2014

The Role of Exercise in Polychlorinated Biphenyl Induced Cardiovascular Disease

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THE ROLE OF EXERCISE IN POLYCHLORINATED BIPHENYL INDUCED CARDIOVASCULAR DISEASE

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

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2014

ABSTRACT OF DISSERTATION

THE ROLE OF EXERCISE IN POLYCHLORINATED BIPHENYL-INDUCED CARDIOVASCULAR DISEASE

Cardiovascular disease remains the leading cause of death in Western societies. Endothelial dysfunction is one of the initiating steps in the development of atherosclerosis. While there is a strong correlation with a person's genetics, lifestyle factors including smoking, physical activity, and diet can significantly increase a person's susceptibility to the development of atherosclerosis. In addition to these lifestyle factors, there is a strong body of evidence linking exposure to environmental pollutants including persistent organic pollutants such as polychlorinated biphenyls to increased cardiovascular disease and mortality. It has been well-established that exercise protects against cardiovascular disease, but whether exercise can modulate PCB-induced cardiovascular inflammation and dysfunction is unknown.

To investigate the effects of exercise on PCB-induced cardiovascular disease, two murine models of atherosclerosis, the ApoE-/- and the LDLr-/- mouse were utilized. Risk factors for cardiovascular disease including adiposity, glucose intolerance, hyperlipidemia, hypertension, oxidative stress, and inflammation, were assessed in these two models as well as mean atherosclerotic lesion size. Exercise positively modulates several risk factors associated with cardiovascular disease including hypertension, hyperlipidemia, adiposity and obesity, systemic levels of oxidative stress, inflammation, and glucose tolerance. Exercise significantly reduced mean lesion size in vehicle-treated animals. To assess the mechanism of protection of exercise in chapter 4, vascular reactivity studies were performed to measure endothelial function after exposure to PCB 77. Exercise prevented PCB-impaired endothelial function implicating the role of superoxide as a cause of impairment. Exercise upregulated phase II antioxidant enzymes. The work in this dissertation demonstrates several protective properties of exercise against PCB-induced cardiovascular disease; however, additional studies are needed to determine if exercise enhances metabolism and excretion of these environmental pollutants.

KEYWORDS: cardiovascular disease (CVD); Polychlorinated biphenyls (PCBs); exercise, oxidative stress, endothelium dysfunction

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In dedication to Margaret O’Bryan Fitts

Acknowledgements

I would like to acknowledge several people who assisted me in the completion of this dissertation. First, I would like to thank my mentor, Dr. Bernhard Hennig who offered me a position in his laboratory after serving as his teaching assistant. Without your guidance, none of this could have occurred. Thank you helping me grow not only in my career as a scientist on a personal level as well.

To my other committee members, thank you for your assistance. Dr. Esser, thank you for being a strong female scientist and true role model. Additionally, thank you for collaborating with us by providing running wheel cages. To Dr. Pearson, thank you for your continued support and enthusiasm about my project and growth as a scientist. To Dr. Cassis, thank you for encouragement and insights into assisting this project along. Your expertise has truly been appreciated in developing dosing regimens as well as the appropriate mouse model to conduct these experiments. Dr. Li, thank you for your critical thought and advice for continuing my work. Dr. Newman, thank you for serving as an outside examiner.

To my fellow labmates, current and previous, I thank you for your continued support, kindness, and advice during this time. Michael Petriello, your academic input has been thoroughly appreciated and necessary. Dr. Zuzana Makjova and Dr. Sung Gu Han, your expertise and training has been invaluable. Katryn Eske, thank you for your kindness and assistance in animal work. To Brad Newsome, thank you for your editing expertise and friendship. The laboratory of Alan Daugherty allowed me to perform and refine many experiments within this dissertation. Jess, thank you for setting up the contractility apparatus and providing me detailed training to conduce these ex vivo experiments. Deborah, your training on aortic root sectioning has been very helpful, you are truly a

master. I am eternally grateful for the day that you and Jess helped me handle a mouse without fear. Anju, thank you for blood pressure training and animal assistance. I would also like to acknowledge Dr. Morris and Manjula Sunkara for their assistance and availability to analyze PCB 77, its metabolites, and F_2-isoprostanes.

To my family and friends, thank you for your continued support, encouragement and love during this time. I am truly grateful for your love and kindness. To Tom Gawriluk, thank you for your continued passion for science. I dedicate this work to my grandmother and namesake, Margaret O'Bryan Fitts, who would have been a brilliant scientist had she lived in a different generation when women had more opportunities.

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Chapter 1: Introduction

1.1 Cardiovascular Diseases and Pathology of Atherosclerosis

Cardiovascular diseases (CVDs) are currently the leading cause of death in the U.S[1]. Atherosclerosis is characterized by the accumulation of lipids and fibrous debris within the large arteries[2]. Early lesions of atherosclerosis consist of cholesterol-laden macrophages in sub-endothelial spaces, known as foam cells[3]. These foam cells and subsequent fatty streaks can be found in the aorta of a human within the first decade of life. Fatty streaks are the precursors of more advanced lesions characterized by the accumulation of lipid-rich necrotic debris and smooth muscle cells (SMCs)[4]. These types of lesions usually have a fibrous cap made up of SMCs and an extracellular matrix that encloses the lipid-rich necrotic core. Although advanced lesions can grow significantly and result in stenosis, the formation of a thrombus or clot is more likely to form resulting in myocardial infarction or stroke, and ultimately death[5].

Epidemiological studies over the past 50 years have revealed several risk factors for atherosclerosis. The main risk factors for atherosclerosis include increased total cholesterol levels with high ratios of low-density lipoproteins (LDL) to high-density lipoproteins (HDL), obesity, diabetes mellitus, and hypertension[6]. While there is a strong correlation with a person's genetics, lifestyle factors including smoking[7], physical inactivity[8] and diet[9] can significantly increase a person's susceptibility to the development of atherosclerosis[10]. In addition to these lifestyle factors, there is a strong body of evidence linking exposure to environmental pollutants including persistent organic pollutants to increased cardiovascular disease and mortality[11].

Cholesterol constitutes a major portion of the atherosclerotic plaque and increased serum levels of cholesterol play a role in the development of atherosclerosis. In fact, the first observable change in the vessel wall is the accumulation of lipoprotein particles and their aggregates in the intima[4]. However, recent evidence has demonstrated that atherosclerosis is a low-grade inflammatory disease rather than a lipid storage disease[2]. Within days, monocytes can be seen adhering to the surface of the endothelium and then transmigrate across the endothelial monolayer to the intima, ultimately forming foam cells[12]. Furthermore, circulating levels of inflammatory mediators including C-reactive protein (CRP) are independent risk factors for atherosclerosis[3]. The

specific involvement of inflammation in plaque initiation and progression will be discussed below.

The formation of an atherosclerotic plaque is a complex process involving several different cell types. A primary initiating event in atherosclerosis is the accumulation of LDL within the sub-endothelial space of the intima, which stimulates endothelial cells to produce a number of pro-inflammatory mediators including adhesion molecules and macrophage colony-stimulating factor (M-CSF)[4]. LDL diffuses passively through gap junctions and its retention within the vessel wall involves interactions with matrix proteoglycans and apolipoprotein B (apoB)[13]. Trapped LDL undergoes oxidation, lipolysis, proteolysis and aggregation which further stimulate inflammatory cytokines[14]. These mediators recruit monocytes and lymphocytes to the arterial wall. The first step in adhesion, the "rolling" of leukocytes along the endothelial surface, is mediated by selectins which bind to carbohydrate moieties on leukocytes[15]. Firm adhesion of monocytes and T cells to the endothelium is mediated by the integrin VLA-4 on the EC, which interacts with both VCAM-1 and the CS-1 splice variant of fibronectin[16]. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine produced by endothelial cells. Mice deficient in monocyte chemoattractant protein (MCP-1) or its receptor CCR2 had significantly reduced atherosclerosis, suggesting a role for MCP-1/CCR2 in monocyte recruitment[17,18]. It has been shown that MCP-1 facilitates monocyte transmigration and retention into the sub-endothelial space in both humans and mice. The rapid uptake of oxLDL particles by macrophages is mediated by two scavenger receptors, SR-A and CD36[19,20]. The resulting cholesterol uptake by macrophages leads to the conversion of a foam cell, which makes up the fatty streak, characteristic of early lesion development[21].

Fibrous plaques are formed by a growing mass of extracellular lipid consisting mainly of cholesterol and its ester, and by the accumulation of SMCs[4]. SMC migration and proliferation as well as extracellular matrix production is regulated by cytokines and growth factors secreted by macrophages and T cells[22]. SMCs form the fibrous cap that encases the necrotic core of the lesion. Additionally, matrix metalloproteinases (MMPs) are produced by macrophages within the plaque and cleave the extracellular matrix. Cleavage of the extracellular matrix aids in the migration of SMCs and contributes to instability of the plaque increasing the risk for thrombosis[23].

1.1.1 The role of endothelium in atherosclerosis

The endothelium is a monolayer which acts as an interface between blood-borne molecules; including circulating nutrients, environmental pollutants, lipoproteins, and cytokines. Endothelial integrity is essential for preserving vascular homeostasis, regulating vascular tone, coagulation, angiogenesis and repair, and inflammatory processes. Nitric oxide (NO) is produced by endothelial cells and has several important roles including regulation of vascular tone, inhibition of platelet aggregation, inducing vasodilation in SMCs, and preventing leukocyte adhesion[24–26]. Endothelial dysfunction, including a decrease in bioavailable NO, is an independent risk factor in cardiovascular disease[27]. Endothelial dysfunction is a condition that includes the upregulation of cellular adhesion molecules (CAMs), which recruit blood mononuclear cells, and increases in endothelial permeability, which facilitates the diffusion of LDL to the intima[28]. The transcription factors nuclear factor-KB (NF-KB) and activator protein-1 (AP-1) regulate the expression of adhesion molecules and cytokines[29,30] and are activated in response to increased levels of reactive oxygen species (ROS)[31]. Circulating leukocytes do not adhere to endothelium unless the expression of CAMs (e.g. VCAM-1, ICAM-1) is present on the cell surface[32]. Similarly, chemoattractant stimuli produced from ECs promote migration of leukocytes into the intima where M-CSF stimulates differentiation from monocytes into macrophages. The macrophages express scavenger receptors which engulf oxidized LDL ultimately forming foam cells. These lipid-laden macrophages secrete a number of inflammatory mediators including IL-1 and TNF-a that amplify inflammation within the vessel wall and contribute to additional leukocyte accumulation, SMC proliferation, and extracellular matrix remodeling[2,4,33].

1.1.2. Mechanisms of Atherosclerosis-Shear Stress and Physical Inactivity

Blood flow-induced shear stress has emerged as an essential feature of the development of atherosclerosis. Shear stress is a biomechanical force that is determined by blood vessel, vessel shape, and fluid viscosity and is expressed as units of dynes/cm^2 [34]. Fluid shear stress has effects on EC morphology. Cells in the tubular regions of arteries are ellipsoid in shape and align in the direction of laminar flow. Cells in arterial areas of curvature are exposed to “disturbed” flow have polygonal shapes and no orientation which increase susceptibility to lesion formation[35]. A sedentary lifestyle results in disturbed vascular flow including irregular and non-uniform flow. The influence

of blood flow in atherosclerosis can be deduced from the presence of vascular inflammation and the distribution of atherosclerotic lesions at lesser curvature of bends and near side branches, where blood flow rate is relatively low.[36] For example, atherogenesis is promoted by low shear stress i.e. <5 dynes/cm^2 because it disrupts numerous cell functions including a reduction in eNOS synthesis, vasodilation, and endothelial cell repair. These disruptions in cell function are coupled to an increase in reactive oxygen species, increased leukocyte adhesion, apoptosis, increased permeability to lipoproteins, smooth muscle cell proliferation, and collagen deposition[35]. A key initial step in this process involves the recruitment and binding of leukocytes to the endothelium. Leukocytes normally do not adhere to the endothelium; however, if the endothelium expresses adhesion molecules, the leukocyte "tethers" through interaction with P- or L-selectin on the microvilli of leukocytes[37]. Low shear stress or reduced flow upregulates the expression of leukocyte adhesion receptors such as intercellular adhesion molecule 1 and vascular cell-adhesion 1[38] as well as chemokines including monocyte chemotactic protein 1[39] under conditions of disturbed flow.

It should be noted that these receptors and signaling molecules initiate and maintain inflammation within the vessel wall leading to the development of CVD. These areas of low or disturbed flow do not cause atherosclerosis by itself, rather it is the systemic inflammation that leads to the development of this disease. Under conditions of low-flow, circulating leukocytes can adhere to the EC surface, transmigrate across the endothelial layer, and initiate the development of the atherosclerotic lesion[40].

1.2. An Overview of PCBs

Exposure to persistent organic pollutants is a risk factor for the development of cardiovascular disease (reviewed in[11]). Polychlorinated biphenyls (PCBs) consist of two benzene rings with 0-10 chlorines attachment sites with the possibility of 209 congeners (Figure 1.1). Most PCB congeners are colorless, odorless crystals. Mass production of PCBs began in 1929, largely through Monsanto Chemical Company under the brand name Aroclor. PCBs were commercially produced as complex mixtures containing multiple isomers at different degrees of chlorination for a variety of applications, including dielectric fluids for capacitors and transformers, heat transfer fluids, hydraulic fluids, lubricating and cutting oils, and as additives in pesticides, paints, carbonless copy paper, adhesives, sealants, and plastics. Their commercial utility was

based largely on their chemical stability, including low flammability, and desirable physical properties, including electrical insulating properties. Their chemical and physical stability has been responsible for their persistence in the environment, despite their ban in 1979 by the U.S. Environmental Protection Agency (EPA) [41].

As early as 1936, occupational exposure was reported to cause acute toxicity leading to workplace threshold limits set. PCB-contaminated cooking oil caused a total of 1,291 "Yusho" patients in Japan. Symptoms of toxicity included low birth weights, chloracne, and pigmentations[42]. In 1966, Jensen reported PCBs in eagles, herring, and other Swedish environmental samples[43]. PCBs have been shown to be nearly ubiquitous environmental pollutants occurring in most human and animal adipose samples, milk, and sediment. PCBs have entered the environment through use and disposal. Because PCBs do not easily degrade and are lipophilic in nature, they are persistent and bioaccumulate. Human exposure to PCBs occurs primarily through low-level food contamination[44,45]. PCBs have been found in nearly all marine plant and animal species including fish, mammals, birds, and humans. Highly PCB-contaminated populations include Native American tribes, communities on Forae Islands[46], and Canadian Inuits[47]. There are several reports of occupational exposure as well as documented cases within communities such as the Monsanto plant in Anniston, AL[48] and the upper Hudson River, NY[49]. Additionally, two cases of accidental ingestion from rice oil were reported, the previously mentioned Yusho incident as well as Yu-Cheng in Taiwan in 1979[50].

PCB levels in human plasma resulting from exposure vary with low ppb concentrations found in the general U.S. population[51]. Yu-Cheng patients in Taiwan were documented to have 99 ppb of PCBs in plasma[50] with levels as high as 1 ppm (3 orders of magnitude difference) after occupational exposure[52].Canadian Inuits, who consume large quantities of fish within their diet have 3.4 fold higher levels of PCBs within their plasma than the U.S. population[53]. High risk groups within these populations include breast-fed children which have been documented to have negative cognitive functions from exposure[54,55].

While acute toxicity of PCBs is a rare phenomenon and limited to symptoms including chloracne[56], a large number of scientific studies (>10,000 published[44]) have linked PCB exposure to chronic disease in humans. PCBs contribute to a variety of pathological conditions within humans. PCBs can act as initiators and promoters of carcinogenesis[57], specifically the congener PCB 3[58,59]. PCBs act as endocrine

disrupters and can alter metabolic processes regulated through the thyroid gland[60]. In addition, developmental exposure to PCBs can result in cognitive dysfunction later in life[61,62].

PCBs can be further classified based on their stereochemistry which affects their biological functions. There are three groups of PCB congeners including non-ortho-substituted coplanar PCBs (e.g. PCB 77 and PCB 126), ortho-substituted non-coplanar PCBs (e.g. PCB 104 and PCB 153) and mixed-inducers with one or two chlorines in ortho positions (e.g. PCB 118)[63]. This dissertation work focuses on the coplanar PCB77, which is an agonist of the aryl hydrocarbon receptor (AhR). The toxicity of the coplanar PCBs correlates with their binding affinity for the AhR which has led to the assignment of a Toxic Equivalency Factor (TEF) for each of the coplanar PCBs. TEF expresses toxicity relative to the most potent AhR ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)[64]. AhR is a cytosolic transcription factor with a basic helix-loop-helix structure that is bound to chaperone proteins including a dimer of heat shock protein 90 (hsp90), and the hepatitis B virus-associated protein 2 (XAP2)[65]. Upon ligand binding (e.g. PCB 77), AhR dissociates from its chaperone proteins and translocates into the nucleus forming a dimer with the AhR nuclear translocator (ARNT)[66]. The newly formed AhR/ARNT complex binds to AhR-response elements (DREs) located in the promoter region of responsive genes, thus serving to modify transcription of targeted genes[66].

There are several targets of the AhR including a family of CYP enzymes and other phase II enzymes such as uridine 5'diphosphoglucuronosyltransferase (UGT) 1A1, and glutathione (GSH)-S-transferase[67]. These gene targets play a role in the detoxification process of hydrophobic compounds by adding an epoxide molecule or other hydrophilic moiety to aid in metabolism[41]. Because AhR upregulates the family of CYP enzymes, this explains some of the toxicity from PCB 77 exposure. It has been shown that upregulation of CYP1A1 by AhR due to PCB 77 leads to its uncoupling, resulting in the production of Reactive Oxygen Species (ROS)[68]. Phase I metabolism of PCB77 forms catechol and hydroquinones which are then converted to quinones by cellular peroxidases[69], which can further contribute to cellular oxidative stress[70]. These reactive intermediates can directly bind to DNA[71] and proteins[72] which may explain some of the toxic effects of PCBs.

1.2.1 Polychlorinated Biphenyls Contribute to Cardiovascular Disease

PCB exposure can lead to the development of CVD. Swedish capacitor workers had increased rates of cardiovascular mortality[73]. In the female population, a National Health and Nutrition Examination Survey (NHANES) demonstrated an association between plasma PCB levels and cardiovascular disease[74]. Within the Yusho patients, elevated levels of total blood cholesterol and triglycerides were reported[50]. A recent report in *Hypertension* revealed that residents in Anniston, AL had increased rates of hypertension, a known risk factor for cardiovascular disease[48]. Exposure to PCBs in human studies has also demonstrated elevated cardiovascular disease risk factors including insulin resistance[75], metabolic syndrome[76], and diabetes[77].

Several mechanisms have been suggested to explain the increased rates of cardiovascular diseases induced by PCB exposure. Yusho patients had elevated plasma cholesterol[78], and similar effects have been reported in animal models after exposure to coplanar PCBs[79,80] including findings reported within this dissertation. Our laboratory has previously shown that PCB 77exposure reduced liver expression of CYP7A1[81], the rate-limiting enzyme in the synthesis of bile acid from cholesterol, which could explain the elevated cholesterol levels in the plasma. Additionally, coplanar PCBs promote adipocyte differentiation as well as upregulating proinflammatory mediators including MCP-1 and TNF-α, thus contributing to the development of obesity and associated inflammation[82] .

However, our laboratory and others have focused on the mechanism of PCB-induced endothelial dysfunction[83,84]. Specifically coplanar PCBs, including PCB 77 bind the AhR leading to upregulation of CYP1A1 within the vasculature[85]. PCB 77 uncouples CYP1A1 increasing ROS production which activates redox sensitive transcription factors NFκB and AP-1. Activation of NFκB can upregulate cellular adhesion molecules including VCAM-1[83,86]. It should be noted that activation of NFκB and AhR through PCB 77 can be reversed with co-treatment of AhR antagonists as well as antioxidants[87,88]. Coplanar PCBs have been shown to disrupt the endothelial barrier[83] and even induce endothelial apoptosis[89]. PCB 77 can phosphorylate eNOS, leading to formation of peroxynitrite ($ONOO^-$) which further increases oxidative stress and formation of nitrotyrosine[90]. Additionally, previous work in our laboratory has investigated the role of caveolae in PCB-induced endothelial dysfunction. Caveolae are a type of lipid raft found in endothelial cells that play a role in signal transduction[91]. PCB 77 exposure increased the formation of caveolae and caveolin-1 protein levels in endothelial cells and PCB 77

was found in the caveolae-rich fraction[92]. This data suggest that caveolae could be a major entry for PCBs into the endothelial cell which can lead to activation of downstream signaling pathways. Cav-1 has been shown to bind AhR, but deletion of Cav-1 prevents upregulation of CYP1A1 and subsequent ROS production as well as preventing downstream activation of NFκB[92]. Furthermore, PCB 77 phosphorylation of eNOS was Cav-1 dependent[90].

1.2.2. Modulation of Polychlorinated biphenyl-induced cardiovascular toxicity through nutrition

An accumulating body of evidence within the scientific literature implicates the role of nutrition in the pathology of atherosclerosis. While a positive energy intake can lead to obesity, an independent risk factor for atherosclerosis[93], specific dietary compounds can modulate cellular signaling in either pro- or anti-atherogenic ways. A diet rich in polyphenols found in fruits and vegetables as well as fish oil have been shown to reduce cardiovascular mortality and reduce the risk of developing cardiovascular disease[94,95]. Extensive studies have demonstrated omega-3 fatty acids, which are a type of polyunsaturated fatty acid (PUFA), to be anti-inflammatory compared to saturated and trans fatty acids which tend to increase cardiovascular risk factors because of their inflammatory nature[96]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are two omega-3 fatty acids primarily found in fish with high anti-inflammatory properties. A large number of epidemiological studies have shown that a diet rich in the consumption of fish or fish oil reduces the risk for cardiovascular disease[97].

Nutrition as a modulator of chronic disease accelerated by environmental pollutant exposure is an exciting area within the field[98]. A majority of these studies have been conducted with PCB being the pollutant studied[99]. Epigallocatechin-3-gallate (EGCG), a compound found in green tea, reduced PCB 77-induced CYP1A1 upregulation while reducing the generation of ROS[87]. EGCG acts as an AhR antagonist by binding to its chaperone protein, hsp90[100]. Dietary antioxidants e.g. vitamin E can reduce PCB-induced activation of NFκB and its subsequent pro-inflammatory signaling[88].

Specifically, our lab has demonstrated that certain polyphenols found in fruits and vegetables can attenuate the PCB-induced pro-inflammatory signaling within cultured endothelial cells. Co-treatment with quercetin prevented PCB 77 upregulation of

CYP1A1 as well as VCAM-1 and E-selectin[101]. Recently, EGCG was shown to protect endothelial cells against PCB 126 induced-inflammation by inhibiting AhR and its associated proinflammatory signaling while upregulating the antioxidant transcription factor, NF-E2-related factor 2 (Nrf2)[102]. Nrf2 is a transcription factor that upregulates phase II enzymes including glutathione S transferase and NAD(P)H: quinone oxidoreductase 1(NQO1)[103]. Additional studies from our laboratory provide evidence that the type of dietary fat can modulate the level of inflammation[104]. Diet enriched with linoleic acid, an omega-6 fatty acid, exacerbated expression of adhesion molecules in the aorta; however, diet enriched in olive oil attenuated this response[104]. Other studies have examined the role of olestra, a sucrose polyester that is non-absorbable and has been shown to decrease the absorption of PCB 77[105]. Fish oil contains a rich source of omega-3 fatty acids including DHA and EPA; oxidized DHA was shown to ameliorate PCB 77 induced proinflammatory signaling by upregulating Nrf2-mediated signaling[106].

Although there is no quick fix to protect against diseases associated with exposure to environmental pollutants including coplanar PCBs, there is strong evidence for the role of nutrition in providing protection against chronic disease associated with environmental toxic insult. However, additional studies are needed in particular epidemiological studies that can demonstrate nutrient protection in human while providing recommendations for an effective dose of nutrient(s). Furthermore, additional studies examining the effects of lifestyle modifications including physical activity on the outcome of chronic disease related to chemical exposure should be included. The second half of this chapter will describe the cardioprotective properties of exercise leading up to the rationale for this dissertation work.

1.3. Exercise

Physical activity is defined as “any bodily movement produced by skeletal muscles that results in energy expenditure beyond resting expenditure.” Exercise is a subset of physical activity that is planned for the purpose of improving one’s physical fitness. The American College of Sports Medicine defines exercise as “Any and all activity involving generation of force by the activated muscles that result in disruption of a homeostatic state”[107]. Exercise can be classified by the type, intensity, and duration of activity. Endurance exercise reflects extended and continuous periods of contractile activity against low resistance compared with resistance exercise (or strength training)

which involves short periods of contractile activity against a high resistance[108]. It has been shown that protection from cardiovascular disease occurs in those who are regularly physically active, however, beginning an exercise program at any age will improve cardiovascular health[109]. Because daily physical activity is considered to be an effective component in preventing cardiovascular disease, most cardiologists prescribe physical activity regimens into primary and secondary prevention programs[110]. Clinical trials have demonstrated that patients who have had a cardiac event but undergo cardiac rehabilitation will have decreased rates of mortality[111–113]. Daily physical aerobic activity is considered an effective prescription in preventing one's risk for cardiovascular disease[10]. It has been well-established that exercise protects against cardiovascular disease, but the complex set of metabolic pathways, hemodynamic effects of exercise on vasculature, and the regulation of genetic expression activated by exercise are not fully elucidated.

1.3.1. The Vascular Response during Acute Exercise

During resting conditions, the heart provides enough blood or cardiac output to sustain basal metabolic needs while central cardiovascular reflex systems maintain blood pressure. Within each tissue, a network of resistance arteries control the amount of resistance needed in order to receive adequate blood supply to meet metabolic needs of that tissue. Within the resistance arteries, vascular smooth muscle (VSM) controls the level of contraction. VSM are also influenced by sympathetic nerve innervation as well as local factors, thus establishing tone and integrating the many inputs that regulate constriction[114].

Initiation of muscle contraction during exercise increases the body's requirement for nutrients and oxygen. Within the skeletal muscle, vascular control mechanisms work in a linear fashion to increase blood flow, which meets this increased metabolic demand. Central control processes also lead to a linear increase in heart rate and cardiac output to match the needs of the contracting skeletal muscle[115]. The increase in cardiac output is supplied by an enhanced venous return which is due to a decrease in visceral blood flow as well as the phenomenon of the muscle pump[116]. The muscle pump refers to the compressive effects of muscle contraction on veins within the contracting skeletal muscle. Vascular resistance is the main control mechanism for blood flow during exercise, which is controlled at the level of vascular smooth muscle cells in resistance arteries of the muscle tissue[117]. There are complex interactions between

vasoconstricting and vasorelaxing factors in the VSM of resistance arteries in active skeletal muscles[118]. The net effect is that central sympathetic stimulation is counterbalanced by an overall decrease in total peripheral resistance of the skeletal muscle e.g. even a 5-fold increase in cardiac output during rigorous exercise mildly increases mean arterial pressure[114].

The local control of blood flow during exercise is mediated by the release of metabolites from the active muscle, the mechanical stimulation of arteries supplying the muscle, vasodilation within the arterial tree, and paracrine signaling from RBCs and endothelial cells[119]. Changes in intravascular pressure during exercise result in the myogenic response. The myogenic response occurs when arterioles dilate passively with increasing pressure until a myogenic constriction occurs (range from 20-120 mmHg)[120]. If pressure continues to increase, the vessel wall will continue to dilate. Relaxation of VSM can also occur from capillaries releasing vasodilators locally to increase their blood flow[120]. An important role of endothelial cells is maintaining vascular tone in large and small arteries by releasing vasconstricting mediators (e.g. endothelin-1) and vasodilating substances (e.g. nitric oxide and prostacyclin). In a healthy individual, the primary effect of endothelial cells appears to be endothelium-dependent dilation (EDD) induced by ligands such as Ach and intraluminal flow-mediated dilation (FMD) of arteries in response to increased blood flow during exercise[121]. The enhanced relaxation of VSM during exercise is due to the release of NO from the endothelium. ECs experience shear stress from the increase in blood flow during exercise which causes NO release from the phosphorylation of eNOS by Akt. The diffusion of NO from the ECs to VSM activates cGMP which induces vasodilation by inhibiting calcium entry into the cell[122].

At the central level, blood flow is controlled by cardiac output and vascular resistance via the autonomic nervous system. Neurons involved in the central control of blood flow are from both sympathetic and parasympathetic systems. Parasympathetic control regulates heart rate while sympathetic effects regulate contractility, vascular resistance and venous compliance through efferent neurons which innervate the vasculature, heart, renal system, and adrenal medulla[123]. At maximal exercise, vasodilation at the active muscle is centrally controlled to prevent overwhelming the heart. A separate, central control of blood flow is necessary to prevent hypotension which could occur from an overly vasodilatory response.

1.3.2. Vascular response to chronic exercise

It has been shown in multiple animal and human models that chronic exposure to physical activity results in improved cardiovascular function through increased maximal oxygen consumption, increased maximal cardiac output, and increased blood flow capacity in skeletal and cardiac muscle. In relation to the prevention of cardiovascular disease, there is a strong argument that exercise is a natural "anti-atherogenic" activity by reducing the risk of certain risk factors including hyperlipidemia, glucose intolerance, and obesity. The molecular pathways responsible for the "anti-atherogenic" characteristics of physical activity are just beginning to be revealed. Although chronic exposure to exercise can influence the molecular pathways of several organ systems, this section will focus on the current findings within the vascular endothelium.

1.3.3. Exercise Reduces Cardiovascular Risk Factors

Over the past thirty years, there has been a rapid increase in caloric intake and sedentary lifestyle in developed countries. As a result of these changes, there has been a parallel increase in cardiovascular disease and metabolic disorders including obesity, type 2 diabetes, and metabolic syndrome. A sedentary lifestyle has been shown to be an independent risk factor for these metabolic disorders that lead to atherosclerosis and cardiovascular disease.[124] Pedersen hypothesizes that physical inactivity leads to an accumulation of body fat within the viscera that activates proinflammatory signaling which promotes the development of these metabolic disorders, described as the "the diseasome of physical inactivity."[125] On the other hand there is a plethora of scientific evidence demonstrating that physical activity reduces and prevents atherosclerotic risk factors as well as the disease itself.

1.3.3.1. Metabolic Disorders

During the late-Paleolithic era (50,000-10,000 BC), hunter-gatherers or ancestors of the human race depended on physical activity to obtain food or survive during hostile encounters. It has been suggested that ancient hunter-gatherers had cycles of feast and famine that required large bouts of physical activity to obtain food because the food supply was inconsistent. Thus, it is proposed that the human genome evolved to ensure that metabolic advantage for these oscillating conditions. A thrifty gene adapted in order to ensure adequate storage of fuel during times of famine including glycogen

conservation and gluconeogenesis while glycogen storage, triglyceride synthesis, and carbohydrate oxidation would predominate during times of feast. These metabolic adaptations enabled our ancestors to hunt for food by performing intense bouts of physical activity during times of famine.[126] Since the late-Paleolithic era, several revolutions (Neolithic, industrial, and telecommunication) have led to alterations within the human environment. Since the latter half of the 20th century, a dramatic increase in sedentary lifestyle has occurred with television viewing, video gaming and employment involving sitting in front of a computer becoming the major activities of society[127]. Furthermore, the food supply is plentiful. These societal changes (sedentary lifestyle and plentiful food supply) have occurred too rapidly for the human genome to adapt. The human genome has remained relatively stable since the arrival of Homo sapiens, suggesting that our genome was not selected for physical inactivity.[128] Based on hypotheses from Booth and Chakravarthy, the combination of physical inactivity and plentiful food supply has led to metabolic storage disorders since certain metabolic genes continue to store fuel with it never being utilized through physical activity[126].

Scientific evidence suggests that metabolism can be maintained without modifying dietary restriction if physical activity is performed to match caloric intake. However, when daily steps are reduced for 2-3 weeks in healthy males, insulin sensitivity decreases and abdominal fat increases suggesting that calories used to maintain muscle mass were partitioned to visceral fat[129]. Previous work has shown that whole body insulin-sensitivity decreases rapidly in athletes when aerobic training ceases or detraining occurs in just 7 days.[130] These studies demonstrate the negative metabolic consequences when physical activity is removed, which makes exercise appear to be a natural regulator of insulin sensitivity. Wild animals do not exhibit metabolic disorders because their physical activity is maintained. In experimental studies, rodents will voluntarily run when placed in a cage with a running wheel; however, when the wheel is removed epididymal fat and abdominal fat mass rapidly increase.[131] In a separate study, cessation of running led to a decrease in insulin sensitivity by reducing expression of the insulin receptor and GLUT4 in skeletal muscle.[132] Collectively, these studies suggest that low physical activity levels can elicit an undesirable metabolic phenotype characterized by reduced insulin sensitivity with subsequent increases in body fat and triacylglycerol levels that lead to the development of cardiovascular disease. A growing body of evidence shows a direct link between newly adopted sedentary activities such

as television watching and risk for metabolic and cardiovascular risk factors in both adolescents and adults[133–135].

1.3.3.2. Insulin Resistance and Glucose Intolerance

Physical activity has been shown to reduce insulin resistance and glucose intolerance in numerous studies. Additionally a review examined 8 studies in patients with type II diabetes who underwent an exercise training program between 12-18 months found that participants lowered their hemoglobin A1c by 0.5-1% on average[136]. The Diabetes Prevention Program has demonstrated the profound effects of exercise compared to metformin in reducing the incidence of type II diabetes. The lifestyle intervention which had participants perform 150 minutes of physical activity each week over 2.8 years had a 58% reduction in incidence compared to 31% in metformin group[137]. The Old Order Amish community, which is a conservative Christian sect, has a low prevalence of type II diabetes. Recent studies have attributed this phenomenon to the fact that the Old Order Amish maintain a much more physically active lifestyle compared to non-Amish, communities within the United States[138,139].

1.3.3.3. Blood Pressure

More than 40 randomized controlled trials including 2,674 participants have examined the effect of exercise on resting blood pressure[140]. The average reduction for systolic was 3.4 mmHg and 2.4 mmHg for diastolic. Vigorous exercise has been shown to acutely reduce systolic blood pressure for up to 12 hours[141]. These studies have not found a relationship between training frequency, duration, or intensity with reduction in blood pressure, suggesting that there is not a dose response curve for exercise and blood pressure; however, exercise may be only therapy needed for mildly hypertensive patients[142].

1.3.3.4. Lipoproteins

The association between serum cholesterol and CVD is well established within the literature, therefore, reductions in serum lipoproteins that promote atherosclerosis are thought to be protective and minimize risk. Low-density lipoprotein (LDL) and apolipoprotein B have been correlated with cardiovascular disease and related events

while HDL has been correlated with risk reduction. A meta-analysis of 52 aerobic exercise training studies lasting >3 months demonstrated an average 5.0% reduction in LDL-C and a 4.6% increase in HDL-C; however, specific dose response relationships could not be established[143]. Within the HERITAGE (Health, Risk Factors, Exercise Training, and Genetics) study, males with low HDL that participated in 5 months of aerobic training demonstrated an increase in HDL (4.9%)[144]. Several groups have reported increases in HDL (e.g. 0.008 mmol/L per mile of running each week[145]) with moderate intensity exercise training[146,147].

1.3.3.5. Exercise Reduces Atherosclerotic Plaque

Recently, studies have begun to assess the effect of exercise on the development and progression of atherosclerosis in humans. Measurement of intima-media thickness (IMT) of the common carotid artery is a common technique used to quantify generalized atherosclerosis[148]. A meta-analysis reviewing the current literature on exercise and carotid intima-media thickness demonstrates that physical inactivity is associated with increased carotid IMT[149]. Hambrecht et al found that leisure physical activity in excess of 1500 kcal/week inhibited the progression of coronary atherosclerotic lesions measured by angiography in CAD patients[150]. Regression of lesions was observed in patients expending greater than 2200 kcal/week. Similarly, another group found a significant regression of coronary atherosclerotic lesions in seven out of eighteen patients with angina who followed a 1 year intervention of exercise[151]. Rauramaa et al. conducted a 6 year random controlled trial examining the effect of exercise on atherosclerosis through the measurement of carotid artery IMT (intima-media thickness). Although the progression of carotid IMT did not differ between the control and intervention groups, a subgroup of patients not taking statins had a 40% reduction in progression in the exercise group[152]. Although these results are promising, it should be noted that coronary angiography and IMT measurements do not provide a comprehensive measure of atherosclerotic plaque burden.

Because accurate assessments of coronary plaque composition and plaque burden remain a challenge, autopsy studies in animal models of cardiovascular disease provide a more thorough portrayal of the development and progression of atherosclerosis. The effect of exercise training on atherosclerosis has been directly measured post-mortem in a variety of animal models including monkeys, pigs, rabbits,

rats, and mice. In the majority of studies, regardless of the animal model tested, animals in the exercise group develop less aortic and/or coronary atherosclerosis than their sedentary counterparts[153]. For example, a long-term exercise study utilizing pigs found that 22 months of treadmill exercise reduced diet-induced atherosclerosis in both the aorta and coronary arteries approximately 10 fold compared with sedentary animals[154]. However, there have been a few studies reporting discrepancies in results. Williams et al. reported no effect of exercise training on the extent of coronary artery atherosclerosis in monkeys, despite improvements in other cardiovascular risk factors including improved left ventricular ejection fractions and improved vasodilation of coronary arteries[155]. A short term exercise study (8 weeks) utilizing pigs reported no change in atherosclerosis, however, this could be due to the duration of the study.[156]

Mice are increasingly being utilized in atherosclerotic studies due to the development of transgenic strains including LDLr-/- and ApoE-/- which develop atherosclerosis at an accelerated rate[157]. More than twenty published studies have examined the effect of exercise training in mouse models of atherosclerosis. Among these studies, regardless of exercise intervention utilized (swimming[158–163], treadmill running[164–170], or voluntary running[171–177]) each of these studies reported a decrease in atherosclerosis following exercise. The implications of each of these studies is profound in that numerous mechanisms were identified that show how exercise exerts its protective effects. These potential mechanisms will be discussed below.

1.3.3.6. Exercise promotes plaque stability

Rupture of atherosclerotic plaque leads to acute myocardial infarction and stroke, two dramatic clinical events that often lead to mortality. In order for plaques to rupture, they must become vulnerable. A plaque is defined as vulnerable when the fibrous cap thins, the smooth muscle content is low while the lipid core is enlarged (>50% surface area), and inflammatory cells have accumulated within the lesion.77 Agents that promote plaque stabilization are attractive therapeutic strategies in the prevention of cardiovascular events. In ApoE-/- mice, swimming for six months led to a stabilization of plaque as evidenced by decreased macrophage content, increased smooth muscle cell content, thicker cap, and lack of adventitia inflammation[159]. In another study that utilized ApoE-/- mice and treadmill running, plaque stabilization occurred with increased collagen content, decreased macrophage and matrix metalloproteinase-2 content, and

increased fibrous cap thickness[178]. Stabilization of plaque composition through exercise is a desirable option as it could lead to a decrease in cardiovascular mortality.

1.4. Potential Mechanisms of Exercise that Protect Against Atherosclerosis: An Implication for the Vascular Endothelium

As mentioned above, there is a plethora of clinical and experimental studies that provide evidence for the cardiovascular protection that exercise provides. These protective mechanisms include modulating the lipid profile, enhancing carbohydrate metabolism and insulin sensitivity, reducing blood pressure, and reducing adipose stores. Chemical signals (e.g. cytokines, metabolites released from muscles, humoral factors) and hemodynamic signals acting on the endothelium (e.g. shear stress, blood pressure and stretch, circumferential stress) are likely candidates that modify endothelial cell (EC) gene expression during bouts of exercise. There is a growing body of evidence that repeated exercise imposes shear stress and stretch on the artery wall can initiate altered gene expression in ECs. A group of exercise physiologists would argue that these gene expression changes induced by exercise training are the "normal" human EC phenotype from our previous history of being hunters and gatherers[124]. This "normal" phenotype is crucial to the maintenance of vascular tone, preventing VSM proliferation and migration seen in atherosclerosis, inhibiting inflammation, and preventing the pro-thrombytic state seen in advanced cardiovascular disease[179]

1.4.1. Shear Stress

The endothelium is exposed to hemodynamic forces including shear stress. Shear stress is an important stimulus as it has been shown to induce gene expression changes involved in NO production, vascular remodeling, and angiogenesis[34]. The increased shear stress at the endothelial surface can result in distortion of the EC monolayer likely due to cytoskeleton rearrangement. Steady laminar but not oscillatory flow increases Na-K-Cl co-transporter mRNA in cultured ECs and also increases the conductance of K+ and Cl- channels, ultimately leading to vasodilation.[180] The authors propose that this transporter may act as a flow-sensor by upregulating transcription of co-transporter proteins. Steady rhythmic flow has impacts on the endothelial lipid bilayer and increases its fluidity. Acting as a transducer, lipid components of the membrane can discriminate changes in shear which initiates MAPK activation in the cell. Prolonged laminar shear stress induced anti-inflammatory gene expression through the

activation of Kruppel-like factor 2 and nuclear factor erythroid-2, which induce transcription of the antioxidant heme oxygenase 1 and eNOS.[181] Other antioxidant genes upregulated by laminar shear stress include superoxide dismutase (SOD), glutathione peroxidase (GPx)[182].

Laminar blood flow from exercise regulates the orientation of EC lining the blood vessels and influences processes such as angiogenesis. These signals from shear stress move through the cytoskeleton to the intimal region of the basal endothelial surface. Integrins are then phosphorylated and activate a multiple complex of non-receptor kinases (FAK, c-Src, Shc, paxillin, and p130CAS) which along with their adaptor proteins (Grb2, Crk) and guanine nucleotide exchange factors (Sos, C3G) activate Ras family.[183] Active Ras then activates Mitogen-activated protein kinase (MAPK) which results in shear stress-induced upregulation of atheroprotective genes that send anti-apoptotic and anti-proliferative signals, by increasing vascular NO bioavailability and vascular remodeling[184]. In contrast, in regions with low and disturbed flow (due to sedentary lifestyle or vessel shape) the atheroprotective genes are suppressed while pro-atherogenic genes including c-Jun NH2-terminal kinases are upregulated, thus promoting atherosclerosis.[185] The vasodilation and platelet inhibition of NO and prostacyclin have been studied extensively. Reduced NO and prostacyclin levels can result in endothelial dysfunction, an initiating step in atherosclerosis[186].

1.4.2. Nitric Oxide

Nitric oxide (NO) is a heterodiatomic free radical generated by oxidation of L-arginine to L-citrulline by the enzyme, Nitric Oxide Synthase (NOS). NO has an important role in the regulation of vascular tone, the inhibition of platelet aggregation, and the control of adhesion molecules[187,188]. In vessels with atherosclerotic lesions, there is a reduction of NO bioavailability which is associated with vasoconstriction, platelet adherence and aggregation, leukocyte adherence to the vascular wall, and increased proliferation of VSMC[189]. NO bioavailability can be indirectly measured by the degree of endothelium-dependent dilation (EDD)[190–193]. It has been more than 20 years since it was described that chronic exercise enhances EDD in canine coronary arteries[194]. This phenomenon has been confirmed many times in a variety of clinical and experimental models (reviewed in [195]).

Exercise training, especially aerobic training, has been shown to improve impaired EDD suggesting an increased bioavailability of NO. Regular exercise has also

been shown to improve endothelial function in patients with CAD[196,197] as well as heart failure[198]. Specifically a 4 week aerobic exercise intervention improved EDD by 54% in patients with CAD[199]. An important study by Hambrecht reported that exercise increased eNOS mRNA expression 2-fold with a 3.2 increase in phosphorylation of eNOS on serine 1177 residue after 4 weeks of regular exercise training in CAD patients[150]. This study suggests that exercise led to an increase in enzymatic activity of eNOS and increased bioavailability of NO since EDD improved.[190]

The increase bioavailability of NO from exercise could result from increased expression of eNOS or prevented degradation of NO by reduction of ROS. Studies utilizing cultured endothelial cells suggest that shear stress increases eNOS expression and activity[200,201]. Further evidence for the role of NO in exercise capacity was discovered in studies utilizing eNOS-/- mice. Running capacity in eNOS-/- is diminished (50-60% less) than age-matched controls[202]. Other studies have shown that the beneficial effects of exercise are negated when one allele is knocked out, suggesting that full eNOS expression is required[203,204]. Additionally, regular exercise has been shown to increase the antioxidant defenses, thus reducing NO degradation[192]. Production of NO by the endothelium can influence expression of antioxidant enzymes and cellular inflammation in vascular tissue.[200] This increase in expression of antioxidant enzymes including superoxide dismutase and glutathione peroxidase, could reduce the NO degradation by reducing the levels of ROS. This increase in antioxidant enzymes and eNOS seems to result from repetitive exposure to increased laminar shear stress during acute bouts of training[114]. In summary, regular exercise increases blood flow and subsequent shear stress which improves NO bioavailability and increases EDD.

1.4.3. Exercise Reduces Expression of Cellular Adhesion Molecules

The activation of endothelial cells by cytokines, oxidized LDL, and ROS can induce the endothelial expression of cellular adhesion molecules including ICAM-1, VCAM-1, E-selectin, and P-selectin which play a major role in the recruitment of leukocytes to the vessel wall[2]. These molecules can be measured in the circulation as soluble adhesion molecules since they are released into the bloodstream and are considered to be important markers of endothelial cell dysfunction and inflammation[205]. In cardiac patients, a 2 week exercise training intervention led to reduced circulating levels of ICAM-1[206]. In patients with heart failure, a 12 week exercise training program decreased the circulating levels of soluble ICAM-1, VCAM-1[207], while a 20 week aerobic

exercise training program decreased circulating levels of P-selectin[208]. Animal experiments support this finding as well. Rabbits underwent exercise training on a treadmill, 5 days per week for 8 weeks and had a significant decrease in circulating levels of P-selectin and VCAM-1[209]. By reducing soluble adhesion molecule expression most likely due to changes in shear stress and downregulation of other proinflammatory cytokines, exercise training may be considered an effective non-pharmacological intervention to reduce endothelial adhesiveness.

1.4.4. Endothelial Progenitor Cells

Endothelial function is dependent on the endothelium's ability to repair after injury. Endothelial progenitor cells (EPC) are circulating bone marrow-derived stem cells that have the capability to differentiate into mature endothelial cells[210]. EPC are mobilized from the bone marrow into general circulation to assist in regeneration of damaged endothelium[211]. Exercise training has been considered one of the most effective interventions in stimulating EPCs from bone marrow[212]. In human studies, both healthy and CAD patients have increased the number of circulating EPCs after an exercise intervention. A 280% increase in circulating EPCs was reported in one study[213]. In an ApoE-/- model, it was found that exercise increased circulating levels of EPCs as well[172]. Current literature supports exercise as an effective therapy in enhancing endothelial regenerative capacity.

1.4.5. Exercise is Anti-inflammatory

Atherosclerosis is recognized as a chronic inflammatory disorder. Because systemic markers of inflammation are elevated in the plasma in atherosclerosis, reducing their levels through exercise or another pharmaceutical therapies (e.g. statins) may be of interest in treating disease. The pathogenesis of atherosclerosis involves several cytokines including IL-1, -4, -6, -8, -10) and macrophage-associated cytokines including TNF-a, IFN-y, and colony stimulating factors[214]. Cytokines are further categorized into pro-inflammatory or anti-inflammatory. The pro-inflammatory cytokines have several roles including the induction of other cytokines and chemokines, the expression of adhesion molecules on EC, the stimulation of cell proliferation and differentiation, the release of matrix-degrading enzymes, and the regulation of the acute-phase reaction[214]. Anti-inflammatory cytokines inhibit pro-inflammatory responses, thus having an atheroprotective role.

The anti-inflammatory effect of exercise has been well-established in human and animal models (reviewed in[215]). During acute exercise, skeletal muscle releases IL-6 which recruits IL-10 and other anti-inflammatory mediators while proinflammatory cytokines such as IL-1 and TNF-a are not increased[216]. Several studies have demonstrated chronic exercise training reduces vascular wall inflammation while increasing levels of IL-10, and reducing levels of proinflammatory cytokines[217–220]. Consistent with these findings, another study reported a 58% reduction in the production of the pro-inflammatory cytokines IFN-y, TNF-a, and IL-1B in cultured mononuclear cells as well as circulating levels of CRP from patients who underwent a 6 month intervention of exercise training[221].

C-reactive protein is an acute phase protein produced by the liver in response to inflammatory cytokines including IL-6[222]. CRP is a biomarker used by clinicians to indicate a person's chronic vascular inflammation[223]. There is evidence that CRP is a proinflammatory mediator that contributes to atherosclerosis by increasing LDL uptake by macrophages[224], increased expression of MCP-1 and cellular adhesion molecules[225], and decreased production of NO by endothelial cells[226]. Several prospective studies have examined the effects of exercise training on CRP level and suggest an anti-inflammatory effect; however, none of these studies were RCTs. In a RCT study, 101 patients with stable CAD were assigned to percutaneous intervention with a stent or to aerobic exercise training. After 24 months of training, there was a 41% reduction in CRP levels[217]. These results were independent from statin therapy In a different study, patients underwent 7 weeks of aerobic physical activity, but the 17.4% reduction in CRP failed to reach statistical significance[227]. These studies suggest that duration of intervention is important to the reduction of inflammation. It has been suggested that exercise reduces CRP levels because of its overall effect on cytokine production (primarily reductions in IL-1 and TNFa[228]). Additionally, reductions in obesity lead to a decrease in adipocyte production of proinflammatory cytokines[229]. The decreased production of cytokines in other tissues could be a potential mechanism as well.

1.4.6. Exercise Increases the Antioxidant Defense System

Under physiological conditions, ROS is removed by the cellular antioxidant systems which include antioxidant vitamins, protein and non-protein thiols, and antioxidant enzymes. Acute aerobic and anaerobic exercise increase vascular oxidative stress and damage cellular proteins, lipids, and nucleic acids. In 1982, Davies et al.

demonstrated that rat skeletal muscle produced free radical after exhaustive exercise[230]. A small study of Tour de France participants were found to have tissue damage induced by xanthine oxidase (XO) from exhaustive exercise[231]. This data was confirmed in marathon runners and demonstrates that XO is a source of free radicals during aerobic exercise. Although high levels of oxidative stress are damaging to cellular function, "mild" levels of oxidative stress such as those produced during endurance exercise, may be beneficial for cellular adaptation. Low to moderate amounts of ROS produced during endurance exercise may be described as a "hormesis," which is characterized by a dose-response relationship in which a low dose is stimulatory and a high dose is inhibitory[232]. In this context, free radicals can be seen as beneficial since they act as signals to increase the antioxidative and defense systems.

Animals exposed to chronic training show less oxidative damage after exhaustive exercise compared to trained animals. This protection occurs because endogenous antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase, and gamma-glutamylcysteine synthetase (GCS) are upregulated[233]. Another study in Yucatan miniature pigs reported upregulation of SOD1 in the aortic endothelium following exercise training[234]. In a study with C57Bl/6 mice , 3 weeks of exercise training had a 3-fold increase in SOD-3[192]. Upregulation of vascular SOD by exercise provides an efficient way to detoxify superoxide and reduces the generation of peroxynitrite, a potent ROS formed from NO and superoxide[184]. A recent clinical trial examined the effects of exercise training in male patients with stable CAD. This study found a significant reduction in NADPH oxidase which is a ROS-producing enzyme. These changes in gene expression were accompanied with a reduced generation of ROS and an improvement in EDD. These results suggest that exercise training increases vascular levels of eNOS and SOD1 while decreasing levels of pro-oxidant enzymes including NOX[235]. Exercise training initially increases levels of oxidative stress but contributes to beneficial changes in vascular gene expression observed after several weeks of exercise training.

There is a growing body of evidence that antioxidant supplementation prevents the hormesis effect of aerobic exercise. In 1971, it was reported that vitamin E reduced athletic performance in swimmers[236]. In triathletes receiving antioxidant supplementation of coenzyme Q10, Vitamin C and E, there were no improvements on maximal oxygen uptake, muscle fatigue, or muscle energy metabolism[237]. In runners receiving Vitamin C supplementation, delayed muscle onset soreness (DOMS) persisted failed to decrease

the recovery process[238]. Within marathon runners, supplementation of Vitamin C decreased improvements in running capacity[239]. In a study utilizing LDLr-/- mice, supplementation with Vitamin E prevented the beneficial effects of exercise by preventing upregulation of catalase and eNOS which led to increased atherosclerotic lesions[167]. These studies indicate that ROS generated by exercise signal to increase the expression and activity of antioxidant enzymes. Furthermore, supplementation with antioxidants has been shown to blunt these cellular adaptations. Because ROS generated from exercise leads to these adaptations, exercise can be considered as an antioxidant.

1.4.7. Nuclear factor erythroid 2-related factor 2 (Nrf2)

Oxidative stress has been implicated in the development of atherosclerosis, particularly within endothelial cells[240]. While the use of direct antioxidant supplementation has been largely unsuccessful in reducing cardiovascular mortality[241,242], physical activity or bioactive metabolites that activate the antioxidant response within the cell may be a non-invasive strategy for protecting against cardiovascular disease. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that responds to increased oxidative stress within the cell. Nrf2 binds to cis-enhance sequence known as antioxidant-response elements (AREs) in the promoters of target genes[243]. Target genes for Nrf2 include a diverse set of antioxidant enzymes and cytoprotective genes and such as heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase-1 (NQO1), thioredoxin, glutathione metabolism genes including glutathione peroxidase 2 (Gpx2), glutathione S-transferases (GSTs), as well as genes involved in cell survival[244].

Under normal physiological conditions, Nrf2 is located in the cytosol and kept dormant by the inhibitory protein, Kelch-like ECH-associated protein 1 (Keap 1), which is a substrate adaptor protein that associates with cullin3 to form a functional E3 ubiquitin ligase complex, which targets Nrf2 for ubiquitination at the proteasome[245]. In response to oxidative stress or other inducers, Nrf2 dissociates from Keap1, translocates to the nucleus, and binds to antioxidant-response elements (AREs) in the promoter region of target genes. Known Nrf2 inducers include flavonoids (e.g. epigallocatechin gallate and quercetin), stilbenes (e.g. resveratrol and piceatannol), diferuloylmethanes (e.g. curcumin) and organosulfur compounds (e.g. allilcin and diallyl trisulfide) which are considered “xenohormetic” phytochemicals[246].

Nrf2 activation can provide protection against a variety of chemical insults. Nrf2 activation has been considered in chemoprevention because electrophilic insult by environmental carcinogenesis is a hallmark of initiation of cancer. Zerumbone, a sesquiterpene derived from tropical ginger and activator of Nrf2, suppressed chemically induced papilloma formation in mouse skin[247]. Another class of electrophilic phytochemicals include isothiocyanates such as sulforaphane, which has been shown to strongly induce carcinogen detoxifying enzymes through activation of Nrf2[248].

Nrf2 signaling is recognized as an adaptive response to environmental stressors, thus it can provide protection against a variety of toxicants including persistent organic pollutants. PCB exposure leads to proinflammatory signaling which is mediated by increased cellular levels of ROS[83] and can be exacerbated by glutathione depletion[89]. Because Nrf2 activation upregulates several genes involved in glutathione metabolism including gamma-glutamylcysteine synthetase, Nrf2 activation might provide protection from PCB and its deleterious effects on the vasculature. Previous studies from our laboratory have shown a reduction in proinflammatory signaling when endothelial cells[102] or C57BL/6 mice[249] were exposed to EGCG, a known Nrf2 inducer. Oxidized DHA prevents pro-inflammatory signaling in endothelial cells exposed to PCB77 due to Nrf2 activation and induction of NQO1, a downstream target[106]. Additionally, PCB metabolism often produces toxic quinones. NQO1, a downstream target of Nrf2, could provide protection from PCB toxicity by detoxifying quinone metabolites[250].

Although high levels of oxidative stress are damaging to cellular function, “mild” levels of oxidative stress such as those produced during endurance exercise, may be beneficial for cellular adaptation. In fact, when redox signaling is blunted through exogenous antioxidant supplementation, many beneficial exercise adaptations including lesion regression[167] are blunted[239]. Low to moderate amounts of ROS produced during endurance exercise are a part of “hormesis”, or a generally favorable biological response to low exposure to toxins and other stressors. Exercise results in increased levels of oxidative stress, thus upregulating antioxidant enzymes in various tissues (described above), including GSH. In response to exercise-induced oxidative stress, Nrf2 dissociates from Keap1 and translocates to the nucleus, and binds to antioxidant response elemends (AREs). There is a growing body of literature suggesting that Nrf2 activation occurs during exercise[251–254]. Research from animal studies have shown a 56% increase in Nrf2 expression in mice after an acute bout of swimming[255]. Nrf2 levels increased 5-fold in skeletal muscle cells of trained male cyclists after an acute bout of

cycling[256]. Moderate intensity exercise in aged C57BL/6 mice led to increased expression of several Nrf2 target genes including GST, GCLC, HO-1, and NQO1 in heart tissue suggesting that moderate exercise training can prevent some of the age-induced ROS that leads to heart disease[252]. In a recent study, Nrf2-/- mice had increased levels of ROS in cardiac tissue that were not reversed with exercise training. The authors suggest that disruption of Nrf2 increased the heart's vulnerability to oxidative damage thus increasing risk for cardiovascular diseases[257].

Since atherosclerosis is a low-grade inflammatory disorder characterized by increased levels of oxidative stress[2], Nrf2 has recently been explored as a pharmacological target in cardiovascular disease. Several studies have demonstrated in cultured endothelial cells exposed to laminar shear stress, an activation of Nrf2 and subsequent upregulation of its target genes, which was implicated to be the cause for reduction in atheroma in these areas of "atheroprotective" flow.[103,258–261] Overexpression of Nrf2 in endothelial cells prevented ROS-induced cytotoxicity and MCP-1 upregulation[262]. Nrf2 activation by pharmacological agents such as phytochemicals and now endurance exercise, are emerging as promising therapies to reduce cardiovascular disease.

1.5 Scope of Dissertation

1.5.1. Aims of dissertation

The main purpose of this project was to determine if voluntary exercise could protect against polychlorinated biphenyl-induced cardiovascular disease. The secondary purpose of this project was to evaluate the mechanism of protection by assessing the role of nitric oxide and Nrf2-mediated signaling pathways.

1.5.2. Rationale

PCB exposure is associated with hyperlipidemia, type II diabetes, obesity, hypertension and cardiovascular mortality. It has become well-established that individuals who engage in regular exercise have a reduced risk of developing chronic disease including atherosclerosis. Exercise is considered a 'natural" anti-atherogenic activity because it prevents atherosclerotic plaque development, decreases inflammation and adiposity, reduces hypertension, improves insulin sensitivity, and preserves endothelial function. Previous findings in our laboratory have positioned nutrition as a non-invasive therapy against polychlorinated biphenyl exposure within the vascular endothelium. Administration of EGCG, a catechin found in green tea, upregulates the antioxidant response through a Nrf2-dependent mechanism. Signaling pathways that are modulated through nutrition intervention are also upregulated during exercise. However, no one has yet examined whether exercise could protect against PCB-induced cardiovascular disease. Furthermore, no studies have examined whether those who engage in regular physical activity have lower body burdens of PCBs and other persistent organic pollutants.

Determining the effects of exercise on PCB exposure will provide insight into signaling pathways mediated by exercise within the vascular endothelium. Additionally, these findings could encourage humans to remain or become physically active in order to prevent the deleterious effects of pollutant exposure. Physicians and registered dietitians maybe more likely to recommend exercise regimens to their patient at high risk for environmental pollutant exposure as more studies reveal the link between environmental exposure and chronic disease.

1.5.3. Hypothesis and Specific Aims:

In the following studies, we have selected PCB 77 as an example of a coplanar PCB that exhibits dioxin-like activity. The general hypothesis of the research described in this dissertation is that exercise will protect against PCB-induced cardiovascular disease Furthermore, we hypothesized that this mechanism of protection is mediated within the vascular endothelium through an increase in bioavailability of nitric oxide. To test these hypotheses the specific aims were proposed:

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Specific Aim 1: To define PCB-induced cardiovascular disease and to demonstrate that exercise protects against this pathology.

Specific Aim 2: To demonstrate that PCB toxicity leads to endothelial cell dysfunction and that exercise prevents endothelial dysfunction through NO signaling and redox status.

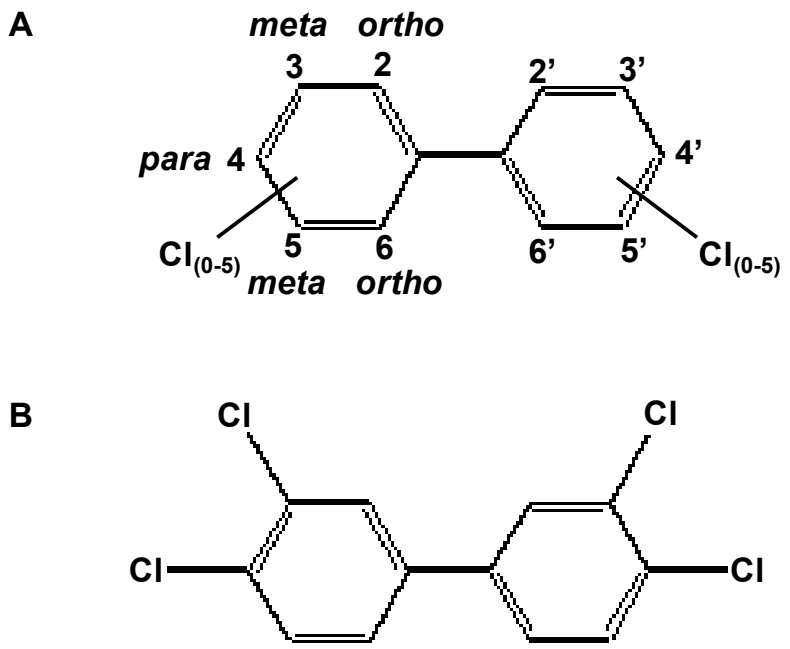


Figure 1.1. Structure and nomenclature of polychlorinated biphenyls.

A) A biphenyl molecule showing the numbering and substitution (ortho, meta, para) system that consists of 209 congeners.

B) Specific structure of PCB77 (3, 3', 4, 4'-tetrachlorobiphenyl) that is studied throughout this dissertation.

Chapter 2: Effect of exercise on PCB 77-induced toxicity in LDL-R-/- mice fed a high-fat diet

2.1 Synopsis

Atherosclerosis, the primary cause of heart disease and stroke, is initiated at the vascular endothelium; and risk factors include exposure to environmental pollutants. Polychlorinated biphenyls (PCBs) are persistent environmental pollutants that promote proinflammatory signaling in the vascular endothelium. Previous work in our laboratory has examined the potential role of nutrition in modulating the toxicity of PCBs in vascular endothelial cells. It has been well-established that exercise can reduce the risk of cardiovascular disease; however, it has not been examined whether or not exercise can modulate PCB-induced cardiovascular inflammation and dysfunction. In the current study, LDLr-/- mice were fed a Western diet (42% fat, 0.02% cholesterol) for 12 weeks to promote an atherogenic phenotype and were divided into sedentary and exercise groups at week 4. Mice in the exercise group were individually housed with a running wheel while their sedentary counterparts were individually housed with no wheel. The mice were further divided into two groups which were intraperitoneally injected with PCB77 at a dose of 170 μM/kg mouse or safflower oil vehicle during weeks 6, 8, 10, and 12. The major finding of this study was that 8 weeks of voluntary exercise led to a reduction in plasma and hepatic cholesterol levels in PCB 77-treated animals as well as a trend towards reduction in atherosclerotic lesions. A surprising finding was that exercise appears to accelerate the inflammatory response in PCB-treated animals and increases liver: body weight ratio suggesting that exercise may accelerate PCB toxicity. Results from this study suggest that exercise has minimal effects on PCB-induced vascular dysfunction using the LDL-R-/- mouse model.

2.2. Introduction

Cardiovascular disease remains the leading cause of death the in developed world. Atherosclerosis is complex disease characterized by chronic low-grade inflammation within the vascular wall[2]. Mouse models of atherosclerosis provide useful tools for studying the development and progression of this disease[157]. Low density lipoprotein receptor-deficient (LDLr-/-) mice are a model of familial hypercholesterolemia with plasma lipoprotein levels mimicking that of humans[263]. There is an accumulating

body of evidence that exposure to environmental pollution (e.g. polychlorinated biphenyls (PCBs) is linked to the development of cardiovascular disease. For example, workers exposed to phenoxy herbicides and PCBs in waste transformer oil had a much higher incidence of cardiovascular mortality[264]. Yusho patients whom accidentally ingested contaminated rice-bran oil had elevated levels of triglycerides and plasma cholesterol, which are risk factors for CVD. A major route of PCB exposure for humans is dietary intake of contaminated food primarily from animal sources[265].

Research from our laboratory and others have shown that PCBs initiate endothelial dysfunction, which is characterized by increased permeability, upregulation cytokines and cellular adhesion molecules which recruit blood mononuclear cells, proinflammatory signaling, and a decreased bioavailability of nitric oxide (NO)[266]. Endothelial dysfunction is an independent risk factor for cardiovascular disease[27]. Coplanar PCBs (e.g. PCB 77) bind to the aryl hydrocarbon receptor (AhR) which is a transcription factor that binds to xenobiotic response elements (XREs) within the promoters of downstream target genes[66]. Target genes of the AhR include cytochrome P450 1A1 (CYP1A1), a phase I enzyme that becomes uncoupled during the metabolism of coplanar PCBs[68], thus producing oxidative stress within the endothelium and subsequent endothelial dysfunction[267].

Research from our laboratory and other groups has shown that nutrition can modulate the toxicity of coplanar PCBs[86,87,102,104,106,249]. Specifically, our lab has demonstrated that certain polyphenols found in fruits and vegetables including quercetin[101] and EGCG[102] can attenuate PCB-induced pro-inflammatory signaling within cultured endothelial cells. Diets high in polyphenols are associated with a reduction in cardiovascular mortality[268]. In addition to diet, it has been highly documented that exercise is an effective prevention against cardiovascular disease. The American Heart Association publishes guidelines for prescribing exercise as primary and secondary interventions in patients with CVD[10]. Exercise improves traditional risk factors of CVD including hyperlipidemia[144], hypertension[142], adiposity[269], inflammation[152], and endothelial function[174]. In LDLr-/- mice, treadmill running for 12 weeks led to a regression of the disease[166].

By using LDLr-/- mice, a well-documented model of atherosclerosis, we studied the relationship between exercise and PCB 77 exposure. We chose a voluntary running model to reduce potential stress of forced activity and the C57BL/6 background possesses a high capacity for nocturnal running activity[270]. Our results indicate that

exercise may protect against PCB 77-induced CVD by reducing atherosclerosis and adiposity. Exercise failed to reduce PCB 77-associated risk factors of CVD including hypertension, elevated LDL levels, and inflammation. Additionally, exercise further upregulated CYP1A1 in PCB 77 exposed animals suggesting enhanced exposure to PCBs as evidenced by higher plasma levels.

2.3. Materials and Methods

2.3.1. Chemicals

PCB77 was purchased from Accustandard, Inc. (New Haven, CT). Tocopherol-stripped safflower oil (vehicle) was obtained from Dyets (Bethlehem, PA). Reverse transcriptase reagents were purchased from Fisher Scientific (Waltham, MA). Reagents used for mRNA isolation and qPCR were purchased from Life Technologies (Grand Island, NY).

2.3.2 Animal treatment

All experimental procedures were approved by the Animal Care and Use Committee of the University of Kentucky. Animals were treated humanely with regard for alleviation of pain. Male LDLr-/- mice (2 months of age, The Jackson Laboratory, Bar Harbor, ME) were given ad libitum access to food and water and housed in a pathogen-free environment with a 12 h light: 12 h dark cycle for 12 weeks. Mice were fed the Western Diet (Harlan TD.88137) for the length of the study. The Western diet is a purified diet wit 21% anhydrous milkfat (butterfat), 34% sucrose, and 0.2% cholesterol. Body weight was measured weekly. Mice were administered vehicle (tocopherol-stripped safflower oil, 0.2 mL)), or PCB-77 (170 μM/kg) by intraperitoneal injection as separate doses during weeks 6, 8, 10, and 12. IP injection administers PCB 77 directly into the peritoneal cavity, thus bypassing potential gut microbiome and intestional absorption effects. At the study end point, mice were euthanized with CO_2 and exsanguinated. Mice were not fasted before euthanasia. Ethylenediaminetetraacetic acid (EDTA) was added to collected blood samples, briefly mixed, and centrifuged at 5000g for 5 min at 4^{o}C to separate plasma. Plasma and tissue samples were frozen in liquid nitrogen and stored at -80^{o}C.

2.3.3. Exercise Protocol

Our model of exercise was the widely used voluntary running-wheel model, previously described[271]. Each mouse randomized to exercise was placed in a modified cage with wheel attached to a magnetic sensing mechanism. This allowed the running activity of each mouse to be tracked by a computer, from which the corresponding distance, speed, and amount of time spent running were obtained via ClockLab software (Actimetrics, Wilmette, IL). The bout threshold was set at 20 rotations/min to measure activity. This meant that any time the mouse was on the wheel and the rate of rotation exceeded 20 r/min the data were included in the analysis. Exercise was completely voluntary; mice were not forced in any way to exercise. The mice predominantly ran at night for a total of 8 weeks. Individually caged sedentary controls were handled for the same procedures and amount of time as the exercised mice.

2.3.4. Echo Magnetic Resonance Imaging

Fat mass and lean body mass were measured during week 12 by an echo nuclear magnetic resonance imaging system (Echo-MRI; Echo Medical Systems, Houston, TX, USA). This system uses the distinct resonance frequency of protons in lipid and water to determine body mass. This system was available through the support of the Center of Obesity and Cardiovascular Disease (COCVD) Center for Biomedical Research Excellence (COBRE) grant from the National Institute of General Medical Sciences (8 P20 GM103527-05) of the National Institutes of Health.

2.3.5. Quantification of PCBs

Tissue samples and serum were flash frozen and stored at -80C until analysis. For the separation of analytes, we used a fully automated Dionex ASE 200 system (Dionex Corporation, Sunnyvale, CA) for assisted solvent extraction and gel permeability chromatography/mass spectrometry. Hexane is pumped into the top of an electrochemical detection cell, which contains the sample and any in-cell cleanup options. The cell is brought to elevated pressure and temperature, and then the extract is forced out of the bottom of the cell and collected in a vial for additional cleanup if necessary. Detection was performed with two microelectron capture detectors; we used Chemstation software (Agilent, Palo Alto, CA) to run the system and interpret the chromatograms. An external standard mixture of PCBs, at known concentrations was

used to test for recovery of the extraction and quantification of PCBs. The limits for detection for PCBs were 0.1 ng/g of tissue (or 0.05 ppm), with coefficient of variability <3.5% and accuracy (error <1.5%).

2.3.6. Plasma cholesterol measurement

Plasma cholesterol concentrations were measured using an enzymatic kit (Wako Chemicals USA, Richmond, VA, USA). Plasma lipoprotein distribution (n=6) was resolved by fast performance liquid chromatography. Eluted fractions of samples from individual mice were collected and measured to determine lipoprotein cholesterol distribution. Lipoprotein cholesterol distribution of very low density (VLDL), low density (LDL), and high density lipoproteins (HDL) was analyzed using Peak-Fit Software 4.1 version (Seasolve Software Inc., San Jose, CA, USA).

2.3.7. Liver cholesterol measurement

Liver cholesterol was measured as previously described[272]. Briefly, livers were homogenized in Krebs-Ringer Solution through repeated low speed sonication for 30 seconds, 10 times. Liver cholesterol concentrations were measured using the enzymatic kit described above. Data are expressed as cholesterol per mg wet tissue weight.

2.3.8. Blood pressure

Systolic blood pressure was measured using a non-invasive tail cuff method (Kent Scientific) as described previously[273]. Briefly, mice were measured on a heated platform at the same time of day for five consecutive days. Twenty measurements for each mouse were obtained each day.

2.3.9 Quantification of atherosclerosis

Frozen aortic root tissues from mice in each treatment group were sequentially sectioned from the origin of the aortic values to the region in the ascending aortic arch. In our studies, we cut 10 um frozen sections of the aortic root. Nine tissue sections of aortic sinus at 80 μm intervals were placed on a single slide. This creates serial sections from the entire aortic root. Lesions were then stained with oil red O and quantified by image analysis software (Image Pro, version 7.) We orientated the section relative to

the disappearance of the aortic valve cusps and represented the lesion throughout the root[274].

2.3.10. Quantification of plasma components

For cytokine and chemokine measurement, blood samples were collected from anesthetized mice by right ventricular puncture. Clotted blood was treated with EDTA and spun at 1,200 g for 5 minutes, and the plasma was obtained and flash frozen and stored at -80C. At analysis, all samples were thawed and multiple cytokines were measured simultaneously by using an 18-plex kit (Millipore, St. Charles, MO) by following the manufacturer's instructions.

2.3.11. Quantification of mRNA using RT-PCR.

Total RNA was extracted from tissues using the TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Reverse transcription was performed using the AMV reverse transcription system (Promega, Madison, WI). The levels of mRNA expression were then assessed by RT-PCR using 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) and SYBR Green master mix (Applied Biosystems). Data analysis was performed using the relative quantification method (ΔΔCt), in which relative mRNA expression for target mRNAs (i.e., VCAM-1 and MCP-1) was compared to a constitutively expressed gene (i.e., β-actin) in the mRNA samples from tissues. β-Actin, CYP1A1, and catalase primer sequences for SYBR Green chemistry were designed using the Primer Express Software 3.0 for RT-PCR (Applied Biosystems) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). β-actin sequences were Forward: TGTCCACCTTCCAGCAGATGT, Reverse: GCTCAGTAACAGTCCGCCTAGAA; CYP1A1: Forward: TGGAGCTTCCCCGATCCT, Reverse: CATACATGGCATGATCTAGGT.

2.3.12. Statistical analysis

Data are represented as mean ± SEM. Two-way ANOVA was used, followed by a post-hoc Tukey's test to measure differences using SigmaPlot (version 12) software. A student's t-test was performed to measure differences among PCB concentrations between sedentary and exercise groups. Differences with a value of $p<0.05$ were considered statistically significant.

2.4 Results

2.4.1. Exercise decreases body weight and fat mass in PCB 77-treated animals

All mice were weighted weekly throughout the study. At baseline, there were no statistical differences among groups. During the first week, all mice were switched to the Western diet and statistical differences were noted among groups (control, exercise versus PCB, exercise; $p<0.05$) after the first week even though no exercise or PCBs had been administered. These trends continued for weeks 2-3 with additional groups being significantly different from each other (vehicle control, exercise compared to vehicle control sedentary; $p<0.05$). At the beginning of week 4, half of the mice began the voluntary wheel intervention. Mice from the control exercise group weighed significantly more than other groups ($p<0.05$) which could be attributed to an increase in lean body mass as Echo-MRI revealed. This trend continued through week 6. At week 6, mice were dosed with 170 μM/kg of PCB 77 or vehicle. At week 7, all groups lost weight most likely due to the intraperitoneal injection; however, there were no statistical differences noted among groups suggesting that the injection itself and not the administration of PCB 77 led to weight loss. During the 8th week, all groups gained weight suggesting recovery from the injection with no statistical differences among groups noted. Another injection was given at week 8 with no observed weight loss. At week 9 exercise groups continued to gain weight with the vehicle control having a significant weight gain compared to PCB77-treated, exercised animals ($p<0.05$). At week 10, all groups continued to gain weight with no statistical differences among each other. During week 10, another injection was administered. Both groups treated with PCB77 lost weight during this week with significant differences between vehicle control, exercised mice noted ($p<0.05$). This trend continued through week 12 with vehicle control, exercise group weighing significantly more than PCB-exercise group and sedentary groups ($p<0.05$). During week 12, an additional dose of 170 μM/kg PCB 77 or vehicle was administered. At the conclusion of the study, vehicle control exercise mice weighed significantly more than PCB 77-treated exercise mice and sedentary groups. Body weight was quite varied throughout the study suggesting potential confounders including Western diet and intraperitoneal injection. It should be noted that 3 mice in the PCB, sedentary group died during the experiment. Autopsy revealed inflammation around surrounding peritoneal injection. Exercise may have protected the PCB 77 group since no deaths occurred in this group during the study.

Body composition was measured using an Echo-MRI system during week 12 of the study. Two-way ANOVA revealed a statistical interaction between PCB77 and exercise. A significant increase in lean mass was found among the exercise groups ($p<0.001$, Figure 2-2A). Among the exercise groups, mice treated with PCB 77 had significantly less fat mass ($p<0.001$) Figure 2-2B). Compared to sedentary animals, animals exposed to voluntary wheel running and PCB 77 had significantly less fat mass as well ($p<0.05$).

2.4.2. Exercise increases liver size relative to body weight in PCB77-treated mice

Liver weights were weighed at the conclusion of the study. Statistical differences were not found among liver weights in different groups; however, when liver size was normalized to body weight, a significant difference was found between PCB77-treated mice who exercised compared to vehicle control, exercised animals as well as sedentary, PCB77-treated mice ($p<0.05$, Figure 2-3).

2.4.3. Running activity

After 4 weeks, 12 week-old male LDLr-/- mice were divided into 2 groups: sedentary and exercise. Wheel-running activity was monitored continuously during the entire 8 weeks of the exercise intervention. The results in Figure 2-3 show that control mice ran approximately 7.2 km/day compared to 6.99 km/day for PCB77-treated mice. Consistent with running distance, control mice ran at higher speeds (0.98 km/hour) compared to PCB77-treated mice (0.87 km/hour). Interestingly, PCB77-treated mice spent more time on average running (7.73 hours/day) compared to control mice (6.84 hours/day). Lerman et al. reported C57Bl/6 mice run approximately 7.5 km/day for approximately 5 hours each day with a speed of 1.32 km/hour. LDLr-/- mice are bred on the C57Bl6 background, but when exposed to PCBs, two aspects of voluntary wheel running were reduced with average daily running distance of 93% and daily running speed of 65% despite a 154% increase in time spent running. Further examination of running data demonstrated no differences in distance or time run on a weekly basis, but PCB-exposed mice did run significantly slower during weeks 4 and 5 (Figure 2-4).

2.4.4. Quantification of PCB77 in exercise and sedentary animals

PCB 77 was quantified through gas chromatography (GC)-mass spectrometry system on frozen tissue samples including liver, soleus, and plasma. Plasma samples

were pooled from 3 animals for each group. PCB 77 levels were undetectable in tissues or plasma from vehicle-treated mice. PCB77 levels in liver and soleus samples did not differ among sedentary or exercise groups. PCB 77 levels were tended to be elevated in the exercise group compared to sedentary (Figure 2-5) suggesting that exercise leads to mobilization of PCB from other tissues into the plasma. These results are consistent with human findings which demonstrated that plasma concentrations of PCBs increased in obese subjects who underwent weight loss.

2.4.5. Exercise reduces atherosclerosis in PCB 77-treated mice

Atherosclerotic lesion area was measured in the aortic root. Due to technical difficulties, a sample size of 3 was examined in all groups. In control, sedentary animals a mean lesion area of 0.206 $mm^{\wedge 2}$ was determined. In sedentary animals administered PCB 77, mean lesion area was 43% higher compared to control, sedentary animals (0.206 $mm^{\wedge 2}$ versus 0.294 $mm^{\wedge 2}$ p=0.06). In control, exercise animals, mean lesion area was 0.137 $mm^{\wedge 2}$ (33% reduction compared to control sedentary, 53% reduction compared to PCB, sedentary). In PCB 77, exercise animals, mean lesion area was 0.200 which is comparable to control, sedentary animals and approximately 32% smaller than PCB, sedentary animals (p=0.051). Exercise led to a significant reduction in mean lesion size regardless of PCB exposure. Due to a small sample size, only trends can be noted.

2.4.6. Exercise reduces total plasma and liver cholesterol in PCB 77-treated mice

Because hypercholesterolemia is associated with PCB 77 exposure in both human and animal studies and exercise has been shown to lower cholesterol, plasma cholesterol concentrations were measured at 12 weeks. Exercise lowered plasma cholesterol concentrations in both control and PCB77-treated animals (Figure 2-7A, p=0.058). Resolution of lipoproteins through size exclusion chromatography followed by nonlinear curve fitting analysis determined that exercise significantly decreased the HDL concentration (Figure 2-7C, $p<0.05$). Exercise lowered LDL fraction in PCB 77-treated mice, however, the results were not statistically significant. PCB 77 exposure significantly elevated hepatic cholesterol; however, exercised animals had significantly lower levels of hepatic cholesterol (Figure 2-7D, $p<0.05$).

2.4.7. Exercise does not attenuate PCB 77 increases in systolic blood pressure

Systolic blood pressure at baseline in all study groups was similar. Although exercise reduced systolic blood pressure in vehicle control-treated animals (Figure 2-8, $p=0.07$), exercise did not lower systolic blood pressure in PCB 77-treated animals.

2.4.8. Exercise increases inflammatory parameters within the plasma of PCB 77-exposed animals

To investigate the effect of exercise on inflammatory parameters in PCB 77-treated mice, a mouse adipokine LINCOPLEX kit was utilized. In mice exposed to both PCB 77 and exercise, significantly higher levels of t-PAI-1, TNF-α, IL-6, and MCP-1 were seen compared to control, sedentary mice (Figure 2-9, $p<0.05$). TNF-a is an acute phase reactant protein and risk factor for cardiovascular disease[275]. T-PAI-1 is a serine protease inhibitor that prevents fibrinolysis and has been shown to accelerate atherosclerosis[276]. IL-6 is a member of the interleukin family and has also been implicated as a risk factor for cardiovascular disease[214]. MCP-1 is a chemokine involved in the recruitment of monocytes from the bloodstream to the intima, a hallmark of the initiation of atherosclerosis[30]. These data suggest that exercise accelerates PCB 77-induced inflammation.

2.4.9. Exercise reduces leptin in PCB 77-treated animals

Leptin is an adipokine that plays a role in regulating energy intake and expenditure and is proportionate to the level of adiposity. Leptin was measured in the plasma and was found to be significantly lower in PCB, exercised animals in comparison to the remaining groups (Figure 2-10, $p<0,05$). Leptin has been shown to be chronically reduced during physical training and is proportional to body adiposity[277].

2.4.10. PCB 77 exposure significantly increases expression of CYP1A1

To examine the effect of exercise on gene expression, CYP1A1 was measured in livers using RT-PCR. CYP1A1 exposure was significantly elevated in PCB, sedentary animals compared to control counterparts (Figure 2-11, $p<0.05$) and even more elevated in PCB, exercise animals compared to PCB, control ($p<0.01$). These findings suggest that exercise may have accelerated phase I metabolism as seen by the elevated expression of CYP1A1 within the liver of these mice.

2.5 Discussion

Exercise has been shown to have a positive effect on primary and secondary prevention of cardiovascular disease. However, the effect of exercise on PCB-induced cardiovascular disease has not been examined. In the current study, we propose that exercise will protect against PCB-induced cardiovascular disease. To test this hypothesis, we utilized male LDLr-/- mice fed a Western diet that were exposed to a chronic dosing of PCB 77 with voluntary wheel running as an intervention. The major finding of this study was that 8 weeks of voluntary exercise led to a reduction in plasma and hepatic cholesterol levels with a trend toward a reduction in atherosclerotic lesion size. A surprising finding was that exercise appears to accelerate the inflammatory response in PCB-treated animals, increases liver: body weight ratio, and elevates CYP1A1, thus suggesting that exercise accelerated PCB toxicity.

When LDLr-/- mice were exposed to PCBs, two aspects of voluntary wheel running were reduced including an average daily running distance daily running speed of 65%. In a previous study it was reported that male LDLr-/- mice ran progressively less throughout the study (6.4 hours and 10 km/day verus 5.5 km/day and 4 hours at end of study[173]. Despite a reduction in average daily running distance, PCB-treated animals had lower levels of plasma cholesterol and lesion size.

To the best of our knowledge, this is the first study to report the effects of exercise against PCB-induced cardiovascular disease. We report a 32% reduction in atherosclerotic lesion size in exercise, PCB-exposed animals compared to sedentary counterparts. Previous studies in LDLr-/- mice have reported a 33% reduction in lesion size[173] compared to a 40% reduction in LDLr-/- mice that underwent forced exercise (i.e., treadmill running[167] or swimming[162].) Additionally, studies in other hypercholesterolemic mice (e.g., the ApoE-/-) report lesion reductions between 30-54%. Because hypercholesterolemia is a risk factor for atherosclerosis[10], we examined hepatic and plasma cholesterol levels. Running led to a small but significant reduction in plasma levels of cholesterol. Additionally exercise reduced hepatic cholesterol content in PCB 77-exposed animals. Few studies are available that describe the effects of voluntary exercise on hepatic lipids in hypercholesterolemic mouse models. It has recently been reported that voluntary exercise reduces plasma levels of cholesterol including VLDL and LDL sized lipoproteins while increasing hepatic lipoprotein lipase with lower hepatic cholesterol storage in LDLr-/- mice[173]. Another group reported reduction in hepatic

triglycerides after treadmill exercise [278]. Control LDLR-/- fed a Western diet have 2.5 times greater hepatic triglyceride and cholesterol contents than chow-fed wild type mice[279]. These previous studies along with our study suggest favorable effects on hepatic lipid storage through exercise.

The protective effects of exercise on cholesterol turnover are not fully understood. In one study, 12 weeks of aerobic exercise led to increase expression of SRBI, LDLr, and Cyp27 within the liver suggesting that exercise could increase cholesterol metabolism[280]. LDLr clears ApoB-containing lipoproteins from circulation[281]; SRB1 promotes selective uptake of HDL-cholesterol within the liver[282], and Cyp27 is the rate limiting enzyme in the conversion of cholesterol to bile acids[283]. This study suggests that a potential mechanism for exercise training is improvement in cholesterol clearance which supports our findings of lower hepatic and plasma cholesterol levels. Increasing cholesterol excretion within the feces is a strategy for preventing atherosclerosis because it reduces plasma cholesterol. Surprisingly, studies describing the effect of physical activity on enterohepatic circulation in humans and animal models are lacking, however there are some early studies in humans demonstrating that exercise increases sterol output and feces production[284]. Interrupting intestinal reabsorption during enterohepatic circulation which causes enhanced excretion of PCB 77 and its polar metabolites is another strategy that has been proposed to reduce body burden and deleterious effects of PCB toxicity[105]. Because these animals were not orally gavaged, initial metabolism was bypassed in the intestine, thus oral gavage may be more appropriate model to investigate whether exercise may have an effect on intestinal reabsorption and excretion of PCB 77.

Because atherosclerosis is an inflammatory disorder[2], we examined the effects of exercise on pro-inflammatory cytokines within the plasma. Circulating levels of IL-6, TNF-α, t-PAI-1, and MCP-1 have emerged as independent risk factors for CVD[275]. The anti-inflammatory effects of exercise have been well-documented in a number of studies (reviewed in [215]); however, few studies have been conducted within the LDLr-/- model. Specifically, 8 weeks of voluntary exercise in the ApoE-/- model demonstrated a significant reduction in the proinflammatory cytokines IL-6, TNF -α, and MCP-1[172]. In another study, aged ApoE-/- mice fed a high fat diet for 4.5 months followed by 12 weeks of voluntary exercise had an 8-fold reduction in circulating IL-6 levels[176]. A recent study reported that 12 weeks of voluntary exercise in LDLr-/- mice had no effect on plasma levels of TNF-α, IFNγ, MCP-1, IL-6, IL-10[173]. This may indicate that within this

model, voluntary exercise will not reduce inflammation even though atherosclerosis lesion size was reduced. Our lab has previously reported that administration of PCB 77 (2 doses of 170 μM/kg body weight at days 1 and 7) upregulates MCP-1 and IL-6 in male LDLr-/- mice fed a standardized diet containing 20% calories from fat[285]. Previous studies have shown that administration of PCB 77 increases aortic expression of vascular cellular adhesion molecule-1 (VCAM-1)[104].

A surprising finding from this study was that exercised animals exposed to PCBs had an increase liver: body fat ratio. Exercise suppresses accumulation of lipids within organs and has been reported to reduce liver weight[286] suggesting that the increase in liver: body weight are due to PCB administration. Our lab reported PCB 77 exposure significantly increased liver-to-body weight ratio in animals on a high fat diet and a concurrent reduction in PPARα signalling[81].

Other groups have reported that exposure to PCB 77 causes lipid peroxidation, hepatomegaly, and increased oxidative stress within the liver[58,287]. Additionally, reduction in PPARα signaling has been linked fatty acid accumulation and cirrhosis[288]. Epidemiological studies from the 2003-2004 NHANES data have demonstrated an association between PCB exposure and nonalcoholic liver disease[289]. LDLr-/- mice are commonly used in studies assessing nonalcoholic steatohepatitis[290] and it has been recently demonstrated that administration of PCBs can accelerate nonalcoholic fatty liver disease in mice fed a high fat diet (42% milk fat)[291]. Inflammation is a hallmark of nonalcoholic fatty liver disease with a number of proinflammatory signaling pathways upregulated including NOD-like receptors, DAMPs as well as the cytokines IL-6, TNF α, and MCP-1[292]. Exercised animals exposed to PCB 77 had much higher levels of inflammation including t-PAI-1, TNF- α, IL-6, and MCP-1 which could indicate steatohepatitis. This suggests that exercise may not overcome the proinflammatory effects of PCB 77 exposure within the LDLr-/- model of atherosclerosis. Additionally, exercise led to a significant upregulation of hepatic CYP1A1 in PCB 77 exposed animals. Although no group has examined the effect of exercise on CYP1A1 expression, flavonoids including quercetin and EGCG have been shown to downregulate PCB 77 induced expression of CYP1A1. A potential explanation for this phenomenon could be the dosing regimen (animals were exposed to PCB 77 24 hours before euthanasia) and enhanced lipolysis from exercise which could have led to increased plasma concentrations of PCB 77 and its metabolites. In obese individuals who underwent gastric bypass surgery, increased serum levels of persistent organic

pollutants were observed. Before weight loss, POP body burden and induction of AhR-target genes including CYP1A1 were elevated as well as deteriorating liver function, and hyperlipidemia. Weight loss led to gradual increases in concentration of POPs, but within 6-12 months of drastic weight loss, the overall body burden of such compounds was significantly reduced by 10-15%[293].

There are several limitations within this study. We found that voluntary exercise reduced adiposity in PCB-treated animals despite consumption of a HFD. Although we did not monitor food consumption, others have reported that exercise leads to an increase in food consumption with a reduction in adiposity likely due to increased energy expenditure from voluntary exercise[269]. Since PCB- treated animals exposed to exercise had a reduction in adiposity but vehicle-treated animals did not, food consumption should be examined in future studies to determine if PCB administration in conjunction with HFD affects appetite. Because of small sample size in atherosclerotic lesion analysis, additional studies are required to substantiate these initial findings. Because sedentary animals gained a considerable amount of weight compared to the exercised animals, we cannot conclude that these findings were caused by exercise alone. Although we observed a reduction in hepatic cholesterol levels, we did not measure total hepatic lipid content or cirrhosis. Future studies should assess the extent of liver cirrhosis as well as monitoring changes in biliary excretion and overall body burden. Although inflammation was not prevented, exercise may decrease overall body burden of these environmental pollutants over time. Future studies should examine the effect of exercise on PCB metabolism/excretion and overall body burden over an extended length of time. An additional limitation could be the potential confounder of high fat feeding. The majority of in vivo work within the fields of exercise and cardiovascular disease utilize the ApoE-/- model and demonstrate positive outcomes in exercised animals related to cardiovascular disease and its associated risk factors. Additionally, the ApoE-/- model does not require high fat feeding for induction of atherosclerosis, which would limit the confounder of high fat feeding. Future studies should examine whether exercise can prevent PCB-induced cardiovascular risk factors including glucose intolerance, endothelial function, antioxidative potential, and hypertension within the ApoE-/- model. This work warrants additional studies to substantiate a new role for exercise.

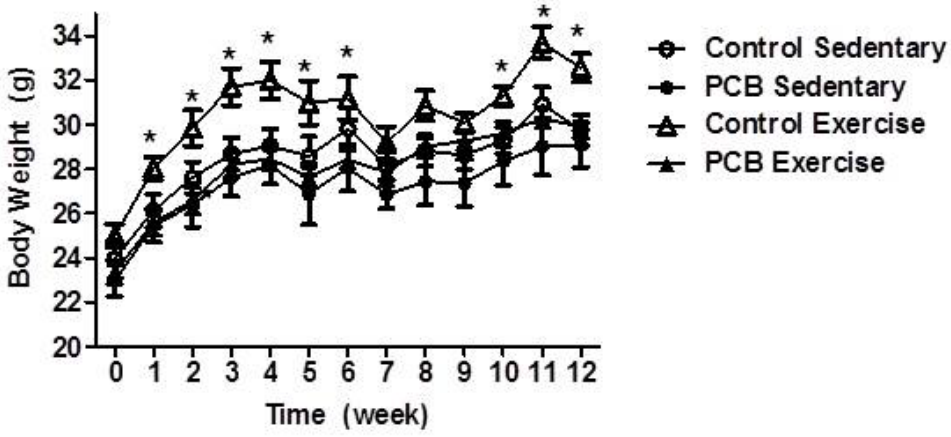


Figure 2-1 Exercise Increases Body Weight in Vehicle-treated Animals

Body weights were measured weekly. Data represent the mean ± SEM. Two-way ANOVA revealed a statistical difference between vehicle control and PCB groups for body weight. (*$p<0.05$ compared to vehicle control for body weight).

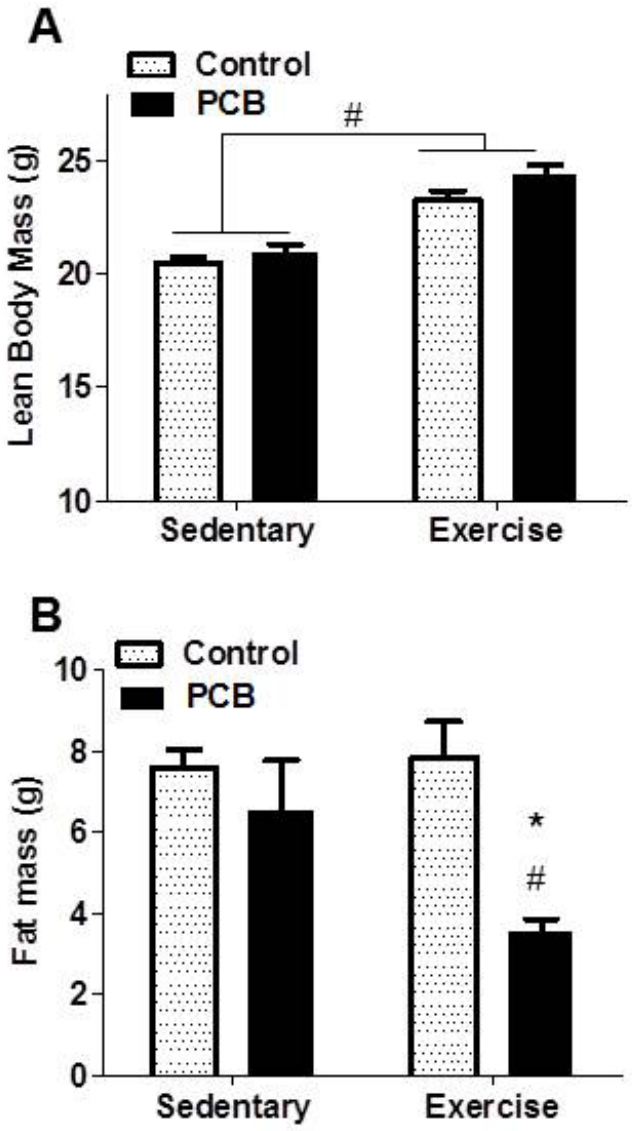


Figure 2-2 Exercise Increases Lean Body Mass and Reduces Fat Mass in PCB-treated animals

A) Lean Mass and B) fat mass were measured through Echo-MRI. Two-way ANOVA revealed a statistical difference between exercise and sedentary groups for lean body mass (#$p<0.001$.) Two-way ANOVA revealed a significant interaction between PCB77 and exercise. *Significantly different compared to PCB77-treated sedentary animals. #Significantly different compared to vehicle control exercise animals.

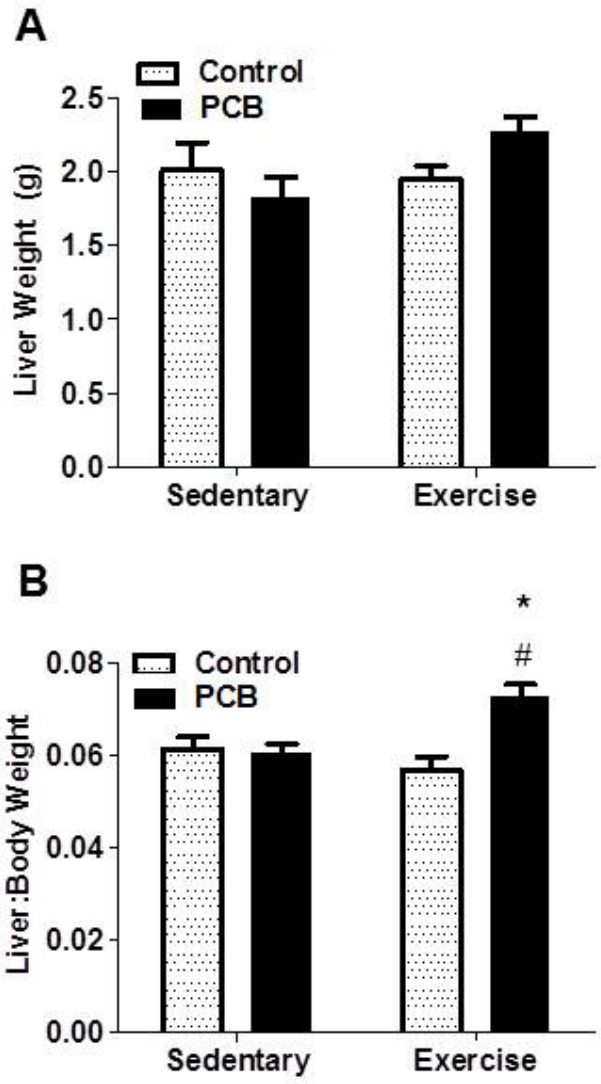


Figure 2-3 Exercise increases liver: body weight in PCB77-treated mice

Liver and body weights were weighed at conclusion of the study. Data represent mean ± SEM. Two-way ANOVA revealed a statistical interaction between PCB77 and exercise. *Significantly different from control, exercise ($p<0.05$) #Significantly different from sedentary, PCB77-treated animals ($p<0.05$).

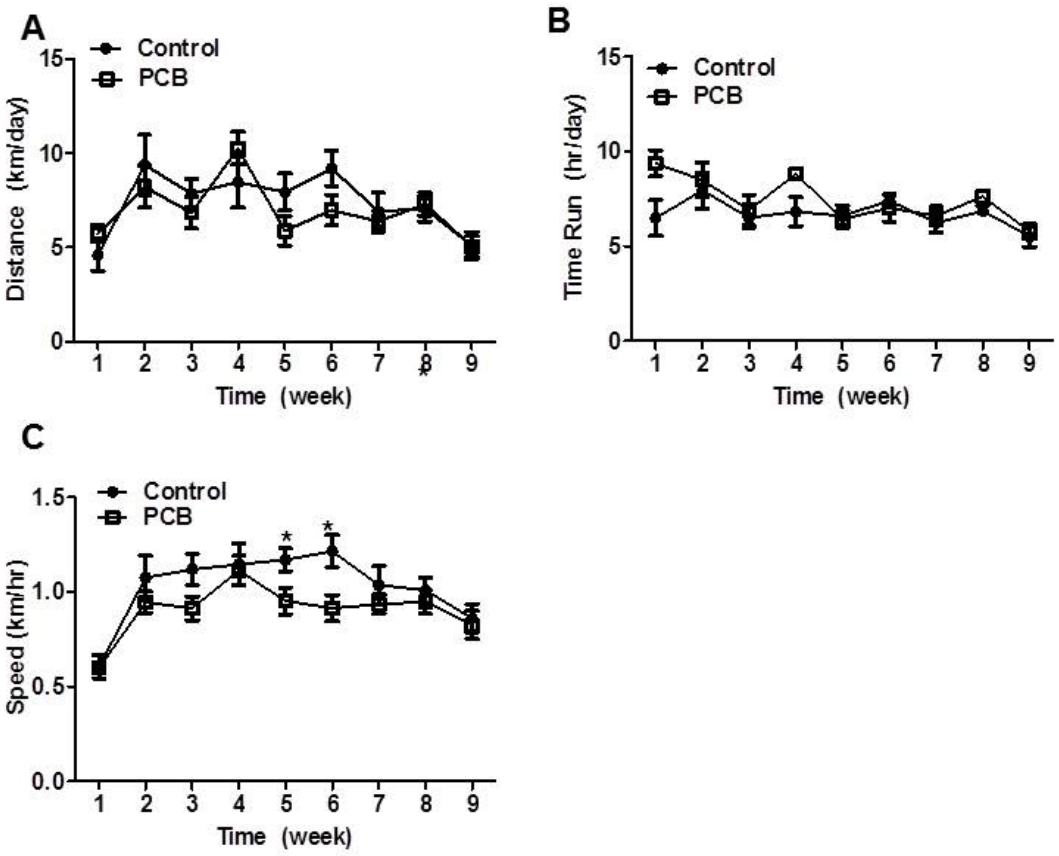


Figure 2-4 Voluntary wheel-running performance in control and PCB77-treated mice

Male LDLr-/- mice were housed singly in cages mounted with a running wheel with one week of acclimation. Wheel-running activity was monitored continuously and analyzed using a Clock-Lab Analysis program. A) Mean daily running distance B) Mean weekly running time C) Mean running velocity (km/h) during the 9 weeks of observation. A student's t-test demonstrated that PCB77-treated mice spent more time running at lower speeds during week 5 and 6 ($p<0.05$).

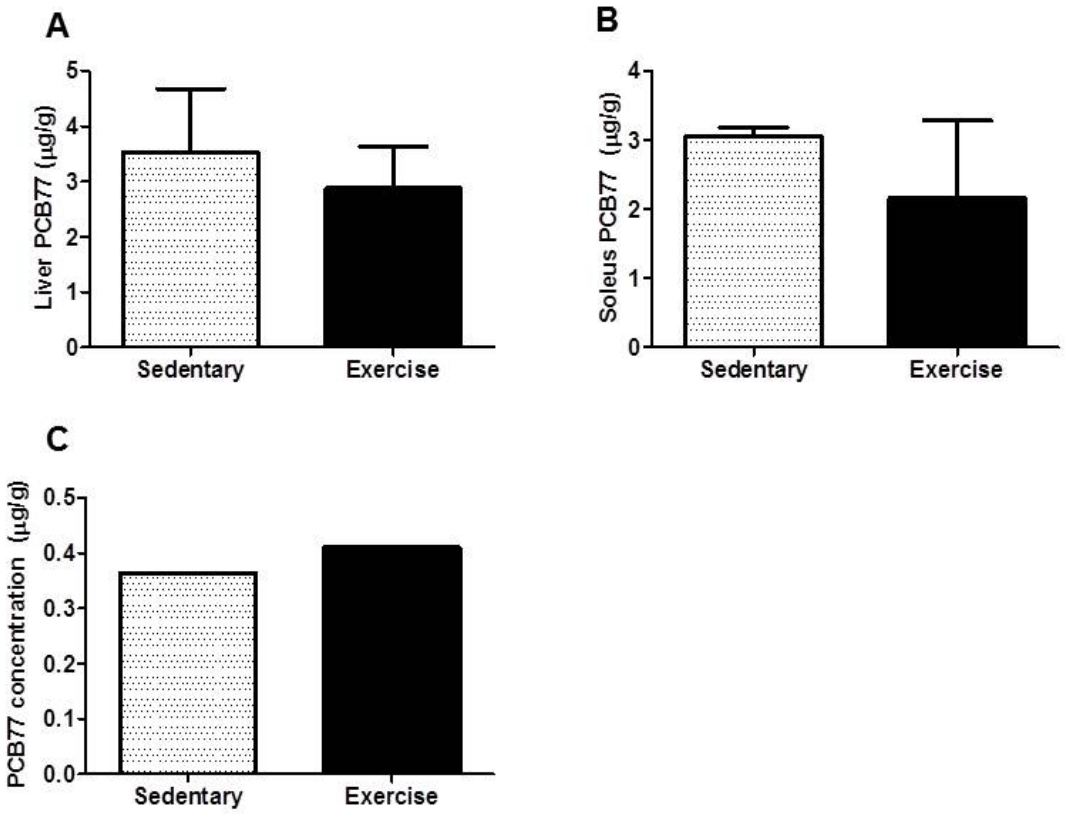


Figure 2-5 Concentrations of PCB in tissues

PCB77 was quantified through gas chromatography (GC)-mass spectrometry system on frozen tissue samples (liver, soleus, plasma). Data are mean ± SEM from 5 animals. Plasma samples were pooled from 3 animals

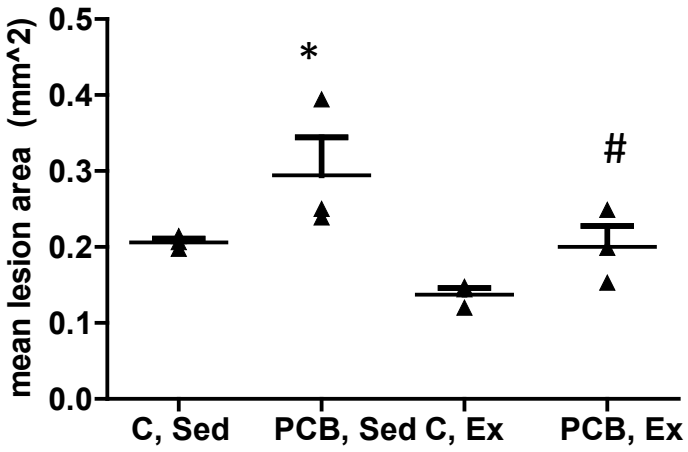


Figure 2-6 Exercise Reduces Mean Aortic Lesion Area.

The aortic root was serially sectioned on a cryostat with 10 μm sections. Lesions were quantified through oil red O. The average lesion area spanning -240 to 240 μm is depicted. Data represent the mean ± SEM (n=3). * compared to vehicle control, sedentary (p=0.061). # compared to PCB77-treated sedentary mice (p=0.051).

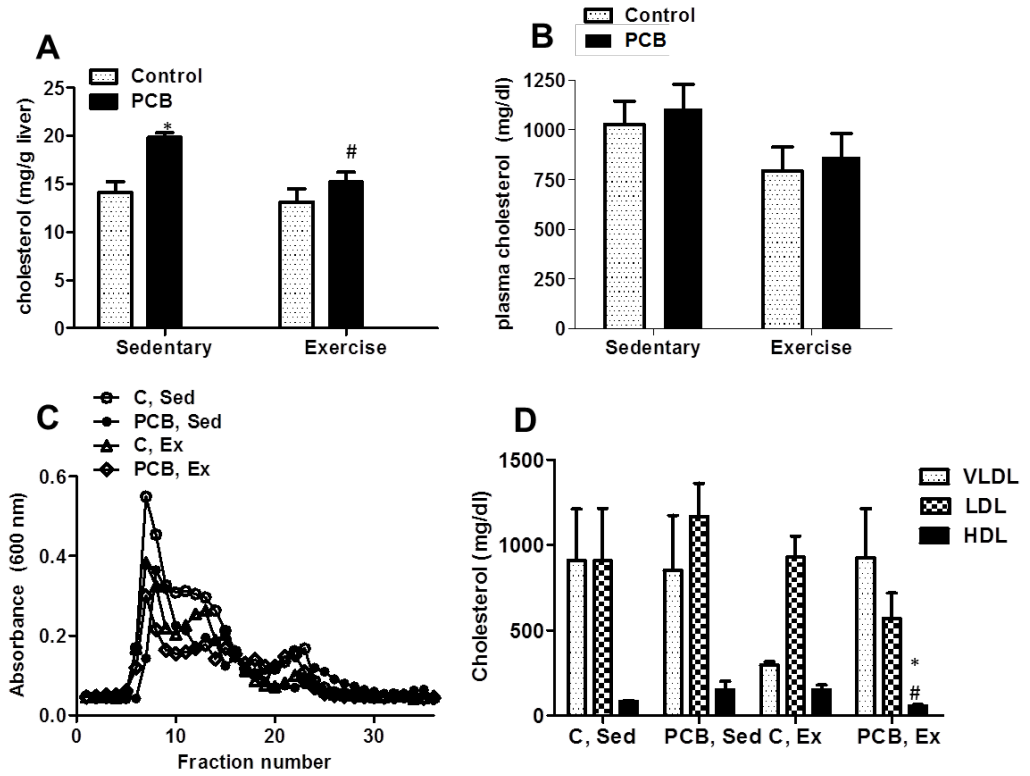


Figure 2-7 Exercise reduced total plasma cholesterol and HDL total cholesterol levels

Liver and plasma cholesterol concentrations were measured at termination. A) Liver B) Plasma cholesterol was measured through enzymatic kit (p=.058 compared to PCB77-treated sedentary mice). C) Lipoproteins were resolved by size exclusion chromatography. D) Plasma cholesterol concentrations of Lipoprotein fractions were calculated using a nonlinear curve fitting approach. Data represent the mean ± SEM of 5 animals. Two-way ANOVA revealed a statistically significant interaction between exercise and PCB77 for HDL total cholesterol levels. * Significantly different compared to vehicle control, exercise ($p<0.05$).# Significantly different compared to PCB77-treated sedentary mice ($p<0.05$).

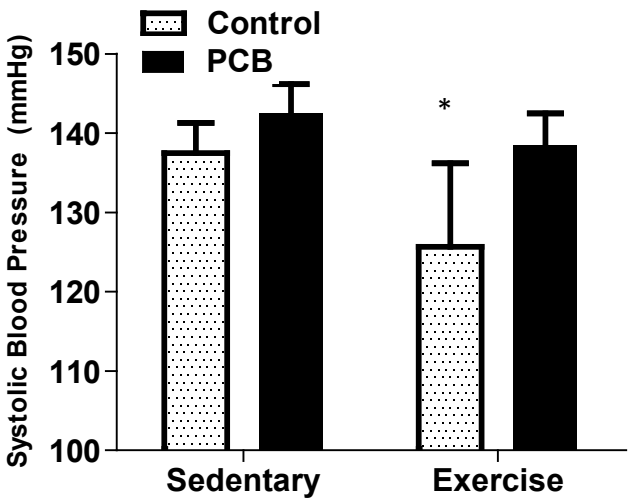


Figure 2-8 Exercise fails to attenuate PCB77 increases in systolic blood pressure

Blood pressure was measured non-invasively through tail-cuff method (Coda). Data represent mean ± SEM of 4 animals. (*p=0.07 compared to control, sedentary).

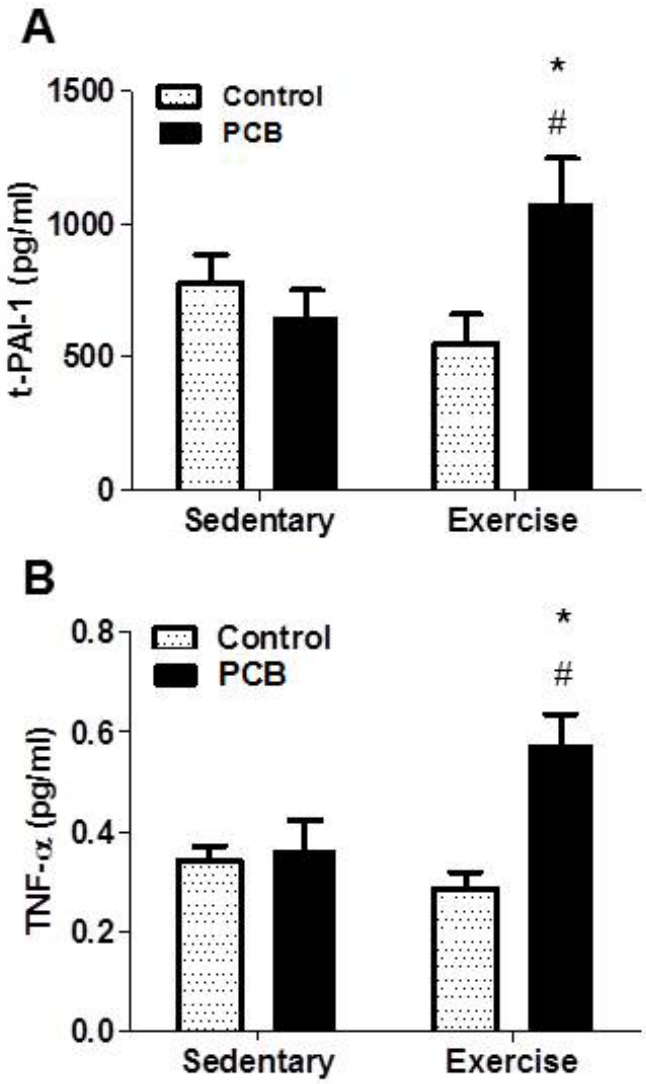


Figure 2-9 Exercise increases serum t-PAI-1 and TNF-α levels in PCB77-treated animals

Plasma samples were analyzed for t-PAI-1 and TNF-α levels using mouse adipokine LINCOPLEX kit. Two-way ANOVA revealed a statistically significant interaction between exercise and PCB77. *Significantly different between PCB, sedentary animals. ($p<0.05$) # Significantly different from control, exercise animals ($p<0.05$)

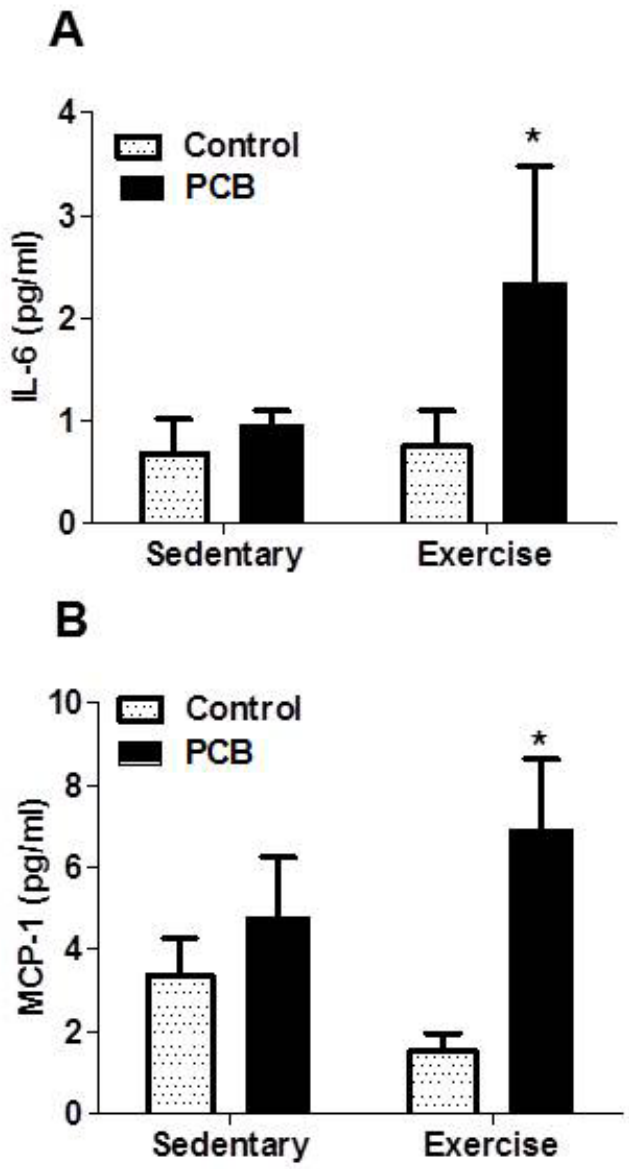


Figure 2-10 Exercise increases plasma levels of IL-6 and MCP-1 in PCB77-treated animals

Plasma samples were analyzed for IL-6 and MCP-1 levels using mouse adipokine LINCOPLEX kit. Two-way ANOVA revealed a statistically significant difference between PCB and Control groups. *Significantly different between PCB, sedentary animals. ($p<0.05$)

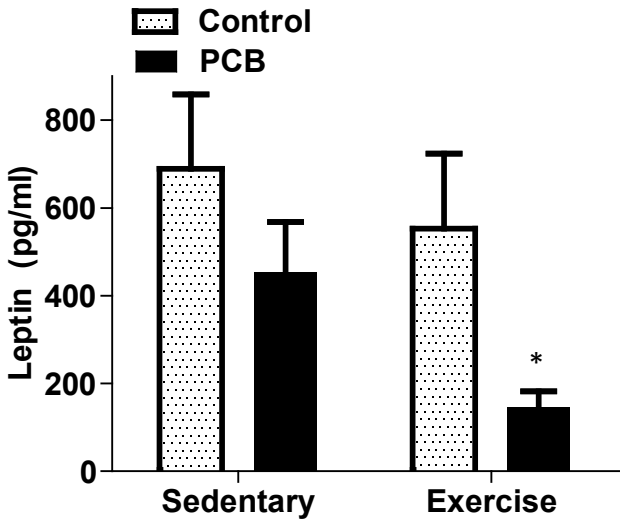


Figure 2-11 Exercise reduces plasma leptin levels in PCB77-treated animals

Plasma samples were analyzed for leptin levels using mouse adipokine LINCOPLEX kit. Two-way ANOVA revealed a statistically significant interaction between exercise and PCB77. * Significantly different compared to control, exercise.

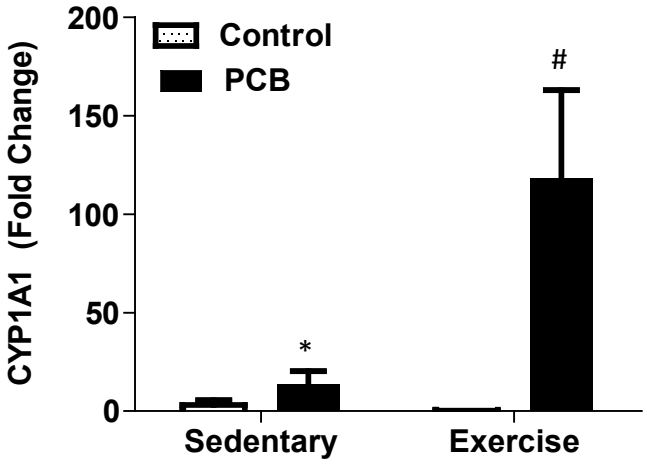


Figure 2-12 Exercise upregulates CYP1A1 in PCB77 treated mice.

Liver mRNA was Isolated and CYP1A1 m RNA levels were measured using RT-PCR. Two-way ANOVA revealed a statistically significant interaction between exercise and PCB77. *Significantly different compared to vehicle control, sedentary ($p<0.05$) #Significantly different compared to PCB77-treated sedentary mice ($p<0.01$)

Chapter 3: The Effects of Physical Activity on PCB-Induced Cardiovascular Disease in ApoE-/- mice

3.1 Synopsis

Cardiovascular disease is the leading cause of mortality in developed countries. Polychlorinated biphenyls (PCBs) are persistent environmental pollutants that contribute to the initiation of cardiovascular disease. There is strong evidence that exercise can reduce the risk of cardiovascular disease; however, whether exercise can modulate PCB-induced inflammation, endothelial dysfunction and atherosclerosis is unknown. In this study we examined the *in vivo* effects of exercise on coplanar PCB- induced cardiovascular disease and associated risk factors including impaired glucose tolerance, hypercholesteremia, oxidative stress, inflammation and endothelium-dependent vasodilation. Male ApoE-/- mice were divided into sedentary and exercise groups (voluntary wheel running) over a 12 week period. Half of each group was exposed to vehicle or PCB 77 (170 μM/kg) at weeks 1, 2, 9, and 10. Exposure to coplanar PCB increased atherosclerosis and several risk factors associated with cardiovascular disease, including glucose intolerance, hyperlipidemia, oxidative stress and systemic inflammation. The 12 week exercise intervention significantly reduced several of these pro-atherogenic parameters induced by PCB exposure. Exercise also lowered PCB-induced oxidative stress and upregulated some antioxidant enzymes including phase II enzymes. There was a trend towards induction of atherosclerotic lesions and protection by exercise.

3.2. Introduction

Cardiovascular disease remains the leading cause of death in developed nations. A number of different factors including environmental and chemical exposures are contributors to cardiovascular diseases. An accumulating body of evidence from epidemiological, *in vitro*, and *in vivo* studies link cardiovascular disease to environmental pollution, including exposure to persistent organic pollutants such as dioxins and polychlorinated biphenyls (PCBs) [11]. Dioxin exposure appears to be associated with mortality from cardiovascular disease [294]. Furthermore, residing near sites contaminated with PCBs is associated with increased rates of hospitalization for coronary heart

disease and acute myocardial infarction [295], and circulating levels of PCBs were associated with atherosclerotic plaques in humans [296].

Because the endothelium is in immediate contact with blood, endothelial cells are particularly vulnerable to environmental contaminants present in the circulation and which can induce inflammation and endothelial dysfunction [83,297]. The majority of pro-inflammatory effects from coplanar PCBs are mediated through the aryl hydrocarbon receptor (AhR) (reviewed in [298]). Activation of the AhR leads to transcription of the detoxifying enzyme cytochrome p450 1A1 (CYP1A1) which when in the presence of PCB can increase the levels of cellular reactive oxygen species (ROS), leading to induction of pro-inflammatory genes and subsequent vascular dysfunction [267]. Our laboratory has demonstrated previously that exposure to PCB 77 increases the expression of vascular cell adhesion molecule-1 (VCAM-1) [299], endothelial-derived monocyte chemoattractant protein-1 (MCP-1) [285], and interleukin-6 (IL-6). Other groups have shown that PCBs impair endothelium-dependent dilation [84] and promote obesity-associated atherosclerosis [82].

As pollutant emissions continue to increase (i.e., manufacturing and agriculture), human exposure to these pollutants will rise, thus leading to the need for physiological buffers to protect against pollutant-induced adverse health effects such as cardiovascular disease. Data from our laboratory and other groups have provided strong evidence that nutrition can modulate cardiovascular toxicity of environmental pollutants [86,87,102,106,300]; however, the effect of other lifestyle modifications such as exercise on health risks associated with exposure to persistent organic pollutants remains relatively unexplored. One such PCB study examined the effects of exercise on the gut microbiome and found that 5 weeks of voluntary exercise attenuated PCB-induced alterations in proteobacteria [301]. Exercise has been well-established as an effective primary and secondary intervention for atherosclerotic cardiovascular disease. Studies in both human and animal models have provided evidence that exercise exerts beneficial effects on atherogenesis and coronary artery disease (reviewed in [302–304]). Exercise improves traditional cardiovascular disease risk factors including, hyperlipidemia, obesity, insulin sensitivity and hypertension, as well as vascular function affected by changes in redox status and inflammation [114]. It has been shown that aerobic exercise decreases atherosclerotic plaque formation [163,213,305] and reduces neointima formation after carotid artery injury [168]. Exercise has also been shown to reduce proinflammatory cytokines in humans with coronary artery disease [218] and in

animal models of cardiovascular disease [209]. In ApoE-/- mice, exercise improves endothelium-dependent vasodilation or relaxation in isolated aortas and decreases vascular oxidative stress [172,174].

By using ApoE-/- mice, a well-documented model of atherosclerosis [157], we studied the relationship between exercise and PCB 77 exposure. Our results indicate that exposure to a coplanar PCB increased cardiovascular disease-related risk factors including oxidative stress, vascular inflammation, hyperlipidemia, and glucose intolerance, which may have contributed to the observed increase in atherosclerosis in mice exposed to PCB. Exercise reduced atherosclerotic lesions as well as associated risk factors in PCB-treated animals.

3.3. Methods

3.3.1. Chemicals

PCB 77 was purchased from Accustandard Inc. (New Haven, CT). Acetonitrile, Oil Red O and other chemicals utilized were obtained from Sigma Aldrich (St. Louis, MO).

3.3.2. Animal treatment & sample collection

Male ApoE-/- mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Each mouse was individually caged, handled, and used in compliance w3ith the Animal Care and Use Committee of the University of Kentucky. Mice were given *ad libitum* access to food (rodent standard chow) and water and housed in a pathogen-free environment for 12 weeks. Body weight was measured weekly. Mice were administered vehicle (0.2 mL tocopherol-stripped safflower oil, Dyets, Inc. Bethlehem, PA), or PCB 77 (170 μM/kg) by oral gavage as separate doses during weeks 1, 2, 9, and 10, and the dosage was based on earlier studies demonstrating glucose intolerance[306]. Our model of exercise was the widely used voluntary running-wheel model, previously described[271]. We chose a voluntary running model to avoid the potential stressor of forced activity, and the C57BL/6 background has been shown to have a high capacity for nocturnal running activity. Each mouse randomized to exercise was placed in a modified cage with the wheel attached to a magnetic sensing mechanism. This allowed the running activity of each mouse to be tracked by a computer, from which the corresponding distance, speed, and amount of time spent running were obtained via ClockLab software

(Actimetrics, Wilmette, IL). Individually caged sedentary controls were handled for the same procedures and amount of time as the exercised mice. Food intake was recorded for 12 weeks. Mice were placed in a metabolic cage system (Techniplast, Inc., Philadelphia, PA) during week 12 to collect urine and feces. Fat mass and lean body mass were measured by an echo magnetic resonance imaging system (Echo-MRI; Echo Medical Systems, Houston, TX). At the study end point, mice were euthanized with CO_2 and exsanguinated. Ethylenediaminetetraacetic acid (EDTA) was added to collected blood samples, briefly mixed, and centrifuged at 5000g for 5 min at 4^{o}C to separate plasma. Plasma and tissue samples were frozen in liquid nitrogen and stored at -80^{o}C.

3.3.3. Glucose tolerance test

Mice were individually housed and fasted for 6 hours prior to the glucose tolerance test performed during week 6 of the study. Blood was collected from the tail vein and tested for glucose concentration with a glucometer (Freedom Freestyle Lite; Abbott Laboratories, Abbott Park, IL). Mice were administered D-glucose (20% in saline, oral gavage) and blood glucose was quantified at the following time points: 0 min, 15 min, 30 min, 60 min, 90 min, and 120 min. Total area under the curve (AUC; arbitrary units) calculates the area below the observed concentrations without the presence of a baseline value[306].

3.3.4. Quantification of plasma cholesterol, lipoproteins and cytokines/chemokines

We determined plasma cholesterol concentrations using an enzymatic kit (Wako Chemicals USA, Richmond, VA, USA). Plasma lipoprotein distribution was resolved by fast performance liquid chromatography. Eluted fractions of samples from individual mice were collected and measured to determine lipoprotein cholesterol distribution. Lipoprotein cholesterol distribution of very low density (VLDL), low density (LDL), and high density lipoproteins (HDL) was analyzed using Peak-Fit Software 4.1 version (Seasolve Software Inc., San Jose, CA, USA)[273]. Cytokines and chemokines were measured simultaneously by using an 18-plex kit (Millipore, St. Charles, MO)[285]. Briefly, analytes were read with a Luminex-200 machine (Invitrogen) according to manufacturer's instructions.

3.3.5. Liver cholesterol measurement

Liver cholesterol was measured as previously described[272]. Briefly, livers were homogenized in Krebs-Ringer Solution through repeated low speed sonication for 30 seconds, 10 times. Liver cholesterol concentrations were measured using the enzymatic kit described above. Data are expressed as cholesterol per mg wet tissue weight.

3.3.6. Quantification of atherosclerosis

Frozen aortic root tissues from ApoE-/- mice within each treatment group were sequentially sectioned from the origin of the aortic values to the region in the ascending aortic arch as previously described[274]. Briefly, 10 µm frozen sections of the aortic root were cut. Nine tissue sections of aortic sinus at 80 µm intervals were placed on a single slide (Probe-On Plus; Fisher Scientific, Pittsburgh, PA). This created serial sections for the entire length of the aortic root. Lesions were then stained with oil red O (Sigma Aldrich, St. Louis, MO) and quantified by image analysis software (Image Pro, version 7). Sections were orientated relative to the disappearance of the aortic valve cusps and represented the lesion throughout the root.

3.3.7. Quantification of PCBs and F_2-isoprostanes

To determine systemic PCB and metabolite concentrations, PCB 77 and its hydroxyl metabolites were isolated from plasma and tissue samples. Briefly, tissues were homogenized (GenoGrinder, Thomas Scientific, Swedesboro, NJ) in deionized water. Homogenates were extracted in acetonitrile with the following internal standards: 10 µM 13C12-labeled PCB126 internal standard (IS) and d6-PCB77 (Cambridge Isotope Laboratories, Tewksbury, MA). Samples underwent sonication and centrifugation at 15,000 rpm for 5 min to remove plasma and tissue debris and repeated twice. Supernatants (3 mL) were dried under N_2 and reconstituted in 99:1 methanol: dI H_2O solvent mixture with 0.5% formic acid and 0.1% 5 M ammonium formate[249].

Measurement of urinary F_2-Isoprostanes (F_2-IsoPs) is considered the gold standard for assessment of *in vivo* oxidative stress[307], and the assay was performed as described by us elsewhere[249]. Briefly, Ethyl acetate:methanol (5:1) + 0.5% acetic acid (v/v) + 10µM 8-iso-PGF2α-D4 (internal standard, Cayman Chemical, Ann Arbor, MI) was added to urinary samples, vortexed, and centrifuged at 15,000 rpm for 5 min. Supernatants were dried under N_2 and reconstituted in methanol and acetic acid before

solid phase extraction (SPE). Supel-Select HLB SPE columns (Sigma-Aldrich, St. Louis, MO) were preconditioned with methanol and 0.5% acetic acid. Reconstituted F_{2}-IsoP samples were loaded onto Supel-Select HLB SPE columns (Sigma-Aldrich, St. Louis, MO) and washed with 0.5% acetic acid followed by a wash containing 0.5% acetic acid and 20% methanol. Analytes were eluted with methanol and dried under N_2 before reconstitution with 50:50 methanol: dl H_2O.

After extractions were performed, plasma, tissue, and urinary levels of PCB 77 and its metabolites were analyzed using a Shimadzu ultra fast liquid chromatography system coupled with an AB Sciex 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode[249]. MRM transitions monitored included 291.9/ 222.1 and 291.9/220 for PCB 77; 338/268.1 and 338/196.1 for 13C12 PCB 126 and 297.9/228.1 and 297.9/226.2 for d6-PCB77. In the MRM ion transition the precursor ion represents the $M^{+\cdot}$ and the product ion represents either $[M\text{-}Cl]^{+}$ or $[M\text{-}2Cl]^{+}$. MRM transitions monitored for PCB 77 metabolites: 352.8/306.9 for hydroxy PCB77 and 368.8/322.9 for dihydroxy PCB 77. The precursor ion of the ion transition is a formic acid adduct [M+FA-H]-, and product ion is [M-H]-. F_{2}-IsoPs were analyzed by integrating peak area i.e., area under the curve, AUC, relative to known internal standard concentrations (AUC/IS). All values were normalized to urinary creatinine values (Cayman Chemical, Ann Arbor, MI) and compared to ion transitions of the internal standard (13C12 PCB126) with known concentration to calculate PCB parent and metabolite pmol concentrations.

3.3.8. Gene expression of CYP1A1 and antioxidant enzymes

Total RNA was extracted from tissues using the TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protoco[249]. RNA concentrations were quantified via the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) and reverse transcription was performed using the AMV reverse transcription system (Promega, Madison, WI). The levels of mRNA expression were assessed by quantitative real-time PCR using 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) and SYBR Green master mix (Applied Biosystems). Data analysis was performed using the relative quantification method ($\Delta\Delta$Ct), in which relative mRNA expression for target mRNAs was compared to a constitutively expressed gene (i.e., β-actin) within mRNA samples from tissues. Primer sequences (see Table 1) for SYBR Green chemistry were designed using the Primer

Express Software 3.0 for RT-PCR (Applied Biosystems) and procured from Integrated DNA Technologies, Inc. (Coralville, IA).

3.3.9. Statistical analysis

Data are represented as mean ± SEM. Two-way ANOVA was used, followed by a post-hoc Tukey's test to measure differences using SigmaStat software (Systat Software, Point Richmond, CA). Differences with a value of $p<0.05$ were considered statistically significant.

3.4. Results

3.4.1. Exercise reduces cardiovascular disease and associated risk factors in PCB77-treated mice

We examined the effects of exercise on specific risk factors associated with cardiovascular disease including obesity, glucose tolerance, hypertension, and hypercholesterolemia. All groups gained weight during the study with sedentary groups gaining approximately 3 grams of body weight and exercise groups gaining 5 grams of body weight during the 12 week study, independently of PCB exposure ($p<0.05$, Supplementary Figure 1A). Body composition was measured using an Echo-MRI during week 12 of the study and a trend was seen in the exercise, PCB-treated group as they exhibited less fat mass than sedentary counterparts ($p=0.053$) with a significant increase in lean mass among exercise groups independent of treatment ($p<.001$ Supplementary Figure 1B). Because all treatment groups gained weight specifically in lean body mass, this dosing regimen did not produce signs of PCB toxicity. Control mice ran approximately 5 km/day for approximately 7 hours per day at a speed of 0.65 km/hour. PCB-treated mice ran less distance during weeks 10 and 11 for a shorter period of time (approximately 6 hours per day and 5 km/day). Speed was not different among groups. The running capacity in ApoE-/- mice is less than that of the LDLr-/- mice. During weeks 10 and 11, both groups had significantly lower distance and time spent running. Actogram analysis revealed disturbances in circadian rhythm which could indicate interrupted light: dark cycles within the facility. Sedentary animals treated with PCB 77 (170 μM/kg) showed a significant increase in blood glucose concentrations, compared with vehicle control and exercise groups, in response to a bolus of administered glucose (Figure 1A, $p<0.05$). The total AUC for blood glucose was

significantly increased in sedentary mice treated with PCB 77 (Figure 1B, $p<0.05$) but exercise attenuated this response ($p<0.05$)

Because hypercholesterolemia and hypertension[82,308,309] are associated with PCB 77 exposure and exercise has been shown by several groups to lower cholesterol as well as hypertension[117,310,311], plasma cholesterol and systolic blood pressure were measured. Exercise significantly reduced plasma cholesterol levels in PCB 77-treated mice (Figure 3-2A, $p<0.001$). Resolution of lipoproteins through size exclusion chromatography followed by nonlinear curve fitting analysis determined that exercise significantly decreased both the VLDL (Figure 3-2b $p<0.001$) and I/LDL cholesterol concentrations ($p<0.01$) in PCB 77-treated mice compared to sedentary counterparts. No differences in HDL concentrations were found among groups. Similarly, exercise significantly reduced hepatic levels of cholesterol in PCB 77-treated mice (Supplementary Figure 4, $p<0.05$). Among sedentary animals, PCB 77 significantly increased systolic blood pressure at the end of the study (Figure 3-3, $p<0.001$). Exercised animals exposed to vehicle treatment displayed significantly lower systolic blood pressure compared to vehicle-treated sedentary animals while animals exposed to PCB 77 during exercise displayed significantly lower blood pressure than PCB-treated sedentary animals (138 mmHg versus 149 mmHg, $p<0.001$) but remained comparable to vehicle, sedentary levels (139 mmHg). Because atherosclerosis is recognized as an inflammatory disease[2], selected inflammatory parameters were investigated. To determine the effect of exercise on inflammation, we measured an array of plasma cytokine and adipokine concentrations. Significant decreases in interleukin 6 (IL-6), monocyte chemoattractant protein 1 (MCP-1), chemokine (C-X-C motif) ligan1 (CXC1 or KC), macrophage colony stimulating factor (M-CSF), and (monokine induced by gamma interferon) MIG were detected in exercised mice exposed to PCB 77 compared to sedentary counterparts (Figure 3-4A-E, $p<0.05$).

Administration of PCB77 tended to increase atherosclerosis more in both sedentary than exercised animals (Figure 3-5). Exercised animals administered vehicle had significantly lower levels of atherosclerosis compared to sedentary animals which supports others' findings ($p<0.05$; 17739.5 μm^2 versus 48511.6 μm^2). Exercise did not significantly reduce mean atherosclerotic lesion size compared to sedentary animals in PCB-treated animals ($p=0.392$) although the mean lesion size was reduced in these animals. Due to the limited sample size within this study, a repeated study with more power would better delineate the effect of exercise on mean atherosclerotic lesion size.

3.4.2. Exercise reduces systemic oxidative stress and upregulates antioxidant enzymes

Because inflammatory diseases, such as atherosclerosis, are redox sensitive, we also assessed *in vivo* systemic oxidative stress. F_2-isoprostanes (F_2-IsoPs), prostaglandin-like eicosanoids formed during fatty acid peroxidation, were measured in urinary samples from all treatment groups. In PCB-treated animals, exercised mice had significantly lower levels of 8-iPF2 α and iPF2 α-VI ($p<0.05$) compared to sedentary animals. Because of an 8-fold reduction of oxidative stress in exercised animals, we next examined expression of antioxidant genes. Overall, exercise significantly upregulated the expression of catalase, glutathione peroxidase (Gpx), glutathione S-reductase (GSR) and glutathione S-transferase (GST) and led to downregulation of the inhibitor of Nrf2, Keap1 (Figure 7).

Additionally, exercised groups exposed to PCB 77 had a significant downregulation of CYP1A1 compared to sedentary counterparts, which could contribute to the lower levels of oxidative stress. To assess whether exercise had an effect on body burden, PCB 77 and its hydroxyl metabolite OH-PCB 77 were quantified in the plasma and several tissues including liver, lungs, soleus, kidney, retroperitoneal white adipose tissue, epididymal white adipose tissue, and subcutaneous white adipose tissue. PCB 77 and OH-PCB 77 levels were undetectable in tissues or serum from vehicle-treated mice or PCB 77 treated mice at the conclusion of the study. OH-PCB 77 levels in feces from sedentary mice were approximately 4 fold higher than those from exercised mice, (Figure 8, $p<0.05$).

3.5. Discussion

There is substantial evidence that exposure to persistent organic pollutants including dioxin and PCBs are linked to the incidence of cardiovascular disease and heart failure which remain the leading cause of death in developed nations[294,296,312] Dioxin and coplanar PCBs exhibit their toxicity by binding to the aryl hydrocarbon receptor (AhR) which causes the upregulation of CYP1A1 expression which leads to an increase in oxidative stress due to the uncoupling of CYP1A1 [68]. We have shown previously that coplanar PCBs, including PCB 77 and PCB 126 are proinflammatory and atherogenic in vascular endothelial cells [83,313]. Coplanar PCBs have also been shown to promote obesity [82], atherosclerosis [79], and diabetes [306,314].

In most people, body burdens of environmental pollutants are prevalent, and prevention against environmental chemical-induced disease pathologies remains a challenge. Positive lifestyle changes such as healthful nutrition and an increase in physical activity tend to protect against the development of inflammatory diseases such as atherosclerosis, obesity and diabetes[315–317]. Evidence from our laboratory suggests that antioxidant nutrients and related bioactive compounds found in fruits and vegetables protect against environmental toxic insult to the vascular endothelium by increasing antioxidant defense and by down-regulation of proinflammatory signaling [98,249]; however, the role of exercise remains largely unknown.

Data from this study provide evidence for the protective properties of physical activity against cardiovascular disease. Exposure to PCB 77 in sedentary animals elevated several risk factors associated with cardiovascular disease including glucose intolerance, hypercholesteremia, hypertension, systemic inflammation, oxidative stress, as well as increased atherosclerosis. Baker et al. have previously reported that coplanar PCBs induce rapid and sustained glucose intolerance in an AhR-dependent manner[306]. We demonstrate glucose intolerance in sedentary, PCB-treated animals that is prevented in exercised animals. In the current study, sedentary, PCB-treated animals had significantly higher levels of liver and plasma cholesterol, predominately in the VLDL and LDL fractions. Our results extend previous findings which have shown that dietary exposure to PCB77 significantly increases hypercholesteremia specifically within the VLDL fraction that is associated with increased atherosclerosis[82]. Voluntary exercise attenuated the hypercholesteremia but failed to significantly reduce the subsequent increase in atherosclerosis. Within this study, voluntary running reduced lesion size by 22% compared to sedentary, PCB-treated animals. In vehicle-treated animals, exercise reduced lesion size by 68% which supports previous findings[159,169]. These findings suggest that exercise can prevent and/or delay the development of atherosclerosis in vehicle-treated animals only. Additional studies that utilize a greater sample size are needed to determine if exercise can prevent PCB-induced atherosclerosis.

Low-grade inflammation is a hallmark of endothelial dysfunction and atherosclerosis[2]. We have demonstrated previously that coplanar PCBs can cause endothelial dysfunction as evidenced by an upregulation of inflammatory mediators including the cytokines IL-6 and MCP-1[83,102,285,297]. In fact, PCB 77-treated animals that were exposed to exercise had levels of inflammation that were similar to control, sedentary animals. Exercise attenuated the PCB 77-mediated induction of inflammatory

cytokines and chemokines including MCP-1, IL-6, and M-CSF (Figure 6). Because inflammation is sensitive to redox changes or an increase in oxidative stress, we also assessed *in vivo* systemic oxidative stress. Results from our study indicate that exposure to PCB 77 leads to a dramatic increase in F_2-isoprostanes in sedentary mice, with much lower levels (8-fold) found in exercised animals, suggesting that exercise protects against systemic oxidative stress associated with PCB 77 exposure.

Mechanisms of protective properties of physical exercise such as voluntary exercise are not simple and may involve induction of phase II antioxidant enzymes[254]. Under normal physiological conditions, Nuclear factor erythroid 2 like 2 (Nrf2) is dormant within the cytoplasm while bound to its inhibitor, Kelch-like ECH-associated protein 1 (Keap1)[245]. In response to oxidative stress, Nrf2 dissociates from Keap1, translocates to the nucleus, and binds to antioxidant response elements (AREs) to upregulate cellular defense genes including GSH-dependent antioxidant enzymes (glutathione peroxidases and glutathione S-transferases)[244]. Exercise training results in increased levels of oxidative stress, which upregulates antioxidant defense mechanisms in various tissues including the liver[318]. This phenomenon is known as hormesis, defined as a generally favorable biological response to low exposure of toxins or other environmental stressors.[319] Our findings demonstrate a downregulation of the inhibitor protein Keap1 with an upregulation of several Nrf2 target genes including phase II antioxidant enzymes Gpx1, GST, and GSR. Additionally, our results show a downregulation of the phase I enzyme, CYP1A1, which has been implicated in contributing to oxidative stress in the presence of coplanar PCBs[68]. Superoxide can uncouple eNOS[320], the enzyme responsible for the production of the potent dilator nitric oxide, thus producing peroxynitrite and reducing endothelial-dependent dilation. We have previously shown in cultured endothelial cells, exposure to PCB 77 leads to an increase in peroxynitrite.[90]

In addition to well-established protective mechanisms of exercise, including down-regulation of inflammation through upregulation of antioxidant genes, our data with PCBs also implicate increased metabolism of lipophilic compounds such as PCBs. Compared to sedentary animals, we found that exercised animals exposed to PCB 77 had significantly less OH-PCB 77 metabolites within their feces. This suggests indirectly that exercise increased drug metabolism and that most of the PCB 77 may have been metabolized and/or excreted prior to the time of measurements (at the end of study). Exercise can alter pharmacokinetics by affecting drug absorption and hepatic and renal clearance of drugs [321]. Four weeks of voluntary exercise in C57Bl/6 mice led to an

upregulation of CYP7A1 and CYP27 which could aid in the secretion of cholesterol into bile acids[322]. Our findings warrant further investigation to determine the effect of exercise on coplanar PCB metabolism and excretion.

Although this is the first study to examine the effect of exercise on metabolism and overall body burden of PCBs, other groups have investigated the role of bioactive nutrients such as olestra[105] and chitosan[323] on excretion of toxicants including coplanar PCBs. These studies demonstrate that these nutrients including charcoal and choric acid[324] inhibit the body from absorbing these compounds while chlolella[325] and chlorophyll[326] promote its excretion from the body by disrupting gastrointestinal absorption. To our knowledge, there is no published work examining the effect of exercise on the metabolism, absorption, and excretion of environmental toxicants; however, other groups have studied flavanones metabolism. Triathletes excrete flavanones five-fold, which the authors propose occurs because of the overactivation of the microbiota metabolism caused by physical exercise[327]. Future studies will investigate levels of PCB 77 and its metabolites in a time course study in an attempt to determine if exercise interferes with the absorption through overactivation of gut microbiome or if exercise accelerates excretion, thus reducing body burden. We propose that exercise may enhance the clearance of coplanar PCBs through an Nrf2-dependent mechanism resulting in upregulation of phase II enzymes leading to enhanced metabolism (Figure 9).

In summary, our study provides experimental evidence that exercise is beneficial for protecting the vasculature against PCB-induced oxidative stress and inflammation. Coplanar PCBs are persistent and a significant risk factor for endothelial injury and associated cardiovascular disease. This is the first study to suggest that exercise can increase the metabolism and clearance of environmental pollutants, thus implying the need for additional studies to determine an effective regimen for protection. More studies are needed to determine if exercise can delay or prevent adverse health effects including atherosclerosis associated with PCB exposure.

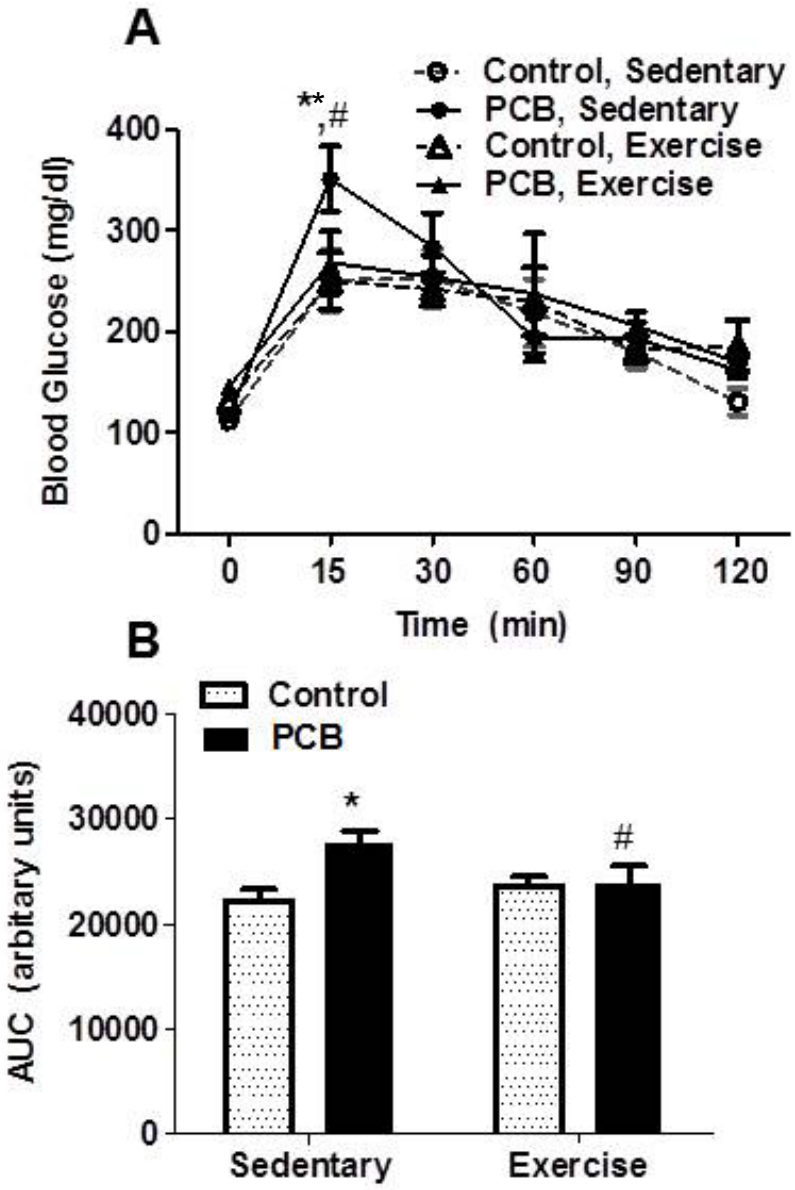


Figure 3-1. Exercise attenuates PCB 77-impaired glucose intolerance.

A) Blood glucose concentrations were examined in mice administered vehicle or PCB 77 (50 mg/kg). B) Total area under the curve (AUC) calculates the area below the observed concentrations. Data present mean ± SEM (n=8). *Significantly different compared to vehicle control, sedentary ($p<0.05$) # Significantly different compared to PCB 77-treated, sedentary mice ($p<0.05$)

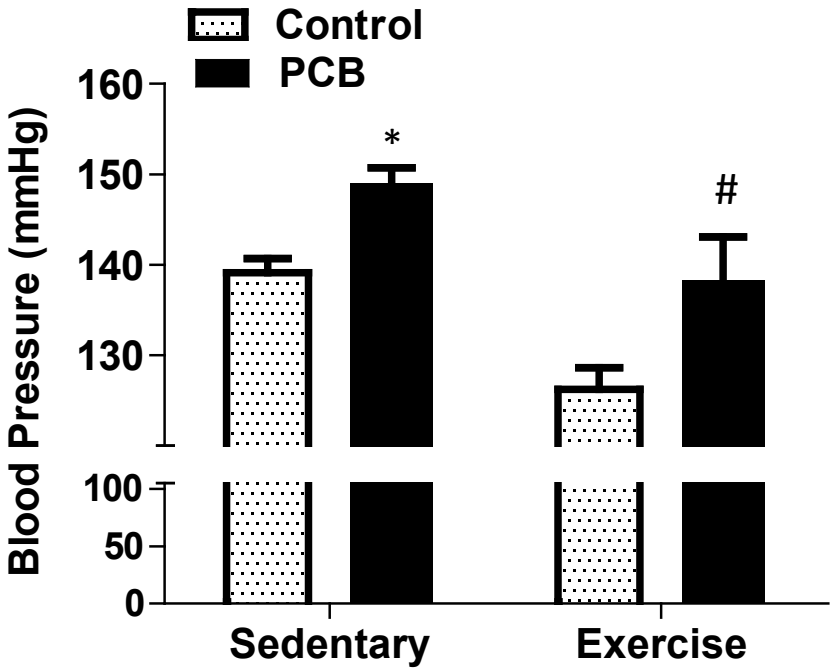


Figure 3-2. Exercise reduces systolic blood pressure in PCB 77-treated mice.

Blood pressure was measured non-invasively via the tail cuff method (Coda). Data represent mean±SEM (n=8). *Significantly different compared to vehicle control, sedentary ($p<0.05$)# Significantly different compared to PCB 77-treated, sedentary mice ($p<0.05$)

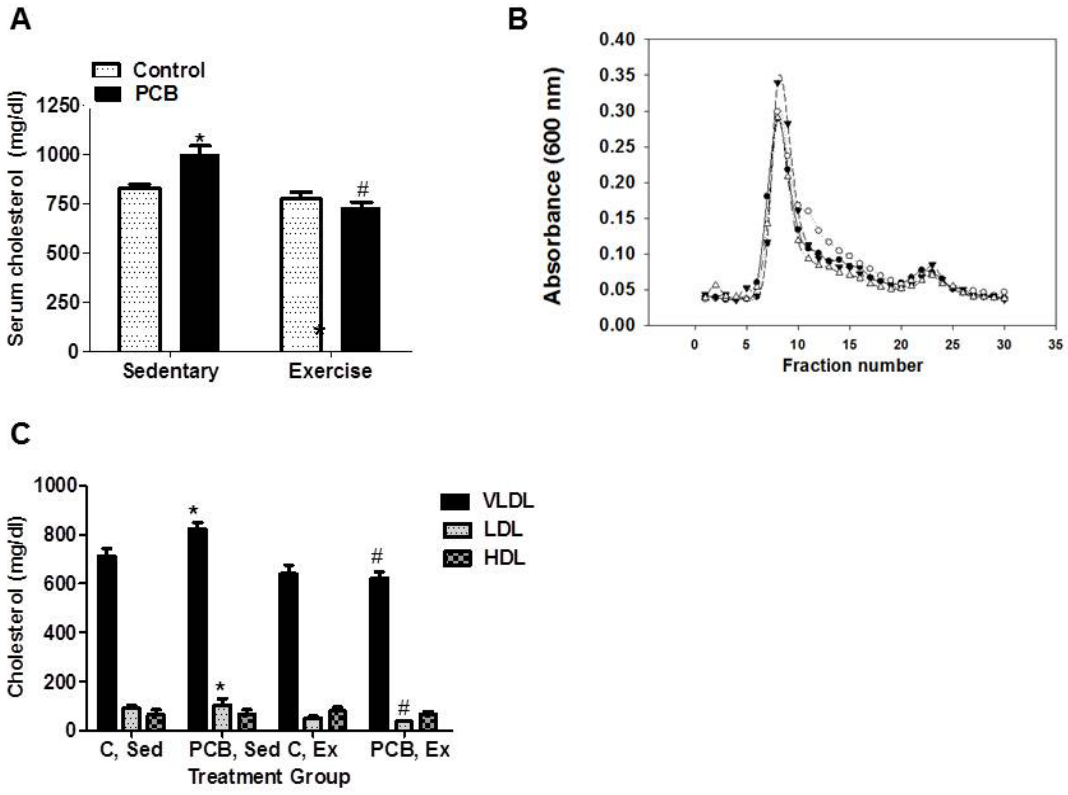


Figure 3-3. **Exercise reduces total plasma cholesterol and VLDL and LDL cholesterol concentrations.**

(A) Plasma cholesterol concentrations were measured at termination (B) Lipoproteins were resolved by size exclusion chromatography. Circles of each point represent mean values of each fraction from six individual mice of each group, and bars are SEMs. (C) Plasma cholesterol concentrations of lipoprotein fractions were calculated using a nonlinear curve fitting approach. Data represent the mean±SEM of 6 animals. Two-way ANOVA revealed a statistically significant interaction between exercise and PCB77. * Significantly different compared to vehicle control, sedentary ($p<0.05$). # Significantly different compared to PCB77-treated sedentary mice ($p<0.01$).

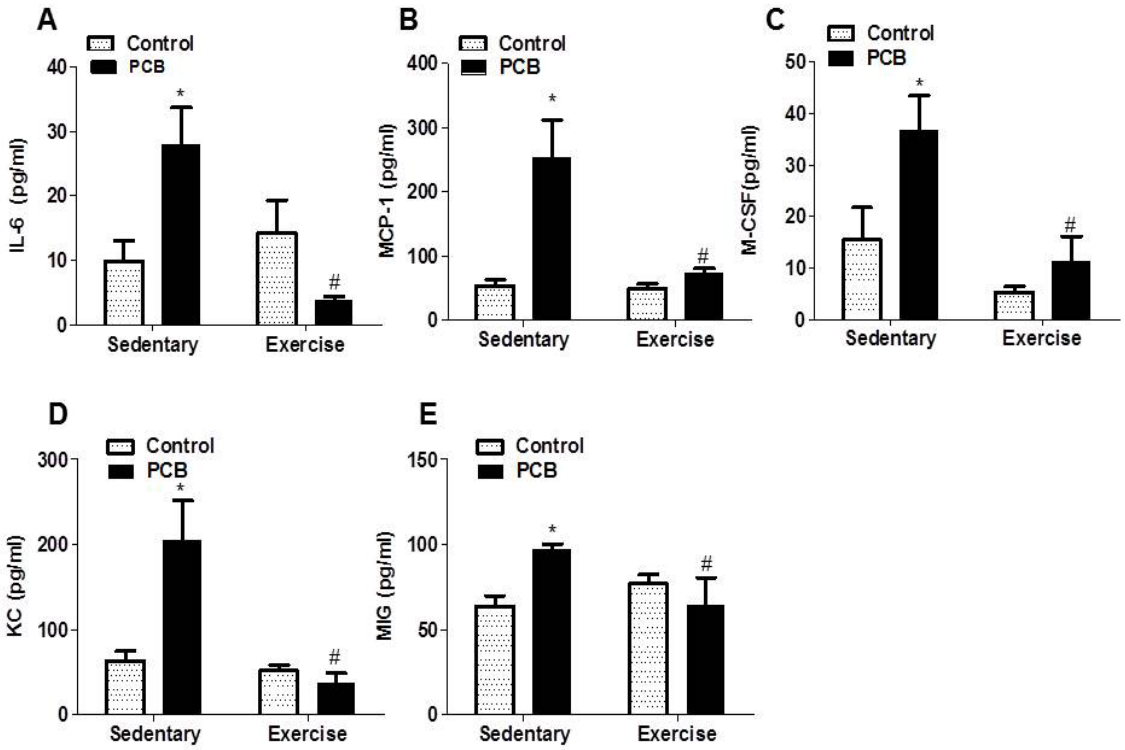


Figure 3-4. **Exercise prevents upregulation of proinflammatory cytokines by PCB77 exposure**.

Plasma samples were analyzed for IL-6, MCP-1, and M-CSF levels using mouse adipokine LINCOplex kit. Data represent the mean± SEM (n=5). * Significantly different compared to vehicle control, sedentary ($p<0.05$). # Significantly different compared to PCB77-treated sedentary mice ($p<0.05$)

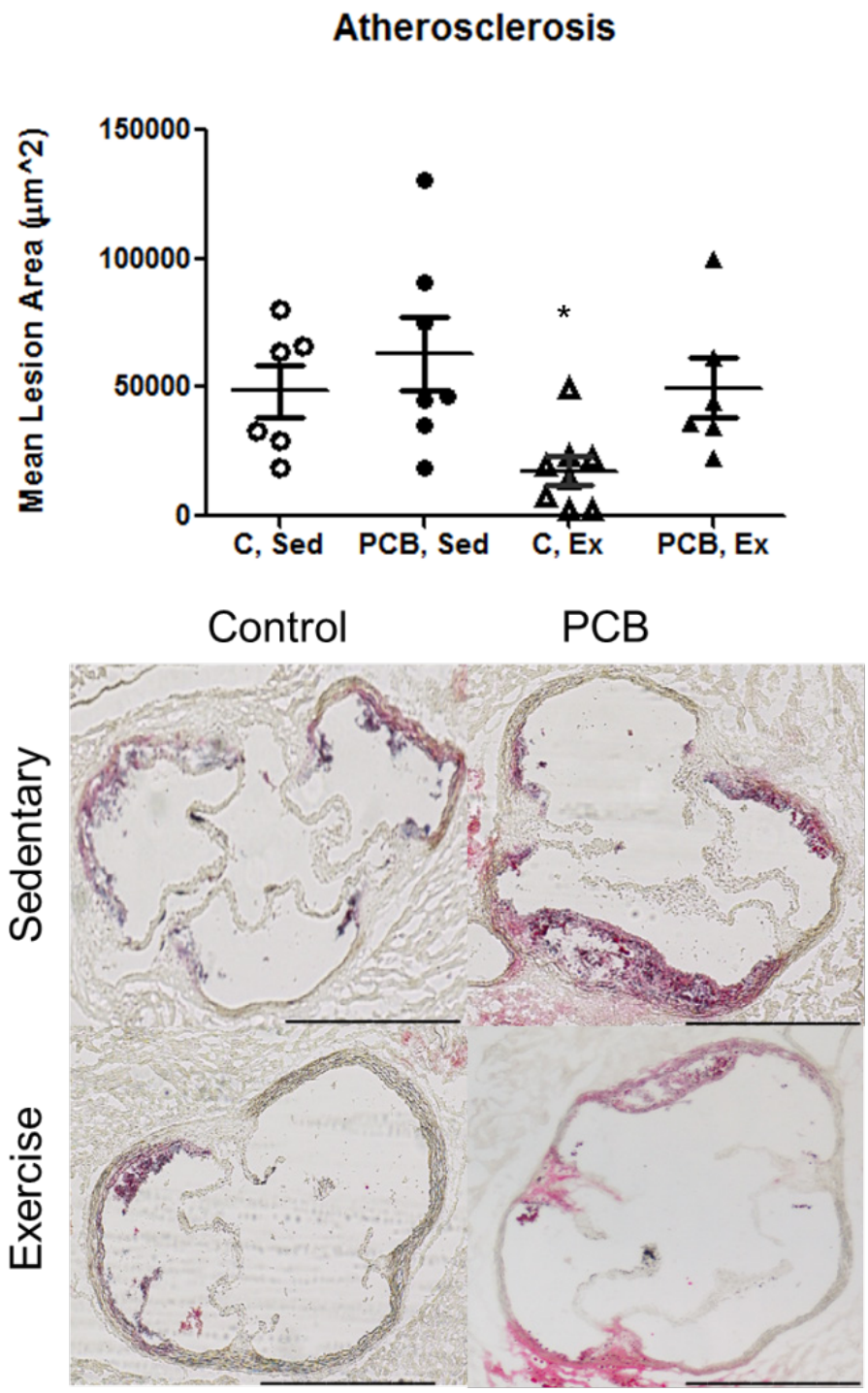


Figure 3-5. **Exercise reduces atherosclerosis in PCB77-treated mice**.
The aortic root was serially sectioned on a cryostat with 10 µm sections. Lesions were quantified through oil red O. The average lesion area spanning -240 to 240 µm is depicted. Data represent the mean ± SEM (n=6-8). Scale bar represents 500 µm
* Significantly different compared to vehicle control, sedentary ($p<0.05$).

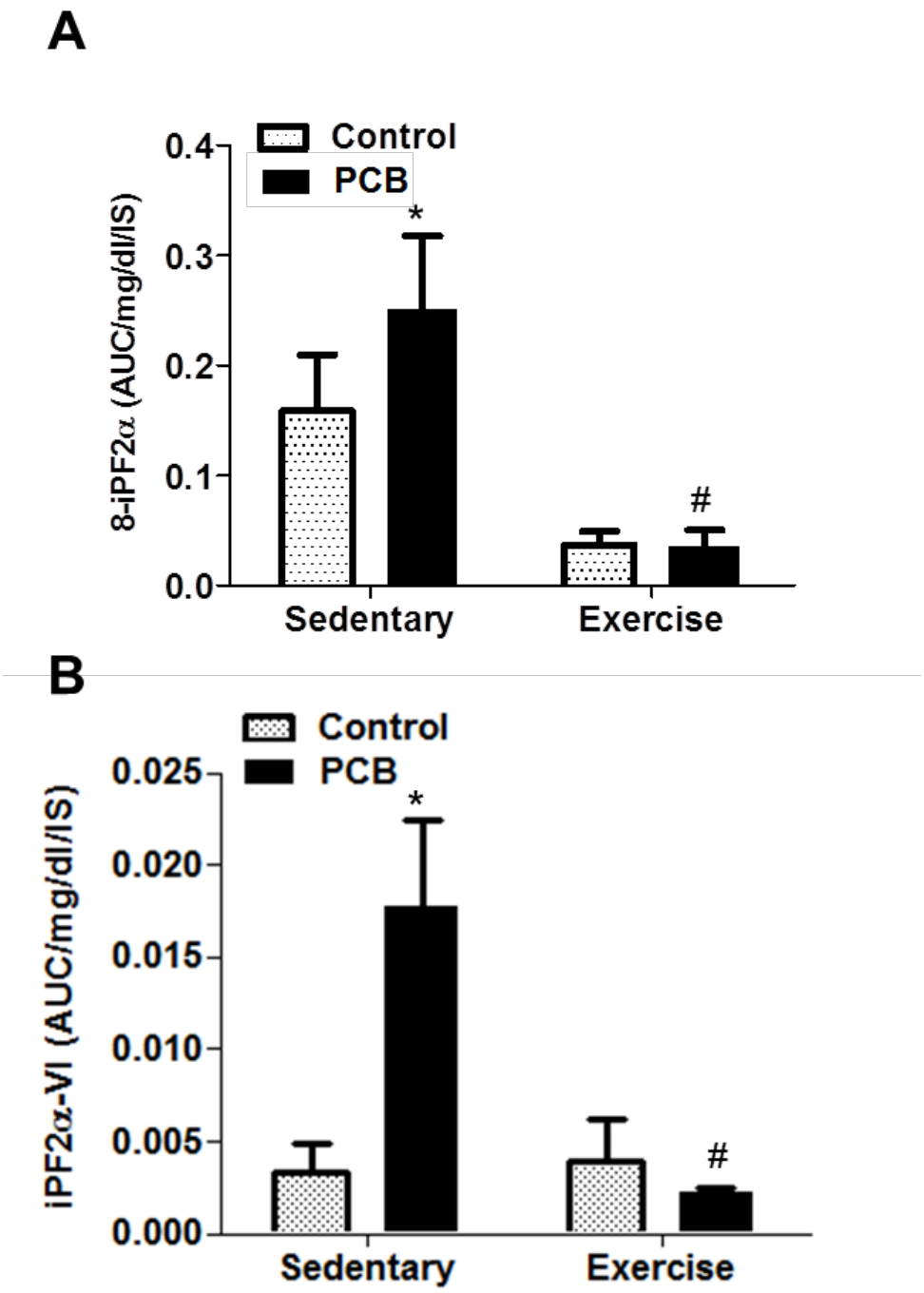


Figure 3-6. **Exercise modulates PCB 77-induced oxidative stress.**

Urine F_2-isoprostane levels were measured by HPLC/MS MS to assess *in vivo* oxidative stress induced by PCB77. All values were normalized to urine creatinine levels and for IS recovery. Data are represented as mean± SEM (n=5). *Significantly different compared to vehicle control, sedentary ($p<0.05$). # Significantly different compared to PCB77-treated sedentary mice ($p<0.05$).

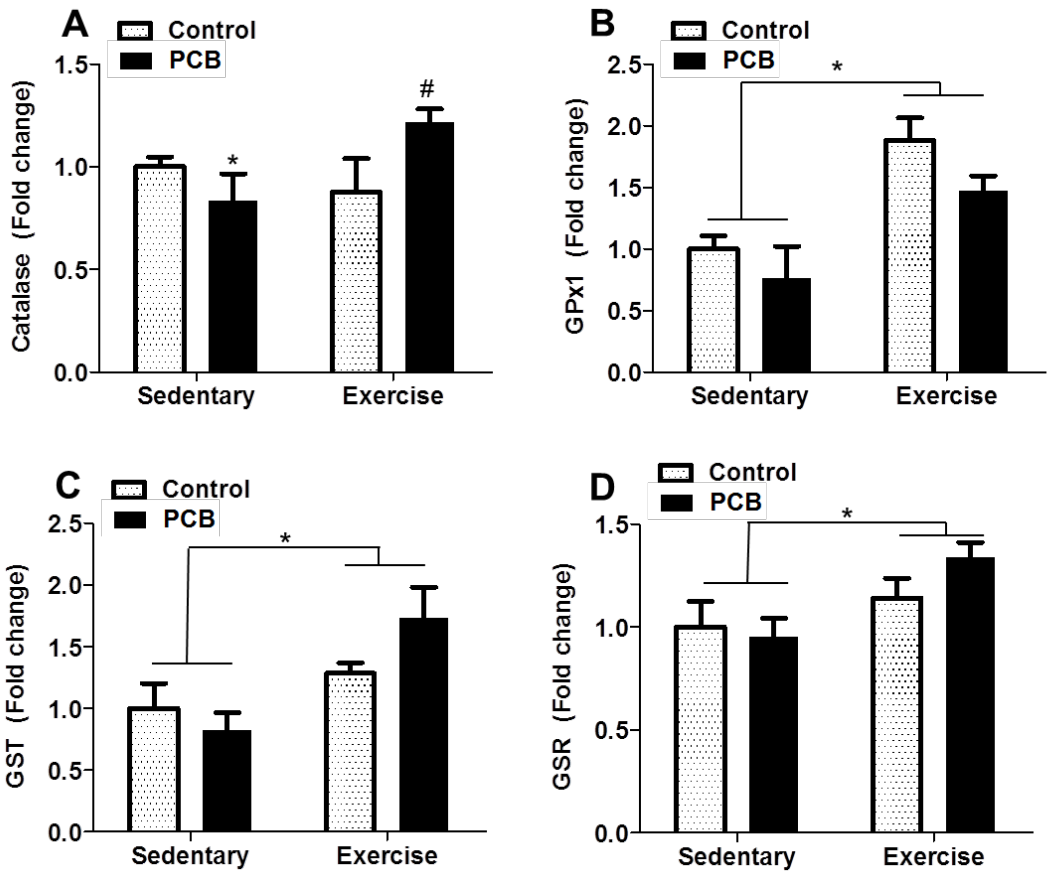


Figure 3-7. **Exercise upregulates antioxidant and phase II enzymes**.
mRNA levels were measured using RT-PCR. Two-way ANOVA revealed a statistically significant interaction between exercise and PCB77. Data are represented as mean ±SEM (n=8). *Significantly different compared to vehicle control, sedentary ($p<0.05$) #Significantly different compared to PCB77-treated sedentary mice ($p<0.05$)

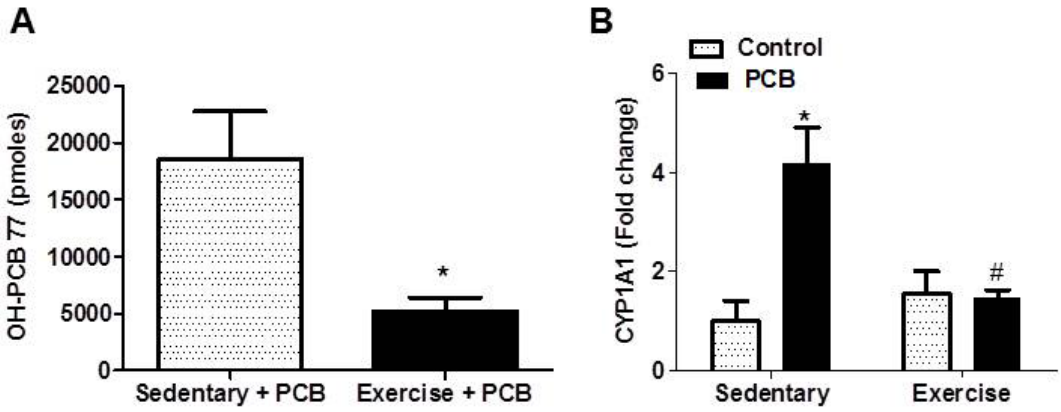


Figure 3-8. **Exercise decreases level of OH-PCB 77 in feces**

PCB 77 and its hydroxy metabolites were measured in plasma and liver by UFLC/MS MS and normalized to IS recovery. Data are mean ± SEM (n=5). A student's t-test revealed statistical differences compared to sedentary animals (*p<0.05).

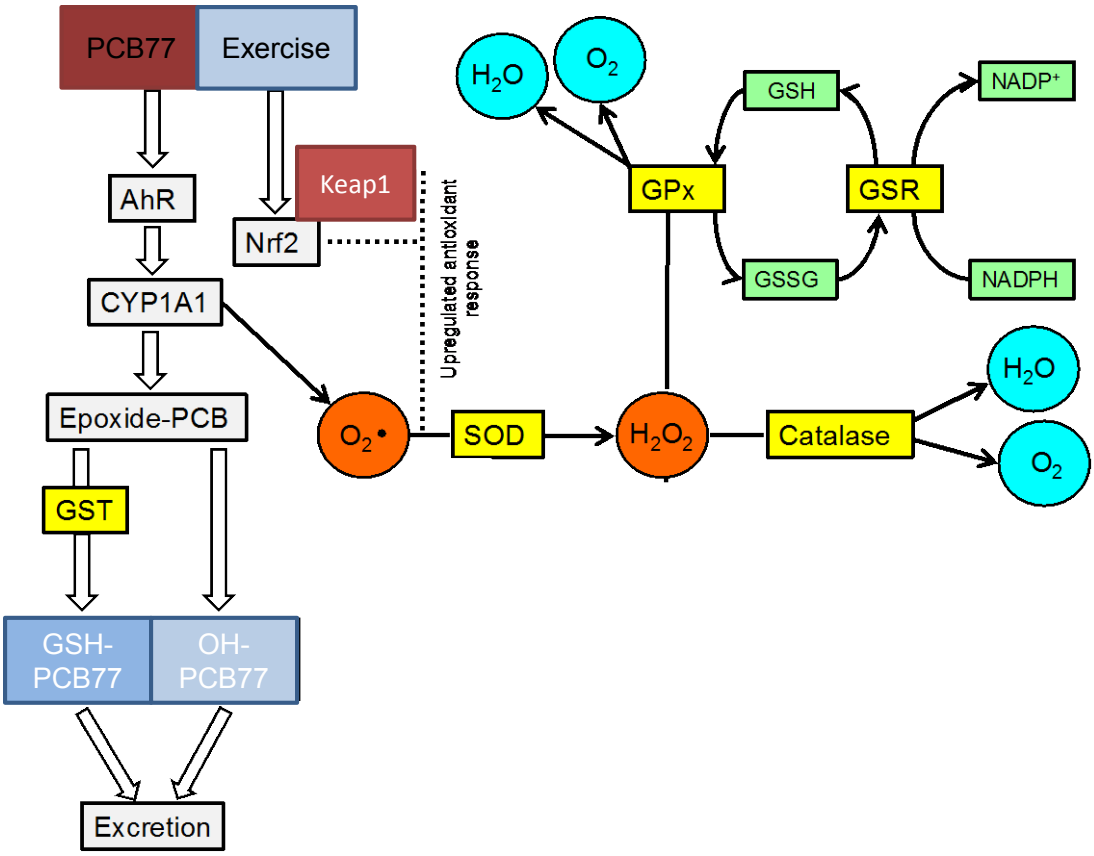
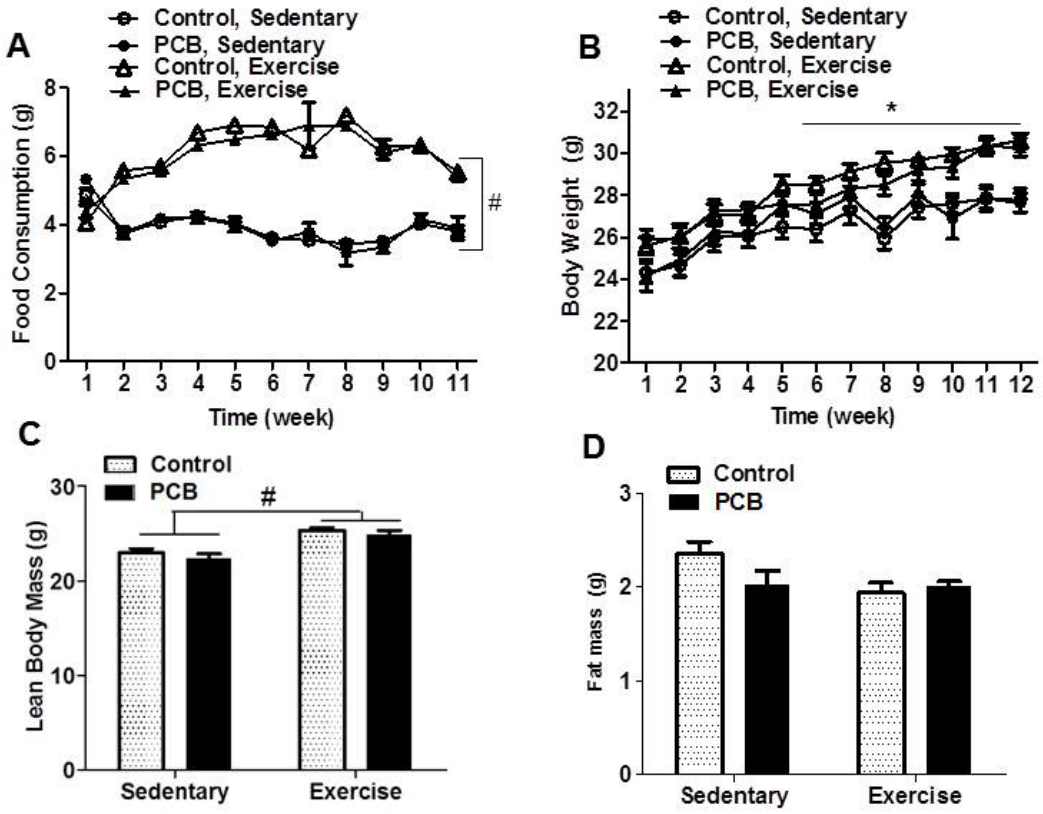
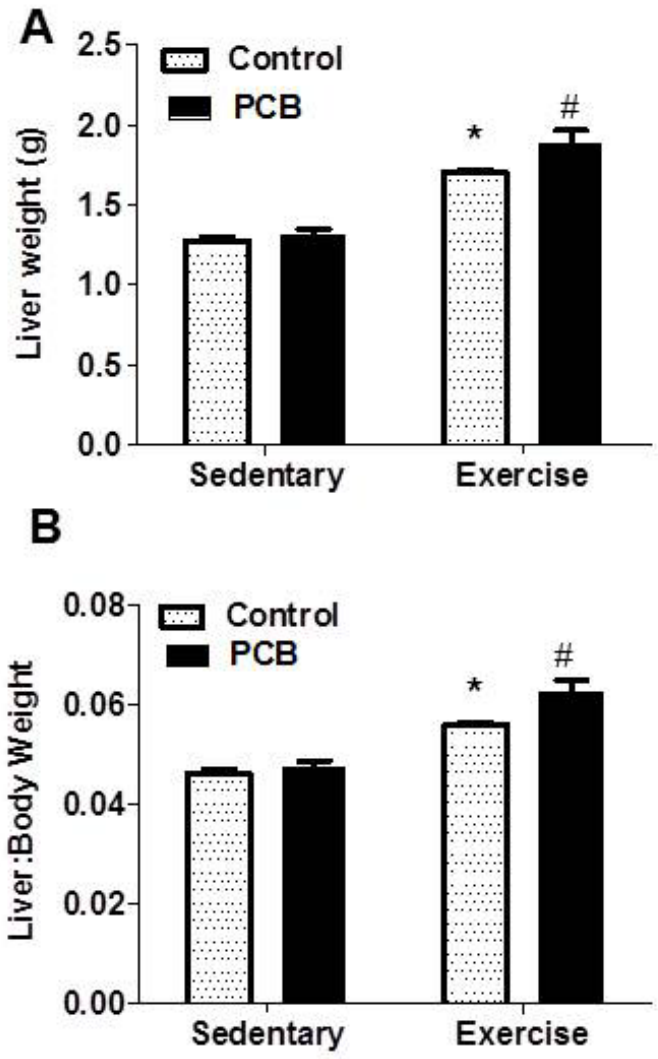


Figure 3-9. Proposed signaling pathway for PCB detoxification in vivo.
PCB 77 is an AhR ligand and causes CYP1A1 upregulation, which when in the presence of PCB 77 leads to superoxide production. Exercise effectively upregulates the antioxidant response in the presence of PCB77 which allows for a more efficient antioxidant response to environmental insult. (Adapted from Newsome et al *JNB*, 2013)

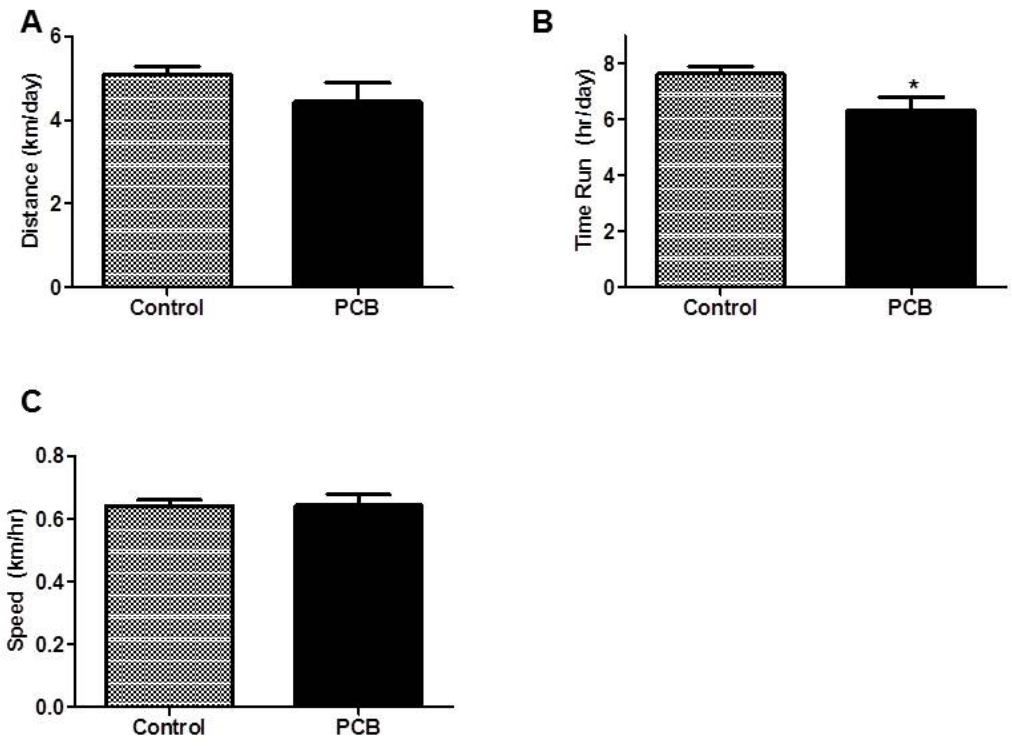


Supplementary Figure 1. Exercise increases body weight and lean body mass. A) Body weights were measured weekly. B) Food consumption was measured weekly. C) Lean mass and D) fat mass were measured through Echo-MRI. Data represent the mean ± SEM. Two-way ANOVA revealed a statistically significant interaction between exercise and sedentary groups. * Significantly different compared to vehicle sedentary groups ($p<0.05$).# Significantly different compared to sedentary groups ($p<0.001$)



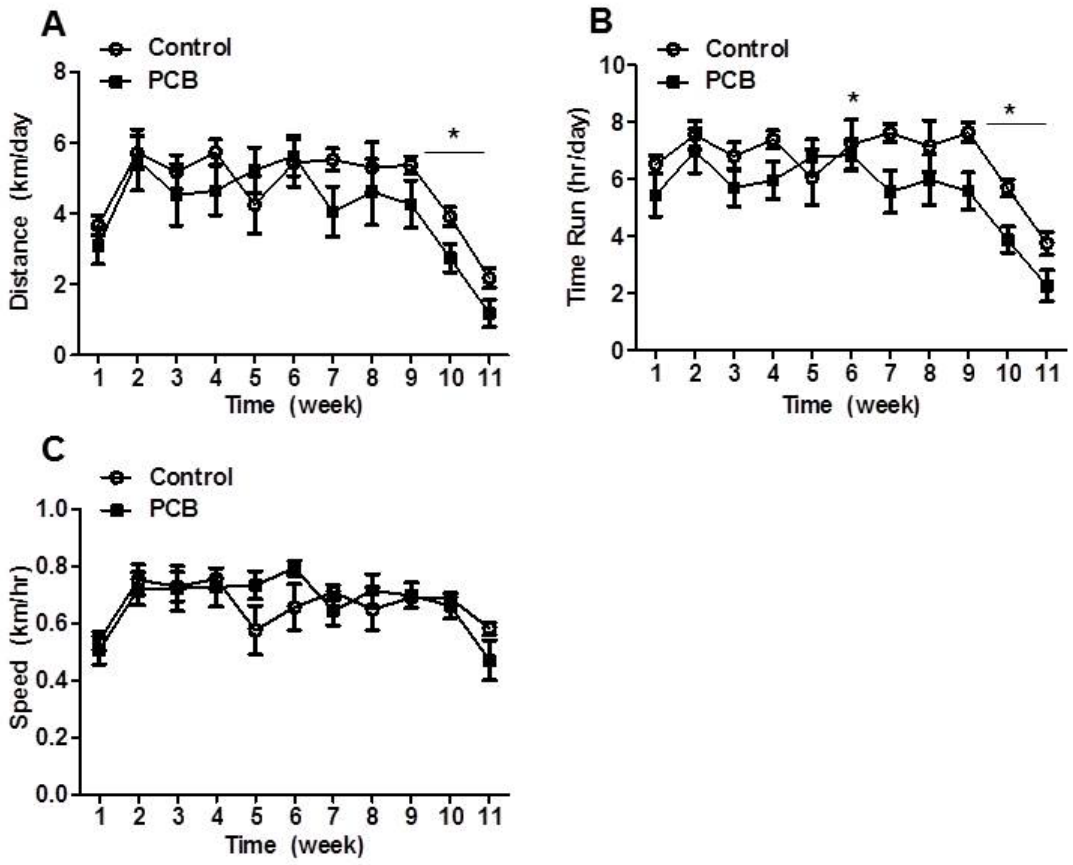
Supplementary Figure 3-2 Exercise increases liver: body weight in PCB77-treated mice

Liver and body weights were weighed at conclusion of the study. Data represent mean ± SEM. Two-way ANOVA revealed a statistical interaction between PCB 77 and exercise. *Significantly different from control, sedentary ($p<0.05$) #Significantly different from sedentary, PCB77-treated animals and control, exercise animals ($p<0.001$).



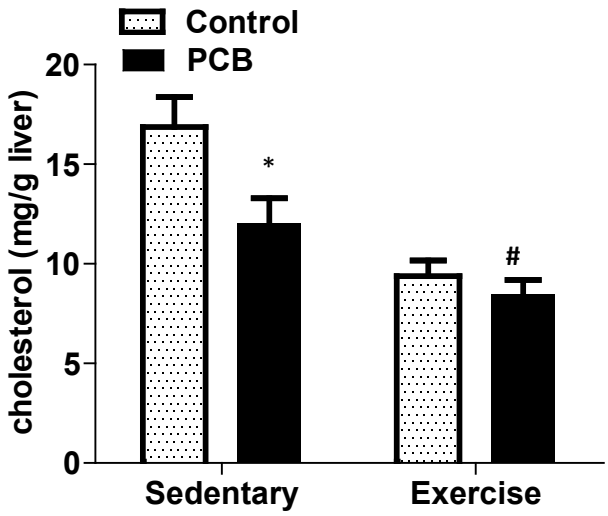
Supplementary Figure 3-3 Voluntary wheel-running performance in control and PCB77 treated mice

8 week old male ApoE-/- mice were housed singly in cages mounted with a running wheel. Wheel-running activity was monitored continuously and analyzed using a Clock-Lab Analysis program. A) Mean daily running distance B) Mean weekly running time C) Mean running velocity (km/h) during the 12 weeks of observation. A student's t test demonstrated a trend that PCB77-treated mice spent less time running ($p<0.05$).



Supplementary Figure 3-4 Voluntary wheel-running performance in control and PCB77-treated mice

Male ApoE-/- mice were housed singly in cages mounted with a running wheel. Wheel lrunning activity was monitored continuously and analyzed using a Clock-Lab Analysis program.A) Mean daily running distance B) Mean weekly running time C) Mean running velocity(km/h) during the experiment. A student's t-test demonstrated that PCB77-treated mice spent less time running during weeks 7, 9, 10, and 11 and covered less distance during weeks 10 and 11 ($p<0.05$).



Supplementary Figure 3-5 Exercise reduces hepatic cholesterol levels.

Data represent mean ± SEM. Two-way ANOVA revealed a statistical interaction between PCB 77 and exercise. *Significantly different from control, sedentary ($p<0.05$) #Significantly different from sedentary, PCB77-treated animals and control, exercise animals ($p<0.05$).

Table 3-1
Primers used for qRT-PCR

Gene name	Forward Primer	Reverse Primer 5'-3'	Fragment size
CYP1A1	TGGAGCTTCCCCGATCCT	CATACATGGAAGGCATGATCTAGGT	100 bp
Nrf2	GAGTCGCTTGCCCTGGATATC	TCATGGCTGCCTCCAGAGAA	100 bp
Catalase	CAGAGAGCGGATTCCTGAGAGA	CTTTGCCTTGGAGTATCTGGTGAT	100 bp
GSR	TCGGAATTCATGCACGATCA	GGCTCACATAGGCATCCCTTT	100 bp
GSTm2	ACACCCGCATACAGTTGGC	TGCTTGCCCAGAAACTCAGAG	118 bp
Gpx1	GTGGCGTCACTCTGAGGAACA	CAGTTCTCCTGATGTCCGAACTG	125 bp

Chapter 4: Effect of exercise on PCB 77-induced endothelial dysfunction in C57BL/6 mice

4.1 Synopsis

Polychlorinated biphenyls (PCBs) are persistent environmental chemicals, and coplanar PCBs can induce oxidative stress and activate pro-inflammatory signaling cascades which are associated with atherosclerosis. Physical inactivity is considered an independent risk factor for CVD and has been shown to cause endothelial dysfunction. Numerous studies in both humans and animal models have demonstrated a beneficial role for exercise in the prevention and treatment of CVD. Thus, we hypothesized that voluntary exercise can modulate PCB-induced endothelial dysfunction. To test this hypothesis, C57BL/6 mice were placed on a voluntary exercise regimen for 5 weeks before administration of PCB 77, 24 hours before euthanasia. *Ex vivo* vascular reactivity studies were performed to measure endothelial function. Sedentary animals exposed to PCB77 exhibited endothelial dysfunction as demonstrated by significant impairment of endothelium-dependent-dilation (EDD), which was prevented in exercised animals. Administration of tempol, a superoxide dismutase (SOD) mimetic restored endothelium-dependent vasodilation implicating increased superoxide levels as a cause of endothelial dysfunction in these animals. Voluntary exercise decreased plasma F_2-isoprostane levels, an *in vivo* marker of oxidative stress. Furthermore, CYP1A1, a phase I detoxifying enzyme was downregulated in exercised animals although liver and plasma levels of PCB 77 were not different between groups. These data suggest that voluntary exercise provides vascular protection by preventing PCB 77-induced endothelial dysfunction.

4.2. Introduction

Polychlorinated biphenyls (PCBs) are environmental pollutants that were manufactured for use in dielectric and coolant fluids, lubricants, and flame retardants due to their chemical stability. PCB production was banned in the United States in 1979 due to their carcinogenic nature[41]. Because of their chemical stability, PCBs continue to persist in the environment. Human exposure occurs primarily through dietary intake of contaminated food and continues to bioaccumulate within individuals[328].

The toxicity of coplanar PCBs is similar to dioxin and is mediated through activation of the aryl hydrocarbon receptor (AhR), an orphan receptor that is classified as a basic helix-loop-helix Per-ARNT-Sim transcription factor[65]. Upon ligand binding (e.g., PCB 77), AhR translocates to the nucleus and binds to xenobiotic response elements (XREs) within the promoters of downstream target genes[66]. Coplanar PCBs increase cellular oxidative stress through an uncoupling of cytochrome P450 (CYP1A1)-mediated uncoupling mechanism[68].

Human exposure to coplanar PCBs has been associated with cardiovascular disease and its associated risk factors including diabetes[77], hypertension[48], dyslipidemia[329], and endothelial dysfunction[84,313,330]. Endothelial dysfunction is an independent risk factor for cardiovascular disease[27] and precedes the development of atherosclerosis[331]. Endothelial dysfunction is very common in patients with atherosclerosis and hypertension[332]. Nitric oxide (NO) is a key regulator of normal endothelial function. NO is produced by endothelial nitric oxide synthase (eNOS) during the conversion of L-arginine to L-citrulline through receptor activation (e.g., muscarinic receptors) or mechanical force (e.g., shear stress)[320]. NO relaxes blood vessels, prevents platelet aggregation and adhesion, limits oxidation of low-density lipoprotein (LDL) cholesterol, inhibits proliferation of vascular smooth muscle cells, and decreases expression of proinflammatory cytokines[187].

Oxidative stress is regulated by mechanisms that keep a tight balance between reactive oxygen species and antioxidant enzymes that remove ROS. Excessive ROS leads to cellular damage of DNA, lipid, and proteins[333]. We have previously shown in cultured porcine aortic endothelial cells, administration of PCB 77 leads to an increase in reactive oxygen species (ROS)[313] and subsequent dysfunctional eNOS signaling[90]. eNOS becomes uncoupled and produces superoxide instead of NO which reacts very rapidly with existing NO to form peroxynitrite leading to endothelial dysfunction and subsequent NFκB-mediated proinflammatory signaling. Endothelial dysfunction is most commonly assessed by vascular reactivity studies that measure the vasodilator response in isolated vessels to various pharmacological agonists[334]. Acetylcholine (ACh) is commonly used to assess endothelial dependent vasodilation because it acts via muscarinic membrane receptors with signal transduction through adaptor proteins that lead to the release of NO. NO diffuses from the endothelial cell to smooth muscle cells where it activates guanylate cyclase to produce cyclic GMP leading to smooth muscle relaxation[335]. *Ex vivo* studies have demonstrated impaired acetylcholine-induced

endothelium-dependent vasorelaxation in hypercholesterolemic animals[158] as well as C57BL/6 mice exposed to dioxin[336]. However, numerous studies have demonstrated the benefits of exercise in restoring endothelial function d[158,164,171,174,337]. This likely occurs by increasing NO bioavailability; however, the mechanisms are not fully understood.

Because ApoE-/- mice do not undergo endothelial dysfunction until later in life[338] and LDL-/- mice require high fat feeding[339], we utilized C57BL/6 mice to avoid such confounders including age and diet. In this study, we hypothesize that exercise will prevent PCB-induced endothelium-dependent dilation by reducing systemic oxidative stress and increasing the bioavailability of NO.

4.3 Methods

4.3.1. Chemicals

We purchased PCB 77 from Accustandard Inc. (New Haven, CT). Phenylephrine, Sodium Nitroprusside, Acetylcholine, Tempol, and L-NG-Nitroarginine Methyl Ester (L-NAME) were obtained from Sigma Aldrich (St. Louis, MO).

4.3.2. Animal treatment

Male C57BL/6 mice were obtained from the Jackson Laboratories. (Bar Harbor, ME). C57BL/6 mice have been shown to exhibit high running behavior[340]. Each mouse was individually caged, handled, and used in compliance with the Animal Care and Use Committee of the University of Kentucky. Mice were given ad libitum access to food (rodent standard chow) and water and housed in a pathogen-free environment for 5 weeks. Body weight was measured weekly. Urine and fecal samples were not obtained in order to minimize stress within these animals. Mice were administered a single dose of vehicle (tocopherol-stripped safflower oil, 0.2 mL)), or PCB 77 (170 μM/kg) by intraperitoneal injection 24 hours before euthanasia. This dose has been shown to produce endothelial dysfunction as measured by impaired endothelial-dependent vasodilation (data not shown). At the study end point, mice were euthanized with ketamine/xylene and exsanguinated. Ethylenediaminetetraacetic acid (EDTA) was added to collected blood samples, briefly mixed, and centrifuged at 5000g for 5 min at 4^{o} C to separate plasma. Plasma and tissue samples were frozen in liquid nitrogen and stored at -80^{o}C.

4.3.3. Exercise

Our model of exercise was the widely used voluntary running-wheel model, previously described[271]. Each mouse randomized to exercise was placed in a modified cage with wheel attached to a magnetic sensing mechanism. This allowed the running activity of each mouse to be tracked by a computer with Clock lab and Mat lab software (Actimetrics, Wilmette, IL) from which the corresponding distance, speed, and amount of time spent running were obtained.

4.3.4. Ex vivo vascular reactivity studies

Forty Male C57BL/6 mice were randomized to exercise or sedentary groups for five weeks based on previous findings implicating a beneficial role of exercise when exposed to PCB [301]. Mice were anesthetized with ketamine/xylene. Aortas from each mouse were perfused with Krebs Henseleit solution via the left ventricle and then removed. Adventitia was carefully dissected free. Measurement of contractile activity was performed using aortic rings as described previously[341]. Briefly, ascending (3 mm) aortic segments were mounted by passing two tungsten wires through the arterial lumen while immersed in Krebs Henseleit solution. Tension (1g) was maintained continuously and recorded on a Micro-Med instrument. Krebs Henseleit solution was refreshed in tissue baths every 10 min. After 30 min of equilibration, tissue viability was tested with 80 mM potassium chloride for 5 min. For dilation studies, vessels were pre-constricted for 3 minutes with 10^{-6} M Phenylephrine and then exposed to a cumulative dose of Acetylcholine (10^{-9} to 10^{-5} M). For endothelium independent studies, vessels were pre-constricted with 10^{-6} M PE and exposed to a cumulative dose of SNP (10^{-9} to 10^{-5}M). Following a 30 min washout period, aortas were pre-incubated with TEMPOL (1 mM) or L-NAME (10 μM) for 3 min prior to repeating the ACh dose response in pre-constricted vessels.

4.3.5. Quantification of PCBs and F_2-isoprostanes

PCB 77 and its hydroxyl metabolites were extracted from plasma and tissue samples to determine systemic PCB and metabolite concentrations. PCB 77 and its hydroxyl metabolites were isolated from plasma and liver samples (plus 10 μM 13C12-labeled PCB126 internal standard (IS) and d6-PCB77, Cambridge Isotope Laboratories, Tewksbury, MA) through extraction with acetonitrile and subsequent sonication and

centrifugation at 15,000 rpm for 5 min to pellet plasma and tissue debris. Supernatants were dried under N_2 and reconstituted in 99:1 methanol: dl H_2O solvent mixture with 0.5% formic acid and 0.1% 5M ammonium formate[249].

Measurement of urinary F_2-Isoprostanes (F_2-IsoPs) is considered the gold standard for assessment of in vivo oxidative stress[307]. We did not collect urine samples for this study, however, plasma was obtained and has been shown to be a reliable index for in vivo oxidative stress[249].For F_2-IsoP analysis, plasma samples were added to 5:1 ethyl acetate:methanol + 0.5% acetic acid (v/v) + 10μM 8-iso-PGF2α-D4 (internal standard, Cayman Chemical, Ann Arbor, MI), vortexed briefly, and centrifuged to pellet debris. Supernatants were transferred and dried under N_2 and reconstituted in methanol and acetic acid before solid phase extraction (SPE). Reconstituted F_2-IsoP samples were loaded onto pre-conditioned Supel-Select HLB SPE columns (Sigma-Aldrich, St. Louis, MO) and washed with 0.5% acetic acid followed by washing with 0.5% acetic acid containing 20% methanol. Columns were eluted with methanol, eluent was dried under N_2 and samples were reconstituted with 50:50 methanol: dl H_2O.

Plasma and tissue levels of PCB77 and its hydroxyl metabolites as well as plasmaF_2-IsoPs were analyzed using a Shimadzu ultrafast liquid chromatography (UFLC) coupled with an AB Sciex 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode. MRM transitions monitored: 291.9/ 222.1 and 291.9/220 for PCB 77; 338/268.1 and 338/196.1 for 13C12 PCB 126 and 297.9/228.1 and 297.9/226.2 for d6-PCB77. In the MRM ion transition the precursor ion represents the $M^{+\cdot}$ and the product ion represents either $[M\text{-}Cl]^+$ or $[M\text{-}2Cl]^+$.MRM transitions monitored for PCB77 metabolites: 352.8/306.9 for hydroxy PCB77 and 368.8/322.9 for dihydroxy PCB77. Precursor ion of the ion transition is a formic acid adduct: [M+FA-H]- and product ion is [M-H]-. F_2-IsoPs were analyzed by integrating peak area (area under the curve, AUC) with regard to known internal standard concentrations (AUC/IS). All values were subsequently normalized according to plasma volume and compared to ion transitions of internal standard (13C12 PCB126) with known concentration to determine PCB parent and metabolite concentrations (pmol/μL plasma).

4.3.6. Plasma and liver cholesterol measurement

Plasma cholesterol concentrations were measured using an enzymatic kit (Wako Chemicals USA, Richmond, VA.). Liver cholesterol was measured as previously

described[272]. Briefly, liver tissue (30-50 mg) was homogenized in Krebs-Ringer Solution through repeated low speed sonication for 30 seconds, 10 times. Liver cholesterol concentrations were measured using the enzymatic kit described above. Data are expressed as cholesterol per mg wet tissue weight.

4.3.7. Quantification of mRNA using RT-PCR

Total RNA was extracted from tissues using the TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol[249]. mRNA concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). Reverse transcription was performed using the AMV reverse transcription system (Promega, Madison, WI). The levels of mRNA expression were then assessed by quantitative real-time PCR using 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) and SYBR Green master mix (Applied Biosystems). Data analysis was performed using the relative quantification method (ΔΔCt), in which relative mRNA expression for target mRNAs was compared to a constitutively expressed gene (i.e., β-actin) in the mRNA samples from tissues. Primer sequences (see Table 1) for SYBR Green chemistry were designed using the Primer Express Software 3.0 for RT-PCR (Applied Biosystems) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

4.3.8. Statistical analysis

Data are represented as mean ± SEM. Two-way ANOVA was used, followed by a post-hoc Tukey's test to measure differences using SigmaStat software (Systat Software, Point Richmond, CA). Differences with a value of $p<0.05$ were considered statistically significant. For vascular function studies, Two-way Repeated Measures ANOVA was performed.

4.4 Results

Exercise training did not significantly change body weight among groups during the study nor did an acute dose of 170 μM/kg of PCB 77 24 hours before euthanasia (Figure 1). After one week of acclimation, mice ran predominantly at night for a total of 5 weeks for 6.2 h/day on average. The average speed of mice was 0.89 km/hour with average distance 6.34 km/day (Figure 2). No differences were noted between

experimental groups. PCB 77 concentration in plasma and liver samples were examined to determine the systemic body burden and to determine whether acute exercise would have an effect on metabolism and excretion of this toxicant. As seen in Figure 3, plasma and liver levels of PCB 77 were much lower than its hydroxyl metabolites. Sedentary animals had approximately 113 pmoles PCB 77 within the liver compared to 118 pmoles in exercised animals. Exercise did not significantly change the amount of parent compound in either liver or plasma levels; however, there was a trend towards less OH-PCB 77 within the plasma compared to sedentary animals (64.6 pmoles in sedentary animals versus 42.4 pmoles in exercised animals; p=0.09). The exercised animals had higher levels of hydroxyl metabolites within the liver (996 pmoles) compared to sedentary animals (609 pmoles). Although there were not significant differences among parent compound, the increase in hydroxyl metabolites within the liver could suggest enhanced metabolism within exercised animals.

4.4.1. Exercise lowers F_2-isoprostane levels

Analysis of F_2-isoprostanes, prostaglandin-like eicosanoids derived from arachadonic acid metabolism, is considered the most reliable marker of in vivo oxidative stress. Plasma samples from mice that were sedentary or exercised and subsequently exposed to PCB 77 were analyzed to determine whether exercise could decrease toxicant-induced oxidative stress. Plasma F_2-IsoP (including 8-isoPF2α, iPF2α-III, PGF2α-IV, PGF2α-V, and PGF2α-VI) and F_2-isoP metabolites (13, 14-dihydro-15-ketoPGF2α) concentrations were determined. 13, 14-dihydro-15-ketoPGF2α and 8-isoPF2α levels were not detectable within these animals. As seen in figure 4-5, exercise led to a significant reduction in F2-isoPs including PGF2α-IV and PGF2α-V in mice exposed to PCB 77, suggesting that exercise has potent antioxidative effects.

4.4.2. Exercise did not lower plasma and liver cholesterol levels

Because PCB 77 administration has been shown to increase plasma and hepatic cholesterol[82,342], we examined whether exercise could mitigate this effect. Acute administration of PCB 77 did not increase plasma or hepatic cholesterol. Male C57BL/6 are resistant to hypercholesteremia unless dietary manipulation occurs[339], thus our findings support this notion.

4.4.3. Exercise restores endothelium-dependent vasodilation in PCB 77-treated mice

To substantiate the PCB-induced dysfunction of the vascular endothelium and subsequent increase in atherosclerosis, vascular functional studies with isolated vessels were performed. PCB 77 impaired ACh-dependent relaxation, an endothelial-dependent event (Figure 7A). Two way ANOVA RM revealed that a statistically significant interaction between treatment groups and concentration ($p=0.002$). Further analysis revealed a statistical difference This reduced relaxation was rescued in vessels derived from exercised but not from sedentary animals. Pre-incubation with Tempol, a superoxide dismutase mimetic, was necessary to improve the PCB-induced relaxation impairment observed in sedentary animals (Figure 7C). Pre-incubation with L-NAME, a NO inhibitor, significantly reduced endothelium dependent relaxation (Figure 7D). As previously reported [336], there were no differences in endothelium independent vasodilation among all treatment groups implicating the importance of the vascular endothelium and not smooth muscle cells in PCB-induced endothelial dysfunction.

4.4.4. Exercise reduces expression of CYP1A1

Cytochrome P450-1A1 (CYP1A1), a phase I metabolizing enzyme, as well as several antioxidant enzymes were analyzed in liver samples. Significant CYP1A1 expression was seen in the presence of PCB 77, which has been shown previously, while exercise attenuated this response (Figure 4-8). Surprisingly, exercise did not lead to the upregulation of any antioxidant enzymes we analyzed including Nrf2 downstream targets: GSR, GST, catalase, SOD-1, NQO1, and HO-1. This data suggests that exercise led to decreased expression of CYP1A1, which could explain the significant reduction in oxidative stress in exercised animals.

4.5 Discussion

Lifestyle changes such as nutrition and physical activity may modulate environmental pollutant toxicity. Exercise has been shown to prevent endothelial dysfunction and subsequent cardiovascular disease[184]. However, the effects of exercise on coplanar PCB-induced endothelial dysfunction are unknown. In the current study, we propose that reduction in oxidative stress and subsequent increased bioavailability of NO is the main mechanism for the protective effects of exercise against PCB-induced

cardiovascular toxicity. To test this hypothesis, male C57BL/6 mice were exposed to voluntary wheel running for 5 weeks. Our previous work has demonstrated that exercise can reduce atherosclerotic lesion development within the aortic sinus (Chapter 3 of this dissertation), thus we isolated aorta from exercised mice to perform vascular reactivity studies in order to examine the effect exercise and PCB exposure have on endothelial-dependent vasodilation.

Endothelial cells play an active role in regulation of vessel tone, blood coagulation, and permeability. Endothelial dysfunction is a critical step in the development of cardiovascular disease including atherosclerosis [333,343] . To further investigate the role of the vascular endothelium in PCB exposure, we performed *ex vivo* vascular reactivity studies. Exposure to PCB 77 led to severe impairment of endothelium dependent vasodilation in sedentary animals. Exercise was able to prevent the PCB-induced impairment. Reduced NO bioavailability is thought to be a primary cause of endothelial dysfunction [332]. Our data show that ACh-induced relaxation is blocked in all groups but PCB 77-treated sedentary animals. Because the relaxation response in exercised and control groups is significantly blunted when treated with the NO inhibitor L-NAME, one could argue that increased bioavailability of NO is one of the protective mechanisms of exercise within the vascular endothelium. In fact, pre-incubation with L-NAME significantly reduced endothelium dependent relaxation in all groups, except in PCB-treated sedentary animals. Because pre-incubation with L-NAME, a NO inhibitor, significantly reduced endothelium dependent vasodilation in all groups, except in PCB-treated sedentary animals, NO-mediated mechanism may also contribute to the protective effects of exercise against PCB-induced endothelial dysfunction. Our work in cultured endothelial cells suggests that PCB can cause dysfunctional NO signaling and subsequent rise in peroxynitrite [90]. Interestingly, our data did not show exercise modulating several cardiovascular disease risk factors including plasma lipids or body weight, but there was a significant improvement in vascular function. A potential explanation for this could be enhanced eNOS activity through phosphorylation of ser1177[344]. Other groups have reported that despite changes in body weight or glucose sensitivity, exercise led to an upregulation of phosphorylation levels of eNOS following exercise[345]. Although we did not measure aortic levels of eNOS or p-eNOS, future measurements would substantiate our proposed mechanism of increased bioavailability of NO especially since exercise has been shown to increase eNOS activity in *db/db* knockout mice[190].

The superoxide mimetic Tempol rescued the impaired vasodilation in PCB-treated sedentary animals, suggesting a relationship between PCB exposure, increased oxidative stress and dysfunction of the vascular endothelium. Previous publications have reported rescue of dioxin-induced endothelial dysfunction by Tempol in sedentary mice [267]. This implies that exercise might normalize the redox status within the vascular endothelium by reducing production of superoxide, thus improving vasodilation. Exercise did not significantly change the amount of parent PCB 77 within liver or plasma; however, there was a trend towards an increase of OH-PCB 77 within the liver and lower plasma levels in these mice. We did not quantify adipose levels of PCB 77, which is a limitation within this work as it has been reported that PCB 77 accumulates within the adipose tissue[306] and whether exercise affects this storage depot remains unknown. Despite similar levels of parent compound of PCB 77 among exercise and sedentary groups, exercise led to a significant downregulation of the phase I metabolizing enzyme, CYP1A1 (Figure 4-8). This downregulation of CYP1A1 could explain the reduction in superoxide levels since PCB 77 administration has been reported to lead to the enzyme's uncoupling and enhanced production of superoxide. Furthermore CYP1A1 KO mice are resistant to dioxin-induced endothelial dysfunction[336]. Surprisingly, our results did not show an antioxidant response in many Nrf2 downstream targets including HO-1, NQO1, GST, and GSR. Because Nrf2 is redox sensitive, this suggests that a lack of oxidative stress in exercised animals (Figure 4-4) prevented its activation and subsequent downstream gene expression. Stimulation of Nrf2 appears to require induction of oxidative stress by a damaging agent such as PCB 77 which has been reported in a similar study examining the effect of resveratrol on PCB 77-induced glucose intolerance[346]. Because CYP1A1 was downregulated in these animals and less oxidative stress was present systematically, Nrf2 may not have had the proper stimuli to become activated. Together this data suggests that exercise attenuates PCB-induced endothelial dysfunction by reducing levels of oxidative stress through downregulation of CYP1A1 expression.

Another mechanism of PCB 77-induced endothelial dysfunction could be an upregulation of Caveolin-1. Our lab has shown that PCB 77 administration increases formation of caveolae and expression of Caveolin-1[92]. A recent study demonstrated in ApoE-/- mice that an increase expression in caveolin-1 protein expression is associated with impaired endothelial dependent vasodilation. Additionally, there was a decreased ratio of p-eNOS to eNOS in thoracic aortas of these mice suggesting a decreased

activation of eNOS[347]. eNOS can directly interact with the scaffolding domain of Cav-1 and become inhibited[348]. Determining whether exercise could modulate caveolae-and its associated signaling would be an exciting area of future research since studies are currently lacking. One study has demonstrated that voluntary exercise can attenuate the upregulation of caveolae-associated NAD(P)H oxidase subunits p47 and gp91 and protects against glutathione depletion in mice exposed to methamphetamine through a Nrf-2 mediated mechanism within cerebral microvessels[340]. Caveolin-1 can interact with Nrf2 leading to inhibition of specific antioxidant enzymes as well as suppress its transcriptional activity[349]. The recent discovery of Nrf2: Caveolin-1 cross talk is a new area of research within our lab and whether exercise plays a role in downregulation of Caveolin-1 thus increasing NO bioavailability through eNOS as well as enhanced activity of Nrf2 is an exciting area to further explore.

Additionally, the same group has examined the effect of exercise on PCB-induced changes in the gut microbiome. Exercise was able to prevent PCB changes of gut bacteria including the decreased population of Proteobacteria. PCB exposure can disrupt intestinal cells and lead to a "leaky" intestinal mucosal barrier by disrupting tight junction proteins which is likely to increase concentrations of PCBs in the systemic circulation and lead to enhanced PCB accumulation in tissues[350]. In addition to mediating changes of the vascular endothelium, exercise could lead to alterations in gut microbiome as well as increased metabolism and excretion of these compounds by preventing intestinal barrier disruption, increasing cholesterol turnover and subsequent PCB clearance. Future studies are needed to further delineate the beneficial effects of exercise on PCB-induced cardiovascular disease.

In conclusion, our results indicate that an acute dose of PCB 77 leads to increased oxidative stress, upregulation of CYP1A1, and impaired endothelial-dependent vasodilation in C57BL/6 mice. Voluntary wheel running prevented these effects and prevented endothelial dysfunction. This study supports a new role for exercise in preventing environmental pollutant-induced endothelial dysfunction by decreasing systemic levels of oxidative stress and downregulation of CYP1A1.

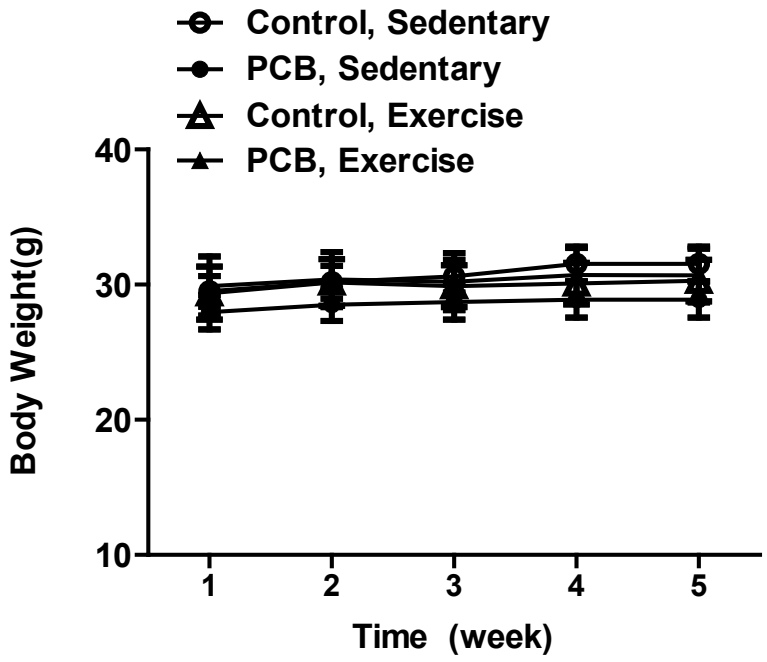


Figure 4-1 Voluntary exercise had no effect on body weight

Body weights were measured weekly. Body weight remained relatively constant in each experimental group throughout the study. Data represent the mean ± SEM.

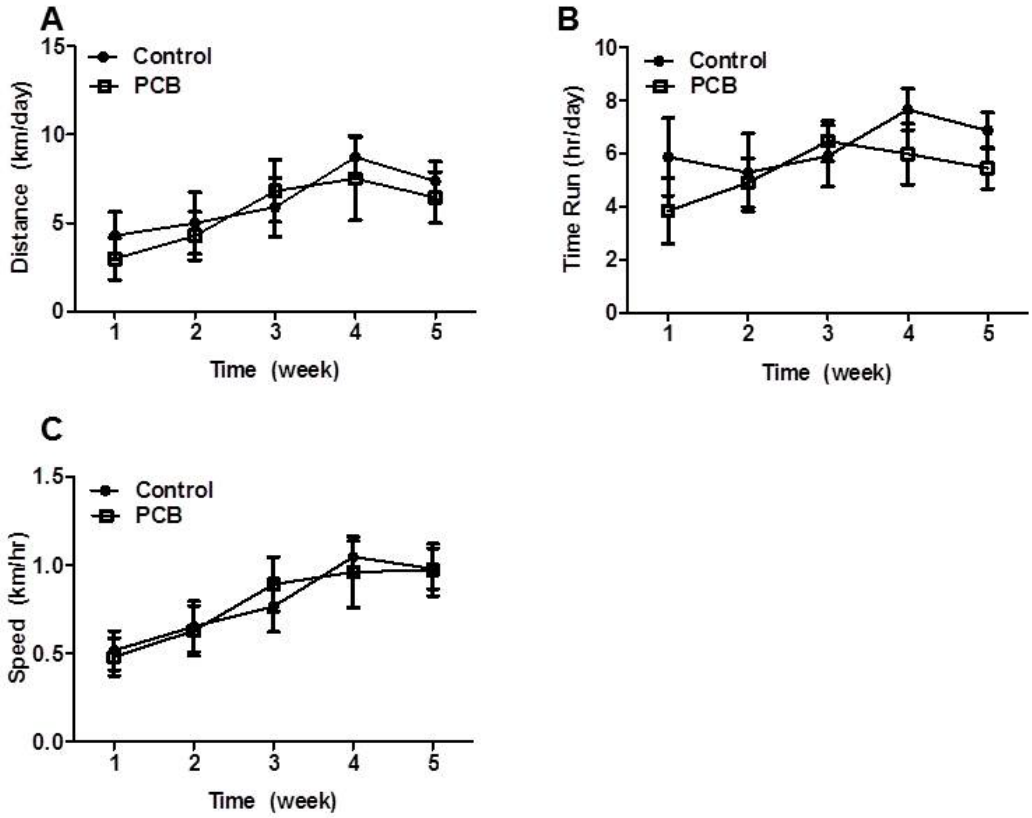


Figure 4-2 Voluntary wheel-running performance

Male C57BL/6 mice were housed singly in cages mounted with a running wheel. Wheel-running activity was monitored continuously and analyzed using a Clock-Lab Analysis program. A) Mean daily running distance B) Mean weekly running timeC) Mean running velocity during the 5 weeks of observation. Data represent the mean ± SEM (n= 8-9).

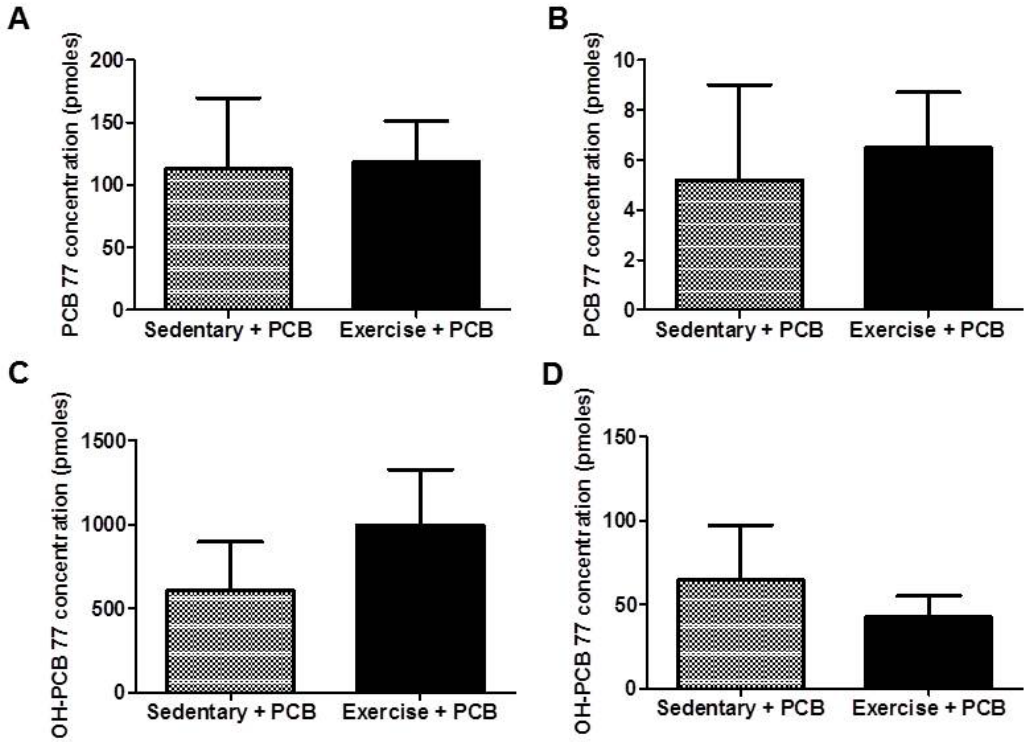


Figure 4-3 The effect of exercise on PCB 77 and OH-PCB 77 concentration. PCB 77 was measured in A) liver and B) plasma by UFLC/MS MS and normalized to sample volume and IS recovery. Hydroxyl metabolites of PCB 77 were measured in C) liver and D) plasma. Exercise did not significantly alter the amount of PCB 77 within liver or plasma; however, there was a trend towards an increase of OH-PCB 77 within the liver and lower systemic levels. Data are represented as mean ±SEM (n=5).

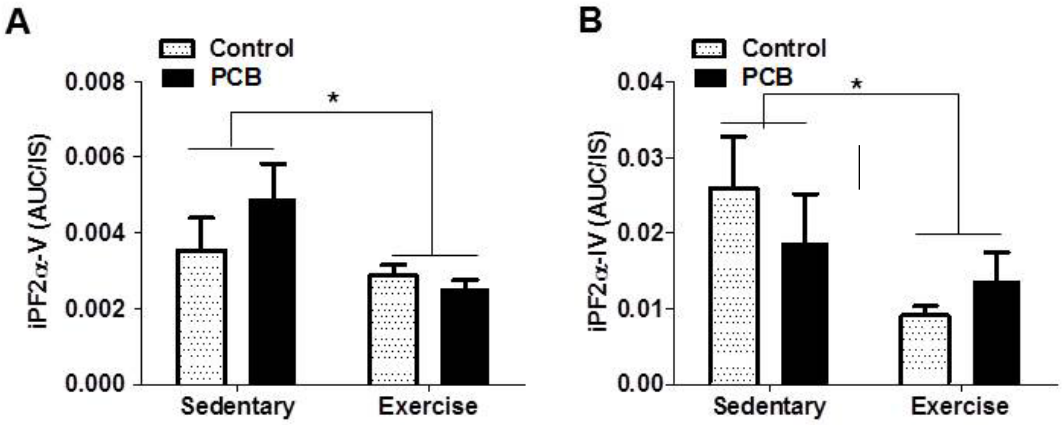


Figure 4-4 PCB 77 induced oxidative stress is reduced in exercised animals. Plasma F_2-isoprostane levels were measured by HPLC/MS MS to assess *in vivo* oxidative stress induced by PCB 77. Data are represented as mean ± SEM (n=5). Exercise groups had reduced oxidative stress levels compared to sedentary groups (*$p<0.05$).

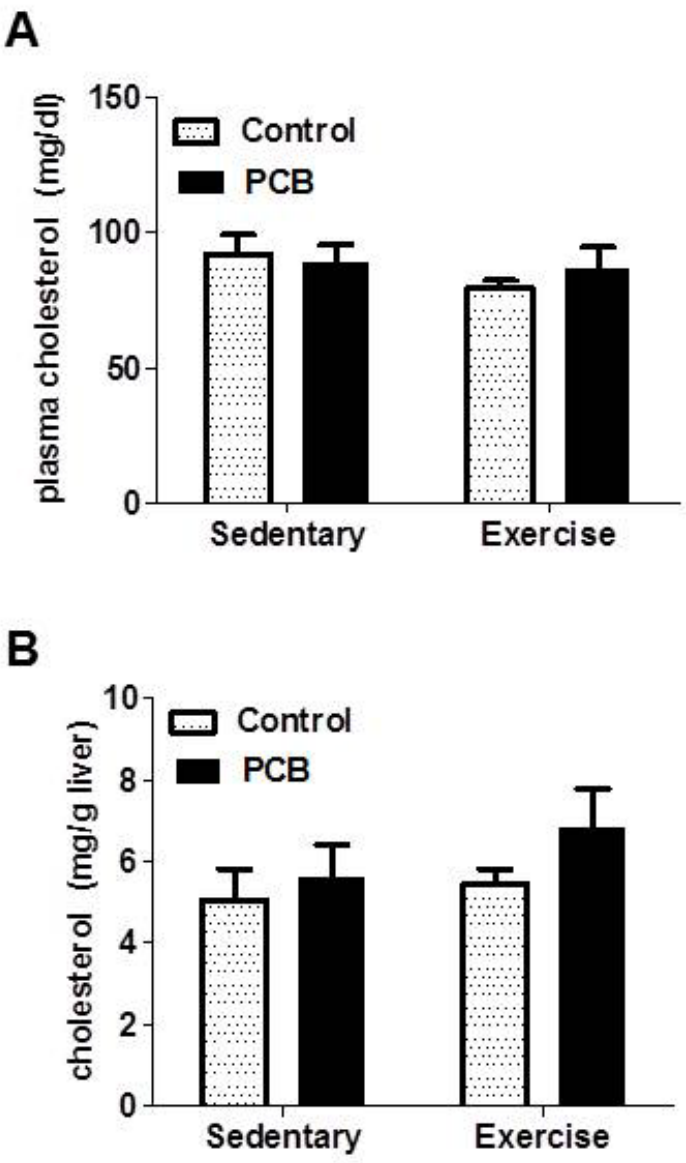


Figure 4-5 Exercise does not reduce plasma or liver cholesterol levels

A) Plasma and B) liver cholesterol concentrations were measured at termination through an enzymatic kit. Data represent the mean ±SEM (n=8-9).

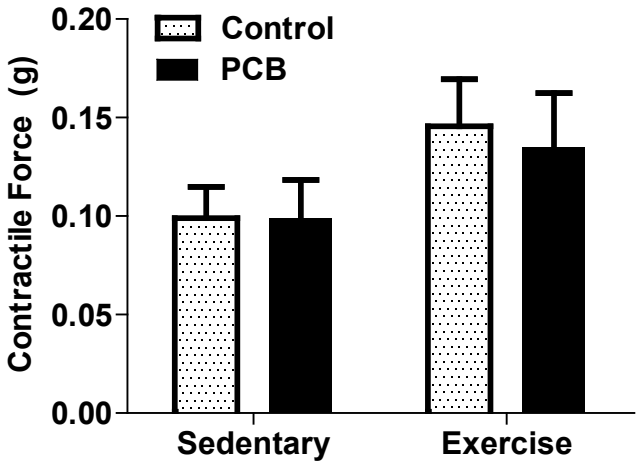


Figure 4-6 Confirmation of Tissue Viability.

Aortic vessels were pre-constricted with KCl to assess tissue viability. Data represent the mean ± SEM.

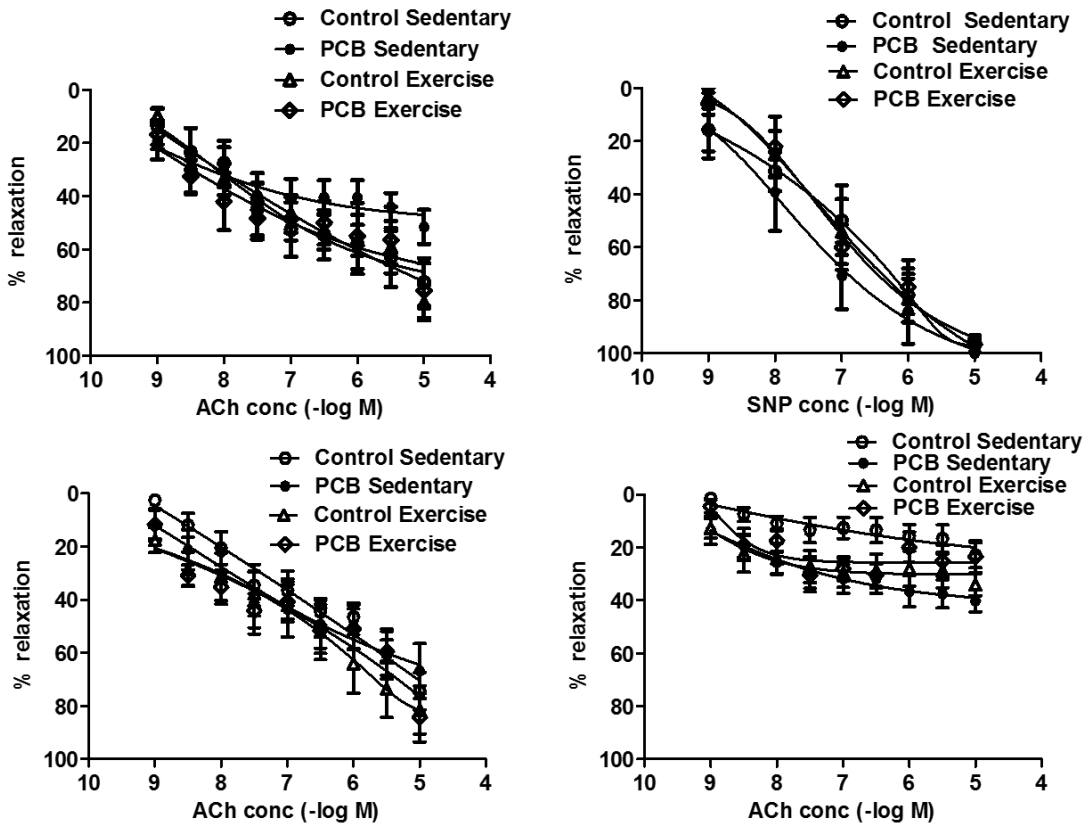


Figure 4.7 Exercise restores endothelium-dependent dilation in PCB77 impaired vessels Isolated aortic rings were dilated in response to ACh in a concentration-dependent manner. A) ACh-induced vasodilation was significantly attenuated in PCB, Sedentary mice. B) No differences in vasorelaxation response to SNP. C) Pre-incubation with Tempol (10^{-3}M) restored vasodilation in PCB, Sedentary animals. (n=7)
D) Incubation of L-NAME (10^{-5}M) significantly reduced ACh-dependent dilation in mice. (n=7)

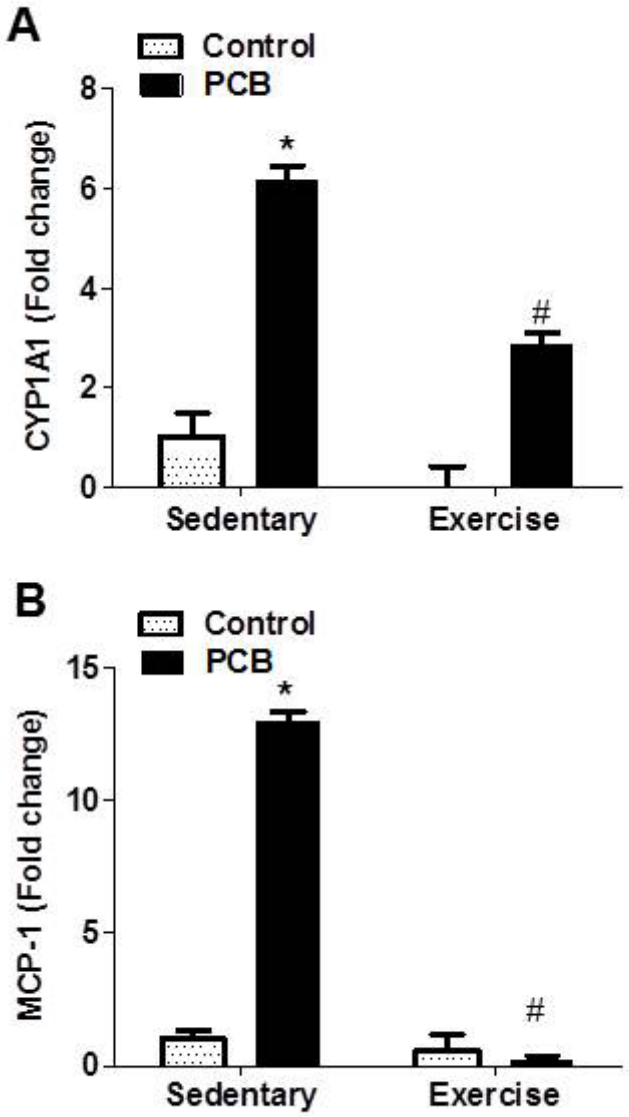


Figure 4-8 Exercise reduces CYP1A1 and MCP-1 levels in PCB 77-treated animals. mRNA levels were measured using RT-PCR. Two-way ANOVA revealed a statistically significant interaction between exercise and PCB77. Data are represented as mean ±SEM (n=5-8). *Significantly different compared to vehicle control, sedentary ($p<0.05$) #Significantly different compared to PCB77-treated sedentary mice ($p<0.05$)

Table 4-1
Primers used for qRT-PCR

Gene name	Forward Primer	Reverse Primer 5'-3'	Fragment size
CYP1A1	TGGAGCTTCCCCGATCCT	CATACATGGAAGGCATGATCTAGGT	100 bp
MCP-1	GCAGTTAACGCCCCACTCA	CCTACTCATTGGGATCATCTTGCT	63 bp
Nrf2	GAGTCGCTTGCCCTGGATATC	TCATGGCTGCCTCCAGAGAA	100 bp
Catalase	CAGAGAGCGGATTCCTGAGAGA	CTTTGCCTTGGAGTATCTGGTGAT	100 bp
GSR	TCGGAATTCATGCACGATCA	GGCTCACATAGGCATCCCTTT	100 bp
NQO1	GGCATCCAGTCCTCCATCAA	GTTAGTCCCTCGGCCATTGTT	100 bp
SOD1	GAAACAAGATGACTTGGGCAAAG	TTACTGCGCAATCCCAATCA	100 bp
GSTa1	AAGCCCGTGCTTCACTACTTC	GGGCACTTGGTCAAACATCAAA	159 bp
GSTa4	TACCTCGCTGCCAAGTACAAC	GAGCCACGGCAATCATCATCA	109 bp
GSTm1	ATACTGGGATACTGGAACGTCC	AGTCAGGGTTGTAACAGAGCAT	349 bp
GSTm2	ACACCCGCATACAGTTGGC	TGCTTGCCCAGAAACTCAGAG	118 bp
GSTm3	CCCCAACTTTGACCGAAGC	GGTGTCCATAACTTGGTTCTCCA	208bp
Gpx1	GTGGCGTCACTCTGAGGAACA	CAGTTCTCCTGATGTCCGAACTG	125 bp
Gpx2	GTGGCGTCACTCTGAGGAACA	CAGTTCTCCTGATGTCCGAACTG	125 bp
Gpx3	CATACCGGTTATGCGCTGGTA	CCTGCCGCCTCATGTAAGAC	80 bp

Chapter Five: General Discussion

5.1 Discussion

5.1.1 Summary

The purpose of this dissertation was to test the hypothesis that voluntary exercise protects against polychlorinated biphenyl-induced cardiovascular disease and that this protection is mediated through enhanced nitric oxide (NO) bioavailability and Nrf2-mediated signaling pathways. The work presented in this dissertation demonstrates a novel approach of how cardiovascular toxicity due to coplanar PCBs can be mediated through aerobic endurance exercise. Within the LDLr-/- mouse model, there is evidence suggesting exercise could reduce PCB-induced atherosclerosis; however, confounding variables including problems from high-fat feeding and intraperitoneal injection led us to switch models to the ApoE-/- mouse. Within this model, we were able to show that exercise improved cardiovascular disease risk factors including glucose intolerance, dyslipidemia, hypertension, systemic inflammation, and oxidative stress compared to sedentary, PCB-treated animals. Additionally, exercise reduced atherosclerotic lesions while upregulating antioxidant enzymes, which are involved in phase II metabolism of PCB 77 and reduce oxidative stress. However, exercise did not significantly reduce mean atherosclerotic lesion in PCB-treated animals. This work suggests that exercise may increase the metabolism; in addition to, excretion of coplanar PCBs. Exercise was identified as a treatment which prevented impaired endothelial vasodilation due to acute exposure to PCB 77, our proven model for coplanar PCB induced endothelial dysfunction. This work demonstrates several protective properties of exercise within these models.

5.1.2. Effect of Exercise on PCB 77-induced toxicity in LDLr-/- mice

Atherosclerosis is the primary cause of myocardial infarction and stroke and remains the leading cause of death within in the United States[10]. Mouse models that mimic human disease including the LDLr-/- model are useful tools for examining atherosclerosis[157]. The LDLr-/- requires feeding of a diet enriched in saturated fat as well as cholesterol (0.15-1.25%), colloquially known as the “Western” diet because it mimics the average dietary composition consumed by humans in the Western hemisphere in order to develop atherosclerosis within the timeframe of our study[157].

Environmental pollutants, specifically polychlorinated biphenyls (PCBs), a type of persistent organic pollutant, are linked to cardiovascular disease[11]. Specifically, exposure to PCBs contributes to cardiovascular-related mortality[73] and accelerate atherosclerotic lesion formation[82]. Within this dissertation, we identify several risk factors associated with cardiovascular disease including hypertension, inflammation, and hyperlipidemia that are accelerated with PCB exposure, as well as provide further evidence supporting that PCBs contribute to CVD.

There have been several mechanisms proposed to explain how PCB exposure leads to CVD. Previous studies from our laboratory and others have shown that PCBs bind to the aryl hydrocarbon receptor (AhR) on endothelial cells which is a transcription factor that binds to xenobiotic response elements (XREs) within the promoters of downstream target genes including cytochrome P450 1A1 (CYP1A1) to initiate endothelial dysfunction, a common first step in CVD[83]. Once CYP1A1 becomes uncoupled from the metabolism of PCBs oxidative stress begins within the endothelial cells[68]. Elevated levels of oxidative stress (e.g. Reactive oxygen species) can lead to the activation of redox sensitive transcription factors including Nκ-KB and AP-1 that upregulate proinflammatory signaling molecules including monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6)[285].

Within this dissertation, male LDLr-/- mice were chronically exposed to PCB 77 (170 μM/kg) during weeks 6, 8, 10, 12. The major finding of this dissertation was that 8 weeks of voluntary exercise led to a reduction in plasma and hepatic cholesterol (Figure 2-7) as well as a trend towards reduced atherosclerotic lesions, after PCB exposure (Figure 2-6). We examined plasma cholesterol levels because cholesterol constitutes a major portion of atherosclerotic lesions and PCB exposure has been associated with hyperlipidemia. We hypothesized that exercise would reduce plasma cholesterol levels within PCB-treated animals. Recently, it has been shown that voluntary exercise reduces plasma levels of cholesterol including, VLDL and LDL sized lipoproteins, suggested to be from increased hepatic lipoprotein lipase that lowers hepatic cholesterol storage in LDLr-/- mice[173]. Our findings demonstrate that exercise reduced plasma cholesterol, thus supporting our hypothesis. This study suggests that aerobic exercise training improves cholesterol clearance that in turn, reduces PCB toxicity, which is supported by data from other labs[173]. Several studies have shown that one strategy to prevent atherosclerosis is to increase cholesterol excretion to the feces because it reduces plasma cholesterol[173,351]. Because PCBs are primarily excreted through feces

upon metabolism into more hydrophilic compounds, a therapeutic approach such as exercise which has been shown to improve cholesterol clearance through enhanced biliary excretion is an exciting candidate. Future studies should further investigate the effects of exercise on cholesterol metabolism and excretion by measuring fecal (e.g. neutral sterols, bile acids, and PCB metabolites) and biliary secretions (e.g. cholesterol, bile acids, and bile flow) in addition to jejunal NPC1I1 expression which is a known cholesterol efflux protein.

A surprising finding was that exercised mice exposed to PCBs had significantly lower levels of HDL. A review of the current literature suggests that exercise slightly raises HDL levels with caveats including diet, reduction in body fat, and numerous single nucleotide polymorphisms (SNPs) in Apo A-I, ABCA1, LPL, CETP, LIPC and GALNT2 that collectively may explain how a person's HDL-C will response to exercise. Additionally, we show that exercise leads to an accelerated inflammatory response in PCB-treated animals and increased liver: body weight ratio compared to sedentary, PCB-treated animals. Because exercise suppresses the accumulation of lipids within the liver and reduces liver weight[286], our findings indicate that the increased liver: body weight ratio is due to administration of PCB 77. Our lab has previously reported PCB 77 exposure significantly increased liver-to-body weight ratio in animals on a high fat diet[81]. Other groups have reported that exposure to PCB 77 causes lipid peroxidation, hepatomegaly, and increased oxidative stress within the liver[58,287]. Although we observed a reduction in hepatic cholesterol levels, we did not measure total hepatic lipid content or cirrhosis. Future studies should assess the extent of liver cirrhosis as well as monitoring changes in biliary excretion and overall body burden. PCB quantification in tissues reveals elevated levels of PCB 77 in the plasma of exercised PCB-treated mice. These findings suggest that exercise enhances lipolysis which leads to increased plasma concentrations of PCB 77 and its metabolites.

Additionally, it should be noted that body weight varies significantly with a high-fat diet alone, prior to treatments. This demonstrates that feeding of the Western diet represents a potential confounding variable within this study; however, we did not measure how PCB-77 affected appetite. Administration of PCB 77 in conjunction with Ang II infusion was examined in both LDLr-/- and ApoE-/- mice to assess the extent of atherosclerosis and abdominal aortic aneurysm (AAA) formation . There was less AAA formation in the LDLr-/- mice and the authors propose that the Western diet, which increases adiposity, may be causing the sequestration of PCBs in the adipose tissue. A

limitation within this study is that we did not quantify PCB 77 within the adipose tissue of these mice so we do not have the results to determine if Western diet feeding led to a redistribution of PCB77 within adipose tissue.

Because of the potential confounding factor of the Western diet, future studies should utilize a different murine model of atherosclerosis, the ApoE-/- mouse. The majority of *in vivo* work within the fields of exercise and cardiovascular disease utilize the ApoE-/- model and demonstrate positive outcomes in exercised animals related to cardiovascular disease and its associated risk factors[159,168,172,175,352]. The ApoE-/- mouse develops atherosclerotic lesions similar to that of humans but does not require high fat feeding for atherosclerosis to occur[338].

5.1.3 The Effects of Physical Activity on PCB-Induced Cardiovascular Disease in ApoE-/- mice

As pollutant emissions continue to increase (i.e., manufacturing and agriculture), human exposure to these pollutants will rise, thus leading to the need for buffers to protect against pollutant-induced adverse health effects such as cardiovascular disease. The most logical being physiological. Exercise has been shown to improve cardiovascular disease risk factors including hyperlipidemia, obesity, insulin sensitivity, hypertension, inflammation as well as the disease itself (reviewed extensively in Chapter 1). In this dissertation, we hypothesized that voluntary exercise protects against PCB-induced cardiovascular disease and its associated risk factors including hyperlipidemia, glucose intolerance, hypertension, inflammation, and oxidative stress,

We utilized the ApoE-/- mouse model and opted for oral gavage administration of PCB 77 in order to prevent injection-associated inflammation. Oral gavage of PCB 77 subjects the toxin to intestinal absorption and the microbiome which may have played a role in metabolism as well. The dosing regimen was modified to a dosage of 170 μM/kg administered during weeks 1, 2, 9, and 10 based on another colleague's data that this paradigm sustained glucose intolerance through week 12[306]. The exercise intervention was lengthened to 12 weeks instead of 8 weeks based on the majority of studies utilizing exercise as an intervention against cardiovascular disease [169,172,173,176,177,279]. In this dissertation, we demonstrate that voluntary exercise prevents several risk factors of cardiovascular disease including glucose intolerance, hypercholesterolemia, hypertension, systemic inflammation, oxidative stress, as well as atherosclerosis. Baker et al. demonstrated that PCB 77 administration leads to impaired glucose intolerance

that is associated with adipose tissue inflammation[306]. Although we did not investigate the signaling pathways associated with adipose tissue or skeletal muscle glucose uptake, exercise enhances insulin signaling and increases expression of GLUT4, a transporter that allows glucose to enter the cell from circulation[353]. We showed that exercise improved glucose tolerance in PCB-treated animals, Sedentary, PCB-treated animals had significantly higher levels of hepatic and plasma cholesterol, predominately in the VLDL and LDL fractions. Our results provide further insight that dietary exposure to PCB77 significantly increases hypercholesteremia specifically within the VLDL fraction that is associated with increased atherosclerosis[82]. Our laboratory previously demonstrated that PCB77 administration leads to decreased expression of ATP-binding cassette A1 which is responsible for cholesterol export from the liver as well as, decreased expression of genes associated with fatty acid metabolism including fatty acid synthesi and lipid transport/export, [104]. There is considerable evidence that exposure to PCBs leads to lipid changes in both plasma and liver tissues[82,104,329,354]. Multiple studies have reported that an increases in liver microsomal lipids (e.g. total lipids, phospholipids, neutral lipids, and cholesterol) after PCB administration[342,354,355]. Voluntary exercise has been shown to lower hepatic content and within our studies, voluntary exercise lowered PCB-induced hepatic and plasma increased cholesterol levels. Epidemiological evidence demonstrates a link between serum PCB level and the prevalence of hypertension, regardless of age[48]. Hence, hypertension is an easily identifiable risk factor for cardiovascular disease. Although we do not specifically investigate mechanisms of PCB-induced hypertension within this dissertation, Chapter four provides data that increased bioavailability of NO improving endothelial-dependent vasodilation in affected vessels may be a possible mechanism.

Atherosclerosis is an inflammatory disorder, leading to the increased expression of specific inflammatory molecules[2]. To determine if inflammation is a result of PCB-exposure, we next assessed the presence of a diagnostic series of inflammatory molecules within the plasma of these animals. Significant decreases in interleukin 6 (IL-6), monocyte chemoattractant protein 1 (MCP-1), chemokine (C-X-C motif) ligan1 (CXC1 or KC), macrophage colony stimulating factor (M-CSF), and (monokine induced by gamma interferon) MIG are detected in PCB77-treated, exercised mice compared to sedentary counterparts (Figure 3-4A-E) suggesting improvements in the inflammatory state. MCP-1 is a chemokine that attracts monocytes into the subendothelial space in early stages of atherosclerosis. The recruitment of monocytes into the artery wall

followed by their differentiation into macrophages and subsequently foam cells is an early event in the pathology of atherosclerosis. Our lab has shown through *in vitro* and *in vivo* studies that PCB 77 significantly upregulates MCP-1. An exercise regimen of low-intensity (10,000 steps, 3 times per week for 8 weeks) among healthy human adults (mean age 45) led to a significant reduction in proinflammatory cytokines IL-6, MCP-1, and TNF-α. Reduction in circulating levels of MCP-1 could be atheroprotective since less recruitment of monocytes would occur. KC is C-X-C chemokine that regulates monocyte arrest (or anchoring to the luminal surface of a vessel) in early atherosclerosis and has been shown to be reduced in aerobic exercise within several different models including hind limb ischemia[356] and acute lung injury[357] further supporting the anti-inflammatory effects of exercise. M-CSF (macrophage-colony stimulating factor) is a cytokine that recruits monocytes to the sites of endothelial injury and is found within atherosclerotic plaques in addition to in the circulation, where it is recognized as a biomarker of disease progression. Deletion of M-CSF leads to reduced atherosclerosis from a reduction in circulating monocytes[358]. The present dissertation provides the first report that aerobic exercise leads to a decrease in circulating M-CSF. MIG or monokine induced by interferon gamma is a chemoattractant expressed in the atheroma that recruits T cells to inflammatory sites[359]. The data demonstrates that exercise significantly reduces expression of MIG which supports previous findings that increased laminar flow (such as during exercise) also reduces MIG expression in endothelial cells[360].

PCB 77 exposure has been associated with increased levels of oxidative stress. To address this, we examined urinary levels of F_2-isoprostanes. F_2-isoprostanes are prostaglandin-like compounds formed from a non-enzymatic mechanism of free radical peroxidation of arachadonic acid[361]. There are several end products of lipid peroxidation including thiobarbituric reactive substances (TBARS), gaseous alkanes, and F_2-isoprostanes; however, measurement of F_2-isoprostanes has proven to be a more accurate marker of oxidative stress *in vivo* in human and other animals[362]. Advantages of mass spectrometry over immunoassays include the high sensitivity and specificity for molecules, which provides resolution to the picogram range (1×10^{-12}). In humans, levels of F2-isoprostanes are ~4-fold higher in atherosclerotic plaques compared to normal vascular tissue[363]. Additionally patients with hypercholesterolemia had 3.4 fold higher levels of F2-isoprostanes compared to normal controls[307] and there was no correlation with serum cholesterol, triglycerides, LDL-c, or arachadonic acid suggesting that patients have increased oxidative stress versus increased lipid substrate. A 12

month random controlled trial that included an intervention of aerobic exercise (60 minutes, 5 days per week) in previously sedentary women demonstrated a decrease in oxidative stress as measured through urinary F2-isoprostane with little change in body mass[364]. Results from this dissertation indicate that exposure to PCB 77 leads to a dramatic increase in F_2-isoprostanes in sedentary mice, whereas, exercised mice have 8-fold lower levels, suggesting that exercise protects against systemic oxidative stress associated with PCB 77 exposure.

In addition to a reduction in risk factors for cardiovascular disease, we demonstrate that exercise significantly reduced mean atherosclerotic lesion size in PCB-treated animals compared to sedentary, PCB-treated animals. Our findings are supported by twenty-three published studies that have examined the effect of exercise training in mouse models of atherosclerosis. Regardless of exercise intervention utilized (swimming[158–163], treadmill running[164–170], or voluntary running[171–177]), each of these studies reported a decrease in atherosclerosis following exercise, providing a basis to test our hypothesis. Our findings are the first to report the beneficial effects of exercise against atherosclerosis accelerated by an environmental pollutant; however, we are not the first group to report that exercise can protect against PCB toxicity[301].

Within this dissertation, voluntary exercise prevented PCB-induced gut microbiome changes by preventing decreases in the population of Proteobacteria. Another group assessed the effects of exercise and dietary supplementation with phytic acid on Cadium toxicity and suggested that exercise provides a protective effect in relation to weight loss, cholesterol levels, and liver damage; however CVD parameters were not investigated[365].

Exercised animals have reduced levels of oxidative stress, thus we assessed several antioxidant enzymes to determine if they provided a potential mechanism of protection. A growing body of evidence suggests that exercise can activate Nrf2[251–254]. Since our lab previously demonstratd the reduction in proinflammatory signaling after EGCG[102 249], a known inducer of Nuclear factor erythroid 2-related factor 2 (Nrf2), we examined downstream Nrf2 gene targets. Nrf2 is a transcription factor that responds to increased oxidative stress within the cell. Target genes for Nrf2 include a diverse set of antioxidant enzymes and cytoprotective genes including heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase-1 (NQO1), thioredoxin, and glutathione metabolism genes including glutathione peroxidase 2 (Gpx2), glutathione S-transferases (GSTs). Our findings demonstrate downregulation of the inhibitor protein, Keap1. We also see

an upregulation of several Nrf2 target genes including phase II antioxidant enzymes Gpx1, GST, and GSR; however, we did not see changes in NQO1 or HO-1 expression. To confirm that these findings are biologically relevant future studies should examine antioxidant expression through Western blot analysis. Additionally, Nrf2 nuclear translocation should be measured to test if exercise increases Nrf2 activation. Electromobility shift assays could be performed to determine the extent of ARE binding in exercised animals which would be another indication of Nrf2 activation.

Our results show a downregulation of the phase I enzyme, CYP1A1, which has been implicated in contributing to oxidative stress in the presence of coplanar PCBs[68]. Superoxide can uncouple eNOS[320], the enzyme responsible for the production of the potent dilator nitric oxide, thus producing peroxynitrite and reducing endothelial-dependent dilation. We have previously shown in cultured endothelial cells that exposure to PCB 77 leads to an increase in peroxynitrite.[90] In future studies, we will further test how exercise regulates NO-mediated signaling and endothelial dependent dilation.

We measured PCB 77 and its hydroxyl metabolites in several tissues to determine if exercise had an effect on metabolism of PCB77. PCB 77 was not detectable in any tissues, and OH-PCB 77 was found in the feces of these animals. Exercised animals had significantly less OH-PCB 77 metabolites in their feces compared to sedentary animals. Because of the rapid metabolism of PCB77, these results are not surprising; however, this suggests indirectly that exercise increased drug metabolism and that most of the PCB 77 may have been metabolized and/or excreted prior to the time of measurements (at the end of study) since the last dose was during week 10. Future studies should utilize a different dosing regimen that accounts for PCB 77's half-life in order to detect potential changes in tissue distribution as well as excretion of PCB and its metabolites.

With regard to toxicokinetics of PCBs, it has been reported that gastrointestinal absorption of individual congeners varies from 66-96% in rats[366]. The distribution of PCBs in the body depends on the dose as well as, the degree of chlorination. Hydroxylated PCBs are the major metabolites with others including arene oxides that highly reactive and converted to phenols, dihydrodiols, and glutathione conjugates which are excreted. Sulfur-containing metabolites (e.g., methyl sulfones) have additionally been identified. A major limitation of our current system is the inability to measure a limited number of metabolites, specifically hydroxylated metabolites. Additional studies

should examine the above metabolites. This would require collaborations with the Superfund group at the University of Iowa which have synthesized internal standards for measuring sulfated metabolites. Excretion of PCB congeners is dependent on their rate of metabolism to more hydrophilic compounds[367]. Most congeners show a biphasic elimination, where the initial half-life is relatively short but the later half-life is much longer and more structure specific. The half-lives of PCB congeners vary from a few days to 450 days depending on degree of chlorination. Within ICR mice, the half-life of PCB 77 was reported at 1.07 days with the majority distributing from serum to adipose, liver, and thymic tissues[368]. Within this study, ICR mice received 8 mg/kg every other day for 10 doses before toxicological endpoints were measured. Because PCB metabolites are eliminated primarily through the bile and feces, feces should be gathered at different time points throughout the study in order to quantify excretion rates between sedentary and exercise animals to determine whether exercise increases metabolism and subsequent excretion of these compounds.

5.1.4. Effect of exercise on PCB 77-induced endothelial dysfunction in C57BL6 mice

Human exposure to coplanar PCBs has been associated with cardiovascular disease and its associated risk factors including diabetes[77], hypertension[48], dyslipidemia[329], and endothelial dysfunction[84,313,330]. Endothelial dysfunction is an independent risk factor for cardiovascular disease[27] and is an initiating step in the development of atherosclerosis[331]. . NO is produced by endothelial nitric oxide synthase (eNOS) during the conversion of L-arginine to L-citrulline through receptor activation (e.g., muscarinic receptors) or mechanical force (e.g., shear stress)[320]. NO relaxes blood vessels, prevents platelet aggregation and adhesion, limits oxidation of low-density lipoprotein (LDL) cholesterol, inhibits proliferation of vascular smooth muscle cells, and decreases expression of proinflammatory cytokines[187]. All of these functions of NO play in role in optimal endothelial health, thus preventing atherosclerosis.

The previous work within this dissertation, specifically Chapter 3, demonstrated the effects of exercise in PCB-induced cardiovascular disease in an observational manner. The aim of this dissertation was to determine a mechanism of protection against PCB-induced CVD within the vasculature. Because our laboratory has shown that PCB 77 exerts toxicity within the vascular endothelium in an AhR-dependent mechanism, we examined the role of exercise in PCB-induced endothelial dysfunction

utilizing the *ex vivo* vascular reactivity assay. Several studies, in both human and animal models, have demonstrated that exercise can reverse endothelial dysfunction[171,369–371]. We hypothesized that exercise protects against cardiovascular disease by preventing endothelial dysfunction through increased bioavailability of NO.

We demonstrate that exposure to PCB 77 led to severe impairment of endothelium dependent vasodilation in sedentary animals (Figure 4-7). Exercise is able to prevent the PCB-induced impairment. Our data show that ACh-induced relaxation is blocked in all groups but PCB 77-treated sedentary animals. The NO inhibitor L-NAME inhibited the relaxation response in both exercised and control groups equally, one could argue that increased bioavailability of NO is one of the protective mechanisms of exercise within the vascular endothelium. In fact, pre-incubation with L-NAME significantly reduced endothelium dependent relaxation in all groups, except in PCB-treated sedentary animals. , NO-mediated mechanism may also contribute to the protective effects of exercise against PCB-induced endothelial dysfunction. Other groups have demonstrated that exercise leads to increased activity of eNOS through phosphorylation of ser1177. A limitation to this study is not confirming aortic expression of eNOS within these animals to substantiate this finding.

The superoxide mimetic Tempol rescued the impaired vasodilation in PCB-treated sedentary animals, suggesting a relationship between PCB exposure, increased oxidative stress and endothelial dysfunction. Previous publications have reported rescue of dioxin-induced endothelial dysfunction by Tempol in sedentary mice [267]. This implies that exercise might normalize the redox status within the vascular endothelium by reducing production of superoxide, thus improving vasodilation.

Examination of tissue distribution demonstrated that exercise did not significantly change the amount of PCB 77 or its metabolites within liver or plasma. Measurement of adipose, skeletal muscle, and fecal concentrations would have allowed us to make a better estimate to the effects of exercise on metabolism, excretion, and body burden. Interestingly, exercised animals had a significant downregulation of CYP1A1 which could explain the reduction of superoxide in these animals. Furthermore, this reduction in superoxide may explain why Nrf2-mediated antioxidants were not stimulated. Stimulation of Nrf2 appears to require induction of oxidative stress by a damaging agent that increases ROS such as PCB 77. A limitation of this work is we did not measure ROS specifically. This data suggests that exercise attenuated endothelial function may occur by a reduction in oxidative stress and decreased CYP1A1 expression.

. The major limitation being this study is the artificial nature of this type of functional analysis where the experimental conditions do not include the physical stresses of shear stress and cyclical wall stretch that endothelial cells within the isolated vessel would normally experience[334]. Although traditional organ-bath pharmacology is artificial, we were able to isolate elements of endothelial function by treating with pharmacological inhibitors including L-NAME and Tempol. Because isolation of aorta to preserve tissue viability was essential to the success of this experiment, I had limited time to collect tissue from these animals. In hindsight, a subset of animals or a co-worker to assist in weighing of liver tissue as well as harvesting skeletal muscle, adipose tissue, kidneys, the remaining thoracic aorta and lungs would have enabled more data to be collected. Additional measurements would include antioxidant enzymes and p-eNOS/eNOS expression within the aorta as well as measuring tissue levels of PCB 77 and its metabolites to determine the effects of voluntary exercise on metabolism, excretion and body burden. Fecal collection would have allowed us to compare the effects of chronic versus acute exercise on PCB excretion rates. An additional limitation to this study is the acute dosing schedule that is not reflective of human exposure. Future studies should examine the effect of exercise on endothelial function in mice that were chronically exposed to PCB 77 (dosing regimen in Chapter 3) to determine if exercise remains protective.

5.1.5 Implications From Different Mouse Models

The data presented in this dissertation support the hypothesis that exercise provides beneficial effects within the vasculature particularly in vehicle-treated animals. Because of the changes in diet, dosing regimen, ways PCBs were quantified, and differences in animal strains it is difficult to accurately compare results between these two studies. Because these different aims utilized different mouse models, a further discussion of differences among strains and recommendations to address these limitations are discussed below.

Several animal models are available to study atherosclerosis including the mouse, rabbits, pigs, and nonhuman primates. Due to the well-established murine models as well as housing costs and feasibility, we utilized the mouse for these studies. Mice are generally resistant to atherosclerosis due to low cholesterol levels (<100 mg/dl) which is present predominately as HDL cholesterol[274]. By genetic and dietary manipulations, specifically by increasing apoB-containing lipoproteins, mice will develop

atherosclerosis. The two major mouse models, the ApoE-/- and the LDLr-/-, are widely accepted within the scientific community. ApoE is mainly produced in the liver and acts as a ligand on the surface of lipoproteins ultimately for their clearance through uptake by the LDLr. Genetic deletion of ApoE delays clearance of lipoproteins and raises plasma levels to 300-400 mg/dl as well as atherosclerosis on a low-fat diet[339]. Feeding a high-fat/high-cholesterol diet can raise plasma cholesterol levels to >1000 mg/dl and accelerates atherosclerosis. The discovery of the LDLr by Brown and Goldstein while studying familial hypercholesterolemia led to the honored Nobel Prize in 1985. LDLr recognizes apoB100 on LDL and apoE on VLDL and chylomicrons for their clearance from the blood. Unlike the ApoE-/- mouse, LDLr-/- mice require a high fat diet (40% calories from saturated fat and 0.1-0.2% cholesterol by weight colloquially known as the Western diet) to develop atherosclerosis and elevated cholesterol levels (>1000 mg/dl). From a practical standpoint, both models are commercially available and are backcrossed to the C57BL/6 background.

The lesion stage and timeframe for development of atherosclerosis has been well-characterized within the literature. LDLr-/- mice fed a Western diet will develop stage I lesions within 4-6 weeks and present with stage III at 16-20 weeks whereas ApoE-/- fed a standard diet present with stage I lesions at 1-2 months and do not develop stage III lesions until 7-9 months of age[372]. Apoe-/- mice fed a Western diet will develop atherosclerosis at a similar rate to the LDLr-/- mice. Stages I-III lesions consist of an abnormal accumulation of lipoproteins, T-cells, and macrophages, which can be measured through staining techniques. Stage IV lesion is considered as an advanced lesion because these lesions consist of a lipid core as well as thickening of the tunica intima. When the lipid core accumulates fibrous material or a cap, this is considered stage V as the lumen becomes narrowed[372].

These discrepancies could explain the differences seen between the two studies. It is important to note that despite different timeframes, the mean lesion size between the two studies was roughly similar (in both LDLr-/- mice and ApoE-/- mean lesion sizes ranged from 0.1-0.3 mm^2, refer to Chapter 2 and Chapter 3). Furthermore, a complete histological analysis of the aortic root was not conducted to better determine which lesion stage these animals were in and whether exercise could decrease macrophage infiltration within the lesion. . We did not see a significant change in lesion area, but that does not necessarily mean that the atherogenic process was the same between PCB, sedentary, and PCB, exercise groups. Additional studies should quantify the infiltration

of macrophages within the aortic root especially since differences were seen in the inflammatory response systemically between the two strains. The amount of technical skill required to collect all of the serial sections spanning a 400 + μm region of the ascending aorta is a major limitation within this body of work. Future studies should utilize an expert with the technical skill to reduce variability and potential human error. Upon statistical consultation, it has been recommended that this additional study should utilize a greater sample size (n=15-20) would account for the inherent variability seen in mean atherosclerotic lesion size and this would allow us to determine if exercise would be an appropriate therapy for reducing body burden and preventing cardiovascular disease. Another consideration to this work is that we examined a relatively short time frame of the disease. Atherosclerosis and body burden of PCBs may be alleviated by continued voluntary exercise for longer periods of time based on other studies that have indicated reduction in POP body burden over a 2 year time frame. An additional factor to account for is the role of diet. Other studies within the literature have demonstrated that a diet consisting of protein and heart healthy fats was required for exercise to protect against dyslipidemia, inflammation, and atherosclerosis in ApoE-/-[176]. Potentially supplementing rodents with olestra or EGCG may offer protective while PCBs are mobilized into the plasma for further metabolism and excretion. Although exercise prevented many of the adverse side effects of PCB exposure, a diet supplemented with phenols may be required protect against the development and progression of atherosclerosis.

Specifically within the LDLr-/- study, systemic inflammation was significantly increased in the PCB, exercised animals. The dosing regimen could account for these changes since mice were exposed to PCB 77 24 hours before euthanasia. Within the ApoE-/- study, systemic inflammation was largely attenuated; however the last dose occurred 3 weeks before plasma was analyzed. To determine the effect of exercise on PCB-induced inflammation, time course studies that examine inflammatory markers throughout the duration of the study would provide insight

Within both studies, exercise led to a reduction in hepatic cholesterol despite increased liver:body weight. This finding suggests that exercise does not fully protect against PCB-associated liver pathologies; however, we did not quantify total lipids or fibrosis in either study. Other difficulties that arise for accurate comparison is missing parameters from the LDLr-/- study including systemic oxidative stress, antioxidant status and glucose tolerance test that were measured in the ApoE-/- study. Having oxidative

stress data would help us better assess why the LDLr-/- mice had significantly elevated CYP1A1 expression which could be merely the result of the dosing regimen but this remains unknown. It is quite interesting that exercise significantly reduced CYP1A1 expression in ApoE-/- but this could be explained by the dosing regimen as well.

In summary, exercise modulates cholesterol particularly in the VLDL and LDL fractions, reduces hepatic cholesterol, and reduces adiposity in both strains exposed to PCB 77. In ApoE-/- mice, exercise reduces systemic oxidative stress, glucose intolerance, inflammation, and hepatic CYP1A1 expression while upregulating phase II enzymes including GST and GSR. Exercised animals had lower mean atherosclerotic lesion size; however, future studies with a larger sample size are needed. The section below outlines future studies that will further delineate the potential protective role of exercise against the adverse health effects of PCB exposure.

5.2. Future Directions

The work presented here demonstrates the cardiovascular toxicity of coplanar PCBs and examines the role of lifestyle modifications including aerobic exercise. Providing guidelines to humans for physical activity based on this project is not recommended due to the infancy of this work. In order to provide clear physical activity guidelines for populations exposed to PCBs and other persistent environmental chemicals, the following questions should be addressed.

In chapter 3, our data indicates that exercise may have an effect on the metabolism of PCB 77, which is a novel finding. One unsolved piece of the puzzle are the pharmacokinetics of the metabolism of PCB 77 and future studies should address it. To assess the effect of olestra on PCB 77 metabolism, Jandacek et al. utilized radio-labeled PCB 77 and determined absorption and excretion rates through fecal samples[105]. Feces should be collected for 48 hours immediately after gavage (if radiolabeled congeners are available and within a reasonable price range) to measure initial dietary absorption (reported as % of dose in 48 hours) and then for an additional 48 hours 7 days after administration to measure enterohepatic circulation. At this endpoint, tissues should be collected to measure distribution of PCB in adipose, plasma, and liver tissue. If radiolabeled congeners are not available, concentration within the adipose tissue could be utilized as an estimate of body burden. This would allow the researcher to make a more thorough report on the potential role of exercise in increasing

metabolism and excretion of PCB 77 through interrupted enterohepatic circulation and enhanced excretion of these compounds.

More recently, Jandacek et al. administered olestra potato chips (15 g olestra/day) to reduce PCB body burden in Anniston residents for 12 months in a double-blind placebo-controlled, 1 year trial. Results from this pilot study demonstrate elimination rate of 37 non-coplanar PCBs to be faster in olestra-consuming patients than before the trial[373]. This study is very important because it demonstrates a dietary intervention that can safely reduce body burden of PCBs. These findings support the need to continue a larger intervention trial to determine whether reduction in PCB body burden will improve metabolic parameters such as hypertension, cardiovascular disease, and diabetes. To date, there is a single case study that reports metabolic improvement (hyperlipidemia and hyperglycemia) in a patient whose PCB body burden was reduced[374].

Additional in vivo studies should examine the effects of chronic exposure of PCB 77 on endothelial dependent vasodilation. Walker and Kopf measured the effect of dioxin exposure (35 days which was the amount of time required for C57BL/6 mice to become hypertensive) on vascular reactivity and found significant impairment[267]. A group of Spolana plant workers in the Czech Republic were heavily exposed to dioxin and vascular function was examined by laser Doppler fluxmetry. Workers were found to have significant endothelial dysfunction in four of the six parameters of microvascular reactivity within the brachial artery (maximal perfusion during hyperemia, time needed to reach maximal perfusion during hyperemia, velocity of perfusion increase, and thermal reactivity)[375]. We hypothesize that chronic exposure of PCB 77 will lead to impaired vasodilation based on our findings regarding increased atherosclerosis, oxidative stress, and inflammation but that exercise will prevent this endothelial dysfunction.

Previous work in our laboratory provides strong evidence that PCB transport requires functional caveolae and its associated scaffolding protein Caveolin-1 (Cav-1), enhancing its toxicity. PCB 77 administration in endothelial cells increases caveolin-1 and PCB 77 accumulates within the caveolae fraction[92]. Because caveolae play a role in the development of atherosclerosis, therapies that decrease caveolae and modulate its associated inflammatory signaling are attractive targets. We did not see changes in Cav-1 expression within the liver of exercised mice; however, future studies should examine expression of caveolin-1 in aorta of exercised mice to determine if exercise reduces its expression. Because eNOS is bound to caveolin-1 in its inhibitory state[348], a

potential scenario is that the lipid bilayer undergoes a conformational change due to mechanotransduction, thus increasing NO bioavailability by freeing eNOS from Cav-1. This hypothesis remains untested and would be an exciting mechanism to explore.

Because shear stress is a potential mechanism with which exercise exerts its cardioprotective effects, porcine endothelial cells should be cultured and exposed to a newly developed system that exerts "physiological" flow instead of the traditional models that apply a controlled level of fluid shear stress. Uzarski et al. have developed a hemodynamic flow model that mimics complex and variable shear fields based on short-term changes in blood flow that were observed in vivo. They compared physiological flow (PF) to static culture and steady flow (SF) that was set at a consistent pulse frequency of 1.3. Hz. Their findings demonstrate that PF led to a significant upregulation of eNOS, upregulation of atheroprotective genes including SOD-1 and downregulation of MCP-1. Additionally these endothelial cells had a lower TNF-α induced HL-60 leukocyte adhesion[376]. This flow model mimics atheroprotective flow and may be a suitable model to mimic flow seen during exercise. Quantification of shear stress in human pulmonary arteries reports that shear changes 19.8 +/- 4.0 to 51.8+/- 6.7 dynes/cm2[377]. If this collaboration cannot occur, a suitable alternative would be collaborate with Hainsworth Shin to utilize his micropipette shear device to administer shear stress to cultured endothelial cells. His work has previously shown that membrane cholesterol influences mechanotransduction of shear stress by PMNLs by affecting membrane fluidity[378] which has implications for testing whether or not exercise can modulate caveolin-1 (a membrane protein) and its associated signaling during PCB administration. These cells then should be exposed to doses of PCB 77 to determine if PF flow (or shear stress representing laminar flow) can prevent proinflammatory signaling mediated by NF-κB and potentially upregulate anti-inflammatory signaling molecules.

5.3 Conclusions

In conclusion, this dissertation demonstrates that voluntary exercise protects against PCB-induced cardiovascular disease risk factors including glucose intolerance, dyslipidemia, hypertension, systemic inflammation, and oxidative stress. Additionally, exercise reduced atherosclerotic lesions while upregulating antioxidant enzymes. This

work suggests that exercise may increase the metabolism and excretion of these compounds as well.

Acute exposure to PCB 77 led to impaired endothelial-dependent dilation, an *ex vivo* measure of endothelial function; however, exercise was able to prevent this PCB-impaired endothelial dependent vasodilation. The mechanisms involved in PCB-induced endothelial dysfunction involve production of superoxide and reduced bioavailability of NO; however the mechanisms involved in eNOS regulation should be studied in further detail including the role of Cav-1.

Lifestyle modifications such as physical activity have been shown to protect against cardiovascular disease. This dissertation provides evidence that exercise can modulate several adverse health effects of PCB 77 but additional studies are required before exercise recommendations can be made for groups residing in Superfund hazardous waste sites.

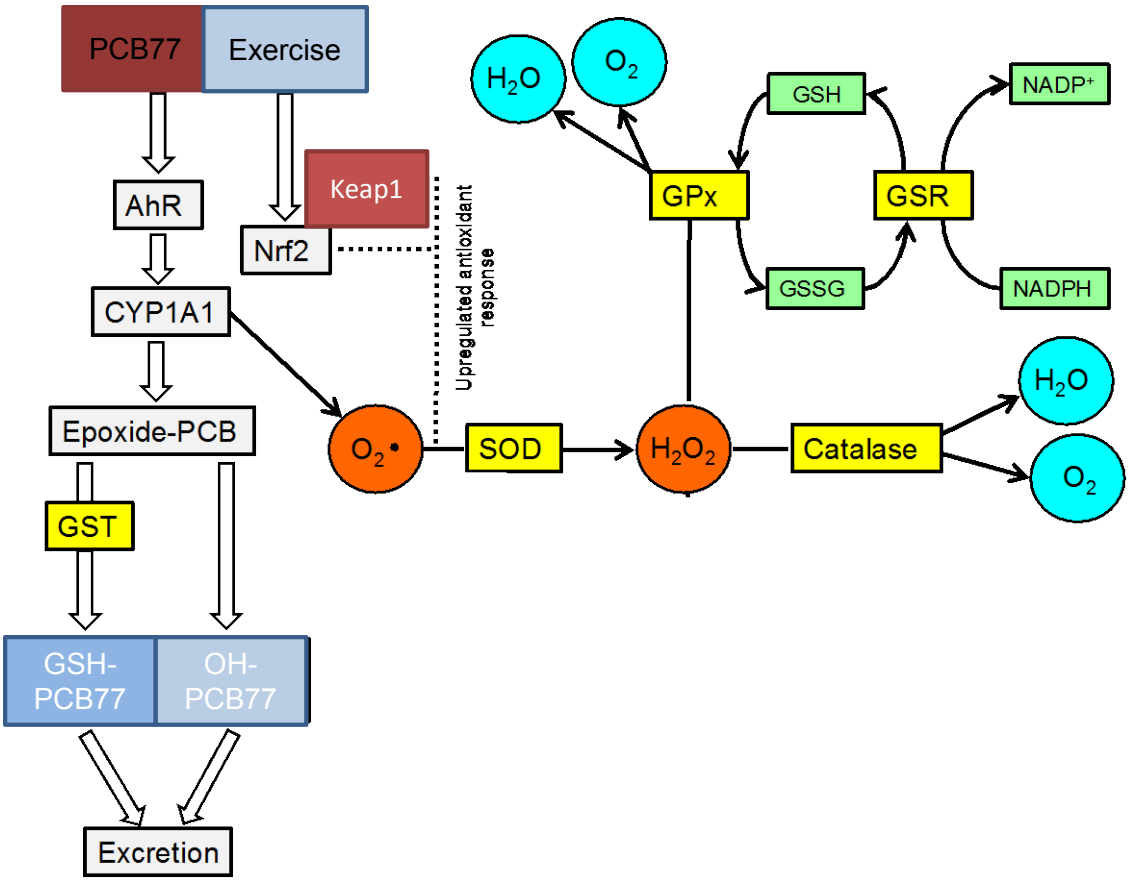


Figure 5 -1. **Proposed signaling pathway for PCB detoxification in vivo**.
PCB 77 is an AhR ligand and causes CYP1A1 upregulation, which when in the presence of PCB 77 leads to superoxide production. Exercise effectively upregulates the antioxidant response in the presence of PCB77 which allows for a more efficient antioxidant response to environmental insult. (Adapted from Newsome et al *JNB*, 2013)

Appendix A: Vascular Reactivity Protocol

Tissue Force Analysis

Before tissue harvest
(Modified)Krebs buffer preparation:
- For 2 liters:
  ~ 1650mls of diH20
  -add NaCl dry chemical and stir ;add dextrose dry chemical stir

-add 60 mls KCl, KH2PO4, MgSo4-7H20 and NaHCO3 stocks and stir for 5 min

- q.s. with diH20

- pour into reservoir; turn on heater and O2/CO2 tank to aerate the buffer. Let buffer aerate ~ 20 mins
- drain solution, add CaCl2-2H20 and re-stir 5 min
- return to reservoir and aerate additional 10 mins before using

80mM KCl solution preparation:
- For 500 mls :
  ~ 300 mls of diH20
- add KCl dry chemical and stir; add dextrose dry chemical stir
- add 15 mls KH2PO4, MgSo4-7H20 and NaHCO3 stocks and stir for 5 min
- q.s. with diH20

- pour into reservoir; turn on heater and O2/CO2 tank to aerate the buffer. Let buffer aerate ~ 20 mins
- drain solution, add CaCl2-2H20 and re-stir
- return to reservoir; aerate an additional 10 mins prior to use.

Balance and calibrate each transducer

Turn on analyzer: the screen will be in the set up mode and read as follows:
1- SET
2- bASE
3- CAL
4- [0.00]

To balance -from set up menu
Press 2 - bASE
Balance/baseline menu will appear as follows:
1 bASE
2 BLANK
3 [current force]
4 [baseline force]

Press 2 - a red light will appear next to button 2, when the red light goes out the transducer is balance. The screen will read as follows:
1 bASE
2 BLANK

3 [0.00]
4 [0.00]

Press button 1 to return to set up mode.

To calibrate - In set up mode

Press 3 - CAL

The calibration menu will appear as follows:

1 CAL
2 [20]
3 BLANK
4 [2.0]

Place the 2 gram weight on the transducer

Press button 3 - a red light will appear next to button three, when the red light goes out the transducer is calibrated

Press button 1 (mode) to return to set up Menu

From set up Menu press Button 1 to enter pre-stimulation mode

The display should read as follows

1 [2.0]
2 BLANK
3 BLANK
4 [00:01]

Remove the 2 g weight

The value in display box 1 should return to zero

(If this value exceeds 0.02g than repeat calibration)

Open up DMSI software on computer; choose “all”, then “force”

- In “window”, choose “tile horizontally” and arrange windows to preference
- When ready to begin experiment, hit “start experiment” to record

<u>Tissue Harvest</u>

1. Anesthetize mice with IP injection of ketamine/rompun/saline cocktail
2. Perform a thoracotomy, drain blood and perfuse the aorta with mod. Kreb’s buffer
3. Carefully remove the entire aorta, with or without the heart, being careful to create no tears in the vessel.
4. Place whole aorta in fresh Kreb’s buffer and remove all adventitia under a microscope
5. Measuring sections of interest:

Arch- 3mm section superior starting from the ascending arch at the base of the heart

Thoracic- 4mm section inferior to the left subclavian artery

Suprarenal- 4mm section from the superior mesenteric artery toward the superior end of the aorta

Infrarenal- 4mm section from left renal artery toward the bifurcation

6) After each segment has been cut, mount the rings on the isometric triangles.

Mounting Tissues in Baths

1. Fill reservoirs with fresh Kreb's buffer.

2. Using the mounting wires, secure each tissue segment to its transducer and fixed point within the glass baths.

3. Create minimal tension (0.5-0.6 g) after tissue segment is successfully mounted

4. When all tissues are mounted, increase tension to 1 g, drain baths and refill with fresh buffer

5. For the next 30 min, readjust tension to 1 g and replace with fresh Kreb's buffer every 10 min to equilibrate tissue segments

Administering Agonists/antagonists

KCL, if being used, should be given first to establish tissue viability and a baseline vaso-reaction for future relaxation trials

**our protocol adds KCL as a solution, so the entire bath is drained of buffer then filled with 80mM KCL for this step. Other drugs/agonists are usually added to the buffer in the baths

The first time a drug dose is added to the chamber, hit "contract" or "relax" (whichever applies) and record the reference force. At each subsequent addition, repeat and record the max. force for that time interval

Tissue should be "washed" at least 3 times after each agonist by filling the baths and draining them

Tissue should be allowed to equilibrate for at least 30 min after the washes (replacing with fresh buffer every 10 min) between agonists

If using antagonists, administer these first and allow equilibrating 30 min before adding the secondary drugs

Drug calculations:

*All drugs dissolved in dH2O unless otherwise noted

*All drugs diluted with fresh Kreb's buffer

*Stocks are good at 4 C for 2-3 days (5HT and PE are light sensitive)

*Aliquots may be frozen for up to 12 weeks - do not freeze/thaw more than once

5HT (serotonin)	MW=212.7
PE (phenylephrine)	MW=203.7

AngII (angiotensin II)	MW=1166
Ach (acetylcholine)	MW=181.66
Cbl (carbachol)	MW=182.6
L-NAME	MW=269.7
Ind (indomethacin)	MW=357.8 *dissolve in ethanol, dilute in dH2O

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Publications

1. Newsome B, Petriello M, Han SG, **Murphy M**, Eske K, Sunkara M, Morris AJ, Hennig B. Green tea diet decreases PCB126-induced oxidative stress in mice by upregulating antioxidant enzymes. *J Nutri Biochem.* 2013 Nov 6. doi: 10.1016/j.jnutbio.2013.10.003.

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