead to muscle weakness, fasciculations, and paralysis(174). Aberrant cholinergic activity within the urinary system can lead to bladder spasms as well as incontinence in some cases(175). Increased cholinergic drive these organ systems contribute to the “SLUDGE” and “DUMBELS” symptoms seen in cases of acute OP insecticide toxicity. These symptoms include salivation, lacrimation,

urination, defecation, GI upset, emesis, diaphoresis, bradycardia, bronchospasm, and bronchorrhea(176).

Chlorpyrifos has also been demonstrated to have unintended effects within these various organ systems, independent of acetylcholinesterase inhibition and below the dose threshold to elicit cholinergic toxicity(177). Chlorpyrifos and chlorpyrifos-oxon are able to bind directly to muscarinic receptor subtypes in the brain and peripheral organs and affect their activity (178, 179). Within the striatum of rats, chlorpyrifos-oxon binding to M2 receptors results in inhibition of adenylate cyclase and alterations in post-receptor signal transduction pathways. Repeated exposure to chlorpyrifos to pups during postnatal days 1-6 resulted in sustained decreases in muscarinic receptor density in the forebrain(180). Additionally, chlorpyrifos has been shown to have adverse effects on the hepatic, reproductive, and endocrine systems(181-184). These unintended toxicities may underly the population-level associations between chlorpyrifos and chronic diseases such as HTN and MetS.

Figure 3.

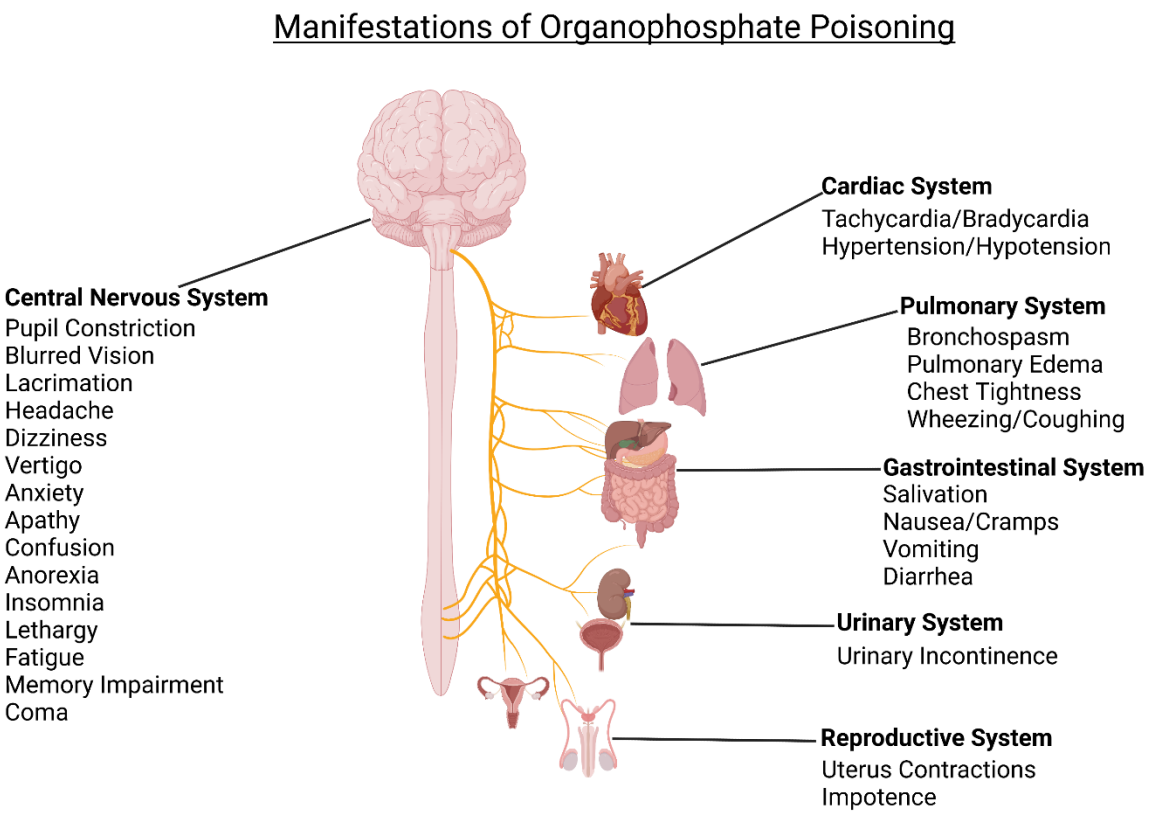


Figure 3: Organophosphate insecticides exert effects on both the central and peripheral nervous system, leading to aberrations in peripheral organ function which can manifest as physical and mental symptoms. The manifestations of toxicity and their severity vary based on the organ systems affected, the duration of OP exposure, and the dose of exposure.

Chlorpyrifos and Hypertension

Current Data Suggesting a Link between Chlorpyrifos and Hypertension

Emerging data suggests a role for OP insecticides such as chlorpyrifos in the initiation, maintenance, and/or progression of HTN, but results from various studies are conflicting, inconclusive, and lack generalizability to the U.S. adult population. A recent systematic review

synthesized evidence on the association between occupational exposure and environmental contamination by pesticides with cardiovascular disease and HTN from 1750 references databases(185). The study found significant associations between heavy metals, malathion, primaphos, and chlorpyrifos and the risk of acute myocardial infarction and HTN. In a study of farmers occupationally exposed to OP insecticides, acetylcholinesterase inhibition was associated with increased odds of HTN, (186). Domestic OP exposure of pregnant women in their first trimesters was associated with a 7% increase in odds of gestational HTN, and a 12% increase was seen in occupationally exposed women(187). Acute exposure to chlorpyrifos has also been associated with risk of myocardial infarction(188). Studies in rats have demonstrated chlorpyrifos' ability to induce HTN and aberrations in heart rate and contractility(189). During heightened spray seasons of OP insecticides, blood pressure elevations were observed among children in Ecuador, and when the spray season ended some blood pressure parameters returned to normal(149).

Chlorpyrifos and Metabolic Dysfunction

As an endocrine disrupting chemical, chlorpyrifos has been demonstrated to alter metabolic homeostasis through a number of proposed mechanisms such as oxidative damage, fatty-acid synthesis, and lipid peroxidation(190, 191). A recent study demonstrates chlorpyrifos' ability to promote obesity and insulin resistance through impacting gut function and the diversity of gut microbiota(93). In mice, chlorpyrifos impairs the integrity of the gut barrier, leading to increased lipopolysaccharide entry into the body and low-grade inflammation. Chlorpyrifos has also been shown to induce hyperglycemia, increase total cholesterol, and lower antioxidative molecules in Sprague-Dawley rats(192). At the population level, chronic

exposure to OP insecticides has been implicated in the risk of metabolic syndrome, where in a study of 600 adults living in Pakistan and Cameroon, malathion, parathion, and chlorpyrifos were associated with elevated Body Mass Index (BMI), insulin, blood glucose, dyslipidemia and HTN(193).

Chlorpyrifos and the Hypothalamus

Experimental Data Showing Effects of Chlorpyrifos on the Hypothalamus

As previously mentioned, the hypothalamus exerts central control over pathways regulating blood pressure and metabolism. The hypothalamus is particularly vulnerable to environmental toxicants, due its properties as a circumventricular organ. The circumventricular organs are highly vascularized structures located around the third and fourth ventricles and characterized by the lack of a blood–brain barrier, as a means to facilitate direct monitoring of metabolites in circulation and the release of hormones directly into the blood stream(194). This adaptation allows the hypothalamus to respond rapidly to changes in homeostasis, but also provides a conduit for exogenous chemicals such as chlorpyrifos to deposit within these structures. Additionally, chlorpyrifos has the ability to cross the blood brain barrier due to its lipophilic properties and small size, and has even been demonstrated to cause disruption of the blood-brain barrier(195).

Chlorpyrifos Effects on the Expression of in Vivo and in Vitro Targets

Previous work done by our lab in mice showed that a daily four-week exposure to 5 mg/kg chlorpyrifos by oral gavage significantly increased hypothalamic mRNA expression of angiotensin type 1A receptor and vasopressin V1A receptor, and increased serum levels of

corticosterone (preliminary results not published). A developmental exposure study found that male offspring of mice exposed to 6 mg/kg of chlorpyrifos between gestational days 15 to 18 had significantly decreased expression of vasopressin(196), and post-natal chlorpyrifos exposure in rats resulted in hypo-sensitized the cholinergic system and down-regulated the mRNA expression levels of the BDNF in the dorsal striatum(197). These outcomes occurred independently of significant brain acetylcholinesterase inhibition. A similar developmental study found that *in utero* exposure is linked to disruptive effects on neuroendocrine axes homeostasis, evidenced by increased expression of the V1A receptor and the estrogen receptor in the amygdala and hypothalamus(198). The developing hypothalamus *in utero* is especially sensitive to the effects of exogenous toxicants like OPs, and exposure may impose lasting effects on the activity of homeostatic processes in the hypothalamus. Given the convergence of blood pressure and metabolism-regulating pathways in the hypothalamus, and chlorpyrifos' tendency to concentrate in the hypothalamus and exert gene expression modulatory effects, it is possible that the pathogenesis of OP-related HTN and MetS can be explained at least in part by chlorpyrifos' toxicity within the hypothalamus.

Study Population (NHANES) and *in Vitro* Model

NHANES

Our lab hypothesized that general population level exposure to OP insecticides is associated with the risk of HTN and MetS (Figure 4). To test this idea, we leveraged data from the National Health and Nutrition Examination Study (NHANES) public database. Currently, most prospective cohort studies and prevalence studies designed to study OP effects on human

health have been largely comprised of occupationally exposed participants. While these studies are important, they do not allow use to estimate health effects of OPs at everyday exposure levels. The benefit to using NHANES is that the estimates derived from these studies can be applied to the general population, and can inform follow-up risk assessment studies. National Health and Nutrition Examination Study is a nationwide census that occurs in two-year cycles. The detailed origin and methods by NHANES can be found in their publicly available archives(199). In short, each two-year cycle contains a sample of roughly 10,000 participants, which are intended to be representative of the civilian, non-institutionalized U.S. population. The census collects data on a wide variety of health outcomes, demographic data, and exposure data. Individuals in supervised care, custody in institutional settings, active-duty military, are excluded. The study utilizes a complex, multistage probability sampling design, and also oversamples certain subgroups to ensure sufficient statistical power (e.g. Hispanic, Black, Asian, and non-Hispanic white and "other" persons aged 60 years or older). This allowed for us to not only test the main effects of OP exposure on HTN and MetS, but also investigate biologically relevant interactions between OP exposure and known risk factors (e.g. age, BMI, race/ethnicity) for HTN and MetS.

Figure 4.

Aim 1/ Aim 2

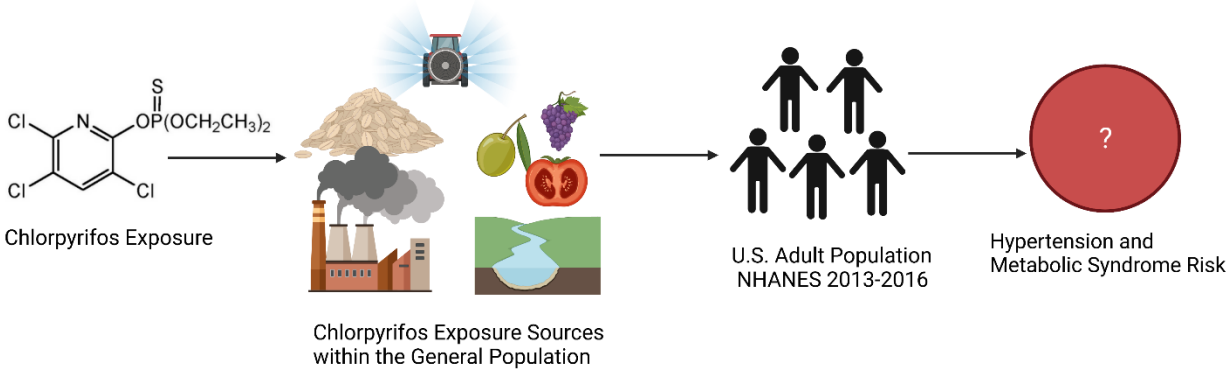


Figure 4: Humans are exposed to chlorpyrifos through several pathways, primarily via contaminated food, water, and air. It's currently unknown what the effect of chronic OP exposure is on the risk of HTN and MetS within the general population. We leveraged data from a nationally representative sample of the U.S. adult population, to quantify levels of OP insecticides and measure their association with HTN and MetS.

Murine Cell Model

We hypothesized that exposure to environmentally relevant concentrations of chlorpyrifos perturbs hypothalamic blood pressure and metabolism regulating pathways. To test the effects of chlorpyrifos on these pathways, we obtained immortalized hypothalamic cell lines from CELLutions®. Due to the difficulty of culturing human primary hypothalamic cultures, commercially available human hypothalamic cell lines are not available. However, murine hypothalamic cell lines are readily available, and the genetic homology between humans and mice allows for relevant extrapolations of biological effects between animal models and humans(200). Adult murine hypothalamic cells were immortalized from two-month-old male C57Bl/6 mice primary cultures by retroviral transfer of SV40 T-Ag. The cells were validated to

express a variety of peptides, enzymatic markers, and biologically active receptors governing metabolic pathways. In Aim 3, we exposed hypothalamic cells to a range of chlorpyrifos and chlorpyrifos-oxon for either 24 hours (acute) or 4 days (subchronic), and subsequently quantified effects on mRNA and protein expression of targets belonging pathways that regulate blood pressure and metabolism (Figure 5).

Benchmark Dose Modeling

The benchmark dose (BMD) model is currently the EPA's preferred risk assessment method, and is an improvement on the traditional practices of no-observed-adverse-effect-level (NOAEL) and lowest-observed-adverse-effect-level (LOAEL), in that it provides range of values for biological outcomes rather than a fixed number. Furthermore, whereas the NOAEL and LOAEL are values within the dataset, the BMD values can be estimated from the data and does not need to be a specific datapoint. This ultimately improves the explanatory power of dosing experiments and provides more biologically relevant endpoints in terms of the risk of adverse outcomes for given exposure doses. The benchmark dose lower confidence limit (BMDL) is a dose where the observed physical effect is less than the predetermined benchmark response (BMR). Using the EPA's BMD modeling software, we assessed the quantitative relationship between chlorpyrifos exposure and mRNA and protein expression. We elected to use an mRNA and protein fold expression cutoff of 0.1 relative to the control as the predetermined change in response to chlorpyrifos and chlorpyrifos-oxon. While the cutoff for biological significance depends on a number of factors including the experimental conditions, and the genes and proteins in questions, a cutoff of 0.1 is regarded as conservative value that

has been validated in many studies, and exceeds the threshold necessary to elicit a biologically relevant effect(201, 202).

Figure 5.

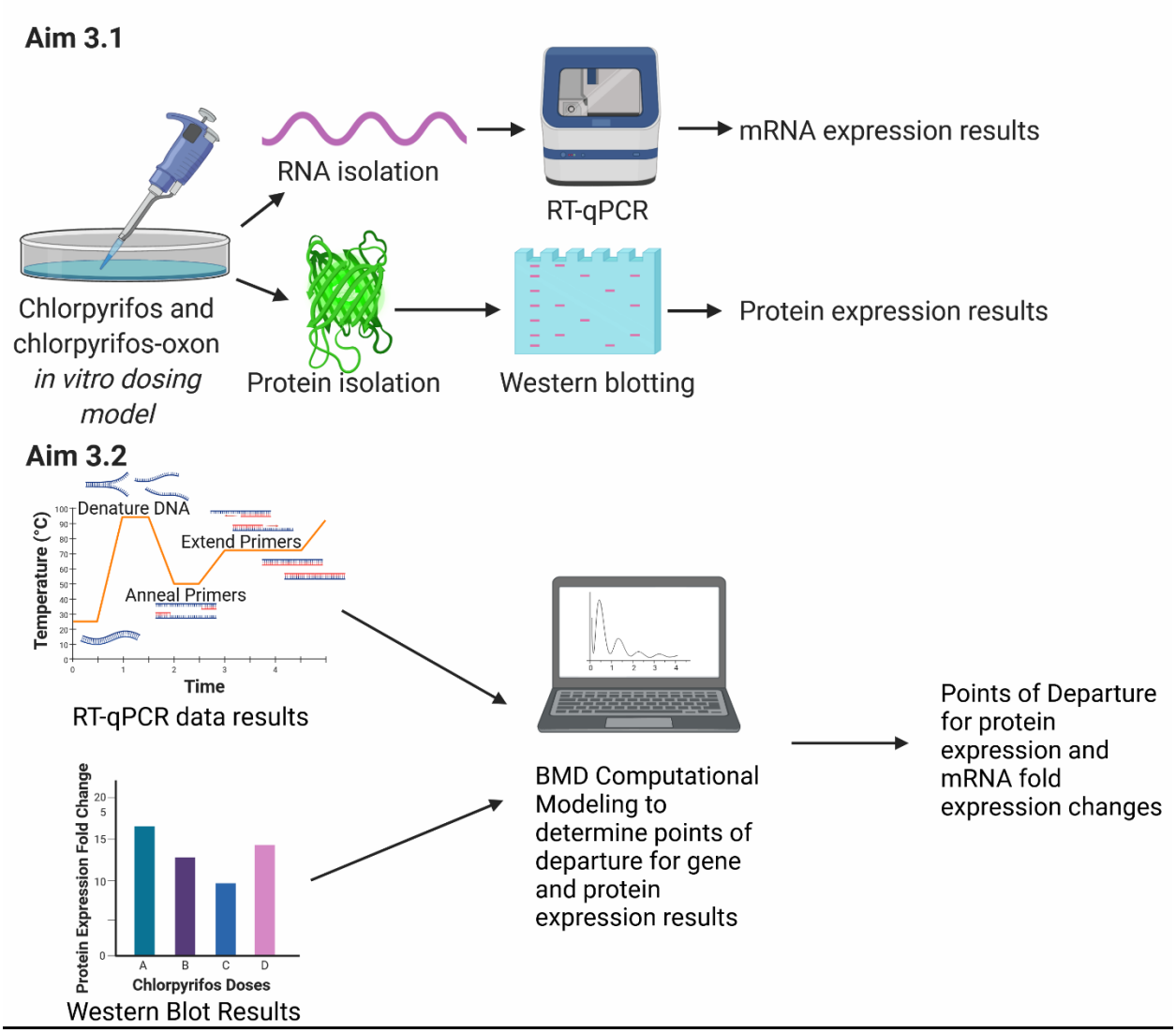


Figure 5: Hypothalamic neurons were exposed to concentrations of chlorpyrifos and chlorpyrifos-oxon ranging from $1.0x10^{-9}$-$1.0x10^{-3}$ M, over a period of 24 hours or 4 days. Protein and mRNA were extracted, and protein and mRNA expression levels for target pathway components were quantified by Western Blotting and RT-qPCR respectively. Lastly, BMD modeling was used to determine points of departure for expression levels at respective concentrations and time points.

Chapter 2: The Association between Organophosphate Insecticides and Blood Pressure Dysregulation: NHANES 2013-2014

Abstract

Background: Organophosphate (OP) insecticides represent one of the largest classes of sprayed insecticides in the U.S., and their use has been associated with various adverse health outcomes, including disorders of blood pressure regulation such as hypertension (HTN). However, the findings from these current studies are conflicting in their results, and it remains unknown what the association is between everyday OP exposure levels and blood pressure.

Methods: In a study of 935 adults from the NHANES 2013-2014 cycle, we examined the relationship between systolic and diastolic blood pressure changes and urinary concentrations of three OP insecticides metabolites, including 3,5,6-trichloro-2-pyridinol (TCPy), oxypyrimidine, and *para*-nitrophenol. These metabolites correspond to the parent compounds chlorpyrifos, diazinon, and parathion, respectively. Weighted, multivariable linear regression analysis while adjusting for potential confounders were used to model the relationship between OP metabolites and blood pressure. Weighted, multivariable logistic regression analysis was used to model the odds of HTN for quartile of metabolites.

Results: We observed significant, inverse association between TCPy on systolic blood pressure (β-estimate= -0.16, $p<0.001$) and diastolic blood pressure (β-estimate= -0.15, $p<0.001$). Analysis with *para*-nitrophenol revealed a significant, positive association with systolic blood pressure (β-estimate= 0.03, $p=0.02$), and an inverse association with diastolic blood pressure (β-estimate= -0.09, $p<0.001$). For oxypyrimidine, we observed significant, positive associations between systolic blood pressure (β-estimate=0.58, $p=0.03$) and diastolic blood pressure (β-estimate=0.31, $p<0.001$). Furthermore, we observed significant interactions

between TCPy and ethnicity on systolic blood pressure (β-estimate=1.46, p=0.0036). Significant interaction terms were observed between oxypyrimidine and ethnicity (β-estimate= -1.73, p<0.001), as well as oxypyrimidine and BMI (β-estimate= 1.51 p<0.001) on systolic blood pressure, and between oxypyrimidine and age (β-estimate =1.96, p=0.02), race (β-estimate = -3.81 p=0.004), and BMI on diastolic blood pressure (β-estimate =0.72, p=0.02). A significant interaction was observed between *para*-nitrophenol and BMI for systolic blood pressure (β-estimate =0.43, p=0.01), and between *para*-nitrophenol and ethnicity on diastolic blood pressure (β-estimate =2.19, p=0.006). Lastly, we observed a significant association between the odds of HTN and TCPy quartiles (OR=0.65, 95% CI [0.43,0.99]).

Conclusion: Our findings support previous studies suggesting a role for OP insecticides in the etiology of blood pressure dysregulation and HTN, and provide estimates for these associations within the general population. Future studies are warranted to corroborate these findings, evaluate dose-response relationships between OP insecticides and blood pressure, determine clinical significance, and elucidate biological mechanisms underlying this association.

Introduction

Hypertension (HTN) poses a significant public health and economic burden, and it is estimated that over 100 million U.S. adults are currently living with HTN(203-205). Hypertension is estimated to cost over $130 billion annually, and is the leading cause of morbidity and mortality associated with cardiovascular diseases and strokes(206). Clinically, HTN can be defined as a systolic blood pressure of 140 mmHg or greater, and/or a diastolic blood pressure of 90 mmHg or greater(207). Recently, studies have shown that even modest deviations from normal blood pressure can significantly increase ones chances of adverse cardiovascular events, and patients with a systolic blood pressure between 120-139 mmHg and/or diastolic pressure between 80-89 are considered pre-hypertensive(208). In over 80% of cases, the exact cause of HTN is unknown, and these situations are classified as primary or "essential" HTN(207). In contrast, secondary HTN describes a situation where a known cause for the pathology has been determined (i.e. side effect of specific medications, genetic conditions such as hyperaldosteronism, organ dysfunction, etc.)(205, 209). While lifestyle factors, diet, aging, and genetic predispositions have been strongly linked with the occurrence of HTN, the influence of exposure to environment chemicals on the initiation and/or progression of HTN has recently gained more attention (193, 210-213).

Historically, a variety of toxicants have been associated with HTN in epidemiological and laboratory studies. Many of these chemicals are classified as persistent organic pollutants (POPs) and include compounds such as dioxin-like and non-dioxin-like polychlorinated biphenyls (PCBs), phthalates, perfluorooctanoic acids (PFOAs), and various organochlorine insecticides like Dichlorodiphenyltrichloroethane (DDT) (214, 215). While some of these

chemicals have been phased out over time and their use restricted, the biochemical properties of POPs including lipophilicity and resistance to biodegradation increase their half-lives in the environment and biological compartments, and thus even restricted or banned chemicals can still contribute to adverse health effects in various populations years later(216). Additionally, newer alternatives that are similar in chemical structure have been shown to have similar deleterious effects on organ systems and overall health, most notably organophosphate insecticides.

In the U.S., organophosphate (OP) insecticides have been manufactured for decades, and millions of kilograms of these insecticides are produced and sprayed annually(217). Currently, OP insecticides constitute over 70% of all insecticides used in the U.S., with the most common OP insecticide being chlorpyrifos(218). Organophosphate insecticides were originally developed as safer alternatives to the organochlorine insecticides, yet their strong association with ADHD and neurodegenerative disorders such as Alzheimer's have raised public health concerns, resulting in their restricted use in many countries(219-221). While parathion has been banned from residential and agricultural use in the U.S. since 2000, and diazinon restricted to agricultural use, metabolites of these insecticides are still readily quantifiable in the general population. This is due to their chemical properties that make them highly lipid soluble, and resistant to biodegradation in certain environments(222). Exposure to OP insecticides can occur via multiple pathways, including household and agricultural use, dietary exposure to herbicide residues, and exposure to agricultural drift(223). Dietary exposure comes primarily from residues in fruits, vegetables, as well as contaminated meat, fish, rice and dairy products. In one study, quantified levels of chlorpyrifos in commonly sold vegetables ranged

from 0.01-3.5 mg/kg(224). Public health initiatives and studies monitor OP metabolites in human samples such as urine, because these concentrations can serve as a reliable proxy for steady-state exposure to parent compounds like chlorpyrifos(225). Several studies have shown that over 90% of the U.S. adult population has measurable levels of a specific metabolite of chlorpyrifos, TCPy, in their urine(226, 227). Additionally, quantification of metabolite concentrations in the general population can give insights into the daily intake of parent compounds reaching systemic circulation. With this information, scientists can model the dose-response relationship between various concentrations of insecticides and health outcomes, and these results help public health experts and regulatory agencies like the Environmental Protection Agency (EPA) set cutoffs and guidelines defining safe doses, as well as providing evidence supporting restrictions on harmful chemicals.

Recent studies have begun investigating the relationship between OP insecticides and the risk for HTN. The primary mechanism of OP insecticides is inhibition of acetylcholinesterase, the enzyme responsible for breaking down acetylcholine(228, 229). With this enzyme inhibited, a robust activation of acetylcholine-dependent (cholinergic) pathways ensues, resulting in overstimulation of cholinergic pathways. Many cholinergic pathways are involved in the central (brain) and peripheral (heart, kidneys, endothelium) control of vascular tone and heart rate, through connections with the sympathetic and parasympathetic nervous systems(230-233). Perturbation of cholinergic pathways through inhibition of acetylcholinesterase has been hypothesized to be one way in which OP insecticides contribute to the pathogenesis of HTN. Chlorpyrifos, the most commonly used OP pesticide in the U.S., and diazinon have been associated with increased risk of gestational HTN in a cohort of migrant farmworkers, as well as

elevations in blood pressure of children exposure to these chemicals during high-spray seasons (234-236). A recent study by Javeres et al. found that chronic exposure to OP insecticides increases risk for metabolic disorders and HTN(193).Additionally, subacute chlorpyrifos exposure in Wistar rats resulted in prolonged HTN and cardiometabolic abnormalities and a prior NHANES study found positive associations between non-specific metabolites of OP pesticides and adverse cardiometabolic health risk(237, 238). In this cross-sectional study, we expand on the current literature to investigate the association between three specific metabolites of OP pesticides and blood pressure.

Research Design/Methods

2.1 National Health and Nutrition Examination Survey (NHANES).

Data analyzed was collected from the NHANES 2013–2014 survey cycle (available from:https://wwwn.cdc.gov/Nchs/Nhanes/2013-2014/TST_H.htm). NHANES is a nationwide survey conducted annually for the purpose of collecting health and diet information from a representative, non-institutionalized U.S. population. NHANES is unique in that it combines interviews, physical examinations, and laboratory evaluations to obtain a large amount of quantitative and qualitative data. Information on NHANES survey methods are described further in detail elsewhere(239). Briefly, the survey examines about 5,000 persons each year from various counties across the country. The country is divided into a total of 30 primary sampling units (PSUs), of which 15 are visited each year. The complex survey design assigns a weight to each individual as a function of their probability of being randomly selected into the study and these weightings are taken into account when building our regression models. All participants provided a written informed consent in agreement with the Public Health Service

Act prior to any data collection. Household questionnaires, telephone interviews, and examinations conducted by healthcare professionals and trained personnel were utilized to collect data.

2.2 Study Participants and Exclusion Criteria.

The 2013-2014 NHANES cycle collected data on 10,175 individuals. We restricted our analysis to adults aged 18 and older. We restricted our analysis to adults due to the fact that HTN in the pediatric population is a rare outcome and would not provide a sufficient sample size for robust analysis. Additionally, pediatric HTN is unlikely to be related to low level, chronic environmental exposures, but rather has been shown to be strongly linked with genetic conditions, and acute, high exposure levels of environmental contaminants(240, 241). From these remaining individuals, analysis was restricted to men and women with valid blood pressure readings, as well as complete information on demographic, anthropometric, questionnaire, and laboratory variables including BMI, alcohol use, diabetes status, education level, hypercholesterolemia status, insurance coverage status, creatinine and albumin concentrations, race, smoking status, and HTN status, resulting in a final analysis sample size of 935.

2.3 Quantification of TCPy, oxypyrimidine, and para-nitrophenol.

Due to the increased cost and technical difficulty in quantifying parent compounds (chlorpyrifos, diazinon, parathion) in the plasma, the NHANES census collected data on readily available and easier to obtain urinary metabolite concentrations. Studies have shown that these metabolites serve as reliable proxies for parent compounds. TCPy, oxypyrimidine, and *para*-nitrophenol were quantified and extracted from the urine matrix of 935 participants using

an automated solid phase extraction system. Selective separation of the analytes was achieved using high-performance liquid chromatography with a gradient elution program. Sensitive detection of the analytes was performed by a triple quadrupole mass spectrometer with a heated electrospray ionization source. Final analyte concentrations were dichotomized to either above the detection limit, a value of 0.15 µg/L, or below the detection limit. A further detailed description on laboratory procedures can be found elsewhere(242).

2.4 Defining Demographic Variables.

Methods for questionnaire data collection are described in the NHANES procedures guide(243). Participants were classified according to highest level of education attainment, insurance coverage status, smoking status, alcohol use, diabetes status, cholesterol status, and HTN status. Highest level of education attainment was based on responses by participants during the home interview. Insurance status and smoking status were recorded as a yes or no response from the home interview. Alcohol use was defined as a yes for individuals who said they drink at least 2 or more alcoholic drinks a day. Diabetes status was defined as a fasting serum glucose greater than 126, having answered yes to taking diabetic medications, or being told by a physician they have diabetes. Hypertension status was defined by at least 4 separate systolic and/or diastolic blood pressure readings greater than 140 mmHg and/or 90 mmHg respectively, having been told by a doctor one has hypertension, or is currently taking hypertension medications. Cholesterol status was defined by whether a person was told he/she has high cholesterol by a physician, or if that person is currently taking hypercholesterolemia medications.

2.5 Statistical Analyses.

Continuous variables were compared using one-way ANOVA, while categorical variables were compared using the Chi-squared test. Multivariable, ordinary least squares regression models were used to measure the association between the urinary concentrations of OP metabolites and blood pressure. We identified potential confounders including race, age, BMI, creatinine levels, diabetes status, education level, smoking status, and hypercholesterolemia based on *a priori* data from literature searches (figure 1). Potential for confounding was empirically assessed, and ultimately we included age, BMI, and race as confounders in our final models. We also included creatine to control for urinary dilution, which can affect urinary metabolite concentrations. Metabolite values were divided into quartiles for logistic regression analysis, to evaluate the association between quartile levels of each metabolite and the odds of HTN. Oxypyrimidine values below the 75^{th} percentile were detected by the analyzer as 0.70, and therefore we divided oxypyrimidine groups into below the 75^{th} percentile and above the 75^{th} percentile. The lowest quartile was used as the reference in each case, and these results are presented as supplementary information.

All statistical analyses were performed using SAS 9.4 and SUDAAN software packages accounting for the complex survey design of NHANES(244). A p-value < 0.05 was used as the criterion for significance.

Results

Demographic tables for our cohort are presented in tables 1-5. The mean age for our cohort was 49.3 ± 0.57, and roughly half of individuals were men vs. women. At least 55% of our cohort received some college degree or above. Table 2 shows the demographic breakdown

of the cohort stratified by quartile of TCPy exposure. We observed a significant difference among smoking status between quartiles of TCPy. Table 3 shows the demographic breakdown by quartile of *para*-nitrophenol exposure, and table 4 shows the demographic breakdown by individuals below the 75th percentile of oxypyrimidine exposure vs. those above the 75th percentile. Table 5 shows the demographic breakdown of the cohort by HTN status. We observed significant differences between hypertensive vs. normotensive individuals for age, race, education, hypercholesterolemia status, mean *para*-nitrophenol concentration, and mean values for systolic and diastolic blood pressure. In our regression analysis of the total cohort, we observed a significant, inverse association between TCPy and systolic blood pressure (β-estimate= -0.16, $p<0.001$) and diastolic blood pressure (β-estimate= -0.15, $p<0.001$). The interpretation of these estimates would be that for every 1 unit increase in TCPy concentration, we would expect a 0.16 mmHg and 0.15 mmHg decrease on systolic and diastolic blood pressure, respectively. Furthermore, we observed significant interactions between TCPy and ethnicity on systolic blood pressure (β-estimate=1.46, $p=0.0036$). This interaction was observed within the Mexican American race when using Caucasian Americans as the reference group. The interpretation for this interaction is that when holding all other variables at zero, we expect an increase on systolic blood pressure of 1.46 mmHg when comparing Mexican Americans exposed to TCPy to Caucasian Americans. Analysis with *para*-nitrophenol revealed a significant, positive association with systolic blood pressure (β-estimate= 0.03, $p=0.02$), and an inverse association with diastolic blood pressure (β-estimate= -0.09, $p<0.001$). A significant interaction was observed between *para*-nitrophenol and BMI on systolic blood pressure (β-estimate =0.43, $p=0.01$), and between *para*-nitrophenol and ethnicity on diastolic blood pressure (β-estimate

=2.19, p=0.006). Significant interaction terms were observed between oxypyrimidine and race (β-estimate= -1.73, p<0.001), as well as oxypyrimidine and BMI (β-estimate= 1.51 p<0.001) on systolic blood pressure. We also observed significant interactions between oxypyrimidine and age (β-estimate =1.96, p=0.02), race (β-estimate =-3.81 p=0.004), and BMI on diastolic blood pressure (β-estimate =0.72, p=0.02). Lastly, we performed multivariable logistic regression to model the odds of hypertension, at quartile levels of each OP metabolite. The lowest quartile was used as the reference in each case. Results from our logistic regression revealed a significant association between the odds of HTN and TCPy (OR=0.65, 95% CI [0.43,0.99]) and no significant associations between urinary concentrations of oxypyrimidine, and *para*-nitrophenol (Supplementary tables 1-3).

Figure 6.

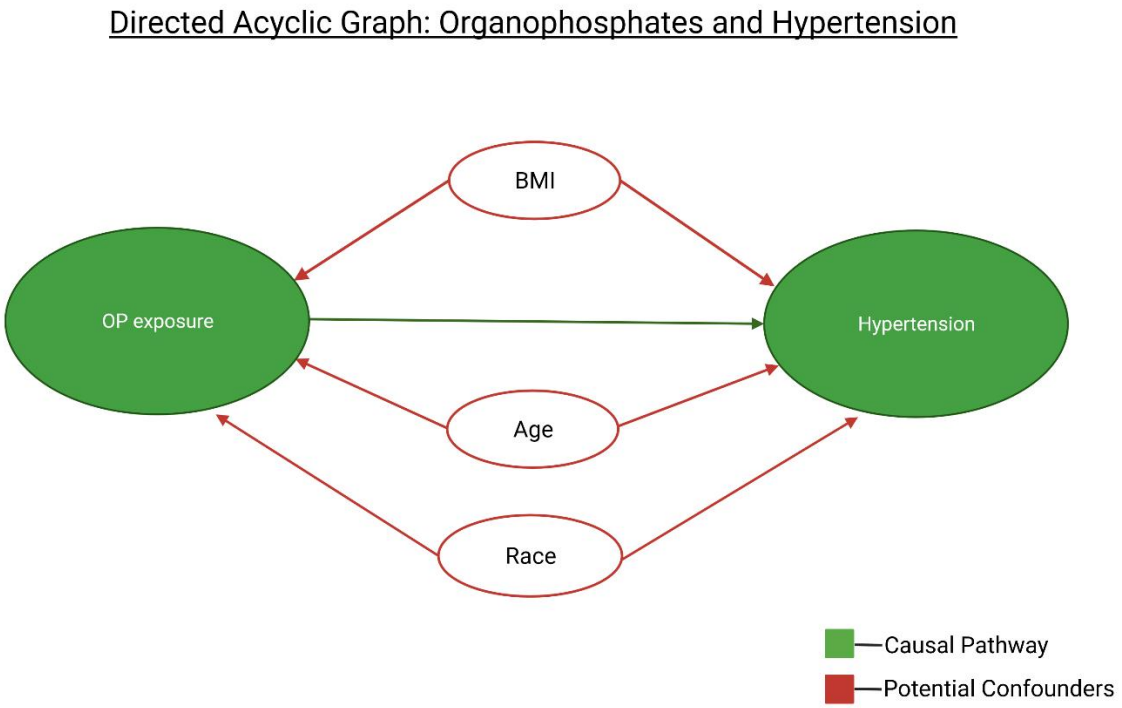


Figure 6: Directed acyclic graph depicting the proposed relationship between OP exposure and HTN. The causal pathway is depicted with the green arrow. Potential founders are depicted along the red arrows. Covariates known to be associated with risk of HTN are along the black arrows.

Table 1. Demographic and Laboratory Data for the Total Cohort

		Total Cohort
		N= 935
Age, Group Class (%)	*18-39*	312 (33.37)
	40-59	322 (34.44)
	60+	301 (32.19)
Mean Age (SEM)		49.3 (0.57)
Gender (%)	*Male*	437 (46.74)
	Female	498 (53.26)
Race/Ethnicity (%)	*Non-Hispanic White*	301 (32.19)
	Non-Hispanic Black	194 (20.75)
	Mexican-American	158 (16.90)
	Other	282 (30.16)
BMI Categories (%)	*18.5-25*	249 (26.63)
	25-30	312 (33.37)
	>30	374 (40.0)
Mean BMI (SEM)		29.6 (0.23)
Education (%)	*Less than 9th Grade*	109 (11.66)
	9th-11th Grade	119 (12.73)
	Highschool Graduate	183 (19.57)
	Some College or AA Degree	288 (30.80)
	College Graduate or Above	235 (25.13)
Smoking Status (%)	*Refused*	1 (0.11)
	Never Smoker	518 (55.40)
	Past Smoker	210 (22.46)
	Active Smoker	207 (22.14)
Alcohol Use (%)	*Non-Drinker*	729 (77.97)
	Drinker	206 (22.03)
Hypertension (%)	*Normotensive*	739 (79.04)
	Hypertensive	196 (20.96)
Diabetes (%)	*Non-Diabetic*	827 (88.45)
	Diabetic	108 (11.55)
Hypercholesterolemia (%)	*Normal Cholesterol*	660 (70.59)
	High Cholesterol	275 (29.41)
Insurance Status (%)	*Covered*	766 (81.93)
	Not Covered	169 (18.07)
Mean Systolic BP (mmHg) (SEM)		125.2 (0.61)
Mean Diastolic BP (mmHg) (SEM)		70.1 (0.38)
Mean Urinary Tcpy (µg/L) (SEM)		1.58 (0.09)
Mean Urinary Oxypyrimidine (µg/L) (SEM)		0.23 (0.05)
Mean Urinary Paranitrophenol (µg/L) (SEM)		1.13 (0.06)
Mean Creatinine (mg/dL) (SEM)		123.4 (2.7)

Table 2. Demographic and Laboratory Data by Quartiles of TCPy

		Q1	Q2	Q3	Q4	P-value
		N=259	N=223	N=237	N=216	
Age, group class (%)	*18-39*	100 (38.61)	64 (28.70)	82 (34.60)	66 (30.56)	0.07
	40-59	82 (31.66)	75 (33.63)	81 (34.18)	84 (38.89)	
	60+	77 (29.73)	84 (37.67)	74 (31.22)	66 (30.56)	
Mean Age (SEM)		48.0 (1.1)	51.2 (1.1)	48.9 (1.1)	49.3 (1.2)	0.89
Gender (%)	*Male*	108 (41.70)	105 (47.09)	115 (48.52)	109 (50.46)	0.15
	Female	151 (58.30)	118 (52.91)	122 (51.48)	107 (49.54)	
Race/Ethnicity (%)	*Non-Hispanic White*	83 (32.05)	66 (29.60)	79 (33.33)	73 (33.80)	0.53
	Non-Hispanic Black	55 (21.24)	45 (20.18)	50 (21.10)	44 (20.37)	
	Mexican American	47 (18.15)	41 (18.39)	30 (12.66)	40 (18.52)	
	Other	74 (28.57)	71 (31.84)	78 (32.91)	59 (27.31)	
BMI categories (%)	*18.5-25*	67 (25.87)	59 (26.46)	69 (29.11)	54 (25.0)	0.66
	25-30	92 (35.52)	72 (32.29)	77 (32.49)	71 (32.87)	
	>30	100 (38.61)	92 (41.26)	91 (38.40)	91 (42.13)	
Mean BMI (SEM)		29.7 (0.5)	29.9 (0.5)	29.1 (0.4)	29.5 (0.4)	0.84
Education (%)	*Less than 9th grade*	32 (12.36)	24 (10.81)	28 (11.81)	25 (11.57)	0.68
	9th-11th grade	36 (13.90)	29 (13.06)	24 (10.13)	30 (13.89)	
	Highschool Graduate	54 (20.85)	45 (20.27)	47 (19.83)	37 (17.13)	
	Some College or AA degree	65 (25.10)	52 (23.42)	61 (25.74)	57 (26.39)	
	College Graduate or Above	72 (27.80)	72 (32.43)	77 (32.49)	67 (31.02)	
Smoking status (%)	*Never smoker*	135 (52.12)	126 (56.50)	136 (57.38)	121 (56.02)	**0.02**
	Past smoker	51 (19.69)	59 (26.46)	53 (22.36)	47 (21.76)	
	Active smoker	73 (28.19)	38 (17.04)	48 (20.25)	48 (22.22)	
Alcohol Use (%)	*Non Drinker*	191 (73.75)	176 (78.92)	192 (81.01)	170 (78.70)	0.45
	Drinker	68 (26.25)	47 (21.08)	45 (18.99)	46 (21.30)	
Diabetes (%)	*Non Diabetic*	230 (88.80)	203 (91.03)	203 (85.65)	191 (88.43)	0.33
	Diabetic	29 (11.20)	20 (8.97)	34 (14.35)	25 (11.57)	
Hypercholesterolemia (%)	*Normal Cholesterol*	174 (67.18)	154 (69.06)	173 (73.0)	159 (73.61)	0.72
	High Cholesterol	85 (32.82)	69 (30.94)	64 (27.0)	57 (26.39)	
Hypertension (%)	*Normotensive*	209 (80.69)	178 (79.82)	174 (73.42)	178 (82.41)	0.07
	Hypertensive	50 (19.31)	45 (20.18)	63 (26.58)	38 (17.59)	
Insurance Status (%)	*Covered*	203 (78.38)	182 (81.61)	199 (83.97)	182 (84.26)	0.95
	Not Covered	56 (21.62)	51 (18.39)	38 (16.03)	34 (15.74)	
Mean Systolic BP (mmHg) (SEM)		124.8 (1.1)	125.1 (1.3)	126.3 (1.2)	124.7 (1.3)	0.95
Mean Diastolic BP (mmHg) (SEM)		70.3 (0.7)	69.1 (0.7)	71.3 (0.8)	69.5 (0.8)	0.28
Mean Tcpy Concentration (SEM)		0.32 (0.01)	0.8 (0.01)	1.4 (0.01)	4.1 (0.3)	**0.0056**
Mean Creatinine (mg/dL) (SEM)		117.6 (4.9)	124.3 (5.2)	120.1 (5.3)	132.6 (5.9)	0.13

Table 3. Demographic and Laboratory Data by Quartiles of Para-nitrophenol

		Q1	Q2	Q3	Q4	P-value
		N=219	N=237	N=220	N=259	
Age, group class (%)	*18-39*	84 (38.36)	78 (32.91)	75 (34.09)	75 (28.96)	0.56
	40-59	75 (34.25)	82 (34.60)	68 (30.91)	97 (37.45)	
	60+	60 (27.40)	77 (32.49)	77 (35.00)	87 (33.59)	
Mean Age (SEM)		47.7 (1.2)	48.9 (1.15)	49.5 (1.2)	50.9 (1.05)	0.39
Gender (%)	*Male*	106 (48.40)	103 (43.46)	102 (46.36)	126 (48.65)	0.43
	Female	113 (51.60)	134 (56.54)	118 (53.64)	133 (51.35)	
Race/Ethnicity (%)	*Non-Hispanic White*	79 (36.07)	82 (34.60)	69 (31.36)	71 (27.41)	0.54
	Non-Hispanic Black	44 (20.09)	45 (18.99)	46 (20.91)	59 (22.78)	
	Mexican American	31 (14.16)	38 (16.03)	34 (15.45)	55 (21.24)	
	Other	65 (29.68)	72 (30.38)	71 (32.27)	74 (28.57)	
BMI categories (%)	*18.5-25*	57 (26.03)	68 (28.69)	59 (26.82)	65 (25.10)	0.83
	25-30	74 (33.79)	76 (32.07)	82 (37.27)	80 (30.89)	
	>30	88 (40.18)	93 (39.24)	79 (35.91)	114 (44.02)	
Mean BMI (SEM)		29.8 (0.5)	29.6 (0.5)	29.5 (0.5)	29.5 (0.4)	0.42
Education (%)	*Less than 9th grade*	27 (12.33)	20 (8.47)	28 (12.73)	34 (13.13)	0.06
	9th-11th grade	34 (15.53)	29 (12.29)	22 (10.00)	34 (13.13)	
	Highschool graduate	36 (16.44)	54 (22.88)	43 (19.55)	50 (19.31)	
	Some college or AA degree	58 (26.48)	55 (23.31)	57 (25.91)	65 (25.10)	
	College graduate or above	64 (29.22)	78 (33.05)	70 (31.82)	76 (29.34)	
Smoking status (%)	*Never smoker*	134 (61.19)	125 (52.74)	115 (52.27)	144 (55.60)	0.17
	Past Smoker	44 (20.09)	59 (24.89)	53 (24.09)	54 (20.85)	
	Active smoker	41 (18.72)	53 (22.36)	52 (23.64)	61 (23.55)	
Alcohol Use (%)	*Non Drinker*	163 (74.43)	181 (76.37)	178 (80.91)	207 (79.92)	0.80
	Drinker	56 (25.57)	56 (23.63)	42 (19.09)	52 (20.08)	
Diabetes (%)	*Non Diabetic*	201 (91.78)	212 (89.45)	193 (87.73)	221 (85.33)	0.28
	Diabetic	18 (8.22)	25 (10.55)	27 (12.27)	38 (14.67)	
Hypercholesterolemia (%)	*Normal Cholesterol*	139 (63.47)	166 (70.04)	161 (73.18)	194 (74.90)	0.08
	High Cholesterol	80 (36.53)	71 (29.96)	59 (26.82)	65 (25.10)	
Hypertension (%)	*Normotensive*	179 (81.74)	185 (78.06)	168 (76.36)	207 (79.92)	0.50
	Hypertensive	40 (18.26)	52 (21.94)	52 (23.64)	52 (20.08)	
Insurance Status (%)	*Covered*	176 (80.37)	192 (81.01)	179 (81.36)	219 (84.56)	0.28
	Not Covered	43 (19.63)	45 (18.99)	41 (18.64)	40 (15.44)	
Mean Systolic BP (mmHg) (SEM)		125.7 (1.4)	124.5 (1.3)	125.1 (1.2)	125.8 (1.05)	0.65
Mean Diastolic BP (mmHg) (SEM)		69.5 (0.8)	70.7 (0.8)	69.6 (0.8)	70.5 (0.7)	0.33
Mean Paranitrophenol Concentration (SEM)		0.22 (0.01)	0.46 (0.01)	0.87 (0.01)	2.75 (0.2)	**0.01**
Mean Creatinine (mg/dL) (SEM)		119 (5.5)	125.8 (5.7)	127.0 (5.3)	121.2 (5.0)	0.95

Table 4. Demographic and Laboratory Data by Oxypyrimidine Percentile

		Below 75th Percentile	Above 75th Percentile	P-value
		N=702	N=233	
Age, group class (%)	*18-39*	236 (33.62)	76 (32.62)	0.63
	40-59	239 (34.05)	83 (35.62)	
	60+	227 (32.34)	74 (31.76)	
Mean Age (SEM)		49.2 (0.6)	49.7 (1.1)	0.13
Gender	*Male*	329 (46.78)	108 (46.35)	0.59
	Female	373 (53.13)	125 (53.65)	
Race/Ethnicity (%)	*Non-Hispanic White*	237 (33.76)	64 (27.47)	0.33
	Non-Hispanic Black	140 (19.94)	54 (23.18)	
	Mexican American	116 (16.52)	42 (18.03)	
	Other	209 (29.77)	73 (31.33)	
BMI categories (%)	*18.5-25*	182 (25.93)	67 (28.76)	0.27
	25-30	244 (34.76)	68 (29.18)	
	>30	276 (39.32)	98 (42.06)	
Mean BMI (SEM)		29.7 (0.3)	29.1 (0.4)	0.15
Education Status (%)	*Less than 9th grade*	80 (11.41)	29 (12.45)	0.62
	9th-11th grade	91 (12.98)	28 (12.02)	
	Highschool graduate	139 (19.83)	44 (18.88)	
	Some college or AA degree	171 (24.39)	64 (27.47)	
	College graduate or above	220 (31.38)	68 (29.18)	
Smoking status (%)	*Never smoker*	383 (54.56)	135 (57.94)	0.07
	Past smoker	162 (23.08)	48 (20.60)	
	Active smoker	157 (22.36)	50 (21.46)	
Alcohol Use (%)	*Non Drinker*	544 (77.49)	185 (79.40)	0.87
	Drinker	158 (22.51)	48 (20.60)	
Diabetes (%)	*Non Diabetic*	624 (88.89)	203 (87.12)	0.38
	Diabetic	78 (11.11)	30 (12.88)	
Hypercholesterolemia (%)	*Normal cholesterol*	491 (69.94)	169 (72.53)	0.33
	High cholesterol	211 (30.06)	64 (27.47)	
Hypertension (%)	*Normotensive*	554 (78.92)	185 (79.40)	0.79
	Hypertensive	148 (21.08)	48 (20.60)	
Insurance Status (%)	*Covered*	577 (82.19)	189 (81.12)	0.54
	Not Covered	125 (17.81)	44 (18.88)	
Mean Systolic BP (mmHg) (SEM)		125.2 (0.7)	125.4 (1.2)	0.25
Mean Diastolic BP (mmHg) (SEM)		70.2 (0.4)	69.8 (0.7)	0.28
Mean Oxypyrimidine Concentration (ug/L) (SEM)		0.07 (0.01)	0.72 (0.21)	0.05
Mean Creatinine (mg/dL) (SEM)		124.5 (3.0)	120.4 (5.7)	0.49

Table 5. Demographic and Laboratory Data by Hypertension Status

		Demographic Tables by Hypertension Status (Total Cohort)		
		Normotensive	Hypertensive	P-value
Age, group class (%)	*18-39*	297 (40.19)	15 (7.65)	**0.001**
	40-59	259 (35.05)	63 (32.14)	
	60+	183 (24.76)	118 (60.20)	
Mean Age (SEM)		45.9 (0.6)	62.1 (1.1)	**0.01**
Gender (%)	*Male*	342 (46.28)	95 (48.47)	0.25
	Female	397 (53.72)	101 (51.53)	
Race/Ethnicity (%)	*Non-Hispanic White*	238 (32.21)	63 (32.14)	**0.0065**
	Non-Hispanic Black	140 (18.94)	54 (27.55)	
	Mexican American	127 (17.19)	31 (15.82)	
	Other	234 (31.66)	48 (24.49)	
BMI categories (%)	*0-18.5*	209 (28.28)	40 (20.41)	0.07
	18.5-25	246 (33.29)	66 (33.67)	
	25-30	284(38.43)	90 (45.92)	
Mean BMI (SEM)		29.4 (0.26)	30.4 (0.48)	0.18
Education (%)	*Less than 9th grade*	73 (9.88)	36 (18.46)	**0.0068**
	9th-11th grade	98 (13.26)	21 (10.77)	
	Highschool graduate	144 (19.49)	39 (20.00)	
	Some College or AA degree	195 (26.39)	40 (20.51)	
	College graduate or above	229 (30.99)	59 (30.26)	
Smoking status (%)	*Never smoker*	405 (54.80)	113 (57.65)	0.63
	Past smoker	171 (23.14)	39 (19.90)	
	Active smoker	163 (22.06)	44 (22.45)	
Alcohol use (%)	*Non Drinker*	568 (76.86)	161 (82.14)	0.52
	Drinker	171 (23.14)	35 (17.86)	
Diabetes (%)	*Non Diabetic*	654 (88.50)	173 (88.27)	0.93
	Diabetic	85 (11.50)	23 (11.73)	
Hypercholesterolemia (%)	*Normal Cholesterol*	555 (75.10)	105 (53.57)	**0.001**
	High Cholesterol	184 (24.90)	91 (46.43)	
Mean Tcpy Concentration (μg/L) (SEM)		1.5 (0.06)	1.7 (0.35)	0.77
Mean Paranitrophenol Concentration (μg/L) (SEM)		1.16 (0.08)	1.02 (0.08)	**0.01**
Mean Oxypyrimidine Concentration (μg/L) (SEM)		0.21 (0.05)	0.3 (0.16)	0.46
Mean Systolic BP (mmHg) (SEM)		118 (0.4)	152.3 (0.98)	**0.001**
Mean Diastolic BP (mmHg) (SEM)		68.6 (0.4)	75.3 (1.1)	**0.001**
Mean Creatinine (mg/dL) (SEM)		129.3 (3.1)	104 (1.1)	**0.001**

Discussion

Our findings support data from previous studies suggesting a link between OP insecticide exposure and blood pressure dysregulation. Here, we have demonstrated significant associations between population-level OP exposure and blood pressure dysregulation. We additionally observed significant interactions between OP exposure and BMI, age, race. It has been demonstrated that OP insecticides and other environmental chemicals commonly found with OPs (i.e. herbicides, heavy metals, PCBs) can sequester within the biological fat compartment, specifically within adipocytes(245, 246). In this case, the fat compartment can serve as a reservoir for continued exposure, beyond the initial time of contact. Thus, studies have shown that for varying levels of BMI, the adverse effects of exposure to a chemical can be significantly more pronounced in individuals with higher BMI, because these individuals trap more chemical within their bodies compared to lower BMI individuals, given a same initial exposure of chemical. The interaction between age and OP exposure is possibly due to the fact that as we age, endogenous levels of protective enzymes and metabolic processes wanes(247). Specifically, levels of liver cytochrome P450 and paroxonase enzymes that are responsible for metabolizing OPs decreases with increasing age, and therefore older individuals might be more likely to experience adverse health effects of OPs(248, 249). Studies have also shown differences in expression levels of parxoxonase enzymes between ethnic groups, and this may in part explain the interaction between race and OP exposure on blood pressure(250). In addition to genetic variability, sociodemographic variables that are known to impact health (e.g. education, access to care, stress, socioeconomic status, etc.) may interact with OP exposure, leading to more pronounced effects on blood pressure.

There are several potential mechanisms that have been hypothesized explaining the association between OP insecticides and blood pressure dysregulation. As mentioned previously, one proposed mechanism is through over-activation of central cholinergic pathways linked to the sympathetic and parasympathetic nervous systems. Increased sympathetic drive from the brain to target peripheral organs like the heart, liver, kidneys, adrenal glands, and endothelium produces a release of vasoactive hormones and physiological responses such as increased heart rate, vasoconstriction, and water retention(51, 251). These sequelae of events ultimately act to increase blood pressure. On the contrary, activation of the central parasympathetic nervous system, and peripheral cholinergic receptors particularly found in the heart act to decrease heart rate, and may ultimately lower blood pressure(173, 252). The duration and dose of exposure to OP insecticides may decide which pathway activation dominates, and ultimately the effect and direction on blood pressure(253, 254).

Additionally, it is important to note that acetylcholinesterase inhibition represents only one part of the complete toxicological profile of OP insecticides, which remains to be fully elucidated. According to the CDC, exposure levels of chlorpyrifos within the general population aren't expected to significantly inhibit acetylcholinesterase and cause overt cholinergic toxicity(255). However, there may be subtle changes occurring with prolonged, chronic OP pesticide exposure, which may contribute to increased risk for HTN. Recent *in vitro* and *in vivo* animal studies suggest that effects on neuronal morphogenesis, neurotransmission, and behavior may occur at systemically nontoxic doses or at doses of chlorpyrifos that do not result in readily apparent changes cholinergic pathways(255). These neuronal pathways (many of which are located in the hypothalamus), rely on the integrity of synapses and neurotransmitter

function to regulate the sympathetic nervous system independent of cholinergic pathways, which in turn regulates blood pressure. Most notably, vasopressin, angiotensin II, and leptin hormones act as key effector hormones within the paraventricular nucleus of the hypothalamus(53, 104). Chlorpyrifos exposure has been shown experimentally to not only increase circulating levels of these hormones, but also bind to their receptors *in vitro*(178, 256). These receptors and neurotransmitters belong to pathways that travel from the hypothalamus to the brainstem, which sends outputs to various peripheral organs to regulate blood pressure. Through these actions chlorpyrifos can affect the activity and expression of these pathways, and ultimately affecting blood pressure.

Organophosphate insecticides like chlorpyrifos, diazinon, and parathion have also been shown to affect expression of numerous micro RNAs (miRNAs) *in vivo* and *in vitro* (257, 258). Micro RNAs are short, noncoding RNA molecules that regulate gene expression at the level of transcription. Many of these miRNAs are targets of genes in cardiac tissue, neural tissue, and skeletal tissue that control homeostatic processes including blood pressure regulation. Our lab previously found that differential expression of several miRNAs (miR-20a-5p, miR-4763-5p, and miR-4709-3p) that regulate vascular remodeling, immune pathways, and cardiac function are implicated in the pathogenesis of hypertension(259). Thus, another possible mechanism through which OP insecticides affects blood pressure is through effects on miRNA-dependent pathways.

It is also important to note that TCPy, oxypyrimidine, and *para*-nitrophenol have their own toxicological profiles in various organ systems, independent of acetylcholinesterase inhibition(260, 261). If the relationship between OP metabolites and blood pressure is due their

direct effects (in conjunction with or independent of parent compound effects), then future studies examining the individual effects of these OP metabolites and the parent compounds on blood pressure are warranted. Lastly, chronic chlorpyrifos exposure has been shown to alter brain development and neuronal morphogenesis of developing fetuses in absence of significant acetylcholinesterase inhibition(262, 263). These *in utero* exposures may also contribute to the effect of OP insecticides on blood pressure and may even predispose individuals to HTN, and future developmental studies are warranted to test this idea.

The present study has several strengths. We incorporated a large number of men and women representative of the general U.S. adult population, and we were able to characterize the association between blood pressure and everyday exposure levels of TCPy, oxypyrimidine, and para-nitrophenol. Unlike this study, many previous studies lack generalizability due to the selection of their study populations, which mostly include occupationally exposed pesticide applicators, and agricultural subpopulations living in areas of high OP pesticide concentrations. Additionally, previous studies have relied on using dialkyl phosphates (DAPs) as proxies for OP exposure. Unlike TCPy, oxypyrimidine, and *para*-nitrophenol, DAPs are not unique to any one parent compound, and are a result from metabolism of a number of OP insecticides, making them a less reliable proxy for parent compound exposure. Another strength lies in the oversampling methods of NHANES, which allowed for sufficient sample sizes of minority populations being recruited (Mexican American, African-American, Asian-American). These groups have been traditionally difficult to include in population-level studies, and when they are included in small numbers there isn't enough power to estimate main effects with confidence. Through oversampling, we were able to examine main effects of OP exposure on

blood pressure within these groups, and examine interaction effects between OP exposure and race/ethnicity on blood pressure.

The current study has several limitations. Due to the cross-sectional nature of this study, we are unable draw any causal relationships between the exposure to TCPy, oxypyrimidine, para-nitrophenol, and blood pressure outcomes. Furthermore, because we are measuring urinary concentrations of metabolites as a proxy for parent compound exposure, we are unable to quantify the true relationship between the parent compounds and blood pressure. Additionally, while TCPy is a specific metabolite of chlorpyrifos, TCPy is relatively stable in the environment, and thus it is likely that quantified TCPy comes not only from direct exposure, but also from a variety of sources such as residues on foods that accumulate overtime. Thus, it is possible that the estimated exposure to chlorpyrifos is overestimated when using TCPy as a surrogate.

Conclusion

Our findings support a potential role for organophosphate insecticide exposure in the pathogenesis of HTN. Results such as these support initiatives to reduce overuse of insecticides, develop safer insecticide alternatives, and to explore alternative avenues for insect control in lieu of insecticides (e.g. bioengineering of insects, crop rotating, etc.). Additionally, improved protocols and safety standards may be beneficial for individuals who use insecticides, as well as farmers and industries whose use of insecticides leads to global exposures at the population level. Future experiments are warranted to elucidate the biological mechanisms responsible for the association between OP insecticides and blood pressure. With better understanding of the

mechanisms contributing to hypertension, improved treatments and therapies may be developed to control blood pressure related to OP exposure.

Supplementary Tables

Table 6. Logistic Regression Results between TCPy Quartiles and Hypertension

Variables		Hypertension	
		OR (CI95%)	*p*
Tcpy	1st Quartile	-	-
	2nd Quartile	0.65 (0.43,0.99)	**0.04**
	3rd Quartile	1.65 (0.97,2.80)	0.06
	4th Quartile	0.82(0.53,1.27)	0.36

Table 7. Logistic Regression Results between Para-nitrophenol Quartiles and Hypertension

Variables		Hypertension	
		OR (CI95%)	*p*
Para-nitrophenol	1st Quartile	-	-
	2nd Quartile	0.87 (0.52, 1.47)	0.59
	3rd Quartile	1.09 (0.61, 1.94)	0.77
	4th Quartile	0.68 (0.37, 1.27)	0.21

Table 8. Logistic Regression Results between Oxypyrimidine Percentiles and Hypertension

Variables		Hypertension	
		OR (CI95%)	*p*
Oxypyrimidine	Below 75th Percentile	-	-
	Above 75th Percentile	0.96(0.59, 1.57)	0.86

Chapter 3: Organophosphate Insecticides are Associated with Blood Pressure Dysregulation and Metabolic Syndrome among U.S. Adults: NHANES 2015-2016

Abstract

Background: Organophosphate (OP) insecticides represent some of the most common environmental contaminants in the United States. Organophosphate insecticide use has been associated with numerous adverse health outcomes, including hypertension (HTN) and metabolic syndrome (MetS), but results from current studies are conflicting and inconclusive.

Methods: In a study of 916 U.S. adults from the 2015-2016 NHANES cycle, we investigated the association between five dialkyl phosphate (DAP) metabolites of OP insecticides and blood pressure parameters (systolic blood pressure, diastolic blood pressure, pulse pressure, mean arterial pressure, HTN), as well the association between total body burden of DAPs with HTN and MetS.

Results: Weighted, multivariable linear regression revealed significant, inverse associations between diethylphosphate and systolic blood pressure (β= -0.16 p=0.02), diethylthiophosphate and systolic blood pressure (β= -0.91 p=0.01), total DAP exposure and systolic blood pressure (β= -0.13, p=0.04), and between dimethylphosphate and diastolic blood pressure (β= -0.15, p=0.0075). No significant associations were observed between total DAP exposure and odds of HTN. We additionally modeled the odds of abnormally high pulse pressure given specific quartile of total DAP exposure. Results showed a significant association between diethylphosphate and odds of abnormal pulse pressure (OR=1.29, 95% CI[1.01,1.65]), and between total DAP exposure and odds of abnormal pulse pressure (OR=1.05, 95% CI[1.03,1.10]). Lastly, we found that adults in the 3rd quartile of OP metabolite exposure had a

3.61 increased odds of having MetS when compared to individuals in the 1st quartile (OR=3.61, 95% CI[1.32,9.85]).

Conclusions: Our findings support data from previous studies suggesting a role for OP insecticides in the pathogenesis of blood pressure dysregulation and MetS. Future studies are warranted to corroborate these findings, and to elucidate potential mechanisms explaining these associations.

Introduction

Insecticides represent a large group of environmentally ubiquitous chemicals, and their use has been crucial for sustaining the growth of communities around the world(122). Since the early 1900s, insecticides have played a key role in protecting crops from insects, as well as lowering the transmission rates of vector borne diseases(123, 264). Additionally, the introduction of insecticides resulted in a surge of economic growth and productivity in many countries, especially those with a large dependency on agriculture(265). Numerous classes of insecticides exist, such as the pyrethroids, carbamates, and organophosphates (OP)(266, 267). Of these, OP insecticides represent the largest class of insecticides in the U.S., and are produced on the order of over 100 million kilograms a year(124). In addition to their mass production, the biochemical properties of OP insecticides including lipophilicity and resistance to photodegradation have allowed them to persist in various environmental compartments for decades(127, 268, 269). As a result, humans are routinely exposed to OPs, primarily through eating foods containing pesticide residues(270).

Despite their utility, numerous studies have found insecticides, most notably OP insecticides, to be associated with adverse health outcomes(271-273). Chlorpyrifos, the most common OP, has been strongly linked to endocrine dysfunction, cognitive delay in children, neurodegenerative diseases, and cardiovascular abnormalities(176). The neurotoxicity of OP insecticides is well established, and occurs primarily through the actions of the oxon form (i.e. chlorpyrifos-oxon)(274). Chlorpyrifos is converted into chlorpyrifos-oxon by oxidative desulfuration in the liver, and the oxon form then binds to and inhibits acetylcholinesterase within the central and peripheral nervous system, as well as on red blood cells(275). With acetylcholinesterase inhibited, excess acetylcholine accumulates within the synaptic cleft of neurons, and leads to robust overactivation of cholinergic pathways. Peripherally, cholinergic pathways in the heart and vasculature help to control heart rate, vascular patency, and ultimately blood pressure(34, 276). In addition to peripheral pathways, acetylcholine is also involved with central regulation of blood pressure, through involvement of gene pathways within the hypothalamus(277, 278). It is hypothesized that through affecting the expression and activity of neurohormonal pathways, OP insecticides may contribute to the pathogenesis of blood pressure dysregulation and hypertension (HTN)(53).

Hypertension is a global public health burden, and it is estimated that nearly half of all U.S. adults are living with HTN(3, 279). Hypertension is estimated to cost the U.S. 130 billion dollars annually, and is the leading cause of morbidity and mortality associated with cardiovascular diseases(6, 11, 12). Clinically, HTN is defined as a blood pressure exceeding 130/85 mmHg on at least two separate office visits, and typically presents without symptoms(280). When symptoms are present, they usually indicate dangerously high blood

pressures, and can include dizziness, shortness of breath, and headaches. While strong risk factors for HTN have been discovered such as high-salt diets, obesity, smoking, and sedentary lifestyle, these factors alone do not explain the complete risk profile of HTN(281-283). Recently, the role of the environment and toxic exposures in HTN pathogenesis has come to light. Numerous studies have found associations between OP insecticides and blood pressure dysregulation, but the overall results have been conflicting and inconclusive(185, 189, 284-287).

In addition to HTN, metabolic syndrome (MetS) is a chronic condition characterized by a cluster of risk factors associated with cardiovascular disease and type 2 diabetes(78). The risk of developing MetS depends on synergy of both genetic and environmental factors(288, 289). The clinical criteria for a diagnosis of MetS requires a patient to have at least 3 of 5 risk factors: a waistline 40 inches or more for men and 35 inches or more for women; a blood pressure exceeding 135/85 mmHg or taken antihypertensive medications; a serum triglyceride level exceeding 150 mg/dL; a fasting serum glucose exceed 100 mg/dL or taking glucose-lowering medications; a high-density lipoprotein level lower than 40 mg/dL for men or lower than 50 mg/dL for women(78). Recent evidence has shown significant associations between environmental contaminants such as insecticides and risk of MetS, but studies representative of exposure levels within the general population are lacking(290, 291). We hypothesized that exposure to OP insecticides increases the risk of blood pressure dysregulation, HTN, and MetS. In this cohort of 916 U.S. adults from the 2015-2016 NHANES survey cycle, we expand on previous literature to investigate the association between general population OP exposure, HTN, and MetS.

Research Design/Methods

2.1 National Health and Nutrition Examination Survey (NHANES).

Data analyzed was collected from the NHANES 2015–2016 survey cycle (available from:https://wwwn.cdc.gov/Nchs/Nhanes/2013-2014/TST_H.htm). NHANES is a nationwide survey conducted annually for the purpose of collecting health and diet information from a representative, non-institutionalized U.S. population. NHANES is unique in that it combines interviews, physical examinations, and laboratory evaluations to obtain a large amount of quantitative and qualitative data. Information on NHANES survey methods are described further in detail elsewhere(239). Briefly, the survey examines about 5,000 persons each year from various counties across the country. The country is divided into a total of 30 primary sampling units (PSUs), of which 15 are visited each year. The complex survey design assigns a weight to each individual as a function of their probability of being randomly selected into the study and these weightings are taken into account when building our regression models. All participants provided a written informed consent in agreement with the Public Health Service Act prior to any data collection. Household questionnaires, telephone interviews, and examinations conducted by healthcare professionals and trained personnel were utilized to collect data.

2.2 Study Participants and Exclusion Criteria.

The 2015-2016 NHANES cycle collected data on 8,373 individuals. We restricted our analysis to adults aged 18 and older. We restricted our analysis to adults due to the fact that HTN and MetS in the pediatric population is a rare outcome, and would not provide a sufficient

sample size for robust analysis. Additionally, pediatric HTN and MetS is unlikely to be related to low level, chronic environmental exposures, but rather has been shown to be strongly linked with genetic conditions, and acute, high exposure levels of environmental contaminants(240, 241). From these remaining individuals, analysis was restricted to men and women with valid blood pressure readings, as well as complete information on demographic, anthropometric, questionnaire, and laboratory variables including DAP metabolites, BMI, serum glucose, serum triglycerides, serum high-density lipoprotein (HDL), waist circumference, creatinine concentrations, alcohol use, diabetes status, education level, hypercholesterolemia status, insurance coverage status, poverty index level, race, smoking status, MetS status, HTN status, and DAP metabolites resulting in a final analysis sample size of 916. When using the survey frequency weighting, these 916 individuals represent 170,550,000 U.S. adults.

2.3 Quantification of Dialkyl Phosphates.

Due to higher cost and technical difficulty in quantifying parent compounds (e.g. chlorpyrifos) in the plasma, the NHANES census collected data on readily available and easier to obtain urinary metabolite concentrations. Studies have shown that these metabolites serve as reliable proxies for parent compounds(292). Five dialkyl phosphates (DAPs) were quantified and extracted from the urine matrix of 916 participants using an automated solid phase extraction system. Selective separation of the analytes was achieved using high-performance liquid chromatography with a gradient elution program. Sensitive detection of the analytes was performed by a triple quadrupole mass spectrometer with a heated electrospray ionization source. Final analyte concentrations were dichotomized to either above the detection limit, a

value of 0.1 ng/mL, or below the detection limit. A further detailed description on laboratory procedures can be found elsewhere(242).

2.4 Defining Demographic and Clinical Variables.

Methods for questionnaire data collection are described in the NHANES procedures guide(243). Participants were classified according to highest level of education attainment, insurance coverage status, smoking status, alcohol use, diabetes status, cholesterol status, HTN status, pulse pressure status, poverty index status, and MetS status. Highest level of education attainment was based on responses by participants during the home interview. Insurance coverage status and smoking status were recorded as a yes or no response from the home interview. Alcohol use was defined as a yes for individuals who said they drink at least two or more alcoholic drinks a day on average. Diabetes status was defined as a fasting serum glucose greater than 126, having answered yes to taking diabetic medications, or being told by a physician they have diabetes. Cholesterol status was defined by whether a person was told he/she has high cholesterol by a physician, or if that person is currently taking hypercholesterolemia medications. Hypertension status was defined by at least four separate systolic and/or diastolic blood pressure readings greater than 130 mmHg and/or 85 mmHg respectively, having been told by a doctor one has HTN, or is currently taking HTN medications. The poverty index ratio is a measure of a household's income to the federal poverty level (FPL). This variable was included as a more sensitive measure of socioeconomic burden, which has been shown to drive adverse health outcomes like HTN. Pulse pressure is emerging as a more sensitive predictor of cardiovascular risk in adults compared to binary cutoffs defining HTN, especially among the elderly(31). The normal range of pulse pressure is 30-40 mmHg, and we

defined abnormal pulse pressure as a value exceeding 50 mmHg. A classification of metabolic syndrome was made if a person had at least three of five abnormal measurements of the following: waist circumference, serum triglyceride levels, serum high-density lipoprotein, blood pressure, blood sugar.

2.5 Statistical Analyses.

Continuous variables were compared using one-way ANOVA, while categorical variables were compared using the Chi-squared test and Fisher's Exact Test when the cell values were 0. Multivariable, ordinary least squares regression models were used to measure the association between the urinary concentrations of OP metabolites and blood pressure parameters. Potential confounders including race, age, creatinine levels, diabetes status, education level, poverty index, smoking status, and hypercholesterolemia based on results from literature searches (figure 1). We then empirically tested these variables potential for confounding. We calculated Spearman correlations and conducted chi-square analyses to quantify continuous and categorical variables' relationship with blood pressure and metabolism outcomes. Through *a priori* literature evidence we believed age, race, gender, and BMI were associated with the outcomes. We evaluated correlations between each potential confounder with the exposure and the outcomes. Potential confounders were iteratively added to the models and the percent change in the crude estimates for the outcome variable were compared. When the addition of a variable that is correlated with both DAP exposure and blood pressure increased the crude estimate by 10% or more, that variable was considered a confounder in the final model. The final models included age, race, and BMI as confounders, and we adjusted for creatinine to account for urinary concentration which can affect metabolite concentrations. The five DAPs

metabolites were summed to represent the total body burden of OP exposure, since there is no evidence suggesting direct effects of DAPs on blood pressure and metabolic function. The total sum of OP metabolites was divided into quartile thresholds for logistic regression analysis, to evaluate the association between quartile levels of each metabolite and the odds of HTN and MetS. The lowest quartile was used as the reference in each case, and these results are presented as supplementary information.

All statistical analyses were performed using SAS 9.4 and SUDAAN software packages accounting for the complex survey design of NHANES(244). A p-value < 0.05 was used as the criterion for significance.

Figure 7.

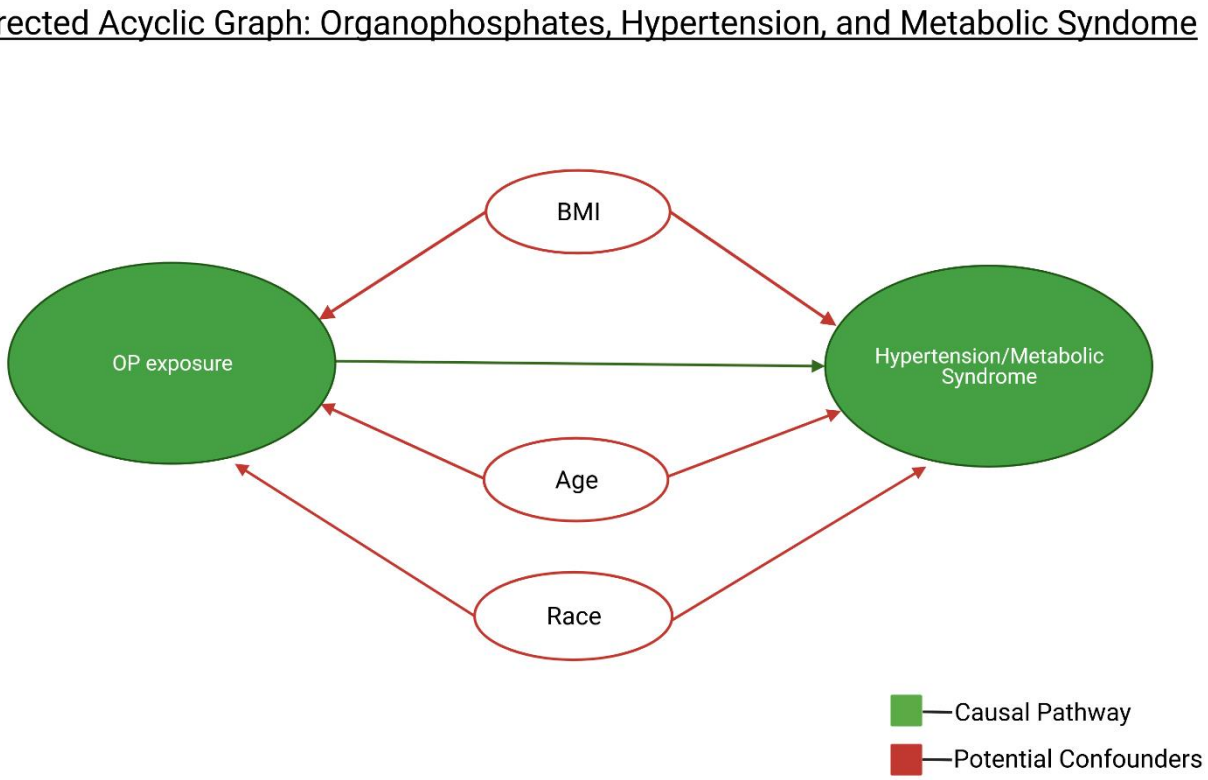


Figure 7: Directed acyclic graph depicted the proposed relationship between OP exposure and HTN/ MetS. Age, BMI, and race were included in the final models as potential confounders.

Results

Table 1 shows the demographic breakdown of our cohort. Table 2 shows the demographic breakdown of our cohort by quartile of DAP metabolite concentration. Tables 3 shows demographic and laboratory data breakdown by number of MetS components. Our study population had a mean age of 48.9 (S.E.M. ± 0.98). There were slightly more women than men

in this cohort (53.82 % women vs. 46.18% men), and approximately 58% of adults obtained some college degree or above. The geometric mean of DAP metabolites was calculated and are reported in tables 1 and 3. When stratified by quartile of insecticide exposure, we noted significant differences in education status between quartiles, mean diastolic blood pressure, and urinary creatinine concentrations. Stratification by number of MetS components revealed significant differences among quartiles of DAP exposure for age, BMI, hypercholesterolemia, HTN, abnormal pulse pressure, means systolic blood pressure, and mean diastolic blood pressure.

Multivariable linear regression while adjusting for potential confounders revealed significant associations between diethylphosphate, diethylthiophosphate, and sum of DAPs and systolic blood pressure (β= -0.16 p=0.02, β= -0.91 p=0.01, and β= -0.13 p=0.04 respectively), and between dimethylphosphate and diastolic blood pressure (β= -0.15 p=0.0075).

Logistic regression to model the odds of HTN given a quartile of total metabolite exposure revealed no significant trends (supplementary table 1). We found significant associations between diethylphosphate and continuous total body burden of DAPs on the odds of abnormal pulse pressure (OR=1.29, 95% CI[1.01,1.65], OR= 1.05, 95% CI[1.03,1.10] respectively). We also observed non-significant, positive trends between quartile of total metabolite exposure and the odds of abnormal pulse pressure (supplementary table 2). When modeling the odds of MetS status for a quartile of OP metabolite, we found a significant increase in the odds of MetS for individuals in the 3rd quartile of exposure (OR= 3.61, 95% CI[1.32, 9.85]) (supplementary table 3). We additionally model the odds of each MetS component for a given quartile of DAP exposure (supplementary tables 4-7). We observed significant associations between the third

quartile of DAP exposure and odds of high triglycerides (OR= 4.52, 95% CI[1.25, 16.31]) and low HDL (OR= 5.95, 95% CI= [1.16, 30.50]).

Our regression models also included interaction terms for age, race, and BMI to examine the relationship between OP exposure and blood pressure and MetS status. Studies have shown that the effects of environmental exposures like insecticides on health outcomes may depend on varying levels of these cofactors. We observed a significant interaction between race and dimethylthiophosphate on systolic blood pressure (β= -1.75 p=0.003), and between dimethylphosphate and race on diastolic blood pressure (β= 0.92 p=0.02). In both cases, individuals who self-identified as Mexican-American were found to have significant interactions with DAPs compared to non-Hispanic white citizens. Additionally, we observed a significant interaction between quartile of total DAP exposure and BMI on MetS, where individuals in the 3rd quartile of exposure and a BMI greater than 30 had 9.54 increased odds of MetS compared to those in the 1st quartile (OR= 9.54, 95% CI= [1.38, 27.2]).

Table 9. Table showing the sociodemographic and laboratory data for the total cohort

		Total Cohort
		N= 916
Age, Group Class (%)	*18-39*	315 (34.39)
	40-59	323 (35.26)
	60+	278 (30.35)
Mean Age (SEM)		48.6 (0.57)
Gender (%)	*Male*	423 (46.18)
	Female	493 (53.82)
Race/Ethnicity (%)	*Non-Hispanic White*	302 (32.97)
	Non-Hispanic Black	207 (22.60)
	Mexican-American	149 (16.27)
	Other	258 (28.17)
BMI Categories (%)	*18.5-25*	242 (26.42)
	25-30	275 (30.02)
	>30	399 (43.56)
Mean BMI (SEM)		29.9 (0.2)
Education (%)	*Less than 9th Grade*	82 (8.95)
	9th-11th Grade	111 (12.12)
	Highschool Graduate	189 (20.63)
	Some College or AA Degree	311 (33.95)
	College Graduate or Above	222 (24.24)
	Refused	1 (0.11)
Smoking Status (%)	*Never Smoker*	515 (56.22)
	Past Smoker	208 (22.71)
	Active Smoker	193 (21.07)
Alcohol Use (%)	*Non-Drinker*	711 (77.62)
	Drinker	205 (22.38)
Hypertension (%)	*Normotensive*	567 (61.90)
	Hypertensive	349 (38.10)
Diabetes (%)	*Non-Diabetic*	796 (86.90)
	Diabetic	120 (13.10)
Hypercholesterolemia (%)	*Normal Cholesterol*	668 (72.93)
	High Cholesterol	248 (27.07)
Metabolic Syndrome (%)	*Normal*	612 (66.81)
	Metabolic Syndrome	304 (33.19)
Abnormal Pulse Pressure (%)	*Normal Pulse Pressure*	638 (69.65)
	High Pulse Pressure	278 (30.35)
Insurance Status (%)	*Covered*	762 (83.19)
	Not Covered	154 (16.81)
Poverty Index (%)	*<1*	259 (28.28)
	1-2	215 (23.47)
	2-4	226 (24.67)
	>4	216 (23.58)
Mean Systolic BP (mmHg) (SEM)		125.5 (0.6)
Mean Diastolic BP (mmHg) (SEM)		70.5 (0.4)
Mean Urinary Dimethylphosphate (ng/mL) (SEM)		3.16 (0.2)
Mean Urinary Diethylphosphate (ng/mL) (SEM)		4.18 (0.25)
Mean Urinary Dimethylthiophosphate (ng/mL) (SEM)		2.6 (0.21)
Mean Urinary Diethylthiophosphate (ng/mL) (SEM)		1.78 (0.06)
Mean Urinary Dimethyldithiophosphate (ng/mL) (SEM)		0.4 (0.04)
Mean Creatinine (mg/dL) (SEM)		131.5 (2.8)

Table 10. Table showing the sociodemographic and laboratory data stratified by quartile of OP metabolite exposure

Quartiles of Total DAP		Q1	Q2	Q3	Q4	P-value
		N= 242	N=208	N= 249	N= 215	
Age, group class (%)	*18-39*	83 (34.30)	75 (36.06)	93 (37.35)	62 (28.84)	0.8
	40-59	86 (35.54)	78 (37.50)	86 (34.54)	73 (33.95)	
	60+	73 (30.17)	55 (26.44)	70 (28.11)	80 (37.21)	
Mean Age (SEM)		41.2 (1.05)	47.8 (1.2)	47.3 (1.1)	51.5 (1.3)	0.48
Gender (%)	*Male*	119 (49.17)	89 (42.79)	117 (46.99)	97 (45.12)	0.38
	Female	123 (50.83)	119 (57.21)	132 (53.01)	118 (54.88)	
Race/Ethnicity (%)	*Non-Hispanic White*	83 (34.30)	77 (37.02)	76 (30.52)	66 (30.70)	0.97
	Non-Hispanic Black	55 (22.73)	43 (20.67)	58 (23.29)	49 (22.79)	
	Mexican American	34 (14.05)	43 (20.67)	38 (15.26)	34 (15.81)	
	Other	70 (28.93)	45 (21.63)	77 (30.92)	66 (30.70)	
BMI categories (%)	*18.5-25*	68 (28.10)	57 (27.40)	60 (24.10)	56 (26.05)	0.87
	25-30	79 (32.64)	60 (28.85)	74 (29.72)	62 (28.84)	
	>30	95 (39.26)	91 (43.75)	115 (46.18)	97 (45.12)	
Mean BMI (SEM)		29.3 (0.4)	30.0 (0.5)	30.1 (0.4)	30.3 (0.5)	0.78
Education (%)	*Less than 9th grade*	20 (8.26)	17 (8.17)	15 (6.02)	30 (13.95)	**0.04**
	9th-11th grade	32 (13.22)	24 (11.54)	27 (10.84)	28 (13.02)	
	Highschool Graduate	54 (22.31)	44 (21.15)	51 (20.48)	39 (18.14)	
	Some College or AA degree	78 (32.23)	77 (37.02)	92 (36.95)	63 (29.30)	
	College Graduate or Above	58 (23.97)	46 (22.12)	64 (25.70)	54 (25.12)	
	Refused	0	0	0	1 (0.47)	
Smoking status (%)	*Never smoker*	141 (58.26)	113 (54.33)	138 (55.42)	121 (56.28)	0.05
	Past smoker	50 (20.66)	46 (22.12)	58 (23.29)	54 (25.12)	
	Active smoker	51 (21.07)	49 (23.56)	53 (21.29)	40 (18.60)	
Alcohol Use (%)	*Non Drinker*	173 (71.49)	160 (76.92)	206 (82.73)	170 (79.07)	0.75
	Drinker	69 (28.51)	48 (23.08)	43 (17.27)	45 (20.93)	
Diabetes (%)	*Non Diabetic*	206 (85.12)	186 (89.42)	215 (86.35)	187 (86.98)	0.96
	Diabetic	36 (14.88)	22 (10.58)	34 (13.65)	28 (13.02)	
Hypercholesterolemia (%)	*Normal Cholesterol*	170 (70.25)	153 (73.56)	185 (74.30)	158 (73.49)	0.63
	High Cholesterol	72 (29.75)	55 (26.44)	64 (25.70)	57 (26.51)	
Hypertension (%)	*Normotensive*	140 (57.85)	124 (59.62)	156 (62.65)	146 (67.91)	0.66
	Hypertensive	102 (42.15)	84 (40.38)	93 (37.35)	69 (32.09)	
Metabolic Syndrome (%)	*Normal*	166 (68.60)	131 (62.98)	167 (67.07)	147 (68.37)	0.97
	Metabolic Syndrome	76 (31.40)	77 (37.02)	82 (32.93)	68 (31.63)	
Abnormal Pulse Pressure (%)	*Normal Pulse Pressure*	173 (71.49)	143 (68.75)	172 (69.08)	149 (69.30)	0.48
	High Pulse Pressure	69 (28.51)	65 (31.25)	77 (30.92)	66 (30.70)	
Insurance Status (%)	*Covered*	196 (80.99)	173 (83.17)	204 (81.93)	188 (87.44)	0.68
	Not Covered	46 (19.01)	35 (16.83)	45 (18.07)	27 (12.56)	
Poverty Index (%)	*<1*	69 (28.51)	62 (29.81)	65 (26.10)	61 (28.37)	0.86
	1-2	54 (22.31)	53 (25.48)	57 (22.89)	51 (23.72)	
	2-4	63 (26.03)	48 (23.08)	58 (23.29)	57 (26.51)	
	>4	56 (23.14)	45 (21.63)	69 (27.71)	46 (21.40)	
Mean Systolic BP (mmHg) (SEM)		126.4 (1.4)	125.7 (1.2)	125.2 (1.2)	124.3 (1.2)	0.19
Mean Diastolic BP (mmHg) (SEM)		72.0 (0.70)	70.3 (0.8)	70.5 (0.75)	68.9 (0.7)	**0.01**
Mean Creatinine (mg/dL) (SEM)		74.7 (3.5)	124.8 (1.9)	152.1 (5.1)	176 (6.2)	**<0.001**

Table 11. Table showing the sociodemographic and laboratory data stratified by number of metabolic syndrome components. Persons in group 0 have 0 features, group 1 have at least one feature, group 2 have at least 2 features etc.

Number of Metabolic Syndrome Features		0	1	2	3	4	5	P-value
		N= 137	N= 224	N= 251	N= 160	N= 113	N=31	
Age, group class (%)	*18-39*	89 (64.96)	89 (39.73)	76 (30.28)	39 (24.38)	19 (16.81)	3 (9.68)	**0.0023**
	40-59	33 (24.09)	66 (29.46)	99 (39.44)	62 (38.75)	49 (43.36)	14 (45.16)	
	60+	15 (10.95)	69 (30.80)	76 (30.28)	59 (36.88)	45 (39.82)	14 (45.16)	
Mean Age (SEM)		36.8 (1.2)	47.6 (1.3)	49.9 (1.0)	52.4 (1.2)	54.4 (1.4)	56.7 (2.6)	**<0.0001**
Gender (%)	*Male*	63 (45.99)	99 (44.20)	121 (48.21)	72 (45.00)	54 (47.79)	14 (45.16)	0.28
	Female	74 (54.01)	125 (55.80)	130 (51.79)	88 (55.00)	59 (52.21)	17 (54.84)	
Race/Ethnicity (%)	*Non-Hispanic White*	53 (38.69)	79 (35.27)	83 (33.07)	46 (28.75)	33 (29.20)	8 (25.81)	0.24
	Non-Hispanic Black	32 (23.36)	66 (29.46)	55 (21.91)	30 (18.75)	19 (16.81)	5 (16.13)	
	Mexican American	14 (10.22)	25 (11.16)	35 (13.94)	41 (25.63)	27 (23.89)	7 (22.58)	
	Other	38 (27.74)	54 (24.11)	78 (31.08)	43 (26.88)	34 (30.09)	11 (35.48)	
BMI categories (%)	*18.5-25*	102 (74.45)	73 (32.59)	48 (19.12)	13 (8.13)	5 (4.42)	1 (3.23)	**<0.001**
	25-30	30 (21.90)	74 (33.04)	86 (34.26)	52 (32.50)	28 (24.78)	5 (16.13)	
	>30	5 (3.65)	77 (34.38)	117 (46.61)	95 (59.38)	80 (70.80)	25 (80.65)	
Mean BMI (SEM)		23.4 (0.3)	28.5 (0.4)	30.6 (0.4)	33.2 (0.6)	33.5 (0.6)	34.2 (0.9)	**<0.001**
Education (%)	*Less than 9th grade*	4 (2.92)	12 (5.36)	21 (8.37)	22 (13.75)	19 (16.81)	4 (12.90)	0.07
	9th-11th grade	11 (8.03)	28 (12.50)	34 (13.55)	22 (13.75)	12 (10.62)	4 (12.90)	
	Highschool Graduate	24 (17.52)	49 (21.88)	60 (23.90)	28 (17.50)	21 (18.58)	7 (22.58)	
	Some College or AA degree	46 (33.58)	76 (33.93)	82 (32.67)	57 (35.63)	41 (36.28)	9 (29.03)	
	College Graduate or Above	52 (37.96)	59 (26.34)	54 (21.51)	30 (18.75)	20 (17.70)	7 (22.58)	
	Refused	0	0	0	1 (0.63)	0	0	
Smoking status (%)	*Never smoker*	77 (56.20)	123 (54.91)	143 (56.97)	90 (56.25)	69 (61.06)	13 (41.94)	0.37
	Past smoker	28 (20.44)	59 (26.34)	52 (20.72)	34 (21.25)	24 (21.24)	11 (35.48)	
	Active smoker	32 (23.36)	42 (18.75)	56 (22.31)	36 (22.50)	20 (17.70)	7 (22.58)	
Alcohol Use (%)	*Non Drinker*	107 (78.10)	179 (79.91)	183 (72.91)	128 (80.00)	88 (77.88)	26 (83.87)	0.32
	Drinker	30 (21.90)	45 (20.09)	68 (27.09)	32 (20.00)	25 (22.12)	5 (16.13)	
Diabetes (%)	*Non Diabetic*	117 (85.40)	187 (83.48)	226 (90.04)	142 (88.75)	98 (86.73)	26 (83.87)	0.31
	Diabetic	20 (14.60)	37 (16.52)	25 (9.96)	18 (11.25)	15 (13.27)	5 (16.13)	
Hypercholesterolemia (%)	*Normal Cholesterol*	126 (91.97)	173 (77.23)	181 (72.11)	109 (68.13)	66 (58.41)	13 (41.94)	**0.01**
	High Cholesterol	11 (8.03)	51 (22.77)	70 (27.89)	51 (31.88)	47 (41.59)	18 (58.06)	
Hypertension (%)	*Normotensive*	137 (100.00)	176 (78.57)	152 (60.56)	66 (41.25)	36 (31.86)	0	**<0.001**
	Hypertensive	0	48 (21.43)	99 (39.44)	94 (58.75)	77 (68.14)	31 (100.00)	
Abnormal Pulse Pressure (%)	*Normal Pulse Pressure*	127 (92.70)	163 (72.77)	176 (70.12)	102 (63.75)	57 (50.44)	13 (41.94)	**<0.001**
	High Pulse Pressure	10 (7.30)	61 (27.23)	75 (29.88)	58 (36.25)	56 (49.56)	18 (58.06)	
Insurance Status (%)	*Covered*	191 (85.27)	205 (81.67)	129 (80.63)	94 (83.19)	29 (93.55)	2 (6.45)	0.84
	Not Covered	33 (14.73)	46 (18.33)	31 (19.38)	19 (16.81)	2 (6.45)	29 (93.55)	
Poverty Index (%)	*<1*	34 (24.82)	59 (26.34)	74 (29.48)	51 (31.88)	35 (30.97)	6 (19.35)	0.87
	1-2	27 (19.71)	47 (20.98)	61 (24.30)	46 (28.75)	27 (23.89)	7 (22.58)	
	2-4	38 (27.74)	56 (25.00)	61 (24.30)	31 (19.38)	33 (29.20)	7 (22.58)	
	>4	38 (27.74)	62 (27.68)	55 (21.91)	32 (20.00)	18 (15.93)	11 (35.48)	
Mean Systolic BP (mmHg) (SEM)		112 (0.8)	120.4 (1.0)	126.1 (1.1)	131.3 (1.5)	136.2 (1.6)	143.3 (2.5)	**<0.001**
Mean Diastolic BP (mmHg) (SEM)		66.2 (0.7)	67.6 (0.7)	72.1 (0.7)	73.5 (0.9)	72.5 (1.1)	75 (2.3)	**0.01**
Mean Total DAP Concentration (ng/mL) (SEM)		10.4 (1.2)	12.8 (1.4)	10.3 (0.8)	11.4 (1.4)	10.1 (1.2)	8.9 (1.9)	0.54
Mean Creatinine (mg/dL) (SEM)		138.2 (7.9)	136.9 (6.1)	128 (5.1)	127.4 (6.3)	131.7 (7.8)	112.3 (10.8)	0.05

Discussion

The etiology of blood pressure and metabolic dysregulation remains to be fully elucidated, and emerging evidence suggests a role for environmental exposures in the pathogenesis of chronic diseases such as HTN and MetS (193, 287, 293). In this study, we expanded on the current literature by investigating the association between OP insecticides and these adverse health outcomes within the U.S. general population. We observed significant associations between exposure to OP insecticides and blood pressure parameters, as well as between OP insecticides and MetS. The predominating effect was found to be inverse associations between DAPs and blood pressure, but also positive associations with abnormal pulse pressure. Previous studies have found positive associations between OP exposure and blood pressure, and while the direction of the association with blood pressure was the opposite of what we expected, the association may speak to general deviations from normal blood pressure normal ranges and blood pressure dysregulation. Ideally, exogenous toxicants such as OPs shouldn't have any effect on blood pressure homeostasis. We also observed that individuals in the 3rd quartile of total body burden of DAPs had a significantly higher odds of MetS compared to individuals in the 1st quartile.

Significant interactions were found between DAP exposure, BMI, and race/ethnicity on blood pressure. A possible explanation for the BMI interaction is that OP insecticides have been demonstrated to increase activity of pathways related to fat accumulation and dyshormogenesis *in vivo*(294, 295). An increase in fat and BMI can lead to an increase in total body burden of insecticides, since they are able to sequester into the biological fat compartment (127). As the total body burden of insecticides increases, not only is the

activation of pathways regulating energy storage, metabolism, and blood pressure increased, but the potential direct effect of OPs on these pathways may also increase(296, 297). In the context of race, it has been shown that certain ethnic groups are more or less likely to live in areas containing higher concentrations of insecticides (e.g. rural/agricultural areas and urban areas in close proximity to manufacturing plants)(298, 299). Additionally, barriers to healthcare within these communities can further increase their susceptibility to insecticide exposure and the severity of disease outcomes(300). Examining interactions between environmental, biological, and sociodemographic factors can help improve both our understanding of the mechanisms driving these outcomes, as well as the variations in health effects of insecticides seen between individuals and between populations. These findings support efforts to identify at-risk populations who may experience higher adverse effects of insecticide exposure as it relates to blood pressure and metabolism. Furthermore, by addressing these biological and social factors we may be able to significantly lower the incidence and prevalence of HTN and metabolic syndrome related to insecticide exposure.

There are several potential mechanisms that may explain the effects of OP insecticides on blood pressure, particularly through cholinergic activation by acetylcholine. As mentioned previously, the primary mechanism of OP insecticides is inhibition of acetylcholinesterase, which leads to increased amounts of acetylcholine both centrally and peripherally. Within the heart, there are various muscarinic receptors that regulate cardiovascular function. Specifically, M2 receptor activation within the heart reduces the force of atrial contractions and slows the heart rate(301). Organophosphates may also modify the activity of nicotinic acetylcholine receptors, which are known to increase vasoconstriction(302). Additionally, within the

hypothalamus acetylcholine plays a key role in activating pre-sympathetic pathways, which ultimately function to increase sympathetic activity centrally and peripherally(53, 277). Muscarinic receptors within the hypothalamus using acetylcholine as a ligand are mediators of these pre-sympathetic pathways. Furthermore, chlorpyrifos-oxon has been demonstrated *in vitro* and *in vivo* to bind directly to muscarinic receptors within the hypothalamus, and potentially modulating receptor activity. Thus, these possibilities may represent several ways in which Chlorpyrifos affects blood pressure regulation in the general population.

The direction of the effect of OP insecticides on blood pressure outcomes may largely depend on the predominating location of cholinergic and non-cholinergic effects (central vs. peripheral), as well as the dose and duration of the insecticide. The understanding of dose-response relationships in the context of environmental exposures has been important for estimating the various effects of chemicals on biological pathways and health outcomes. For example, the vasculature contains a wide variety of muscarinic and nicotinic receptors(303-305). Activation of nicotinic receptors by acetylcholine largely results in fasciculations and blood vessel constriction, though some studies have demonstrated their ability to induced vasodilation in hypertensive animals via acetylcholine-dependent mechanisms(306). Activation of the M2 receptor subtypes within the vasculature generally leads to vasodilation(276). Additionally, acetylcholine activates endothelial nitric oxide synthase and prostaglandin production, the production of both ultimately resulting in vasodilation(307). Future studies that test the specific effects of chlorpyrifos exposure within target organ systems at varying doses are warranted to test these hypotheses.

In addition to cholinergic toxicity, chlorpyrifos and chlorpyrifos-oxon have non-cholinergic effects which may lead to changes in blood pressure and metabolism. It has been shown that total body burden of insecticides is associated with hormonal dysregulation and metabolic dysregulation, at concentrations of chlorpyrifos that do not cause cholinergic toxicity. These concentrations may be more relevant for individuals exposed in the general population. Additionally, chlorpyrifos has been demonstrated to induce morphogenic changes within hypothalamic neurons and affect levels of neurotransmitters involved in blood pressure regulation(196). Many of the pathways that regulate energy metabolism and storage (e.g. glucose, lipids, proteins) converge in the hypothalamus(308). Thus, it is possible that the effects that chlorpyrifos has on these hypothalamic pathways may in turn affect blood pressure and broader metabolic processes.

Strengths

The current study has several strengths. To our knowledge, this is one of the first studies to evaluate the association between OP insecticides, blood pressure, and MetS within the general U.S. adult population. Furthermore, the estimates for OP exposure reflect everyday exposure levels through common pathways such as the diet, soil, and performing general daily activities. Traditionally, most OP insecticide studies have used cohorts that are occupationally exposed at high concentrations. While these studies are important, their relevance to the general population is limited. Another strength lies in the oversampling methods of NHANES. By oversampling minority groups, the study recruited sufficient sizes of African American and Mexican American citizens, who traditionally have been difficult to include in studies in large numbers. This allowed us to not only measure the main effects of OP insecticides within these

groups, but also to examine potential interactions of biology, race, and sociodemographic factors on the relationship of OPs, blood pressure, and metabolism.

Limitations

There are important limitations worth mentioning. Since we are using DAPs as a proxy for OP insecticide exposure, we are not able to measure the direct effect between parent compounds such as chlorpyrifos and blood pressure. Additionally, due the cross-sectional nature of the study, we are unable to draw any casual relationships between OP exposure, HTN, and MetS. Lastly, we are unable to measure dose-response relationships between OP insecticides and blood pressure. It is likely that for a give dose and duration of exposure, we could observe differential effects on blood pressure and risk of metabolic syndrome. This could be due to changes in the expression of metabolism-regulating gene pathways, which have been demonstrated *in vitro* to display dose dependency in response to drugs and environmental chemicals. Future studies that include a prospective cohort design would be warranted to evaluate causal effects, as well as *in vitro* and *in vivo* studies that include varying doses of OP exposure.

Conclusion

We found significant associations between exposure to OP insecticides, blood pressure, and metabolic dysregulation. These findings support efforts to improve our understanding of how environmental exposures are related to chronic diseases, and also efforts to consider safer alternatives to OP insecticides and reduce population-level exposure. Future studies are

warranted to corroborate these findings, and to elucidate potential biological mechanisms underlying these associations.

Supplementary Tables

Table 12. Logistic regression results modeling the odds of hypertension for a given quartile of OP metabolite

Variables		Hypertension	
		OR (CI95%)	*p*
Sum of total DAPs	1st Quartile	Ref.	Ref.
	2nd Quartile	1.11 (0.42,2.92)	0.83
	3rd Quartile	0.84 (0.40, 1.78)	0.63
	4th Quartile	1.71 (0.66, 4.46)	0.25

Table 13. Logistic regression results modeling the odds of abnormal pulse pressure for a given quartile of OP metabolite

Variables		Abnormal Pulse Pressure	
		OR (CI95%)	*p*
Sum of total DAPs	1st Quartile	Ref.	Ref.
	2nd Quartile	1.89 (0.70, 5.11)	0.19
	3rd Quartile	1.92 (0.50, 7.37)	0.32
	4th Quartile	1.93 (0.55, 6.37)	0.29

Table 14. Logistic regression results modeling the odds of metabolic syndrome for a given quartile of OP metabolite

Variables		Metabolic Syndrome	
		OR (CI95%)	*p*
Sum of total DAPs	1st Quartile	Ref.	Ref.
	2nd Quartile	1.15 (0.18, 7.39)	0.88
	3rd Quartile	3.61 (1.32, 9.85)	**0.01**
	4th Quartile	1.45 (0.46, 4.51)	0.51

Table 15. Logistic regression results modeling the odds of high serum glucose for a given quartile of OP metabolite

Variables		High Glucose	
		OR (CI95%)	*p*
Sum of total DAPs	1st Quartile	Ref.	Ref.
	2nd Quartile	2.44 (0.29, 20.40)	0.39
	3rd Quartile	2.35 (0.57, 9.65)	0.22
	4th Quartile	2.79 (0.67,11.65)	0.15

Table 16. Logistic regression results modeling the odds of abnormal waist size for a given quartile of OP metabolite

Variables		Abnormal Waist Size	
		OR (CI95%)	*p*
Sum of total DAPs	1st Quartile	Ref.	Ref.
	2nd Quartile	0.76 (0.08,7.44)	0.80
	3rd Quartile	3.49 (0.49, 24.99)	0.20
	4th Quartile	1.30 (0.18, 10.98)	0.74

Table 17. Logistic regression results modeling the odds of high serum triglycerides for a given quartile of OP metabolite

Variables		High Triglycerides	
		OR (CI95%)	*p*
Sum of total DAPs	1st Quartile	Ref.	Ref.
	2nd Quartile	1.10 (0.37,2.39)	0.85
	3rd Quartile	4.52 (1.25,16.31)	**0.02**
	4th Quartile	2.25 (0.79, 6.40)	0.12

Table 18. Logistic regression results modeling the odds of abnormally low HDL for a given quartile of OP metabolite

Variables		Low HDL	
		OR (CI95%)	*p*
Sum of total DAPs	1st Quartile	Ref.	Ref.
	2nd Quartile	2.44 (0.61, 9.86)	0.19
	3rd Quartile	5.95 (1.16, 30.50)	**0.03**
	4th Quartile	1.35 (0.21, 8.59)	0.74

Chapter 4: Chlorpyrifos Influences the Expression of Hypertension and Metabolic Syndrome-Related Pathway Targets in a Hypothalamic Cell Line

Abstract

Background: Hypertension (HTN) and metabolic syndrome (MetS) are chronic conditions that significantly increase the global morbidity and mortality associated with cardiovascular disease. While strong risk factors including aging, sedentary lifestyle, high-salt diet, and smoking have been identified, the complete etiology of HTN and MetS remains to be elucidated. In recent years, growing attention has been placed on the contribution of environmental exposures such as organophosphate (OP) insecticides to the pathogenesis of HTN and MetS. Organophosphate insecticides are environmentally ubiquitous, neurotoxic chemicals that have been strongly linked to disorders of the central nervous system, including autism, cognitive delay, and Alzheimer's. In addition to these disorders, recent studies have demonstrated associations between OP exposure and risk of HTN and MetS, but the mechanisms underlying these associations are unknown. *In vitro* and *in vivo* data have clearly demonstrated OP insecticides accumulate in high concentrations within the hypothalamus, which acts as the central regulator of energy and metabolism in the body. Thus, it is possible that the population-level associations seen between OP exposure, HTN, and MetS are driven by actions of OPs within the hypothalamus, at concentrations below the threshold to induce cholinergic toxicity. Our group hypothesized that OP exposure dysregulates the expression of hypothalamic blood pressure and metabolism-regulating pathway targets, actions that may contribute to the incidence and prevalence HTN and MetS.

Methods: Using a primary culture cell line of murine hypothalamic cells, we exposed neurons to a range of environmentally relevant concentrations of chlorpyrifos and chlorpyrifos-

oxon ($1.0x10^{-9}$-$1.0x10^{-3}$ M), and quantified their effects on mRNA and protein expression of targets within the hypothalamus.

Results: Results from our immunoblotting and RT-qPCR revealed significant dose and time dependent effects on the expression of hypothalamic proteins and genes that regulate blood pressure and metabolism pathways. Lastly, we identified biologically relevant points of departure of mRNA and protein expression using benchmark dose response (BMD) models. We were able to successfully calculate the benchmark dose and benchmark lower limit values, which ultimately are used to calculate daily reference doses.

Conclusion: Our findings provide relevant and unique contributions to the toxicological profile of OP insecticides beyond their notorious neurocognitive effects, and support previous data suggesting OP contribution to the pathogenesis of HTN and MetS. The central nervous system (particularly the hypothalamus) may provide an important target for restoration of blood pressure and energy homeostasis in the context of OP-related HTN and MetS. Future studies are warranted to corroborate these findings, test these effects in whole organism models, and assess more components of the pathways we proposed to fully elucidate the mechanism of OP-related HTN and MetS.

Introduction

Hypertension (HTN) and metabolic syndrome (MetS) are common chronic conditions that significantly increase the morbidity and mortality associated with cardiovascular diseases(10, 71). Globally, HTN and MetS account for 10-20% of deaths annually(6). In the U.S., the prevalence of HTN and MetS among adults ranges between 30-40%, and in special subpopulations such as minorities and the elderly, these estimates can exceed 50%(9, 74, 282, 309). In addition to having detrimental effects on personal health, diagnoses of HTN and MetS pose significant economic burdens as they significantly increase individual and national health care costs(11, 72). Furthermore, HTN and MetS have been shown to significantly lower ones overall quality of life(310). There are several strong identified risk factors for HTN and MetS, including sedentary lifestyle, high-salt diets, obesity, alcohol, and smoking(117, 208, 311). However, the knowledge of these risk factors alone doesn't fully explain the etiology of HTN and MetS, and this incomplete knowledge may contribute to the continued increases in incidence and prevalence of these conditions, as well as difficulties in therapeutic management. In recent decades, the contribution of the environment and chemical exposures has come to light in the context of HTN and MetS pathogenesis(214, 290). Most notable environmental toxicants including heavy metals (e.g. lead, arsenic, copper), polychlorinated biphenyls (PCBs), and insecticides have been implicated in the etiology of HTN and MetS, but data from these studies are inconclusive, and biological mechanisms underlying these associations remain to be fully elucidated. Of these environmental contaminants, insecticides are the most common in terms of their frequency, distribution, and concentrations within the environment(124).

Insecticides are a group of pesticides comprised of various classes of chemicals, designed to improve crop yield, reduce the transmission of vector-borne diseases, and control pests in residential and agricultural settings(122). Of the insecticide classes, organophosphates (OP) are the most used in the U.S., and have been in use since the 1900s(312). Many of their biochemical properties such as lipophilicity and resistance to photodegradation confer the ability to not only accumulate within compartments such as the soil, water, and tissues of organisms, but also to be transported long distances to unintended sites(133, 269). The primary mechanism of action of OPs is acetylcholinesterase inhibition, which is the enzyme responsible for breaking down a potent neurotransmitter acetylcholine(177). With acetylcholinesterase inhibited, robust activation of acetyl-choline dependent ("cholinergic") pathways ensues both in the central nervous system, as well as peripherally at the neuromuscular junctions and organ systems. While older OP insecticides have been phased out since the early 2000s due to strong evidence for nervous system damage, chlorpyrifos is still registered for use in dozens of countries(150, 313). Chlorpyrifos is the most used OP insecticide, and one of the most used insecticides globally. As of February 2022, phaseouts for chlorpyrifos use have begun in the U.S., where restrictions have been placed on its use in both residential and agricultural sectors(139). Despite these sanctions, chlorpyrifos remains a popular insecticide in dozens of countries worldwide, and through continued manufacturing for use on crops and insect control, global exposure levels to OPs will still be relevant through importation/exportation of goods, biomagnification within food chains, and pesticide drift(128).

There are several organ systems that play a crucial role in the homeostasis of blood pressure and metabolism(94, 172). Most commonly, the cardiovascular system and renal

systems dynamically regulate basal blood pressure, while the liver, GI tract, and adipose tissue are important regulators of glucose, lipids, and energy storage and expenditure(32, 36, 39, 51, 83). In addition to control at the peripheral level, top-down control of blood pressure and metabolism occurs through actions of the central nervous system(95, 314). In particular, pathways from peripheral organs converge within the hypothalamus, an organ that exerts central control over blood pressure and metabolism via the autonomic nervous system(51). The hypothalamus is a key regulatory hub for blood pressure and metabolic control, through its influence on the activity of cholinergic and sympathetic nervous system pathways(57). The hypothalamus is composed of several distinct nuclei that contain the cell bodies of specialized neurons that control the production and release of various proteins and hormones (e.g. vasopressin and cortisol). These effector molecules can then increase the activity of blood pressure and energy expenditure pathways(54). Of note, the paraventricular (PVN), arcuate, and posterior nuclei have important roles in blood pressure regulation and energy metabolism(56, 57). In particular, brain-derived neurotropic factor (BDNF) signaling within the PVN regulates sympathetic signaling in the context of blood pressure, as well as energy expenditure(102). Angiotensin II and vasopressin signaling strongly influences blood pressure increases through the initiation and propagation of sympathetic nerve stimuli, as well as increasing activity of the Renin-Angiotensin-Aldosterone System (RAAS)(38, 60-62, 113). Aberrations in hypothalamic signaling within these pathways have been implicated several hypertensive phenotypes, regardless of the etiology(53, 104).

Organophosphates have been shown to preferentially accumulate within the CNS in high concentrations(315). Thus, it is possible that chlorpyrifos deposition within the

hypothalamus is linked to changes in expression of the aforementioned pathways. Previous studies have shown chlorpyrifos exposure can modulate the expression of hypothalamic genes, where developmental exposure to chlorpyrifos was demonstrated to downregulate expression of vasopressin in young mice(196, 316). In another study, chlorpyrifos exposure was shown to increase expression of orexigenic genes, impair feeding control, and lead to weight gain in mice(317). Given the fact that chlorpyrifos deposits readily in the hypothalamus and has been shown to affect gene pathway regulation in prior studies, we hypothesized that exposure to environmentally relevant concentrations of chlorpyrifos and chlorpyrifos-oxon affects the expression of several blood pressure and metabolism regulating pathway components. To test this idea, we exposed a primary culture cell line of murine hypothalamic cells to a range of environmentally relevant concentrations of chlorpyrifos and chlorpyrifos-oxon, and subsequently quantified the relative protein expression of target receptors and mRNA.

Methods

In vitro model/ Cell Culture

Immortalized hypothalamic cell lines were obtained from CELLutions®. Adult mouse hypothalamic cells were immortalized from two-month-old male C57BL/6J mice primary cultures by retroviral transfer of SV40 T-Ag. These cell lines were validated to express numerous peptides, enzymatic markers, and biologically active receptors relevant to obesity and HTN. Following receival of the cells, the cell vial was thawed quickly in a 37°C water bath, and the cells were initially incubated in a 100mm tissue culture plate. On the same day four hours later, the medium was replaced with fresh media to remove the preserving agent dimethyl sulfoxide (DMSO), which is toxic to cells.

After the initial thawing, cells were cultured in 1x Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 25 mM glucose and 1% penicillin/streptomycin. The incubator was maintained at 37°C with 5% CO2, and to ensure healthy growth and proliferation capacity, the cells were split once reaching 70-90% confluency, at a plate ratio of 1:4. Cells were passaged via trypsinization using trypsin-EDTA (1 mL per 100mm plate) at 37°C for 1-5 minutes, followed by washing/resuspension in warm growth medium. For all assays and dosing experiments the passage number did not exceed 10, to minimize genetic drift and confounding by the effects of age and quiescence on cellular pathways(318).

MTT Cytotoxicity Assay

To measure the cytotoxicity of chlorpyrifos and chlorpyrifos-oxon, we utilized an MTT assay developed by Sigma Aldrich®. Viable cells contain functioning NADPH-dependent cellular oxidoreductase enzymes which can convert a tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into an insoluble formazan(319). MTT is originally a yellow tetrazole, and is converted into a purple formazan product which can be read at a wavelength of 600 nm on an ELISA plate reader, using the BioTek® Gen5 software. The methods for the assay as recommended by the manufacturer are described in detail elsewhere(320). Hypothalamic cells were cultured in Corning CellBind™ 96-well flat bottom plates at initial densities of 2x 10^3 cells/well. Chlorpyrifos and chlorpyrifos-oxon 100mg quantities were obtained from Chem Service Inc.® Stock solutions of chlorpyrifos and chlorpyrifos-oxon were prepared by diluting 100mg of insecticide in 1mL DMSO, and then into a final volume of 100 mL of culture medium. Serial dilutions were prepared from the stock solutions to create a range of

concentrations from $1.0x10^{-9}$-$1.0x10^{-3}$ M. These concentrations represent estimates of the population-level exposures in both acute and chronic settings according to data from the EPA, based on cohort, *in vitro*, and *in vivo* studies(321-323). The next day, concentrations of chlorpyrifos and chlorpyrifos-oxon ranging from $1.0x10^{-9}$- $1.0x10^{-3}$ M were applied to each well in replicates of five. The cells were incubated in the insecticide solutions for 24 hours or four days. At the end of the timepoints, 10 µL of MTT labeling solution were added to each well, and incubated for 2hrs in the dark at 37°C. Following the incubation, 100µL of stop solution were added to each well, and the plate was incubated overnight in the dark at 37°C. The absorbance intensities are proportional to the number of viable cells, and were read at a wavelength of 600 nm. Relative intensity of absorbance was reported which each sample absorbance normalized to the control absorbance. A total of five biological replicates of independent experiments were obtained.

Acetylcholinesterase inhibition assay

Acetylcholinesterase inhibition assays were obtained from Sigma Aldrich® and performed according to the recommended protocol by the manufacturer. The percent acetylcholinesterase inhibition at each time point and concentration of chlorpyrifos-oxon was measured, as this gives insights into whether that concentration would be expected to induce cholinergic toxicity. Generally speaking, at least 70% acetylcholinesterase inhibition is required to induce cholinergic toxicity, and levels below this would suggest primarily non-cholinergic mechanisms of toxicity. Samples of acetylcholinesterase containing serum were diluted in 1X reaction buffer. A positive control of acetylcholinesterase was prepared to a final concentration of 0.2 U/mL. Reaction buffer without acetylcholinesterase was used as a negative control.

100µL of samples were added to 100µL of solution containing Amplex Red™ reagent, horse radish peroxidase, choline oxidase, and acetylcholine into individual wells on a flat-bottom 96 well plate. The reactions were incubated for 30 minutes in the dark at room temperature. Fluorescence was read at 590nm on a plate reader.

Chlorpyrifos and Chlorpyrifos-Oxon Dosing

We elected to compare the effects of chlorpyrifos the parent compound and the oxon form, to investigate if one form of the insecticide has the same or different effect on gene and protein expression within the hypothalamus. Hypothalamic cells were cultured Corning CellBind™ 100mm flat bottom culture dishes, and exposed to concentration ranges of chlorpyrifos and chlorpyrifos-oxon for either 24 hours or 4 days. The rationale for these time points was to compare acute effects vs. subchronic effects of insecticide exposure on these pathways. During the 4-day exposure, fresh chlorpyrifos and chlorpyrifos-oxon were applied at the beginning of each day to ensure consistent concentrations for the entire exposure. A total of three biological replicates of independent experiments were obtained for both chlorpyrifos and chlorpyrifos-oxon.

RNA and Protein Isolation

Following termination of either a 24hr or 4-day exposure, RNA and protein were isolated simultaneously from cells using RNeasy™ kits from Qiagen® and methods optimized in our lab. The culture media was removed from each plate, and cells were washed with 500 µL of phosphate-buffered saline solution. A cold solution of 0.5 mL trizol was added to each plate, and cell scrapers were used to remove the cells from the plate bottoms. The cells were transferred to 1mL centrifuge tubes on ice. Following transfer, the tubes were vortexed to lyse

the cells, and 100 µL of chloroform was added to each centrifuge tube. The tubes were centrifuged for 10 seconds, and allowed to sit at room temperature for three minutes. The tubes were then centrifuged at 12,000 x RPM for 15 minutes at 4°C. The upper aqueous phase containing RNA was transferred for a Qiashredder™ tube, and the remaining DNA and protein containing phase was stored on ice. The tube was centrifuged for 2 min at 15,000xRPM at room temperature, and 350µL of ethanol was added to the flow-through. The sample was transferred to a RNeasy spin column, and centrifuged for 15 seconds at 10,000xRPM. The flow through- was discarded, and the 700 µL of buffer RW1 was added to the column. The column was centrifuged again for 15 seconds at 10,000xRPM, and the flow-through was discarded. 500 µL of buffer RPE was added to the column, centrifuged for 15 seconds at 10,000xRPM, and the flow-through discarded. 500 µL of buffer RPE was added to the column, centrifuged for 2 minutes at 10,000xRPM, and the flow-through was discarded. The column was then placed into a new collection tube and centrifuged for 1 minute at 15,000xRPM. The column was then placed in a new 1.5mL Eppendorf tube and 30 µL of RNAse-free water was added to the column. The column was centrifuged for 1 minute at 10,000xRPM to elute the RNA. In the tube containing DNA and protein, the remaining white DNA containing layer was removed, and the protein layer was suspended in cold methanol. The protein was incubated at room temperature, then centrifuged for 10 minutes at 12,000xRPM at 4°C. The supernatant was removed, and the protein pellet was washed with 500µL of cold methanol. The protein was centrifuged again for 10 minutes at 12,000xRPM at 4°C, then the supernatant was removed, and the pellet was resuspended in 150 µL Caudle homogenization buffer(324). Using an electric

pestle, the protein was sonicated into solution. Samples of RNA and protein were stored at -80°C until quantification.

RNA and Protein Quantification (Nanodrop and BCA Assay)

The concentrations of RNA were quantified using the Nanodrop® according to the manufacturer's specifications. A 1µL sample of RNA was added to the reader, and the concentration in ng/µL was recorded. To quantify protein concentrations, we used the Pierce™ BCA protein assay according to specifications by the manufacturer. A standard curve was prepared with protein concentrations ranging from 2,000 µg/mL to 0 µg/mL. Samples of protein were diluted in 1:10 ratios of protein to homogenization buffer. Samples were plated in triplicates of 25µL on a 96 well flat-bottom plate. 200µL of working reagent was added to each well, and the plate was incubated in the dark at 37°C for 30 minutes. Following incubation, the plate was read on an ELISA plate reader, using the BioTek® Gen5 software at 560 nm wavelength. The concentrations of protein are directly proportional to the absorbance values, as understood through the Beer-Lambert's Law.

Immunoblotting

Primary antibodies for the angiotensin II type 1A, vasopressin 1A, trkB, and leptin receptors were obtained from Enzo laboratories. The activity of these receptors drives the effects of sympathetic pathways in the hypothalamus that regulate blood pressure and metabolism. Western blotting was done according to an optimized protocol by our lab(325). Protein samples were loaded onto Invitrogen Bolt™ 4-12% bis-tris plus gels at target concentrations of 40µg per lane. Gel electrophoresis was performed at 200V for 35 minutes in 1X MOPS running buffer at room temperature. Transfer of the proteins onto a polyvinylidene

difluoride (PVDF) membrane was performed at 45V for 30 minutes at room temperature. Following transfer, PVDF membranes were blocked in 1% BSA solutions for two hours at room temperature with agitation. PVDF membranes were incubated in primary antibody solutions of 1:5,000 dilutions antibody in blocking solution overnight at 4°C with agitation. The following day, membranes were washed three times in 1X TBST solutions for 10 minutes at room temperature with agitation. Membranes were then incubated for 1 hour and 30 minutes at room temperature with secondary antibodies of 1:50,000 dilutions antibody in 1X TBST. Membranes were then washed three times in 1X TBST solutions for 10 minutes at room temperature with agitation. Chemiluminescence was performed by incubating the membrane in Immobilon® western chemiluminescent HRP substrate for 30 seconds at room temperature, and imaging of the membranes was done using the ChemiDoc™ molecular imager. Relative intensity of protein bands normalized to the controls were reported. All samples were normalized to beta-actin.

cDNA preparation

Two micrograms of cDNA were converted from isolated RNA samples via SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), using the Veriti 96 well thermal cycler per protocols optimized by our lab(326). An initial DNAse step was performed for 47 minutes. Following the DNAse step, a reverse transcriptase step was performed for 70 minutes. Samples were stored at -20°C until RT-qPCR.

RT-qPCR

Messenger RNA expression of BDNF, NFkB, COMT, TGF-β, and TLR-4 was quantified using real-time quantitative polymerase chain reaction (RT-qPCR). The PCR cycle conditions

were performed by recommended manufacturer protocols: 94°C-2:00; 35 cycles: 94°C-0:15, 55°C-0:30, 68°C-1:00; 4C-hold. Each plate was loaded with appropriate controls, treatment groups, and a GAPDH internal standard. $^{\Delta\Delta}$Ct values were computed for each treatment group, and results are expressed relative to gene expression for the nontreated control groups.

Statistical Analyses

Results from the MTT assay, acetylcholinesterase inhibition assay, immunoblotting, and RT-qPCR were compared between treatment groups using one-way ANOVA in GraphPad/Prism™, followed by post hoc analysis (Tukey test) for the comparison of means to determine the significance of difference among the treatment groups and the control. Mean and standard error (SE) values were determined for all the parameters, and the results were expressed as mean ± SE. A p value below 0.05 was considered statistically significant.

Benchmark Dose Modelling

Results from our immunoblotting and RT-qPCR were imported into benchmark dose (BMD) models developed by the Environmental Protection Agency (EPA). Benchmark dose modeling is an improvement on the traditional uses of no-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL) to assess chemical toxicity. The results from a BMD analysis include a benchmark dose value, and a benchmark dose lower limit value, which are used to calculate daily reference doses, to inform the maximal amount of ingestion before adverse health effects occur.

Results

MTT-Assay

We observed dose and time-dependent effects of chlorpyrifos and chlorpyrifos-oxon on cell viability. At the 24-hour timepoint, chlorpyrifos concentrations of $1.0x10^{-9}$,$1.0x10^{-6}$,$1.0x10^{-5}$, and $1.0x10^{-4}$ M increased cell viability relative to the control group, whereas the $1.0x10^{-3}$ M showed a significant reduction in cell viability. Chlorpyrifos-oxon at 24 hours showed significant reductions in cell viability at concentrations of $1.0x10^{-5}$, $1.0x10^{-4}$, and $1.0x10^{-3}$ M. After 4 days, we observed significant reductions in cell viability for chlorpyrifos concentrations of $1.0x10^{-8}$,$1.0x10^{-6}$, $1.0x10^{-5}$, $1.0x10^{-3}$ M and increased viability at $1.0x10^{-4}$ M. Chlorpyrifos-oxon after a 4 day exposure revealed increases in viability for concentrations $1.0x10^{-9}$-$1.0x10^{-4}$ M, and a decrease in viability for $1.0x10^{-3}$ M. Observed decreases in cell viability could be due to direct toxic effects on chlorpyrifos and chlorpyrifos-oxon on hypothalamic neurons. Chlorpyrifos has been demonstrated to increase cell pyroptosis and increase susceptibility of cells to oxidative damage(327). Cell proliferation could be indicative of the response to oxidative stress induced by chlorpyrifos and chlorpyrifos-oxon, where oxidative stimulation has been shown to stimulate mitogenic pathways(328).

Acetylcholinesterase Inhibition Assay

Acetylcholinesterase inhibition of at least 70% has clinically been defined as the threshold necessary to induce overt cholinergic toxicity. We observed dose and time-dependent effects of chlorpyrifos-oxon with respect to acetylcholinesterase activity. After 24 hours of chlorpyrifos-oxon, we observed statistically significant reductions in the concentration range of $1.0x10^{-7}$-

$1.0x10^{-3}$ M. However, it is important to note that not until $1.0x10^{-4}$ M and $1.0x10^{-3}$ M do we observe reductions of 74% and 49%, respectively. After 4 days of chlorpyrifos-oxon we observed significant inhibition in the range of $1.0x10^{-8}$ M to $1.0x10^{-3}$ M. However, only at concentrations of $1.0x10^{-4}$ M and $1.0x10^{-3}$ M did we observe inhibition below 70%, at values of 63% and 40%, respectively. Given this information, we would expect that mRNA and protein expression effects in the $1.0x10^{-9}$ M to $1.0x10^{-5}$ M range are occurring primarily through noncholinergic mechanisms, whereas in the $1.0x10^{-4}$ M and $1.0x10^{-3}$ M range we would expect cholinergic toxicity to play a role to a significant degree.

Immunoblotting

We elected to quantify the expression of three well-known receptors (angiotensin II type 1A, vasopressin V1a, leptin, and trkB receptors) that integrate hypothalamic blood pressure and metabolic pathways. Within the 24 chlorpyrifos group, we observed overall increased expression of the angiotensin II receptor in each concentration, and statistically significant increases in the $1.0x10^{-9}$ M and $1.0x10^{-8}$ M groups. Vasopressin receptor expression was increased in all groups compared to the control, but nonsignificant. For the trkB receptor we noted increasing trends in expression for all concentration groups except for $1.0x10^{-5}$ and $1.0x10^{-3}$ M, where we observed decreased expression. For the leptin receptor, we observed nonsignificant increases in the $1.0x10^{-9}$-$1.0x10^{-7}$ M groups, following a decrease in the $1.0x10^{-6}$ M group, return to baseline in the $1.0x10^{-5}$ M group, and ending with a relative increase in the $1.0x10^{-4}$ and $1.0x10^{-3}$ M groups.

In the 24-hour chlorpyrifos-oxon group, we noted increasing trends in expression of all four receptors relative to the controls, with statistically significant increases for angiotensin II type 1A receptor at $1.0x10^{-3}$ M, vasopressin 1A receptor at $1.0x10^{-6}$ M, and the leptin receptor at $1.0x10^{-7}$ and $1.0x10^{-4}$ M concentrations.

In the 4-day chlorpyrifos groups, we observed significant increases in the $1.0x10^{-9}$ and $1.0x10^{-5}$ M groups, and significant decreases in the $1.0x10^{-7}$ and $1.0x10^{-6}$ M groups. Significant increased expression of the vasopressin 1A receptor was observed in the $1.0x10^{-5}$ M group, and significant decreases in trkB receptor expression in the $1.0x10^{-6}$ and 1.0x10-4 M groups. Expression of the leptin receptor was significantly increased in the $1.0x10^{-4}$ and $1.0x10^{-3}$ M groups.

Following a 4-day exposure to chlorpyrifos-oxon, we observed increasing trends of angiotensin II, vasopressin, and trkb receptor expression, though nonsignificant. For the leptin receptor, we observed significant increased expression in the $1.0x10^{-9}$-$1.0x10^{-4}$ M groups.

RT-qPCR

As done for our immunoblotting targets, the mRNA expression of hypothalamic genes known to regulate blood pressure and metabolism following chlorpyrifos and chlorpyrifos-oxon exposure were quantified. Additionally, we included three genes expressing components of hypothalamic inflammatory pathways. The immune system has been well established to play a role in the pathogenesis of HTN and MetS, and hypothalamic inflammation is implicated in the etiology of numerous forms of HTN(104, 308). At 24-hour chlorpyrifos exposure, significant decreases in bdnf mRNA was observed in the $1.0x10^{-4}$ and $1.0x10^{-3}$ M groups. For a 24-hour chlorpyrifos-oxon exposure, TGF-β mRNA was significantly increased in the $1.0x10^{-3}$ M group.

Benchmark Dose Modeling

The EPA's benchmark dose modelling software was used to fit our data to a series of linear and polynomial functions to compute benchmark dose values. We set a value of 0.1 change in expression relative to the control as the benchmark dose response of interest. Benchmark dose modeling performs best with monotonic data, and after assessing our data for monotonic trends we chose to calculate benchmark dose response values for the 4-day chlorpyrifos acetylcholinesterase inhibition assay and the 4-day chlorpyrifos TGF-β mRNA expression. The benchmark dose lower limit values are reported in figures 28 and 29, and these values would ultimately be used to calculate reference doses for oral exposures.

Figure 8.

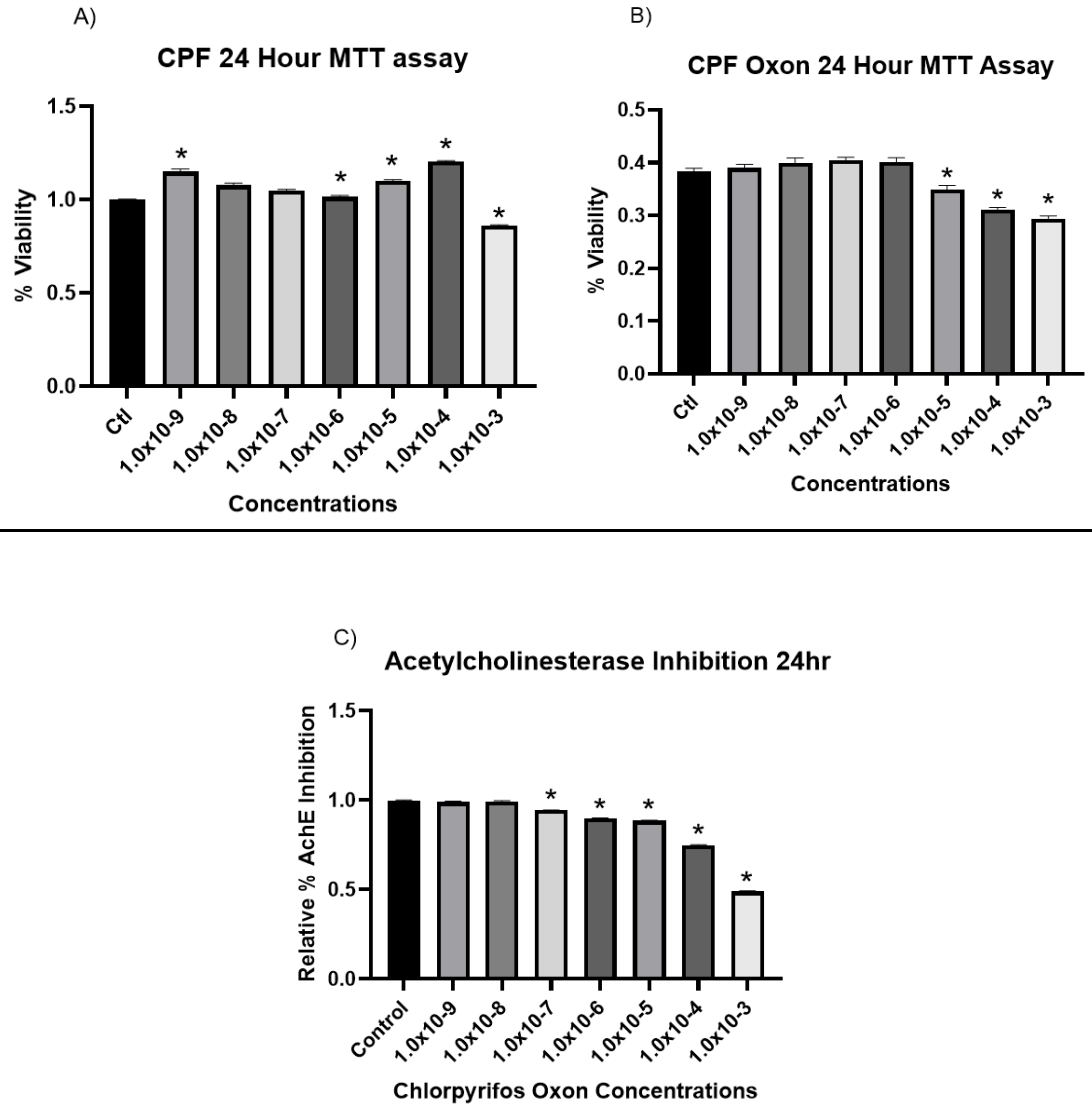
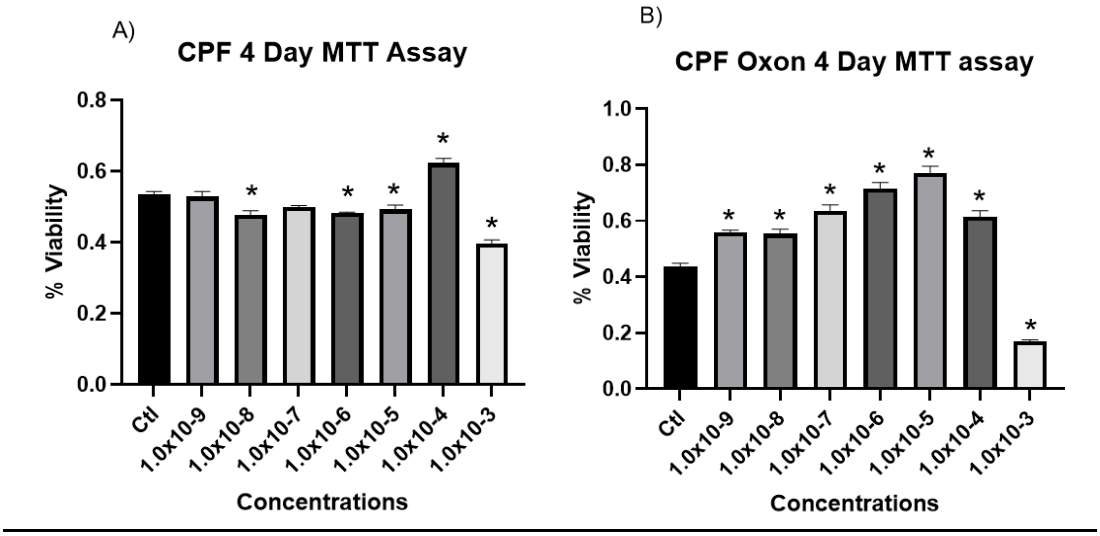


Figure 8: Figures A, B, and C showing the percent viability and percent acetylcholinesterase inhibition by chlorpyrifos and chlorpyrifos-oxon at 24 hours. N=5 and a p value <0.05 was threshold set for statistical significance.

Figure 9.



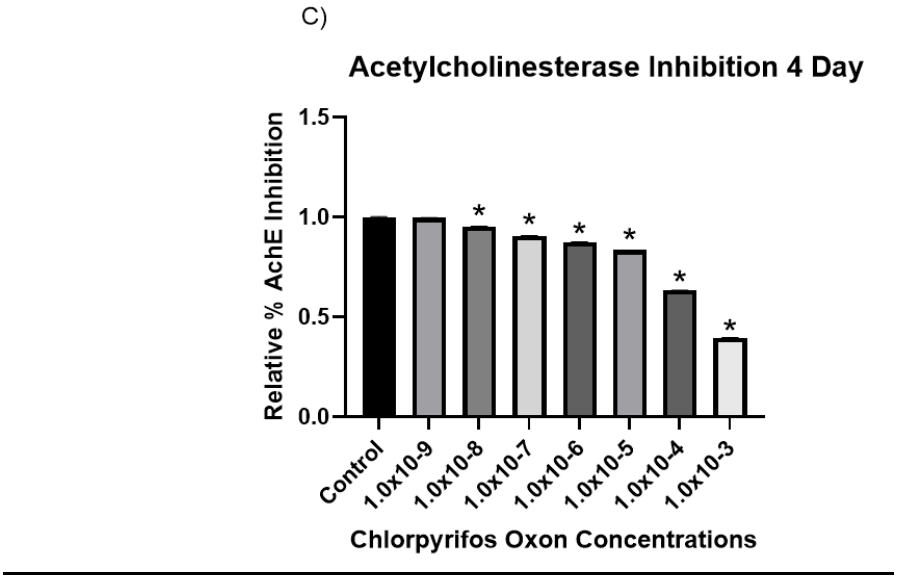


Figure 9: Figures A, B, and C showing the percent viability and percent acetylcholinesterase inhibition by chlorpyrifos and chlorpyrifos-oxon at 4 days. N=5 and a p value <0.05 was threshold set for statistical significance.

Figure 10.

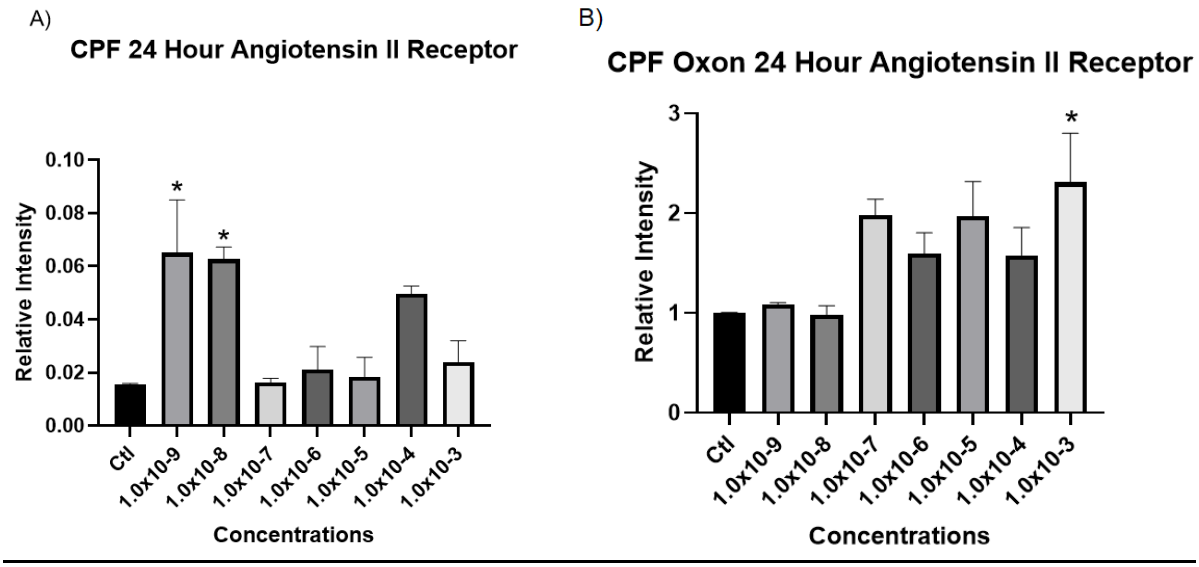


Figure 10: Figures A and B show the relative protein expression of the angiotensin II receptor respectively, following a 24-hour exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 11.

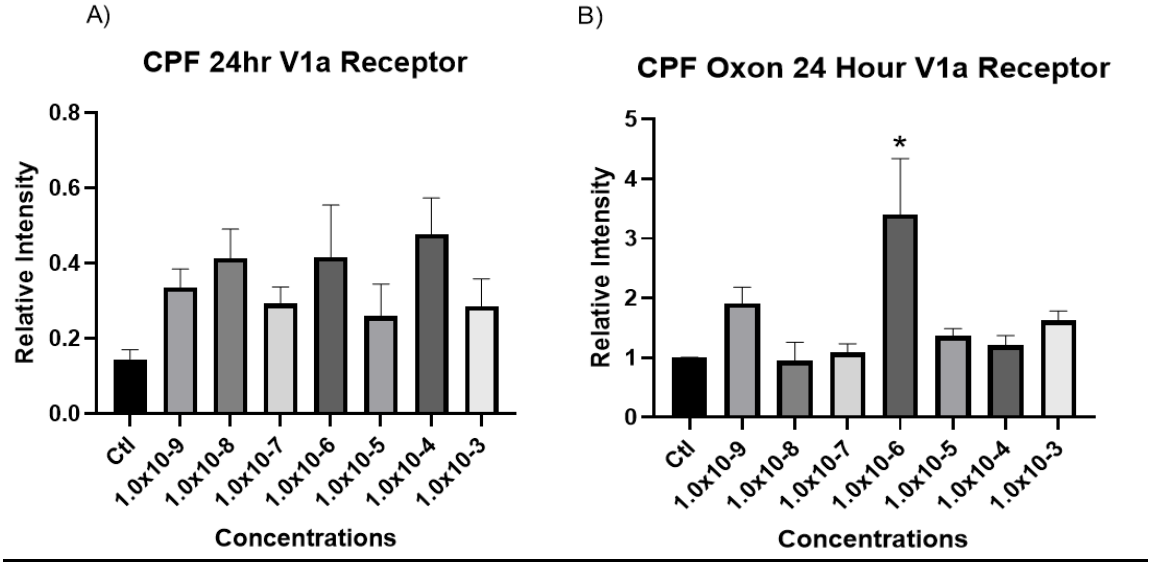


Figure 11: Figures A and B show the relative protein expression of the V1A receptor following a 24-hour exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 12.

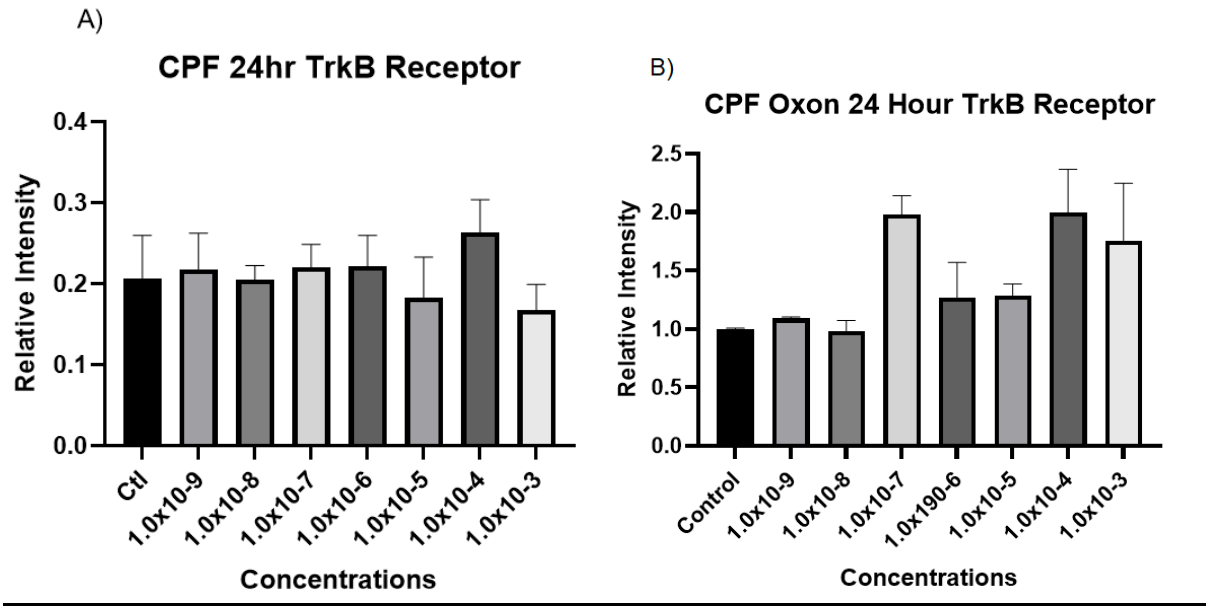


Figure 12: Figures A and B show the relative protein expression of the TrkB receptor following a 24-hour exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 13.

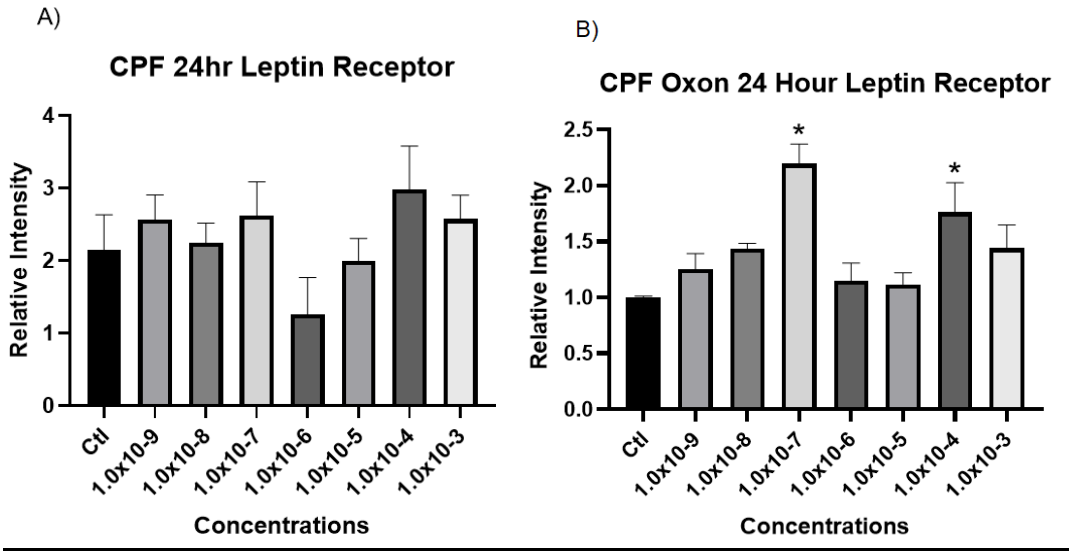


Figure 13: Figures A and B show the relative protein expression of the leptin receptor following a 24-hour exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 14.

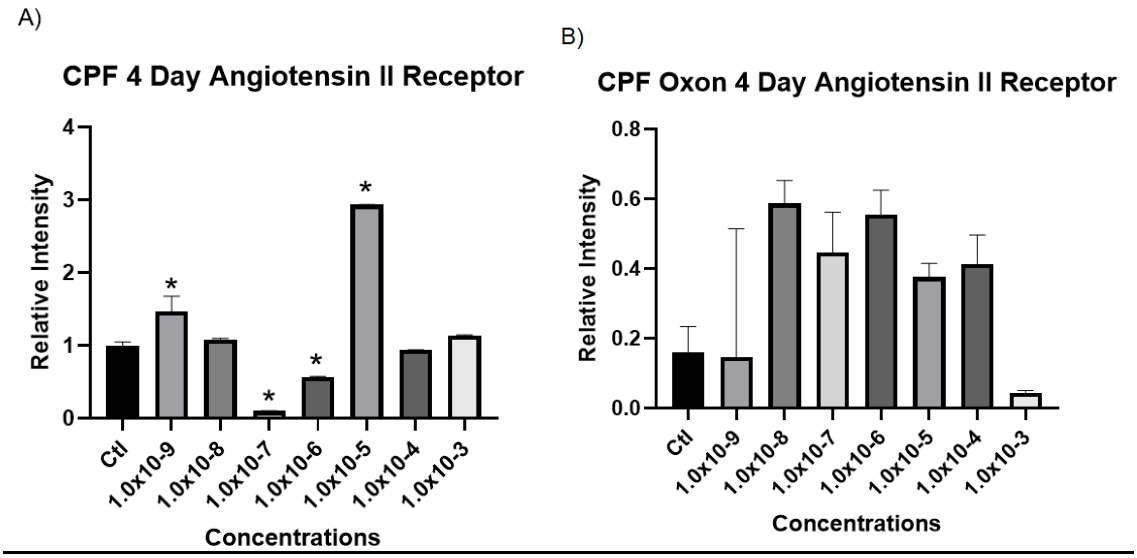


Figure 14: Figures A and B show the relative protein expression of the angiotensin II receptor following a 4-day exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 15.

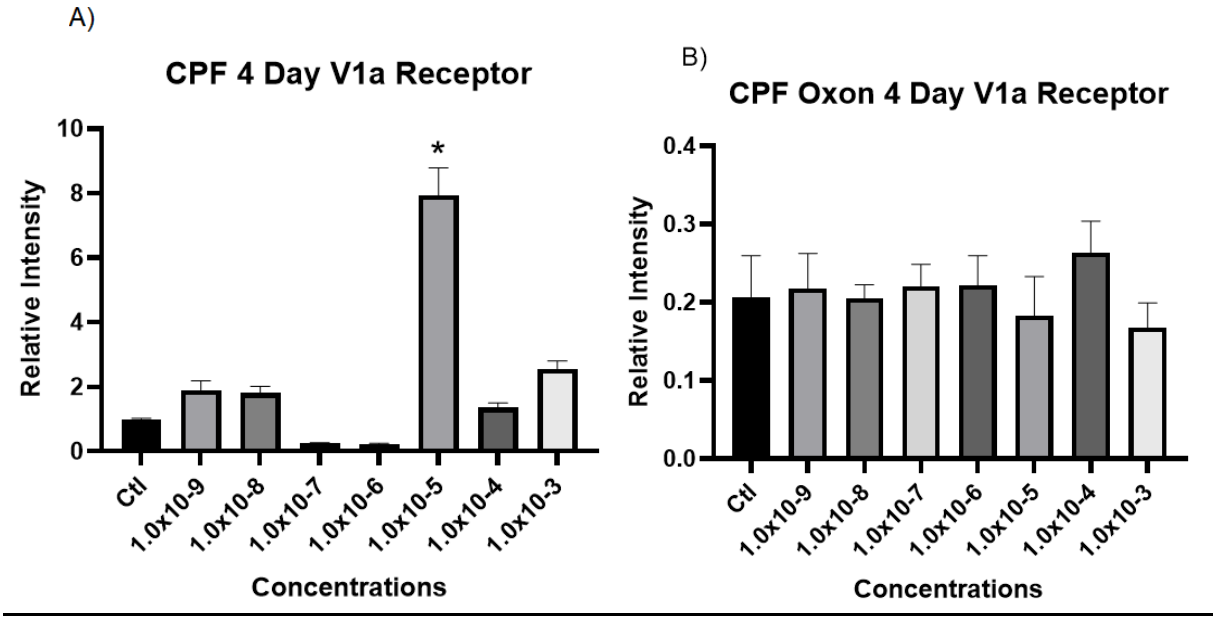


Figure 15: Figures A and B show the relative protein expression of the V1A receptor following a 4-day exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 16.

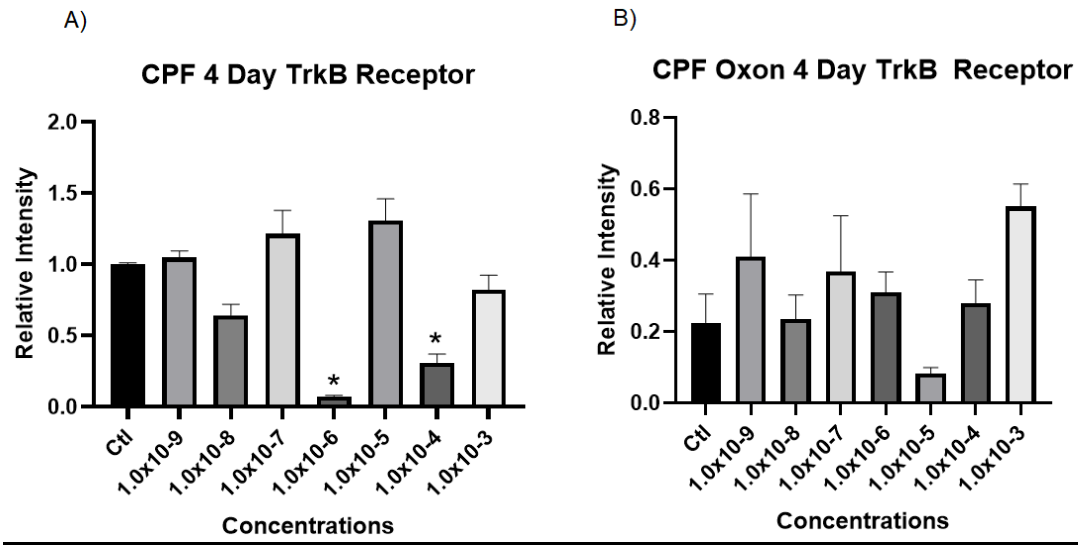


Figure 16: Figures A and B show the relative protein expression of the TrkB receptor following a 4-day exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 17.

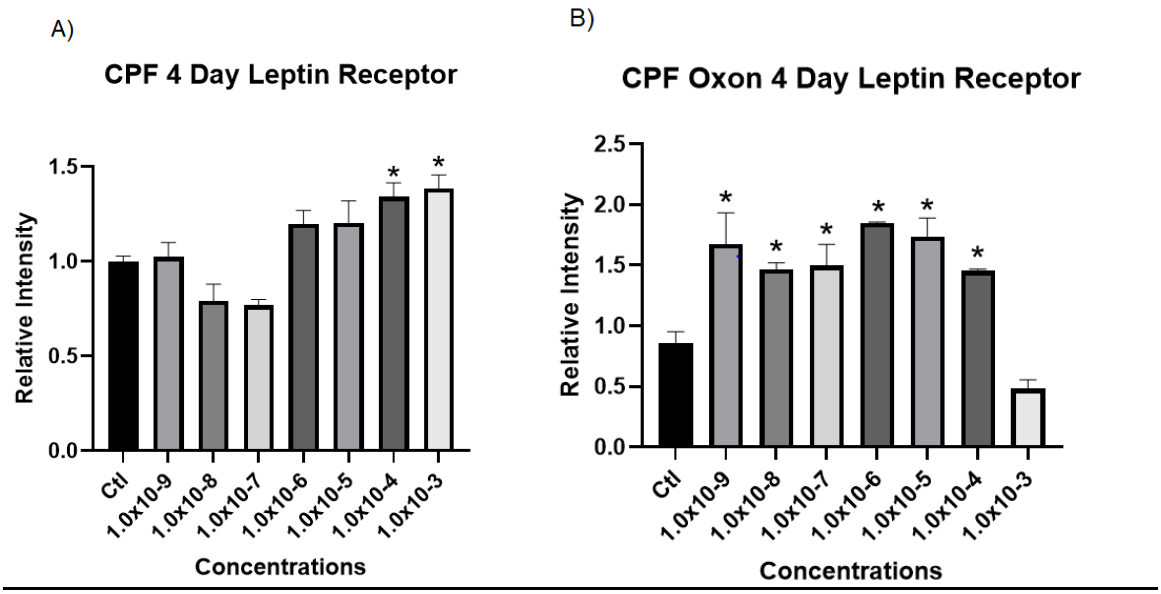


Figure 17: Figures A and B show the relative protein expression of the leptin receptor following a 4-day exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 18.

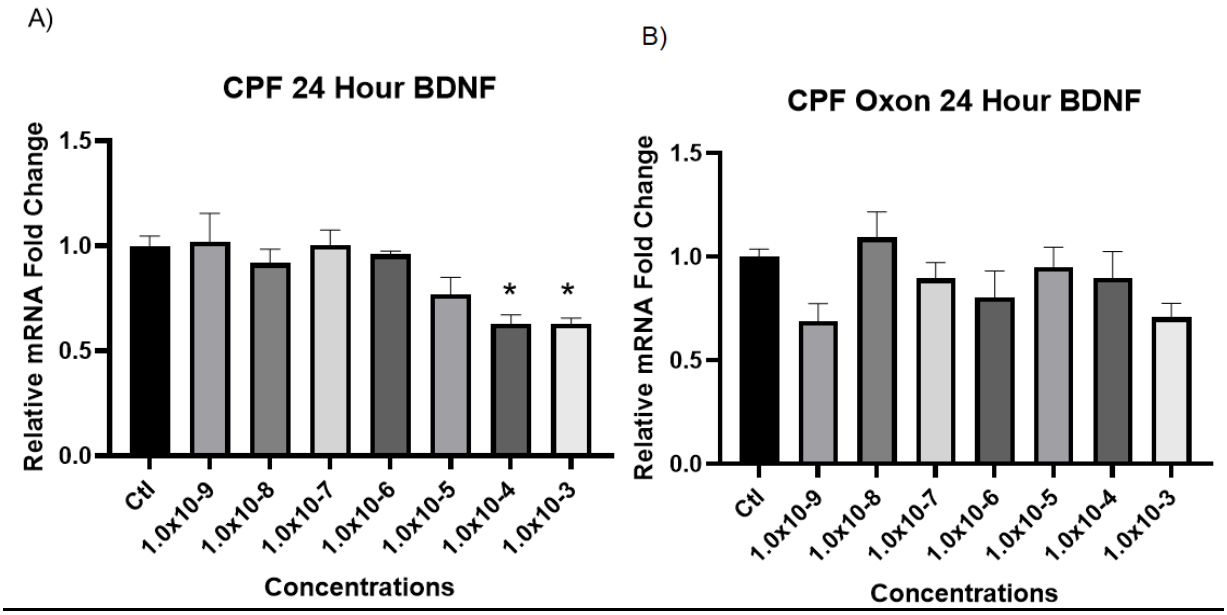


Figure 18: Figures A and B show the relative mRNA expression fold change of the BDNF gene following a 24-hour exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 19.

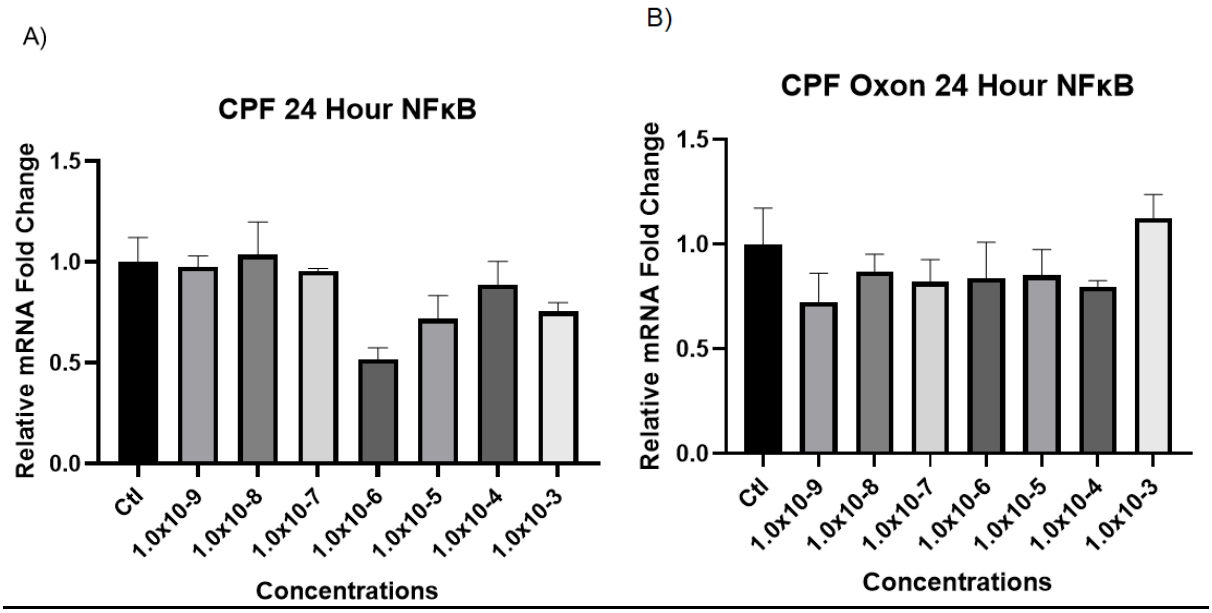


Figure 19: Figures A and B show the relative mRNA expression fold change of the NFkB gene following a 24-hour exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 20.

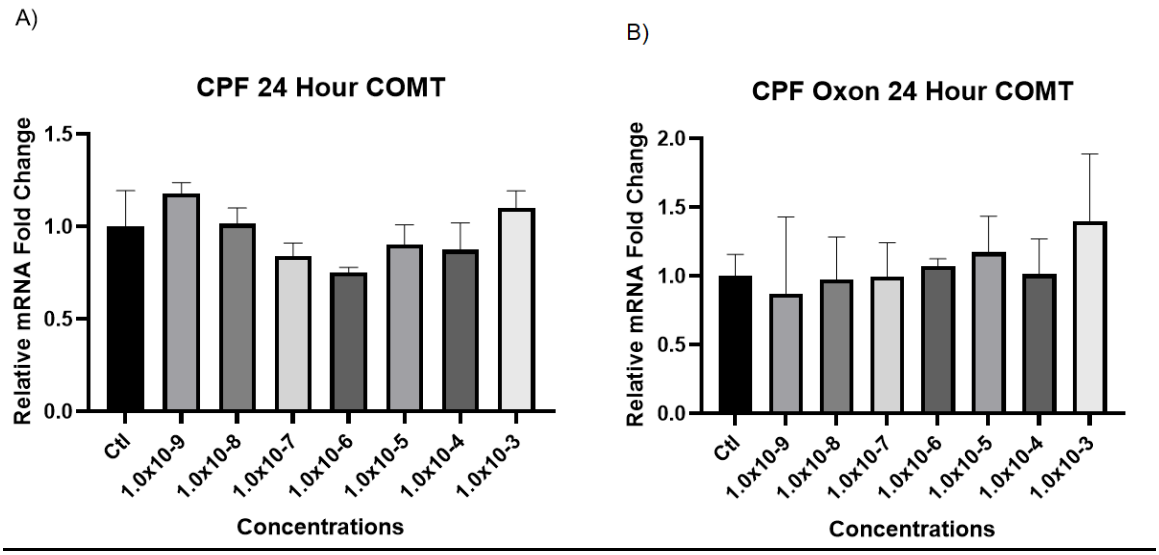


Figure 20: Figures A and B show the relative mRNA expression fold change of the COMT gene following a 24-hour exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 21.

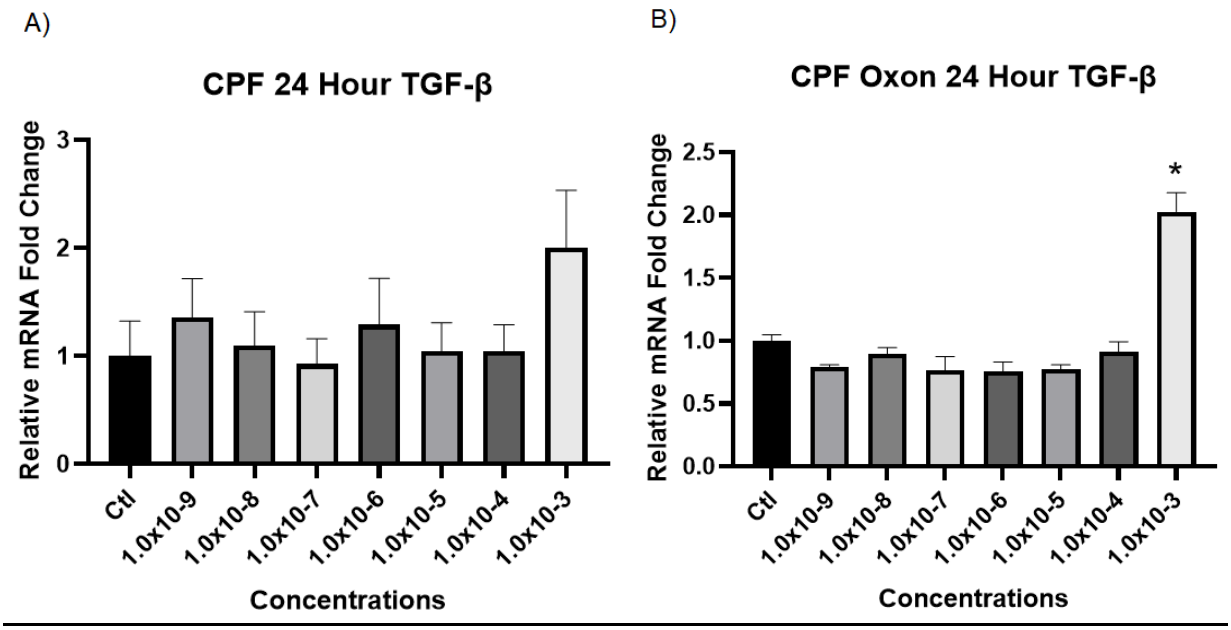


Figure 21: Figures A and B show the relative mRNA expression fold change of the TGF-β gene following a 24-hour exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 22.

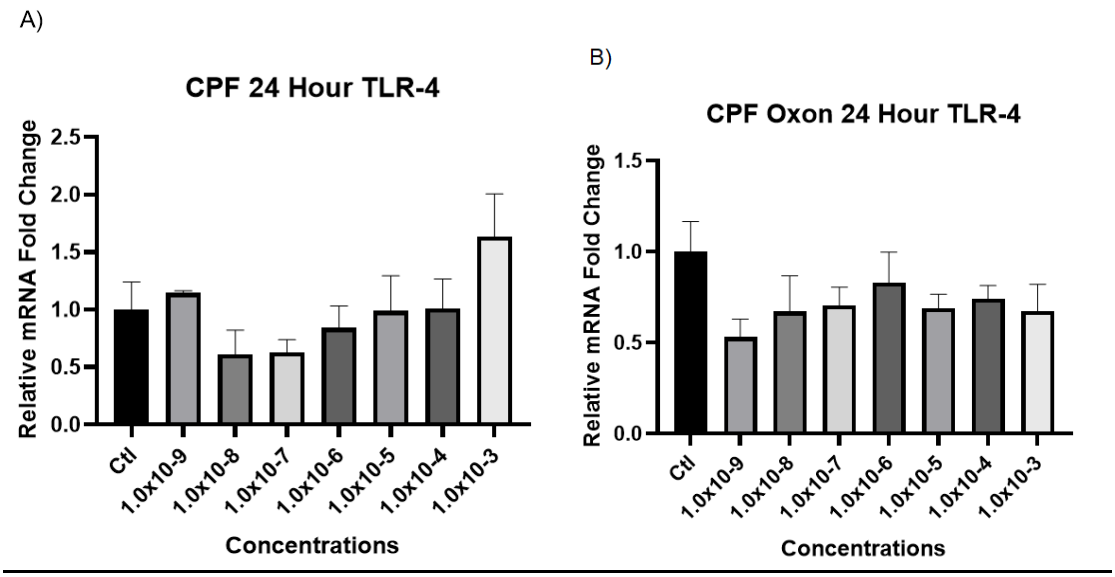


Figure 22: Figures A and B show the relative mRNA expression fold change of the TLR-4 gene following a 24-hour exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 23.

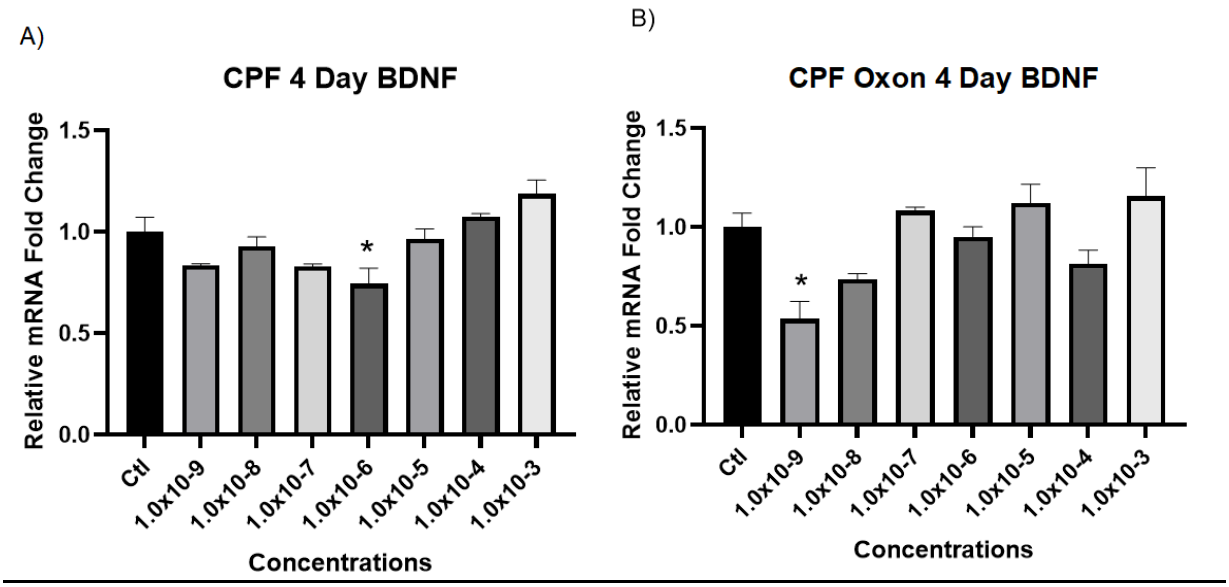


Figure 23: Figures A and B show the relative mRNA expression fold change of the BDNF gene following a 4-day exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 24.

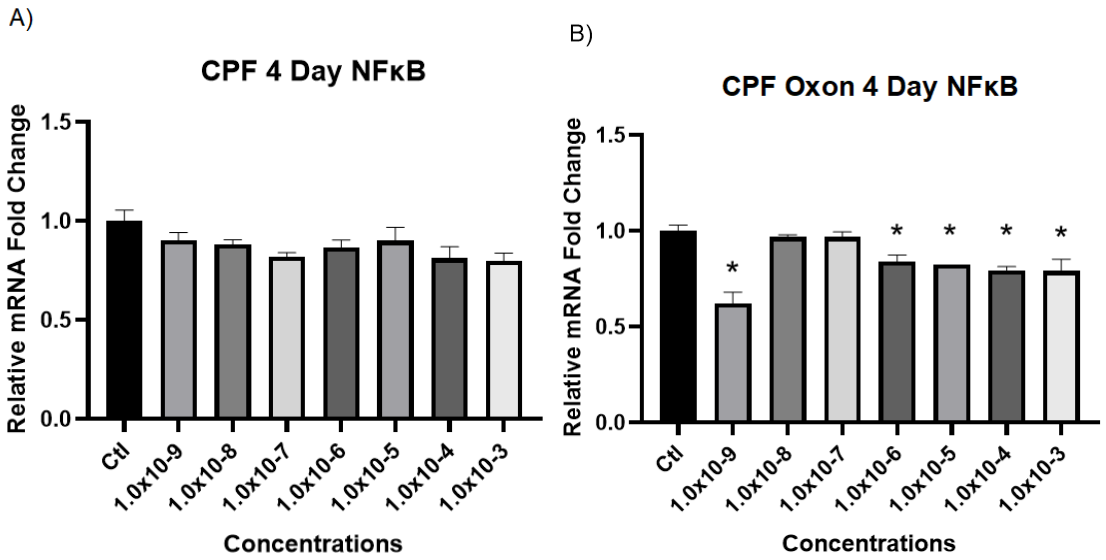


Figure 24: Figures A and B show the relative mRNA expression fold change of the NFkB gene following a 4-day exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 25.

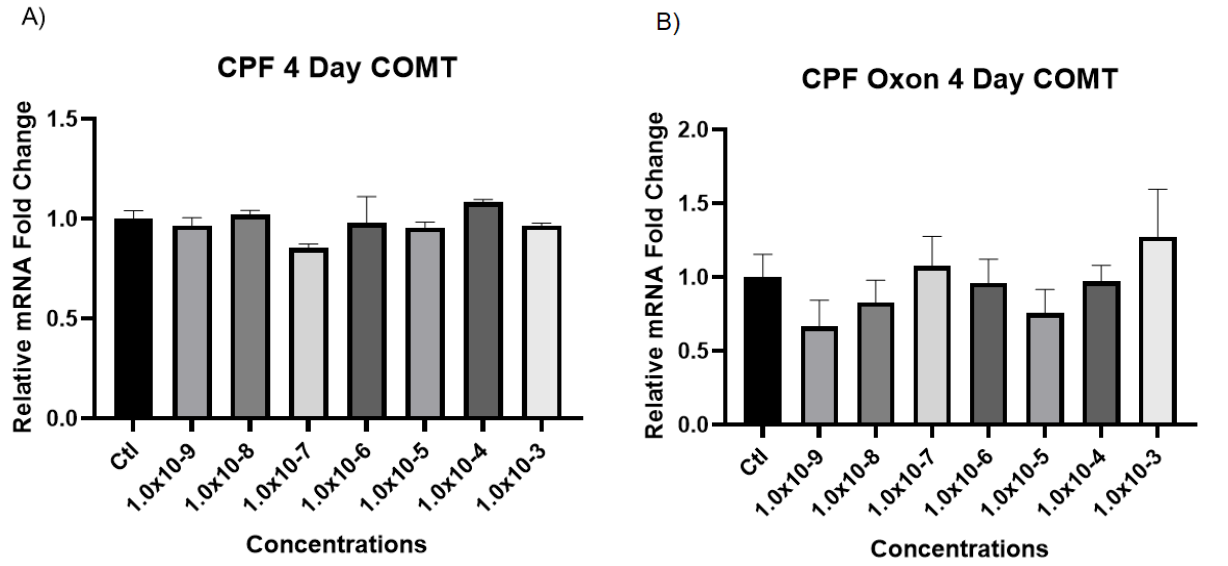


Figure 25: Figures A and B show the relative mRNA expression fold change of the COMT gene following a 4-day exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 26.

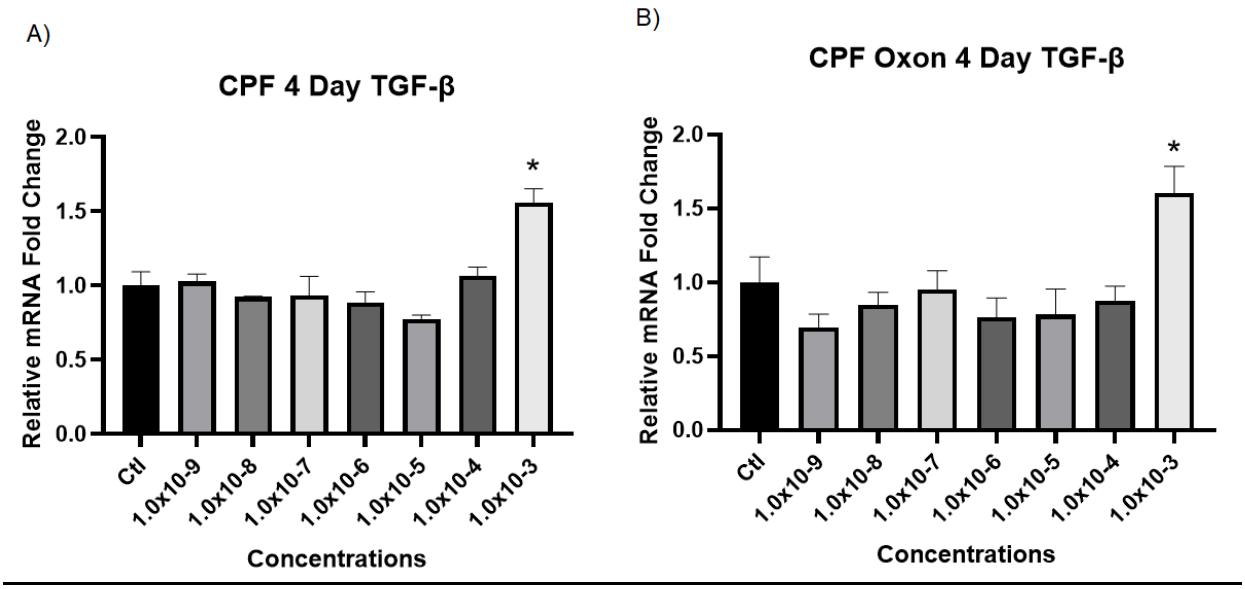


Figure 26: Figures A and B show the relative mRNA expression fold change of the TGF-β gene following a 4-day exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 27.

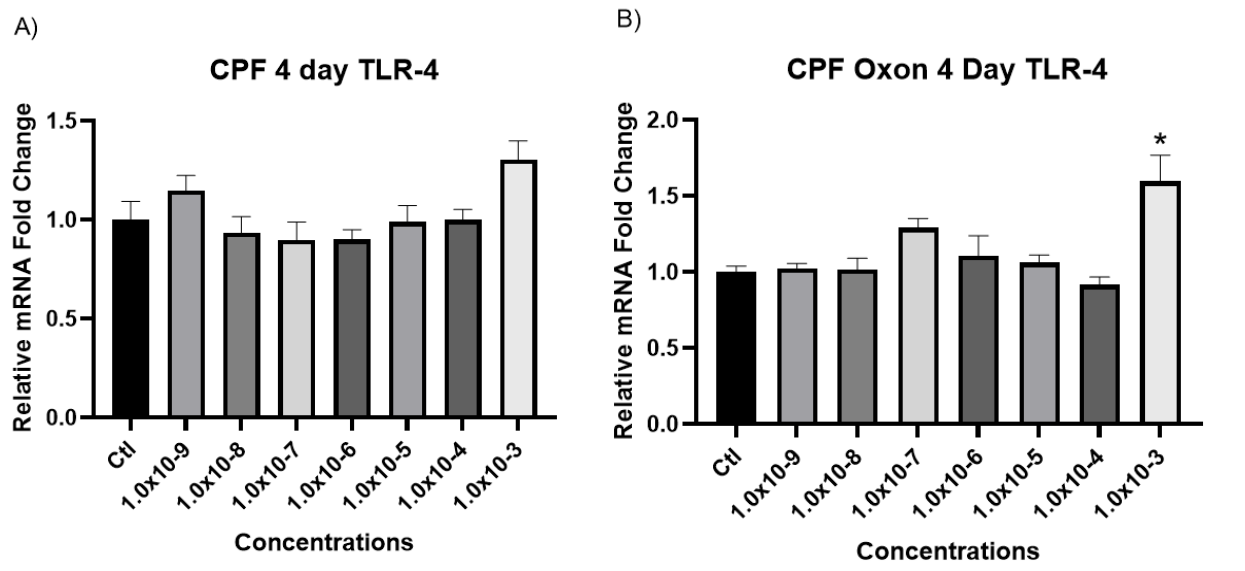


Figure 27: Figures A and B show the relative mRNA expression fold change of the TLR-4 gene following a 4-day exposure to chlorpyrifos and chlorpyrifos-oxon. N=3 and a p value <0.05 was threshold set for statistical significance.

Figure 28.

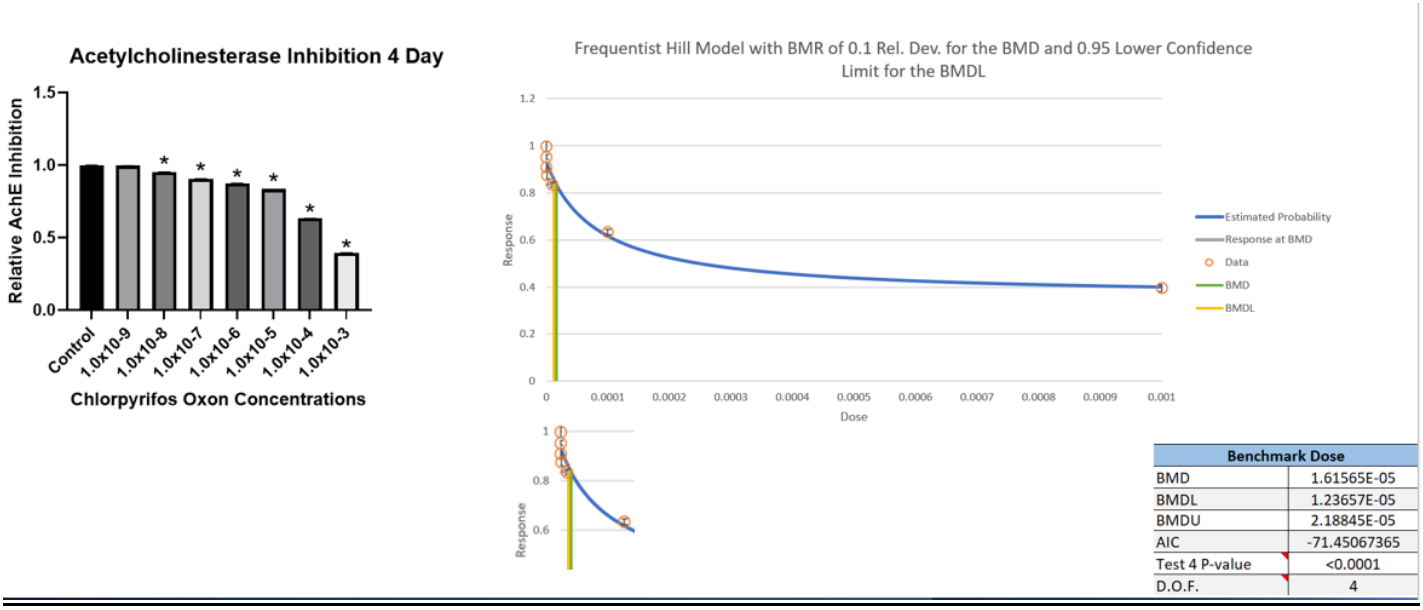
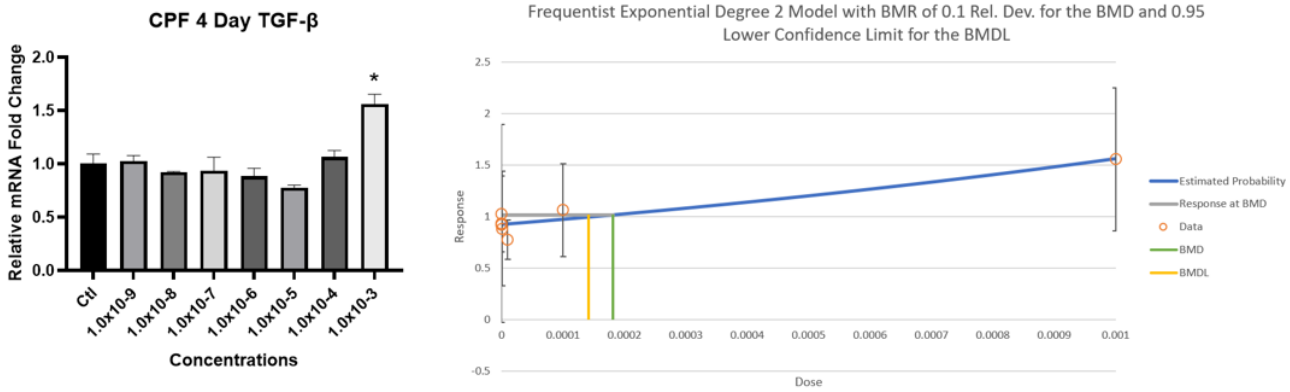


Figure 28: Benchmark dose response model for acetylcholinesterase inhibition following a 4-day exposure to chlorpyrifos-oxon. A predetermined value of 0.1 was set as the benchmark dose response.

Figure 29.



Benchmark Dose	
BMD	0.000181545
BMDL	0.000141482
BMDU	0.00025886
AIC	-2.681075693
Test 4 P-value	0.58773377
D.O.F.	5

Figure 29: Benchmark dose response model for TGF-β gene expression following a 4-day exposure to chlorpyrifos. A predetermined value of 0.1 was set as the benchmark dose response.

Discussion

The incidence and prevalence of HTN and MetS are expected to increase over the ensuing decades, and the contribution of the environment in their pathogenesis is becoming increasingly apparent. Currently, the biological mechanisms explaining the associations between OP exposure and risk of HTN and MetS remain to be fully elucidated. Here, we have demonstrated chlorpyrifos and the oxon form's ability to induce gene and protein expression of hypothalamic products known to regulate blood pressure and metabolism, even at concentrations below what is necessary for cholinergic poisoning. The following hypothalamic

targets have been implicated in HTN and MetS in various *in vitro*, *in vivo*, and population studies.

Hypothalamic Angiotensin II/Type 1A receptor signaling

Angiotensin II is a potent vasoconstrictive hormone that has both peripheral and central effects. Within the hypothalamus, angiotensin II acts primarily through the type 1A receptor to elicit alterations in blood pressure, fluid intake, and hormone secretion(329). The angiotensin 1A receptor functions as a G protein coupled receptor, and its expression is increased by a number of stimuli including circulating angiotensin II, and stimulation of intracellular p44/42 MAPK and JNK signaling pathways(330). Organophosphates have been demonstrated to induce ERK, JNK, and p38-MAPK activation, which can lead to oxidative stress and apoptosis in various tissues. However, the activation of MAPK signaling pathways may differ depending on the type of OP, the concentration of OP, duration of exposure, and the type of cells exposed(331). In addition to effects on blood pressure, the angiotensin II receptor is also involved in regulating feeding behavior through anorexigenic corticotropin-releasing hormone pathways in the hypothalamus(332). Thus, aberrations in expression of the angiotensin II receptor by chlorpyrifos may be one mechanism that contributes to HTN and MetS.

Vasopressin/V1A signaling

Elevated vasopressin has been implicated in the etiology of HTN, though the exact role vasopressin plays at the cellular level remains to be fully understood. Vasopressin is produced by magnocellular neurons in the PVN and supraoptic nucleus of the hypothalamus, and stimulates water reabsorption in the kidney to help maintain blood pressure(104). Within the

CNS, increased hypothalamic expression of vasopressin has been observed in borderline hypertensive rats(333). In the 2K1C rat model of renovascular hypertension, vasopressin contributes to HTN via a PVN-specific salusin-β AVP-V1 receptor-dependent (angiotensin II-independent) pathway(334). This evokes increased sympathetic activation, and subsequently HTN via downstream signal transduction and V1A receptor activation in the rostral ventrolateral medulla.

Vasopressin signaling in the hypothalamus is also involved in feeding behaviors and energy expenditure(335). Central administration of vasopressin was shown to decrease food intake in both rats and pygmy goats, actions mediated by the V1A receptor(336, 337). In contrast, orexigenic effects of neuropeptide Y administration in the arcuate nucleus were enhanced in V1A receptor knockout mice(338). Vasopressin is also involved in the maintenance of glucose, lipids, and fatty acids. Studies have shown that V1A receptor activity can both induce hyperglycemia and also improve glucose tolerance(339, 340). Vasopressin has also been demonstrated to have both lipolytic and anti-lipolytic actions, depending on the experimental condition. Under starved conditions, constant infusion of vasopressin induced fatty acid release from adipose tissue by a direct anti-lipolytic effect in adipose tissue in rats(340). In another study, pitressin, a synthesized form of vasopressin, induced lipolysis in rat adipose tissue *in vitro(341).* The dose, frequency, and duration of chlorpyrifos exposure may determine which combination of effects vasopressin exerts on blood pressure and metabolism.

BDNF/TrkB signaling

Brain-derived neurotropic factor has a variety of actions in the CNS, including mediating pathways that regulate blood pressure and metabolism. BDNF has been implicated in the initiation and maintenance of high-salt mediated HTN via downregulation of PVN KCC2 through activation of its trkB receptor pathway(342). Additionally, impairments in inhibitory signaling are mediated by bdnf, leading to increased excitability of hypothalamic vasopressin-secreting neurons(343).

Leptin Signaling

Leptin is an important hormone involved in the regulation of blood pressure and energy expenditure. Once released from white adipose tissue, leptin acts within the hypothalamus via intracellular AMP-activated protein kinase (AMPK) signaling pathways to evoke sympathoexcitation and HTN(344). These effects are mediated by the leptin receptor, located in highest densities in the arcuate and paraventricular nuclei. It has been demonstrated that leptin also activates the mammalian target of rapamycin (mTORC1) via a phosphoinositide 3-kinase (PI3K) pathway, and mTORC1 activity is believed to be necessary to mediate leptin-induced increases in renal sympathetic nerve activity (RSNA) and blood pressure(345). In mice, increased expression of the leptin receptor is associated with resistance to dietary-induced obesity in female C57BL/6J mice(346).

COMT signaling

The catechol-O-methyl transferase (COMT) gene encodes an enzyme that degrades catecholamine neurotransmitters including dopamine, norepinephrine, and epinephrine to

inactivate them. Catecholamines act on a number of central and peripheral receptors (e.g. alpha and beta receptors), to increase cardiovascular dynamics and vasoconstriction(42). Recently, the importance of the *COMT* gene in HTN and MetS pathogenesis has become more apparent, where polymorphisms in the *COMT* gene have been shown to differentially affect blood pressure, glucose, and lipid profiles(347). Specifically, Val/Val genotype expression in the *COMT* gene appears to be associated with a higher prevalence of increased systolic BP compared with the Met/Met or Met/Val genotypes (348).

NFkB signaling

The immune system and inflammation are heavily implicated in the initiation, maintenance, and progression of HTN and MetS(107, 308, 349). In particular, increased hypothalamic expression of proinflammatory cytokines is closely associated with the pathophysiology of HTN(350). For example, nuclear factor-κB (NFκB),a key transcription factor, governs inflammatory processes and plays a critical role in mediating inflammation-induced HTN(351). Experimental blockade of NFκB within the PVN has been shown to significantly attenuates angiotensin II-induced hypertension. Another study found that eight weeks of aerobic exercise training decreased blood pressure in hypertensive rats by reducing production of proinflammatory cytokines through TLR4/MyD88/NF-κB signaling within the PVN, and delayed the progression of 2K1C renovascular HTN(352).

TLR-4 signaling

Activation of toll-like receptors induces expression of proteins involved in the immune response, and TLR-4 activation has been shown to contribute to increased BP and low-grade inflammation in the spontaneously hypertensive rat(353). In another animal model, blockade of TLR-4 signaling within the hypothalamus attenuated blood pressure and inflammation(354).

TGF-β signaling

Transforming growth factor-β is a pleiotropic cytokine that plays an important role in wound healing, angiogenesis, and immunoregulation(355). The activity of TGF-β has also been implicated in protection from HTN, where removal of TGF-β or blocking its signaling before HTN induction accelerated HTN progression, and supplementation of TGF-β1 substantially suppressed neuroinflammation and blood pressure(110).

Strengths

To our knowledge, this is the first study incorporating a range of environmentally relevant concentrations of chlorpyrifos and chlorpyrifos-oxon to measure effects on gene and protein expression within the hypothalamus, specifically pathways related to the pathogenesis of HTN and MetS. It is possible that the expression modulatory effects of chlorpyrifos on these pathways may in part play a role in the pathogenesis of HTN and MetS. By using both the parent compound and its primary active metabolite, were able to make side by side comparisons of both chlorpyrifos and chlorpyrifos-oxon, an observe any similarities or differences in their effects in hypothalamic signaling. Additionally, we were able to computationally model points of departure for mRNA and protein expression changes using

BMD modeling. as improvements on traditional no-observed-adverse-effect-level (NOAEL) and lowest-observed-adverse-effect-level (LOAEL) endpoints.

Weaknesses

The present study has important limitations. Since we are not evaluating these effects in a whole organism model, we were unable to link these expression changes to biological endpoints such as blood pressure, lipid profiles, weight gain, and glucose levels. It is possible that chlorpyrifos induces cellular stress resulting in increased expression of these receptors through various pathways, but not in a manner that increases the function of the genes and receptors. It is also possible that chlorpyrifos' effects seen within the hypothalamus are contingent on the interplay with feedback loops between peripheral organ systems. Chlorpyrifos likely has direct effects on peripheral organs in the setting of whole organisms, and compensatory biological mechanisms may determine what effect chlorpyrifos has on the hypothalamus, blood pressure, and metabolic profiles. Additionally, our cell lines were derived from male mice, and studies have demonstrated potential for sex-differences in the sensitivity to chlorpyrifos exposure(356). Lastly, BMD modelling suits data that has monotonic patterns, where the data is either continuously increasing on continuously decreasing. For a number of the mRNA and protein expression data, the inflections in the data made it difficult to produce statistically reliable BMD models. Future studies that include pathway components upstream and downstream of these targets, as well as whole organisms are warranted.

Future Directions/Conclusion

We have demonstrated chlorpyrifos and chlorpyrifos-oxon's abilities to affect the expression of hypothalamic pathway components relevant to HTN and MetS. Future studies are warranted to corroborate these findings and expand on these pathways, test these associations in whole organisms, and include biological endpoints like blood pressure and metabolic profiles.

Chapter 5: Summary

Summary

Through an in-depth investigation using the NHANES cohort dataset, we observed significant associations between everyday exposure levels of OP insecticides and indications of blood pressure dysregulation, as well as between OP insecticides and MetS prevalence. Many of our analyses also revealed important null associations between OP exposure, risk of HTN, and metabolic parameters. Results from our targeted *in vitro* model revealed significant effects of chlorpyrifos and chlorpyrifos-oxon on the mRNA and protein expression of hypothalamic blood pressure-regulating and metabolism-regulating pathway components. It is possible that the associations between OPs such as chlorpyrifos, HTN, and MetS are due to effects within convergent hypothalamic pathways, as the hypothalamus regulates energy metabolism and homeostasis through shared pathways of blood pressure.

Organophosphate insecticides have peripheral cholinergic and non-cholinergic effects in addition to the central effects within the hypothalamus. Thus, some of the null findings of OP exposure on blood pressure in Aims 1 and 2, as well as on the expression of hypothalamic targets in response to chlorpyrifos may indicate that chlorpyrifos acts on both central and peripheral systems to affect blood pressure. Chlorpyrifos has been shown to directly target peripheral metabolic organs such as the pancreas, liver, and kidneys. As discussed previously, the maintenance of blood pressure and metabolism is governed by a number of organ systems, and direct effects of chlorpyrifos within those systems may better explain the association with HTN and MetS. The concurrent dysregulation of peripheral and central pathways, paired with upregulation of compensatory mechanisms may result in a return to blood pressure and metabolic homeostasis, resulting in null associations at the population level. However, these

subtle effects on pathway activation may increase the set points out of normal ranges, and persons who are predisposed to HTN and MetS, or accumulate various other risk factors overtime may be susceptible to developing HTN and MetS when the threshold is crossed. Additionally, the dose and duration at which people are exposed to chlorpyrifos may determine which pathways (peripheral vs. central) are most affected. Future studies including measuring blood pressure and metabolites as an outcome, while controlling for central and peripheral actions of chlorpyrifos are warranted.

It's important to mention that OPs are usually found in high associations with other environmental contaminants, that may or may not also affect blood pressure. Notable examples include polychlorinated biphenyls, DDT, and arsenic(214). It is possible that through synergistic or antagonistic effects, mixture affects may distort or exacerbate the effect of OPs seen on blood pressure, HTN, and MetS risk.

A large focus of the study was centered on measuring the effect of chlorpyrifos on cholinergic and sympathetic pathways within the hypothalamus and how their perturbation may contribute to HTN and MetS. While increased activation of acetylcholine-dependent pathways has been linked to HTN, other studies have demonstrated acetylcholine to have antihypertensive qualities by increasing parasympathetic activity, and blunting the immune response by inhibiting the release of cytokines from macrophages(357). Donepizil, a centrally acting acetylcholinesterase inhibitor, significantly reduced the plasma levels of circulating inflammatory mediators TNF-α, IL-6, and IFN-γ and lowered blood pressure in spontaneous hypertensive rats. This effect was not seen with physostigmine, a peripherally acting

acetylcholinesterase inhibitor, which substantiates the importance of the brain and hypothalamus in the maintenance and progression of HTN.

Another possibility for the null findings is that under different physiological states, the effect of chlorpyrifos on biological pathways may be the opposite of would be expected under basal conditions. For example, in states of hypoxia, bilateral blockade of the angiotensin II type 1A receptors in the medulla leads to an increase in blood pressure and renal sympathetic nerve activity, whereas an additional GABA receptor block in the medulla results in a decrease in renal sympathetic nerve activity and blood pressure(358). Under normoxia, the blockade has no effects on blood pressure. Thus it is possible that in cellular states of hypoxia and stress, the effects of insecticides on gene expression and ultimately blood pressure regulation may vary.

Hypertension is often accompanied by metabolic abnormalities (e.g. abnormal lipid profile, hyperinsulinemia, obesity) and it has been shown that biomarkers such as amino acids can serve as predictors of HTN before measurable increases in blood pressure. These biomarkers such as glycine, carnitine, ornithine, phenylalanine, and tyrosine have been shown to differentiate between hypertensive and normotensive patients with a sensitivity and specificity greater than 85%(1). Future studies that include examining the effects of chlorpyrifos exposure on the profile of metabolic biomarkers may give additional insights into the pathways affected.

Our findings are important for individuals not only in the general population of the U.S., but a number of special subpopulations who are exposed to OPs in high concentrations. One example would be populations living in agricultural and rural settings, and settings in close

proximity to industrial plants. As expected, studies have shown that individuals living in rural and agricultural communities have higher exposure levels of OP insecticides, because these communities depend on insecticides to cultivate crops. Furthermore, water, soil, and air contamination has been shown to be significantly higher in communities near insecticide manufacturing plants. Special populations of individuals who have lower paroxonase and cytochrome P450 enzyme activity may also be at increased risk of adverse health events related to OPs. This is due to the fact that P450 enzymes catalyze the reactions necessary to detoxify OPs, and studies have shown that polymorphisms in P450 expression can significantly affect the half-life of OPs within the body. Additionally, certain minority groups within the U.S., specifically communities of Blacks and Mexican-Americans, have the highest prevalence of HTN. In understanding the causes of health disparities, it's important to be sensitive to the statistical, conceptual, and historical complexities associated with race. It was previously thought that the association between chronic diseases such as HTN and race/ethnicity was largely due to genetic factors. As our understanding has since evolved, and emerging evidence supports the idea that the process of racialization is largely responsible for the disparities seen between racial groups. For centuries racialization as a whole has dictated if and where certain people can obtain healthcare and the quality of that healthcare, limited the options for where people can live, their economic and education attainment opportunities, and even creates barriers to a healthy diet. As a result, many minorities find themselves in situations with high exposure to environmental chemicals (e.g. outdated housing with poor air-quality, insecticide residues, foods containing high concentrations of insecticides), and it is possible that these reasons contribute even more to the incidence and prevalence of HTN within these communities.

The environmental hazards posed by insecticides are well-documented, and their safe use, storage, and disposal have become increasingly challenging. The course of chlorpyrifos has been controversial, where its regulation and bans have been approved and overturned several times over the last decade. On April 29, 2021, the United States Court of Appeals for the Ninth Circuit ordered EPA to issue a final rule concerning the chlorpyrifos tolerances by August 20, 2021. Based on the currently available data and taking into consideration the currently registered uses for chlorpyrifos, the EPA was unable to conclude that the risk from aggregate exposure from the use of chlorpyrifos meets the safety standard of the Federal Food, Drug, and Cosmetic Act (FFDCA), and chlorpyrifos will be phased out beginning in February 2022. While chlorpyrifos has been banned, thousands of new unregulated chemicals are produced annually with potentially similar toxicological profiles. It is important to be vigilant in the risk assessment of these chemicals to provide accurate public health information. Additionally, while diazinon and parathion have been banned since the early 2000s, we identified appreciable levels of their metabolites within the general population. It is possible that a combination of their long half-lives and use in parts of the world where they are unrestricted is leading to their persistence in the environment. Furthermore, it is unknown what long-term effects exposure to OPs has had on biological processes governing blood pressure and metabolic function. Developmental studies have shown that early exposure to OPs may lead to long-lasting effects on the gene and protein expression of various pathways. Particularly, *in utero* chlorpyrifos has been shown to affect the expression of oxytocin and vasopressin in young mice. Future studies to examine the effects of early life exposures to chlorpyrifos and other OPs are warranted.

Findings such as these support decisions made by the EPA to restrict chlorpyrifos use, and reinforce the need to develop safer chemical and non-chemical alternatives to OP insecticides. Additionally, the development of updated guidelines and procedures for the general public to reduce exposure to OP insecticides (e.g. public health information on which foods contain the highest concentrations of various insecticides to help lower the total body burden of insecticides), and novel farming strategies such as crop rotation and use of biopesticides may provide more beneficial to health outcomes. A number of alternative studies are warranted to further investigate the effects of chlorpyrifos on blood pressure and metabolism pathways. These studies may include high-throughput methods of gene and protein expression analyses such as microarrays and metabolomic and proteomic profiling. Additionally, the concurrent administration of peripheral blocking muscarinic agents with central administration of chlorpyrifos, or central blocking muscarinic agents with peripheral administration of chlorpyrifos can help determine whether the biological endpoints measured are due to central, peripheral, or both effects.

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