POLYCHLORINATED BIPHENYL-INDUCED ENDOTHELIAL CELL DYSFUNCTION AND ITS MODULATION BY DIETARY LIPIDS

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ABSTRACT OF DISSERTATION

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The Graduate School
University of Kentucky
2010
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ABSTRACT OF DISSERTATION

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

By
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Lexington, Kentucky

Director: Dr. Bernhard Hennig, Professor of Animal and Food Sciences
Lexington, Kentucky
2010
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Cardiovascular diseases are the number one cause of death in Western societies. Endothelial dysfunction is an early event in the pathology of atherosclerosis, which is an underlying cause in the majority of cardiovascular events. Exposure to persistent environmental pollutants, such as polychlorinated biphenyls (PCBs), is a risk factor for the development of atherosclerosis.

First, we tested a hypothesis that coplanar PCBs, dioxin-like chemicals with affinity for aryl hydrocarbon receptor (AhR), can stimulate up-regulation of monocyte chemoattractant protein-1 (MCP-1), an endothelium-derived chemokine that attracts monocytes into sub-endothelial space in early stages of atherosclerosis. Coplanar PCBs 77 and 126 increased expression of MCP-1 in endothelial cells, and this effect was dependent on activation of AhR and increased levels of cytochrome P450 monoxygenases. Subsequent rise in the levels of reactive oxygen species (ROS) led to a downstream stimulation of redox-sensitive kinases and transcription factors. Lipid rafts, and particularly caveolae, are enriched in endothelial cells, and down-regulation of caveolin-1, a key structural protein of caveolae, decreases the progression of atherosclerosis. Studies using deletion of caveolin-1 in vitro and in vivo demonstrated that intact caveolae were required for up-regulation of MCP-1 and pro-inflammatory interleukin-6 (IL-6) by PCB77.

Nutrition can modulate adverse outcomes of human exposure to environmental chemicals. Fish oil-derived long-chain omega-3 polyunsaturated fatty acids, such as docosahexaenoic acid (DHA, 22:6ω-3), can alleviate inflammatory responses and the risk of cardiovascular disease. Cyclopentenone metabolites produced by oxidation of DHA contribute to these protective effects. Endothelial cells were pre-treated with oxidized DHA (oxDHA), prepared by incubation of the fatty acid with a free radical generator. Subsequent up-regulation of MCP-1 by coplanar PCB77 was markedly reduced. DHA-derived cyclopentenones increased nuclear translocation and DNA binding of a transcription factor NF-E2-related factor-2 (Nrf2), as well as expression levels of its target, antioxidant enzyme NAD(P)H:quinone oxidoreductase (NQO1). This stimulation
of antioxidant responses prevented ROS production and inflammatory responses induced by PCB77. These data support the concept that nutrition prevents toxicity caused by environmental pollutants; thus, nutrition and can be a sensible approach to alleviate chronic pathologies associated with these chemicals.

KEYWORDS: Endothelium; monocyte chemoattractant protein-1; cardiovascular disease; polychlorinated biphenyls, docosahexaenoic acid
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Chapter one: Introduction

1.1 Background

1.1.1 Cardiovascular diseases and etiology of atherosclerosis

Cardiovascular diseases (CVDs) are currently the most prevalent underlying cause of death in the U.S. [1]. Atherosclerosis is a chronic build-up of lipids and cells in arterial walls [2]. Atherosclerotic plaque (atheroma) growth may result in stenosis and limited blood flow through coronary arteries (ischemic heart disease), while plaque disruption with subsequent thrombosis can lead to myocardial or cerebral infarction, and subsequently death [3]. This way, atherosclerosis accounts for nearly three fourths of all deaths from CVDs [1]. Evaluation of risk factors for cardiovascular events has helped to identify the populations at risk, and also to decipher the mechanisms involved in atherosclerotic lesion development. The main risk factors for the development of coronary artery disease include increased total cholesterol levels and high ratios of low-density lipoproteins (LDL) to high-density lipoproteins (HDL), obesity, diabetes mellitus, and hypertension [2]. While genetic factors clearly play a role in a susceptibility to atherosclerosis [4], lifestyle choices including smoking [5], a lack of physical activity [6], as well the composition of one’s diet [7] can significantly modify the pathology of atherosclerosis. In addition to these factors, exposure to certain environmental chemicals, including persistent organic pollutants [8], can increase cardiovascular risk and mortality.

Cholesterol is a major component of atherosclerotic plaque, and plasma cholesterol levels, as well as its processing by various tissues, clearly play a key role in the pathology of atherosclerosis [9]. However, a low-grade inflammation was found to be an integral mechanism in the process of plaque development, and an independent risk factor for coronary artery disease [10]. To support the hypothesis that inflammation is an integral mechanism in atherosclerosis development, a 73% reduction in atherosclerosis formation was observed in immunodeficient mice, and it could be reversed by passive transfer of immunocompetent CD4+ T-cells [11]. The levels of circulating inflammatory mediators, in particular C-reactive protein (CRP) and tumor necrosis factor-α (TNF-α)
are independent predictors of coronary heart disease [12]. A build-up of inflammatory cells is a prominent feature of atheroma, and early atherosclerotic plaque consists largely of leukocytes; in particular monocytes, and to a lesser extent T-cells [13]. The specific inflammatory reactions involved in the plaque initiation and growth will be discussed in the following section.

Formation of an atherosclerotic plaque is a result of the interaction among several different cell types and organ systems [2]. LDLs, lipoprotein particles carrying liver-synthesized cholesterol into peripheral tissues, can at high plasma levels cross the endothelial layer and accumulate in the sub-endothelial space of the intima [14]. In the absence of plasma-derived anti-oxidants, LDLs become oxidized (oxLDL) by the action of vascular wall cells, including endothelial cells and macrophages [15, 16]. In turn, the exposure of endothelial cells to oxLDL; and other blood-borne agents; including bacterial wall lipopolysaccharide (LPS), inflammatory cytokines, such as TNF-α and interleukin-1β (IL-1β) [17], or toxic chemicals [18]; can up-regulate endothelial expression of adhesion molecules and chemokines. Monocytes from the bloodstream recognize adhesion molecules, primarily vascular cell adhesion molecule-1 (VCAM-1) [19], and become attached through a VCAM-1 counter-receptor, very late activation antigen-4 (VLA-4) [17].

Monocyte chemoattractant protein-1 (MCP-1), a chemokine produced by endothelial cell, binds its cognate receptor C-C chemokine receptor 2 (CCR2) on monocytes, and facilitates their transmigration and retention in the sub-endothelial space [20]. While monocytes are the predominant inflammatory cells in the early plaque, other subsets of leukocytes contribute to the pathology of atherosclerosis. T helper 1 (Th1) cells and dendritic cells can facilitate its progression, while regulatory T-cells (Treg) inhibit plaque formation [13]. Once inside intima, monocytes differentiate into macrophages through the action of colony-stimulating factor-1 (CSF-1) produced by vascular cells [21]. Macrophages specifically recognize and uptake oxLDL particles through binding to their scavenger receptors type 1 and 2 (SR-A1 and SR-A2) [22]. The resulting cholesterol accumulation leads to macrophage conversion to foam cells characteristic of fatty streak, an early atherosclerotic lesion [23].
Vascular smooth muscle cells (VSMCs) contribute to plaque growth by increased proliferation rates and synthesis of extracellular matrix. Recent evidence suggests that they can also synthesize and release pro-inflammatory mediators [24]. However, they are also responsible for formation of a fibrous cap that stabilizes a necrotic core of the plaque, composed mainly of lipids and apoptotic foam cells, and thus prevents plaques rupture and potentially fatal event. Meanwhile, matrix metalloproteinases (MMPs), endopeptidase enzymes produced mainly by plaque macrophages, cleave the extracellular matrix, facilitating the migration of vascular smooth muscle cells by the breakdown of elastic lamina and plaque growth, and also contribute to plaque instability and potential thrombus formation by degradation of the fibrous cap [25]. Plaque formation can be also exacerbated by cytokines produced by remote tissues. Adipose tissue-derived adiponectin can alleviate plaque formation; while resistin and leptin aggravate CVDs development [26]. In addition to its major role in cholesterol synthesis, the liver, in response to interleukin-6 (IL-6) synthesizes acute phase response proteins, such as CRP and serum amyloid A (SAA), which are associated with higher cardiovascular risk [27].

1.1.2 The role of vascular endothelial cells in atherosclerosis development

The endothelial lining provides an active interface between blood-borne molecules; including lipoproteins and cytokines, circulating nutrients and environmental pollutants; and peripheral tissues. Endothelial functions in vascular homeostasis include regulation of vessel tone, coagulation events, angiogenesis and repair, and inflammatory responses. Nitric oxide (NO) produced by endothelial cells induces vasodilatation in VSMCs, thus maintaining systemic blood pressure [28]. NO also plays a protective role in the atherosclerosis development by inhibiting leukocyte adhesion [29] and platelet aggregation [30]. The enzyme responsible for NO production in endothelial cells, endothelial nitric oxide synthase (eNOS), is plays a role in the development of atherosclerosis. Endothelial dysfunction, including endothelial activation and decreased NO availability, is an independent predictor of cardiovascular events [31].

As mentioned previously, endothelial cells play an active role in leukocyte recruitment. Transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), are central regulators of expression of adhesion molecules and
cytokines [32, 33]. Interestingly, an extensive body of research suggests that oxidative stress plays a significant role in the pathology of atherosclerosis [34] and both of these transcription factors are activated in the response to increased cellular levels of reactive oxygen species (ROS) [35]. Other important signaling regulators in the endothelial inflammatory reaction are mitogen-activated protein kinases (MAPKs); signaling proteins that regulate cellular responses to various environmental stimuli and play a significant role in the regulation of various human pathologies. The best characterized MAPK subfamilies include extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 kinases [36], and all of them can respond to cellular redox status [37]. JNK and p38 in particular are important pro-atherogenic mediators [38]. Specifically, JNK and p38 can elicit their pro-inflammatory actions by targeting down-stream transcription factors AP-1 and NF-κB, respectively [39, 40]. Both AP-1 and NF-κB transcriptionally up-regulate of the MCP-1 gene [33]. Endothelial MCP-1 is a critical mediator in the development of atheroma. MCP-1 can facilitate monocyte infiltration into atherosclerotic lesions in mice [41] and humans [42], elevated MCP-1 levels in humans are associated with an increased risk of coronary heart disease [43], and its plasma levels in transgenic mouse models correlate with the plaque severity [44, 45]. Recently, the regulation of endothelial pro-inflammatory signaling at the level of plasma microdomains called caveolae has been proven important in the pathology of atherosclerosis [46].

1.1.3 Caveolae–mediated regulation of endothelial functions

Cellular membranes are comprised of lateral domains with a distinct lipid and protein composition, and varying size and half-life. Membrane rafts are defined as “small (1-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes” [47]. Caveolae are a subgroup of lipid rafts abundant in endothelial cells that were proposed to play a role in regulation of various endothelial functions [48, 49]. Caveolae were identified as early as in the 1950’s as morphologically distinct invaginations in plasma membranes [50, 51]. Similar to lipid rafts, caveolae are relatively rich in sphingolipids and cholesterol [52], but unlike lipid rafts, they are structurally supported by major cholesterol-binding proteins called caveolins [53], and the recently identified cavins [54].

4
Caveolin-1 (Cav-1) was initially identified as a tyrosine-phosphorylated substrate of v-src [55] and later cloned [56]. To this date, three caveolins have been described. While Cav-1 and caveolin-2 (Cav-2) are present in most terminally differentiated cells, and in particular in endothelial cells, adipocytes, and type II pneumocytes [57]; caveolin-3 (Cav-3) is muscle specific [57]. Cav-1 and Cav-3 are highly homologous [58] and required for caveolae formation in their respective cell types, while caveolae can form in the absence of caveolin-2 [59]. A number of different functions have been attributed to caveolae and caveolins over time. Caveolae-mediated endocytosis is involved in macromolecule uptake [60], as well as in the activation of associated signaling pathways [61]. Cav-1 can directly bind lipophilic molecules, such as cholesterol [62] and fatty acids [63], which allows for intracellular transport of these molecules. As a result of numerous signaling molecules being associated with caveolae and/or directly-bound by caveolins (reviewed in [64]), the “caveolae signaling hypothesis” has been proposed where compartmentalization of these signaling molecules within caveolae allows for coupling of activated receptors and downstream effector systems [65].

The majority of the information about the role of caveolae in specific physiological processes was derived from studying transgenic mice. While mouse models deficient in any of the three caveolins are viable and fertile [59, 66-68], they bear various abnormalities, in particular in their cardiovascular system. Cav-1 knockout can result in cardiac hypertrophy and heart failure [69], pulmonary hypertension [70], and angiogenesis [71]. With regard to atherosclerosis, even though the Cav-deficient (Cav-1<sup>−/−</sup>) mice had a pro-atherogenic lipoprotein profile, atherosclerotic lesion development was reduced by ∼ 70% [72]. These Cav-1<sup>−/−</sup> mice also had lower levels of the class B scavenger receptor CD36 and the adhesion molecule VCAM-1 in the aorta [72], suggesting decreased uptake and transcytosis of lipoproteins and monocytes. Because Cav-1 directly binds and inhibits eNOS [73], Cav-1<sup>−/−</sup> mice also tend to have increased NO bioavailability, while exhibiting an abnormal regulation of vascular tone [66]. All of these observations sparked an interest in utilizing caveolae and Cav-1 as a pharmacological target in the regulation regulation of cardiovascular disease [74].
As mentioned previously, endothelial cells have some of the highest levels of caveolae and Cav-1. Consequently, most of the vascular, cardiac, and pulmonary alterations in Cav-1−/− mice [75], including reduced atherosclerotic plaque formation [46], seem to be caused by Cav-1 deficiency specifically in the vascular endothelium. Genetic deletion of Cav-1 in atherosclerosis-prone mice inhibited the progression of atherosclerosis, while re-expression of Cav-1 specifically in the endothelium reversed lesion formation [46]. In addition, Cav-1 expression was stronger in atherosclerotic lesions in hypercholesterolemic rabbits and apolipoprotein E-deficient (apoE−/−) mice as compared to normal rabbits and mice [76], as well as in endothelial cells isolated from arteries of smokers [77]. These studies strongly suggest that endothelial Cav-1 levels are correlated with endothelial dysfunction and atherosclerosis development. Several mechanisms could explain this effect.

Endothelial eNOS, localizes to caveolae [78] as a result of its myristoylation and palmitoylation [79], and it also binds Cav-1 scaffolding domain, which inhibits its enzymatic activity [73]. Although increased NO production is generally associated with atherosclerosis prevention, eNOS activation in the absence of Cav-1 is not accompanied by compensating levels of its cofactor tetrahydrobiopterin (BH₄) which results in eNOS uncoupling and a release of superoxide (O₂⁻) and other ROS. This can facilitate cardiac stress and even heart failure [80]. In turn, in vivo delivery of the Cav-1 scaffolding domain prevented overt NO production and reduced inflammation [81]. Similarly to eNOS, other enzymes and signaling proteins can localize to caveolae and/or directly bind to Cav-1 through its scaffolding domain (residues 82-101) [82]. Examples include heterotrimeric G proteins, adenylyl cyclase, Src kinases, phosphatidylinositol 3-kinase (PI3K), protein kinases A and C, and H-Ras [64]. Many important endothelial G protein-coupled receptors, e.g., endothelin-1 receptor ET_B [83], bradykinin receptor B2R [84], and angiotensin II type I (AT1) receptor [85], also localize to caveolae and thus Cav-1 can modulate activation of their downstream targets.

NK-κB activation by TNF-α in endothelial cells is also a subject to regulation by Cav-1 [86], and Cav-1-deficient mice exhibit decreased NK-κB activation in response to exposure to LPS [87]. Reduced NF-κB activation could explain the lack of VCAM-1
expression observed in Cav-1-deficient mice [72]. Since NO production by eNOS is increased in the absence of Cav-1, this could be another plausible mechanism of decreased NF-κB activation in Cav-1-deficient models [88]. Furthermore, caveolae can contribute to the cellular redox status, thus affecting endothelial signaling, including NF-κB. As mentioned before, eNOS activation in Cav-1−/− models in the presence of low levels of BH4 leads to eNOS uncoupling and ROS production [80]. NO can form the highly active peroxynitrite (ONOO−), when combined with O2− from other cellular sources, such as NAD(P)H oxidases or various cytochrome P450s (CYPs) [89]. Non-phagocytic NAD(P)H oxidases are another significant source of ROS in vascular endothelial cells [89]. Caveolae endocytosis is involved in the recruitment of the NAD(P)H subunit Nox2 and co-activator Rac1 into a new organelle, redoxosome, that allows for compartmentalized production of ROS and NF-κB activation [90]. These mechanisms implicate caveolae in regulation of cellular redox status. In turn, Cav-1 levels can be increased in response to ROS [91], which could exacerbate inflammation and atherogenesis.

Caveolae play a role in endocytosis and transcytosis of various compounds. Endothelial transcytosis of macromolecules, such as albumin and LDL, can facilitate the development of atherosclerotic plaque, which was one of the stated reasons for reduced atherosclerosis in Cav-1-deficient animal models [92]. Cholesterol molecules directly bind Cav-1 [62] and cholesteryl ester is taken up by the caveolae-localized scavenger receptor BI (SRB1) and transported intracellularly while complexed to Cav-1 [93]. Caveolae can accept cholesterol from HDL, which counteracts cholesterol depletion and eNOS inactivation by oxLDL [94]. Interestingly, caveolae seem to play an important role in cellular uptake of small lipophilic molecules, such as certain flavonoids [95], and also persistent organic pollutants [96]. The significance of caveolae-mediated uptake and signaling in the toxicity of environmental pollutants and the interactions with dietary compounds will be discussed further.

1.1.4 Polychlorinated biphenyls – an overview

Exposure to persistent organic pollutants is a contributing factor to the development of atherosclerosis [8]. Polychlorinated biphenyls (PCBs) are man-made
chemicals and complex mixtures of 209 congeners, formed by 0-10 chlorines attached to two connected benzene rings (Figure 1.1). Mass production and commercial use of PCBs in the U.S. started in 1929, predominantly by the Monsanto Chemical Company under a brand name Aroclor. Because of their heat resistance and excellent electric insulating properties, they became widely used in particular in electrical equipment, such as transformers and capacitors. They were also utilized in a variety of consumer products, including plastics, paints, and caulking compounds. With the rise of the environmental movement in the 1960s, the persistence of PCBs in the environment, as well as potential adverse effects on wildlife and humans, became better understood. Eventually, PCB production was banned by the U.S. Environmental Protection Agency (US EPA) in 1979. By that time, about 1.1 billion pounds of PCBs had been produced in the U.S. [97].

Although PCBs are not produced any more, many of the PCB-containing products are remaining, for example more than one million capacitors and 14,000 transformers are still in use in the U.S. [98]. Furthermore, because of chemical stability, PCBs are environmentally persistent and thus became one of the most widespread environmental contaminants. They can be transported long-distance by wind and water [99]. In the environment, they accumulate in hydrophobic matrices, including living organisms. Due to their lipophilicity and resistance to metabolism, they also become enriched in successive trophic levels in the food chain in a process called biomagnification [100]. As a result, the major sources of PCBs in human diet are products of animal origin, and particularly fish [101].

The pharmacokinetics of PCBs in various species has been relatively well characterized [102-105]. Due to their lipophilicity, they are carried in plasma by albumin and lipoproteins [106, 107]; and they partition mainly into adipose tissue with an adipose:plasma partition coefficient of 190:1 in humans [108]. Consequently, weight loss can lead to increased plasma levels in exposed populations [109]. Only negligible amounts of PCBs are excreted as parent compounds [103]. PCB metabolism is initiated by CYP monooxygenases, specifically CYP 1A1, 1A2, 2B1, and 2B2, and proceeds through the formation of an unstable epoxide intermediate [110], as documented by the existence of a chlorine shift in PCB77 metabolites [102]. The epoxide can be converted
to either hydroxylated metabolites (OH-PCBs), or to sulfur containing metabolites via the mercapturic acid pathway. OH-PCBs can be either excreted directly in feces [102], or further form glucuronide and sulfate conjugates [110].

Due to the propensity of PCBs to bioaccumulate in animal tissues, the majority of PCB exposure in the general population occurs by ingestion [101], although in some specific cases inhalation of PCB-contaminated air can contribute significantly [111]. PCBs have been identified, at some level, in roughly one-third of the sites listed on the National Priorities List (NPL), a list associated with the Superfund program [97]. Highly PCB contaminated populations include some of the traditionally fish-eating communities, such as certain groups Native American tribes [112], Canadian Inuits [113], and communities on Faroe Islands [114]; the workers who were exposed occupationally during the years of PCB production, particularly in capacitor production [115]; and also communities who live in close proximity to former PCB-producing factories in the U.S., such as the Monsanto plant in Anniston, AL [116], and on the upper Hudson River, NY [117]. Additional sources of exposure include two cases of accidental ingestion of relatively large amounts of PCBs from rice oil were reported; Yusho in Japan in 1968 and Yu-Cheng in Taiwan in 1979 [118].

Accordingly, PCB levels in human plasma resulting from these exposures vary widely. Low ppb concentrations are present in plasma of the general U.S. population [98]. Yu-Cheng patients had 99 ppb of total PCBs in blood [118], and levels as high as 1 ppm were found in their plasma after occupational exposure [119]. Inuits as a result of their fish oil-rich diet, have 3.4-fold higher plasma PCB levels than Caucasian population [120]. High risk groups also include children exposed to PCBs prenatally through cord blood [120], or during postnatal development by breast milk [121]. Prenatal exposure to PCBs was found to have a negative effect on cognitive functions in childhood [122].

While acute toxicity of PCBs is low and generally limited to chloracne, as documented in the case of Yusho patients [123]; a large number of epidemiological and mechanistic studies unraveled a contribution of PCBs to the development of various chronic human pathologies. PCBs can act as initiators and promoters of carcinogenesis [124], in particular PCB3, a common pollutant in indoor air [125]. Neurobehavioral
changes resulting from PCB exposures are likely caused by modification of ryanodine receptors in brain [126]. PCBs might act as endocrine disruptors and can alter thyroid homeostasis [127]. Developmental exposure to PCBs is likely to result in negative outcomes later in life [128, 129] and the mechanisms involve epigenetic modifications [130].

PCBs in the environment are present as complex mixtures with varying degrees of chlorination and individual congeners that vary in their biological effects. Based on their chlorination pattern and biological activities, three sub-groups of PCB congeners are recognized: non-ortho-substituted coplanar PCBs (e.g. PCB77 and PCB126), ortho-substituted non-coplanar PCBs (e.g. PCB104 and PCB153), and so-called mixed inducers with one or two chlorines in ortho positions (e.g. PCB118) [131]. The mechanism of toxicity is the best understood for the coplanar PCBs, which are agonists of the aryl hydrocarbon receptor (AhR). Their toxic potency seems to correlate to their ability to bind and activate the AhR, and for the purpose of risk assessment, each of the coplanar PCBs is assigned a toxic equivalency factor (TEF) which expresses its toxicity relative to that of the most potent AhR inducer, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [132]. AhR is a basic helix-loop-helix (bHLH) transcription factor [133]. In its unliganded form, AhR resides in the cytoplasm as part of a complex containing two molecules of 90-kDa heat shock protein 90 (hsp90) [134], and the hepatitis B virus X-associated protein 2 (XAP2) [135]. Ligand binding causes AhR translocation to the nucleus, release of hsp90, and dimerization with AhR nuclear translocator (ARNT). The AhR/ARNT complex recognizes and binds AhR-response elements (AREs) located in the promoter region of responsive genes, thus modulating transcription [133].

AhR targets include CYP enzymes and some phase II enzymes, such as glutathione (GSH)-S-transferase (GST) Ya and uridine 5′-diphospho-glucuronosyltransferase (UGT) 1A1, that mediate PCB metabolism [136]. While AhR activation results in cross-talk with a variety of cellular targets [137], the CYP up-regulation and subsequent metabolism of PCBs in fact explains many of the toxic effects observed after PCB exposure. For example PCB77, while up-regulating cellular levels of CYP1A1, subsequently binds and uncouples the enzyme, thus resulting in production of
ROS [138], a likely culprit of the defective signaling associated with many PCB-induced pathologies. Meanwhile, phase I of PCB metabolism that is catalyzed by CYP enzymes often results in catechols and hydroquinones [139]. These metabolites are subsequently converted to quinones by cellular peroxidases [140], and undergo redox cycling thus contributing to cellular oxidative stress [141]. In addition, these reactive intermediates directly bind biomolecules, such as DNA [140] and proteins [142].

1.1.5 Cardiovascular toxicity of polychlorinated biphenyls

PCB exposure can facilitate the development of CVDs. Increased cardiovascular mortality was observed in Swedish capacitor workers [143] and National Health and Nutrition Examination Survey (NHANES) revealed an association between PCB levels and cardiovascular disease in females [144]. High levels of PCBs in epidemiological studies coincide with known cardiovascular risk factors, for example non-coplanar PCBs were associated with insulin resistance [145] and coplanar PCBs with the metabolic syndrome [146]. Elevated levels of total blood cholesterol and triglycerides were found in Yusho patients [147]. A similar blood lipid pattern was observed in fish-eating communities in the U.S. and was associated with high PCB levels and CVD risk [112]. In addition, exposure to TCDD, a toxic chemical that shares a mechanism of action with coplanar PCBs, increased the risk of heart disease after occupational exposure [148, 149]. Dioxins were also correlated with the incidence of diabetes mellitus [149-151] and hypertension [149, 151] among Vietnam War veterans.

Several mechanisms have been proposed to explain the cardiovascular toxicity of PCBs. Yusho patients had elevated plasma cholesterol [147], and a similar effect was observed in an animal model after an exposure to coplanar PCB126 [152]. This might be caused by decreased flow of bile acids, a major excretion route for cholesterol, which was observed after treatment of rats with Aroclor 1248 [153]. Recently, it was found that PCB77 treatment reduced liver expression of CYP7A1, the rate-limiting enzyme in the synthesis of bile acid from cholesterol [154], which would be a plausible mechanism for reduced cholesterol clearance after PCB exposure. In addition, low concentrations of coplanar PCBs promote adipocyte differentiation and their production of inflammatory
cytokines, including MCP-1 and TNF-α, thus contributing to obesity development and inflammation in mice [155].

However, so far the most widely studied mechanism of PCB-induced atherogenesis is an endothelial dysfunction induced by coplanar PCBs [18]. The AhR pathway is expressed in the vasculature [156] and endothelial cells exhibit an induction of CYP1A1 in response to AhR agonists [157]. PCB77 uncouples the catalytic cycle of CYP1A1 [138] which results in increased ROS production. As stated above, transcription factors NF-κB and AP-1 are redox sensitive [35]. Increased NF-κB mediated transcription can cause up-regulation of adhesion molecules, such as VCAM-1 in vitro [158] and in vivo [18] after exposure to coplanar PCB77. In turn, most of these effects can be reversed by AhR antagonists and antioxidants [159, 160]. Coplanar PCBs can also disrupt the barrier function of endothelial cells [161] and, under conditions of antioxidant depletion, even induce endothelial apoptosis [162]. Furthermore, NF-κB activation by PCB77 leads to up-regulation of inducible cyclooxygenase (COX-2) with a subsequent increase in pro-inflammatory eicosanoid prostaglandin E\(_2\) (PGE\(_2\)) [158]. Recently it was found that PCB77 can stimulate eNOS phosphorylation and NO production, which contributes to cellular oxidative stress, and formation of harmful ONOO\(^-\) that forms nitrotyrosine adducts on proteins [163].

Later it was found that ortho-substituted non-coplanar PCBs, such as PCB104, can cause endothelial toxicity as well. PCB104 exposure leads to increased ROS production, although the mechanism is unclear at this point [164]. It increases transcriptional activity of both NF-κB and AP-1 in endothelial cells [165]; and consequently boosts the levels of adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and E-selectin, as well as release of MCP-1 [164]. A recent study suggested that PCB153, the most abundant non-coplanar PCB in the environment, can also disrupt signaling in microvascular endothelial cells [166]. It increased expression of both VCAM- and ICAM-1, and Src kinases were involved in the regulation of this event. Interestingly, lipid raft disruption by cholesterol-depleting agent prevented the changes in cellular signaling induced by PCB153; supporting the notion that PCB toxicity can be modulated at the level of plasma membrane.
1.1.6 The role of caveolae in endothelial dysfunction induced by polychlorinated biphenyls

Lipophilic molecules, such as PCBs, associate in the plasma mainly with albumin [107] and lipoproteins [106]. Lipophilic membranes of endothelial cells, and in particular caveolae, are likely to be the cellular receptors of these compounds, because the albumin receptor gp60 is localized to caveolae [167]. After PCB exposure, coplanar PCB77 was distributed mainly to the caveolae-rich fraction in cultured endothelial cells [96]. This suggests that caveolae are a major point of entry for PCBs at the cellular surface. Thus, lipid rafts, and caveolae in particular, present an intriguing regulatory platform for PCB uptake, and the activation of downstream pathways in endothelial cells.

In addition, exposure to PCB77 increased Cav-1 protein levels in endothelial cells [96]. This could potentially contribute to the increased plaque formation that was observed after PCB exposure [155] by two main mechanisms. First, LDL accumulation in the sub-endothelial matrix is critical for lesion formation in the early stages of atherosclerosis [2]. Caveolae actively regulate the endothelial transcytosis of macromolecules [168] and mice with selective deletion of Cav-1 in endothelial cells had lower rates of intimal LDL accumulation [46]. Secondly, the presence of Cav-1 was associated with increased VCAM-1 expression in aorta [72], and caveolae play an active role in trans-cellular migration of T-lymphocytes across vascular endothelium [169]. These mechanisms can lead to increased inflammation and monocyte recruitment to the lesion in the early stages of atherosclerosis.

Caveolae have been also emerging as mediators of PCB-induced ROS production. Cav-1 was found to bind AhR, and consequently in absence of Cav-1, CYP1A1 up-regulation and ROS production was decreased [96]. PCB77-mediated induction of eNOS in endothelial cells was caveolae-dependent as well, and down-stream NF-κB activation was reduced in cells lacking Cav-1 [163]. Caveolae also play a role in the assembly and activation of NAD(P)H, another source of ROS in the vascular endothelium [90]. NAD(P)H oxidase activation by the non-coplanar PCB153 and its role in up-regulation of adhesion molecules was studied in microvascular endothelial cells [166]. PCB153 increased recruitment of the NAD(P)H oxidase subunit p47phox to lipid rafts, but Cav-1
silencing did not prevent downstream adhesion molecule up-regulation. This led to the conclusion that lipid rafts regulate PCB153 toxicity in this system. Because coplanar PCBs seem to produce relatively more oxidative stress in endothelial cells than non-coplanar PCBs [18], their effect on NAD(P)H oxidase in endothelial cells should be explored as well.

In summary, the changes in cellular signaling induced by various PCB congeners can facilitate development of atherosclerosis in humans. The current evidence suggests that the induction of chronic inflammatory reactions in the vascular endothelium likely contributes to this process, and it could be regulated by caveolae. The following passage will address some strategies aimed to prevent the toxic outcomes of PCB exposures.

1.1.7 Dietary in modulation of polychlorinated biphenyl toxicity

An overwhelming number of studies supports the notion that composition of diet is a major risk factor for development of atherosclerosis [7]. While a total calorie intake plays a role in obesity and associated cardiovascular risk [170], specific diet-derived compounds can affect cellular signaling and thus either prevent or facilitate atherosclerosis development. Certain nutrients, in particular polyphenols found in fruits and vegetables [171], and fish oil enriched in omega-3 fatty acids [172], can alleviate cardiovascular mortality and associated risk factors. In human studies, saturated and trans fatty acids tend to exacerbate cardiovascular risk factors, while long-chain polyunsaturated fatty acids (PUFAs) have some beneficial properties [173]. In terms of PUFAs, it is now evident that the position of the final carbon-carbon double bond has some significance. Specifically omega-3 PUFAs, such as fish oil-derived eicosapentaenoic acid (C20:5ω-3, EPA) and docosahexaenoic acid (C22:6ω-3, DHA), are highly anti-inflammatory [174]. Consequently, a substantial number of studies demonstrated that regular consumption of fish and/or EPA and DHA results in lower cardiovascular mortality [175].

The emerging concept of using nutrition as a modulator of chronic diseases induced by environmental pollution has acquired much interest recently [176]. Much of the data regarding mechanistic interactions between a toxicant and dietary molecules was
obtained by studying PCBs [177]. Green tea-derived (-)-epigallocatechin-3-gallate (EGCG) and dietary flavonoid quercetin reduced PCB77-induced CYP1A1 up-regulation and subsequent increase in oxidative stress [159]. Later it was clarified that EGCG inhibits AhR-mediated transcription through binding to AhR chaperone hsp90 [178]. Dietary antioxidants, such as vitamin E, can reduce PCB-induced NF-κB activation in endothelial cells [160]. In addition, various dietary polyphenolic can prevent endothelial inflammation induced by benzo[a]pyrene (B[a]P) [179].

Data from our laboratory provide ample evidence that the type of dietary fat can affect the outcome of PCB exposure [180]. Diet enriched with linoleic acid (C18:2ω-6, LA) in the form of corn oil exacerbated expression of adhesion molecules in mouse aorta, while similar levels of olive oil-derived oleic acid (C18:1ω-9, OA) led to reduced inflammation. While LA can amplify endothelial toxicity of PCBs [161], omega-3 PUFA α-linolenic acid (C18:3ω-3, ALA) decreased endothelial inflammation induced by PCB77 [158]. Some studies also suggest that dietary supplementation with non-absorbable fat, a sucrose polyester olestra, can reduce absorption [181], and potentially facilitate excretion [182] of persistent organic pollutants, such as PCBs. Much information is still needed, in particular reliable epidemiological studies, in order to 1) prove the concept of dietary prevention of the toxicity of environmental chemicals in humans, and 2) establish the effective levels of specific nutrients required in order to gain benefits. Eventually this area of research should provide a variety of safe options to alleviate toxic outcome to environmental chemicals.

1.1.8 Nutrition and a regulation of caveolae-associated signaling

Membranes, and specifically caveolae, have proven themselves to be a viable target for nutrient-induced changes in cellular signaling. Much like PCBs [96], and potentially other persistent organic pollutants [183], lipophilic flavonoids are thought to utilize caveolae for their interaction with the cells. Functional caveolae were required for resveratrol uptake and chemoprevention [95]. Free fatty acids in plasma are carried mainly by albumin, while esterified fatty acids are incorporated into lipoproteins as triglycerides and cholesteryl esters [184], so they are likely to be targeted towards caveolae during their interaction with the cellular surface. Additionally, Cav-1 can bind
free fatty acids directly [63, 185], and caveolae have been implied in uptake [186] and intracellular transport [185] of fatty acids.

Dietary fatty acids can also modulate caveolae composition and function [187]. Caveolae membranes are esterified mainly with saturated fatty acids that allow for a liquid ordered state of caveolae, and an effective organization of the associated signaling molecules [188, 189]. On the other hand, the incorporation of unsaturated fatty acids can change lipid rafts’ stacking and their natural liquid ordered state [188]. Enrichment of the membranes with dietary unsaturated fatty acids, such as long-chain omega-3 PUFAs, thus can lead to the displacement of Cav-1 and cholesterol from caveolae, resulting in decreased activation of the associated signaling pathways [187]. Pre-treatment of endothelial cells with the omega-3 PUFA ALA prevented TNF-α-induced co-localization of Cav-1 and TNF receptor 1 (TNFR1) and down-stream inflammatory signaling [190]. In endothelial cell cultures, both DHA [191] and EPA [191] displaced eNOS from the caveolae fraction and enhanced NO production, which could be an important mechanism for their cardioprotective behavior. DHA was also found to decrease caveolae cholesterol levels in vitro, resulting in displacement of Src kinases Fyn and c-Yes from caveolae and decreased VCAM-1 levels in endothelial cells [192].

Dietary polyphenols in fruits and vegetables are likely responsible for cardioprotective properties of diets rich in fruits and vegetables [193]. Endothelial Cav-1 facilitates atherosclerosis [46], and thus modulation of Cav-1 levels can be an underlying mechanism of flavonoid-induced cardioprotection in vascular endothelium. Cav-1 levels were increased in response to ROS production by activation of oxidative stress sensitive p38 MAPK [91]. Consequently, antioxidant flavonoids, such as EGCG [194] and quercetin [195] decreased Cav-1 expression levels. Similarly, the anti-oxidant properties of ALA might explain its inhibitory effect on TNF-α-induced Cav-1 expression in endothelial cells [190]. The anti-oxidant vitamin E also prevented up-regulation of Cav-1 and ROS-associated premature cellular senescence [196]. Taken together, the antioxidant potential of diet-derived molecules might correlate with a decrease in Cav-1 levels, as well as with endothelial protection.
In conclusion, caveolae are involved in the uptake of various dietary compounds that can have protective effects on the vascular endothelium. Accumulation of flavonoids, fat-soluble vitamins, and fatty acids in caveolar membranes can result in changes of caveolae composition and function of associated proteins. Exposure to flavonoids leads to changes in Cav-1 levels. Most flavonoids decrease Cav-1 expression, possibly as a result of their anti-oxidant capacity. A decrease in Cav-1 levels will result in reduced caveolae formation, and down-regulation of caveolae-associated pathways and endocytosis.

Similarly, incorporation of highly unsaturated omega-3 PUFAs into caveolae membranes leads to displacement of Cav-1 and cholesterol and subsequent down-regulation of caveolae-associated signaling. Because up-regulation of endothelial Cav-1 seems to be pro-atherogenic, the down-regulation of Cav-1 and caveolae-associated signaling may be a viable mechanism for dietary intervention in prevention and treatment of cardiovascular pathologies.

1.1.9 Fatty acids and their metabolites in cardiovascular disease development

The relative ratios of dietary fatty acids have clearly an effect on the subsequent cardiovascular risk. Trans fatty acids tend to increase LDL cholesterol and should be substituted by other forms of fat [197]. Replacing saturated with unsaturated fatty acids can also improve cardiovascular risk factors, including blood lipoprotein profile, insulin resistance, and hypertension; as well as the overall rates of CHD [198]. At the level of vascular inflammation, it was suggested that the anti-inflammatory activity of FAs in endothelial cells is proportional to the number of double bonds [199, 200] with DHA being particularly potent. The most common forms of polyunsaturated fatty acids are omega-3 and omega-6 PUFAs, denoted based on position of the last double-bonded carbon as counted from the methyl end. Essential dietary precursors of the omega-3 and omega-6 series are ALA and LA, respectively.

While the majority of fatty acids in tissues is utilized as an energy supply or as building blocks of cellular structures; it is now clear that PUFAs are a subject to both enzymatic and non-enzymatic reactions leading to a production of potent signaling molecules; and their formation can mediate responses to extracellular stimuli [201]. The enzymatic reactions involved in the conversion of arachidonic acid (AA, 20:3ω-6) to
bioactive eicosanoids are by far the best understood, partially because AA is the most common highly unsaturated fatty acid in humans [202]. The initial step is a release of AA from membrane phospholipids by the action of phospholipases, primarily cytosolic phospholipase A2 (PLA2). Subsequently it is enzymatically processed by COX, lipoxygenase (LOX), or CYP-mediated pathways [203].

The first step in the cyclic pathway of eicosanoid metabolism; that yields prostaglandins (PGs), prostacyclins, and thromboxanes, is the formation of bicyclic endoperoxide PGH2 catalyzed by the action of COX (prostaglandin H synthase) [204]. To date, three COX isoforms were identified, with COX-1 and COX-3 being constitutively expressed and COX-2 acting as an inducible mediator of inflammatory reaction [205]. While COX enzymes are fairly ubiquitously expressed, the downstream enzymes determine which eicosanoids are produced in specific tissues and their biological outcome. For example, prostacyclin synthase produces prostacyclin (PGI2) in endothelial cells. Thromboxane (TXA2) is the main product of platelets and is formed by thromboxane synthase. PGD2 and PGF2 are formed from PGH2 by their respective synthase enzymes in a variety of cell types, and PGE2 can be produced both by enzymatic (PGE2 isomerase) and non-enzymatic pathways [206]. In the vasculature, and the development of CVDs, there is a particularly important balance between PGI2 and platelet-derived TXA2. PGI2 binds it cognate receptors on VSMCs and inhibits contraction. In contrast, TXA2 is a vasoconstrictor and facilitates thrombosis [207].

5-lipoxygenase (5-LOX) catalyzes the conversion of AA to an unstable intermediate 5,6-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid (leukotriene (LT)A4) [208], which can be further converted into LTB4 by LTA4 hydrolase [209], or conjugated with GSH by LTC4 synthase to form LTC4 [210]. LTB4 is a potent chemoattractant for neutrophils, eosinophils and monocytes, while LTC4 and its metabolite, LTD4, are bronchoconstrictors and increase vascular permeability, rendering LO-5 and LT receptors attractive targets for treatment of chronic inflammatory diseases, such as atherosclerosis [211]. In contrast, processing of AA by 12-LOX and 15-LOX leads to production of lipoxins that play a role in the resolution of inflammation and their contribution to atherosclerosis development is currently under study [212].
AA can be also subject of enzymatic conversion by CYP monoxygenases to form hydroxyeicosatetraenoic acids (HETEs) [213] or epoxy-eicosatrienoic acids (EETs) [214]. 20-HETE is the main HETE produced in the vasculature, is a potent vasoconstrictor [215], and facilitates inflammation in endothelial cells by activation of NF-κB pathway [216]. On the other hand, EETs are endothelium-derived hyperpolarizing factors (EDHFs) [217] with anti-inflammatory properties [218]. Increasing EETs’ levels by inhibition of soluble epoxide hydrolase (sEH), an enzyme responsible for degradation of EETs, have been shown to be atheroprotective [219]. Most of the enzymes involved in the processing of arachidonic acid are useful pharmacological targets in inflammation, pain management, and cardiovascular diseases [203].

Many epidemiological and experimental studies have demonstrated cardioprotective properties of omega-3 PUFAs, and fish oil-derived very long-chain EPA and DHA are considered to be the most effective [220]. Although ALA can be elongated by mammals into the longer omega-3 PUFAs, only 5% of ALA is converted to EPA and <0.5% of ALA is converted to DHA in humans; and therefore dietary intake of only ALA is considered insufficient to provide protection [221]. As demonstrated by intervention studies in humans, EPA and DHA can incorporate into lipids in the atheroma, and here they decrease macrophage infiltration and improve plaque stability [222]. Omega-3 supplementation can improve arrhythmia threshold in the heart and thus decrease the probability of sudden cardiac death [223]. Diet enriched in fish oil prevented platelet aggregation [224], and higher doses of omega-3s decreased blood pressure [225]. Also, while the changes in blood cholesterol and lipoproteins resulting from omega-3 supplementation were inconclusive, the lowering effect on plasma triglyceride (TG) levels have been shown consistently [226]. A prescription form of EPA and DHA supplement has been approved recently by the U.S. Food and Drug Administration (US FDA) for the management of very high TG levels [227].

It is now understood that omega-3 PUFAs can be a substrate for some of the same eicosanoid-producing enzymes as AA. Interestingly, some of the EPA-derived eicosanoids are less pro-inflammatory than AA-based products of the same pathway, and their competition for the same enzymes is considered a mechanism for some of the anti-
inflammatory effects observed with omega-3 supplementation [202, 228]. Resolvins and protectins are produced from omega-3 PUFAs by sequential metabolism of CYP or COX-2 enzyme and LOXs [212]. DHA and EPA metabolism results in formation of D- and E-series resolvins and protectins, respectively. Both resolvins and protectins are thought to facilitate a resolution of the inflammatory reaction, and can probably contribute to cardioprotective properties of omega-3 PUFAs. For example, resolvin E1 counteracts the pro-inflammatory action of LTs at the level of their receptors [229], and protectin D1 can inhibit NF-κB activation and leukocyte infiltration in the brain [230]. Indeed, the 12/15-LOX–deficient (12/15-LOX−/−) mouse model displayed increased plaque formation and the products of 12/15-LOX-mediated metabolism, resolvin D1, and protectin D, were atheroprotective [231]. In the light of this evidence, further studies should focus on the role of omega-3 metabolites in the pathology of atherosclerosis.

1.1.10 Fatty acid-derived isoprostanes and their role in atherosclerosis

As early as 1975, it was hypothesized that PUFAs containing three or more double bonds can be converted into prostaglandin-like compounds by a non-enzymatic free radical-initiated reaction that progresses through a formation of bicyclic endoperoxide [232, 233]. Subsequently, AA-derived compounds containing F-type prostane ring, F₂-isoprostanes (IsoPs), were identified in rat plasma using gas chromatography-mass spectrometry techniques [234]. A treatment with CC1₄, a potent initiator of lipid peroxidation, led to 200-fold increase in F₂-IsoPs formation, while the COX inhibitor indomethacin had no effect; thus demonstrating that these IsoPs are formed by free radical-initiated and no by an enzymatic mechanism [234].

Subsequently, more kinds of IsoPs were identified as products of a free radical-initiated oxidation of long-chain PUFAs [235, 236]. The nomenclature generally follows that of PGs. The substituents on the cyclopentane ring determine the type of IsoP, for example A/J-IsoPs contain α,β-unsaturated ketones, D/E-IsoPs possess β-hydroxy ketones, and F-IsoPs are 1,3-diols. The numerical subscript refers to the number of double bonds contained on the side chains of the cyclopentane ring and is generally determined by the number of unsaturated bonds in the parent fatty acid. Therefore, AA forms F₂-IsoPs, whereas EPA gives rise to F₃- IsoPs, DHA produces F₄-IsoPs, etc.
Subsequently, regioisomer classes can be distinguished based on the carbon number on which the side chain hydroxyl group is attached with the carboxyl group being 1 [237].

As stated above, the formation of IsoPs proceeds through PGH$_2$-like bicyclic endoperoxide intermediates (Figure 1.2). These H-IsoPs are either reduced to form F-IsoPs, or they can undergo isomerization leading to production of unstable E/D-IsoPs, These tend to be subsequently dehydrated into A/J-IsoPs [236]. This process was found correct for AA [236], EPA [238], and DHA [239]. DHA-derived IsoPs are specifically called neuroprostanes (NPs), because they are particularly abundant in brain tissues, similarly to the parent fatty acid DHA [240].

IsoPs can be structurally distinguished from PGs because IsoPs have side chains that are predominantly oriented cis to the prostane ring, while PGs possess mainly trans side chains [241]. Unlike PGs, IsoPs do not need to be hydrolyzed prior to oxidation [242]. As a result, the majority of IsoPs in the body exist in their phospholipid-bound form, corresponding to the fact that the majority of FAs in the body are conjugated. They can be released by the action of certain phospholipases, e.g., both plasma and intracellular platelet-activating factor (PAF) acetylhydrolase participate in the release of phospholipid-bound F$_2$-IsoPs [243]; however, specific phospholipases responsible for processing of esterified NPs have not yet been identified. Emerging new evidence suggests that phospholipid-bound IsoPs are quite capable of the activation of cellular signaling [244].

Many of the products of the IsoP pathway have a pronounced biological activity. For example, AA-derived 8-epi-PGF2α, in the low nanomolar range, is a potent renal vasoconstrictor [234]. Interestingly, some of the omega-3 PUFAs-derived IsoPs have signaling properties that would explain the cardioprotective effects observed after dietary supplementation with EPA and EHA. In fact, in some studies oxidized DHA and EPA prevented cytokine production in endothelial cells, but parent fatty acids were ineffective [245]. The most compelling evidence exists for the anti-inflammatory effects that are shared by PGs and IsoPs due to their similar structural features, in particular a cyclopentenone group in A/J compounds.
It was discovered that only PGs that contain the cyclopentenone group, such as 15-deoxy-Δ12-14-PGJ2 (15dPGJ2), and a product of a dehydration and isomerization of PGD2, were potent inhibitors of NF-κB pathway [246]. A clue for the mechanism involved in this reaction was given by the fact that a cysteine in the activation loop of upstream I-κB kinase β (IKKβ) was required for a covalent modification by these cyclopentenone PGs. Later it was confirmed that the unsaturated α,β-carbonyl moiety in the prostane ring of cyclopentenone IsoPs renders these compounds electrophilic and prone to Michael addition with cellular nucleophiles, in particular protein thiol groups [236]. Specifically, omega-3-derived A3/J3-IsoPs and A4/J4-NPs were found to suppress NF-κB-mediated inflammatory reactions [247], thus suggesting that these compounds could contribute to cardiovascular prevention, as observed in human studies after omega-3 intervention. Interestingly, recent evidence suggests that the same mechanism could be involved in stimulating cellular antioxidant defenses by omega-3 metabolites [248].

1.1.11 Nuclear factor erythroid 2-related factor 2 protects against polychlorinated biphenyl-induced endothelial cell dysfunction

Oxidative stress plays a significant role in the development of atherosclerosis, and in particular endothelial inflammation [249]. While the use of direct antioxidants as possible means for cardiovascular prevention has been studied intensely and was largely unsuccessful [34], some suggest that dietary compounds or pharmaceuticals that activate innate antioxidant responses in the cell could be a viable strategy for cardiovascular prevention [250]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that plays a major role in responses to oxidative stress and chemoprevention. It binds a cis-acting enhancer sequence known as ARE (antioxidant response element) [251] in promoters of genes involved in electrophile and oxidant metabolism, such as GSTs, UGTs, and NAD(P)H:quinone oxidoreductase 1 (NQO1), as well as some genes involved in cell survival and tissue regeneration [252].

The activation of Nrf2 signaling is recognized as an adaptive response to environmental and endogenous stressors. Under basal conditions, Nrf2 is sequestered in the cytoplasm by its binding partner Keap1 [253], which is a substrate adaptor protein that associates with cullin3 to form a functional E3 ubiquitin ligase complex, thus
targeting Nrf2 for ubiquitination and subsequent proteosomal degradation [254]. Dissociation from Keap1, generally a result of its modification by Nrf2 inducer, leads to Nrf2 stabilization, and subsequently nuclear translocation and increased transcription of target genes [255]. Known Nrf2 inducers include an antioxidant tert-butylhydroquinone (tBHQ) [256], a dithiolethione drug oltipraz [257], a dietary isothiocyanate sulforaphane [255], and triterpenone drugs, such as 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), the most potent Nrf2 inducer known to date [258].

Nrf2 activation can provide protection against a wide range of toxic chemicals. Since electrophilic insult by environmental carcinogens is a common first step in the development of cancer, Nrf2 activation has been considered primarily in cancer chemoprevention [259]. Oltipraz stimulated the expression of phase II enzymes and alleviated benzo[a]pyrene induced tumor formation, but was ineffective in Nrf2-deficient (Nrf2\textsuperscript{−/−}) mice, documenting that Nrf2 is a critical mediator in the observed protection [260]. In the absence of Nrf2-induced adaptive responses, cells become much more susceptible to the insult by toxic chemicals. Liver damage by a free radical precursor CCl\textsubscript{4} was exacerbated in Nrf2-deficient mice, and the healing process was hindered [261]. Nrf2 was found to be protective against arsenic-induced tissue damage and DNA hypomethylation [262]. Also, Nrf2 levels and activity in tissues are important determinants of drug-induced toxicity [263].

Many of the toxic effects of PCBs are mediated by ROS [264, 265], and can be exacerbated by GSH depletion [162, 264]. Considering that Nrf2 activation improves the cellular ability to replenish GSH levels by up-regulation of enzymes such as gamma-glutamylcysteine synthetase [252], increased Nrf2 activation might therefore provide protection from PCB toxicity, and brief evidence of this process has been provided recently [266]. In addition, PCB metabolism often results in the formation of toxic quinones [267]. NQO1, which is one of the most widely recognized targets of the Nrf2 pathway, could provide protection by both replenishing the levels of general cellular antioxidants, and also by detoxification of these quinone metabolites [268].

Since oxidative stress is a major mediator of inflammatory signaling during the development of atherosclerosis, it is not surprising that regulation of Nrf2 has been
explored recently as a pharmacological target for atheroprotection. Nrf2 seems to be highly susceptible to activation by unidirectional shear stress, which was implicated in the reduced atheroma formation in protected regions of aorta [269]. Nrf2 overexpression in endothelial cells can prevent ROS-induced cytotoxicity and MCP-1 up-regulation [270]. Nrf2 activation by pharmacological agents, such as sulforaphane, is therefore emerging as a promising strategy to decrease expression of adhesion molecules in the aorta [269]. The newer and highly potent pharmacological inducers, including CDDO-Im [258], should be explored for their potential to reduce the development of atherosclerosis.

A wide variety of diet-derived compounds, such as sulforaphane, are capable of activating Nrf2-mediated responses. This is generally recognized as a precursor for the potency of these nutraceuticals in chemoprevention [271]. Considerable efforts have been devoted to understanding which structural determinants make a molecule a Nrf2 inducer. It has been proposed that the potency of a chemical to induce adaptive responses in the cell and to up-regulate antioxidant enzymes is parallel to its ability to act as a Michael reaction acceptor [272]. As described previously, cyclopentenone IsoPs are very susceptible to this type of reaction and it was observed that omega-3 PUFAs-derived IsoPs are potent inducers of Nrf2 by means of binding to thiol groups on Keap1 and thus destabilizing the Keap1 association with Cullin3, the E3 ligase scaffold protein [248]. Hence, it would be relevant to explore the potential of these omega-3 PUFAs metabolites to reduce the observed PCB toxicity by inducing adaptive antioxidant response in endothelial cells.

1.2 General hypothesis and specific aims

In the following studies, we have selected PCB77 as an example of coplanar PCBs and a persistent environmental pollutant that exhibits dioxin-like toxicity. The general hypothesis of the research described in this dissertation is that PCB-induced endothelial dysfunction is mediated by caveolae and can be modulated by dietary very long-chain omega-3 PUFAs. To test this hypothesis, the following specific aims were proposed:
**Specific Aim 1**: To test the hypothesis that MCP-1 is critical mediator of early progression of atherosclerosis and can be induced by PCB77 in the vascular endothelium.

**Specific Aim 2**: To test the hypothesis that caveolae-associated pathways are an integral regulatory platform that mediates pro-inflammatory signaling induced by coplanar PCBs.

**Specific Aim 3**: To test the hypothesis that metabolites of omega-3 PUFAs produced by free-radical mediated oxidation prevent endothelial toxicity induced by PCB77 by promoting cellular antioxidant defenses controlled by Nrf2.

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Figure 1.1. Structure and nomenclature of polychlorinated biphenyls.

A biphenyl molecule showing numbering and substitution (i.e. ortho, meta, and para) system (A). A specific structure of PCB77 (3,3’,4,4’-tetrachlorobiphenyl) (B).
Figure 1.2. Formation of 7-series neuroprostanes from DHA.

The reaction is initiated by a free radical-mediated abstraction of hydrogen atom from DHA. Then the pentadienyl radical combines with an oxygen molecule to produce a racemic peroxy radical, and an addition of another oxygen molecule results in a formation of a bicyclic endoperoxide intermediate (H$_4$-NP). H$_4$-NP can be reduced to form F$_4$-NP, or isomerized to E$_4$- and D$_4$-NPs. E$_4$-NPs and D$_4$-NPs can be dehydrated to create A$_4$-NPs and J$_4$-NPs, respectively. ( Adopted from [239] and [238])
Chapter two: Up-regulation of endothelial monocyte chemoattractant protein-1 by coplanar polychlorinated biphenyl 77 is caveolin-1-dependent

2.1 Synopsis

Atherosclerosis, the primary cause of heart disease and stroke, is initiated in the vascular endothelium; and risk factors for its development include environmental exposure to persistent organic pollutants. Here, we tested the hypothesis that coplanar polychlorinated biphenyls (PCBs) can induce endothelial expression of monocyte chemoattractant protein-1 (MCP-1), a chemokine that attracts monocytes into the sub-endothelial space in the early stages of atherosclerosis development. In primary endothelial cells, coplanar PCBs 77 and 126, as well as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), increased MCP-1 expression. MCP-1 up-regulation by PCB77 was prevented by an aryl hydrocarbon receptor (AhR) antagonist α-naphthoflavone (α-NF), as well as an antioxidant N-acetyl-L-cysteine (NAC). Also, MCP-1 up-regulation by PCB77 was prevented by inhibiting p38 and c-Jun N-terminal kinase (JNK), but not extracellular signal-regulated kinases (ERK) 1/2, suggesting regulatory functions via p38 and JNK mitogen-activated protein kinase (MAPK) pathways. Caveolae are membrane microdomains involved in the regulation of pro-inflammatory signaling pathways in vascular endothelial cells. Silencing of a major caveolae structural protein, caveolin-1 (Cav-1), abolished MCP-1 up-regulation in endothelial cells. To test the hypothesis that caveolae are required for PCB-induced inflammation in vivo, atherosclerosis-prone low density lipoprotein receptor-deficient (LDL-R\(^{-/+}\)) mice (control) or Cav-1\(^{-/-}\)/LDL-R\(^{-/-}\) mice were treated with PCB77. PCB77 induced aortic mRNA expression and plasma protein levels of MCP-1 in control, but not Cav-1\(^{-/-}\)/LDL-R\(^{-/-}\) mice. Pro-inflammatory cytokine interleukin-6 (IL-6) exhibited the same pattern of expression. Thus, our data demonstrate that coplanar PCB77 can induce MCP-1 expression by endothelial cells and that this effect is mediated by AhR, as well as p 38 and JNK MAPK pathways. Intact caveolae are required for these processes both in vivo and in vitro. This finding supports a key role for caveolae in vascular inflammation induced by persistent organic pollutants.
2.1 Introduction

Endothelial activation is one of the earliest events in the development of atherosclerosis [273]. Exposure to circulating persistent organic pollutants, such as PCBs, can facilitate this process [18]. Coplanar PCBs, for example 3,3’,4,4’-tetrachlorobiphenyl (PCB77), bind to the AhR in endothelial cells, causing an up-regulation of cytochrome P450 1A1 (CYP1A1) [274]. Subsequent uncoupling of CYP1A1 by PCB77 leads to overproduction of reactive oxygen species (ROS) [275], activation of oxidative stress-sensitive signaling pathways, and up-regulation of inflammatory mediators [160].

MCP-1 is an endothelium-derived chemokine that plays an essential role in the recruitment of leukocytes to the site of injury during inflammation. The recruitment of monocytes into the artery wall, followed by their differentiation into macrophages and foam cells, is also one of the earliest events in the pathology of atherosclerosis [276]. It has been reported that AhR ligands, for example polycyclic aromatic hydrocarbon benzo[a]pyrene (B[a]P) [277] or the strongest known AhR ligand, TCDD [278], have the ability to induce MCP-1 production. Understanding mechanisms of MCP-1 up-regulation by coplanar PCBs would help to dissect mechanisms responsible for the increased incidence of atherosclerosis observed after PCB77 treatment in vivo [155], as well as epidemiological evidence implicating PCBs in increased cardiovascular risk in exposed populations [112, 143].

Many endothelial functions, including signal transduction, seem to be regulated through caveolae [49, 279], which are 50-100 nm membrane microdomains enriched in cholesterol and sphingolipids, as well as its major structural protein Cav-1 [280]. Interestingly, a significant reduction in the size of atherosclerotic lesions has been observed in apolipoprotein E-deficient (ApoE−/−) mice deficient in Cav-1 [72]. Recent evidence from our laboratory implicate caveolae as a regulatory platform involved in endothelial activation by environmental contaminants, namely B[a]P [183] and coplanar PCBs [96, 163]. Cav-1 was required for endothelial nitric oxide synthase (eNOS) activation by PCB77 [163], and AhR binding to Cav-1 seems to play a role in up-regulation of downstream AhR targets including CYP1A1 [96].
Thus, the current study was designed to test the hypothesis that functional caveolae are required for MCP-1 up-regulation by coplanar PCB77 in endothelial cells. Our data provide clear evidence that PCB77 increases MCP-1 expression through AhR signaling and that Cav-1 is a possible biomarker of inflammatory events mediated through AhR.

2.3 Materials and Methods

Materials and chemicals

The inhibitors α-NF, NAC, SB203580 and PD98059, as well as sterile, endotoxin-tested, dimethyl sulfoxide (DMSO), were purchased from Sigma-Aldrich (St. Louis, MO). PCB77 was a generous gift from Dr. Larry W. Robertson, University of Iowa, Iowa City, IA. 3,3’,4,4’,5-pentachlorobiphenyl (PCB126) and TCDD were purchased from AccuStandard, Inc. (New Haven, CT).

Cell culture

Primary porcine endothelial cells were isolated from pulmonary arteries as described previously [281]. Cells were cultured in medium 199 (M199) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT). Prior to treatments, cells were grown until confluent and then synchronized by maintaining them in 1% FBS for 16 h. All vehicle controls and treated cultures contained the same amount of DMSO (0.1% v/v). This concentration of DMSO did not have any effect on MCP-1 expression.

Animals

All animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care-certified animal facilities at the University of Kentucky. For our experiments mice were used with low LDL-R-deficient background. These mice are a preferred model for atherosclerosis studies because they mimic human lipoprotein levels and atherosclerosis development [282]. We also have demonstrated previously that PCB77 increases aortic adhesion molecule expression in LDL-R−/− mice (Hennig et al., 2005). LDL-R−/− mice on a C57BL/6 background and Cav-1-deficient (Cav-1−/−) mice
were purchased from The Jackson Laboratory (Bar Harbor, ME). Cav-1−/− mice were generated in the Sv129 strain and backcrossed onto a C57BL/6 background. Mice were bred at the University of Kentucky to generate LDL-R/Cav-1 double null mice (LDL-R−/− Cav-1−/− mice). At 8 weeks of age, mice were placed on a standardized diet containing 20% calories from fat (Dyets Inc., Bethlehem, PA). After 2 weeks, mice were injected intraperitoneally with PCB77 (170 μmol/kg body weight) or vehicle (olive oil) and then 6 days later they were injected again. This dose of PCB77 was previously sufficient to induce aortic adhesion molecule expression *in vivo* [18, 180], as well as the development of atherosclerotic lesions over the course of 6 weeks [155]. 24 h after the last treatment, mouse tissues were harvested.

**Cav-1 small interfering RNA (siRNA) and transfection**

Cav-1 protein levels in endothelial cells were silenced using siRNA technique as described previously [163]. Briefly, the cells were transfected with a mixture of two siRNAs targeted against Cav-1 (40 nM each) or control siRNA (80 nM), synthetized by Dharmaco (Lafayette, CO) according to previously published sequences [283], and by using GeneSilencer transfection reagent (Genlantis, San Diego, CA) in OptiMEM media (Invitrogen). Subsequently, cells were treated with vehicle (DMSO) or PCB77.

**Real-time PCR (RT-PCR)**

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Mouse aortas were cleaned of peri-adventitial tissue and stored in RNAlater RNA stabilizing reagent (Qiagen, Valencia, CA) at −80°C. Total mRNA was purified using the RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer’s instructions. Reverse transcription was performed using the AMV reverse transcription system (Promega, Madison, WI). The levels of mRNAs expression were then assessed by RT-PCR using 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) and SYBR Green (in endothelial cells) or TaqMan (in tissues) master mix (Applied Biosystems). MCP-1 or IL-6 mRNA levels were divided by β-actin (internal control). β-Actin and MCP-1 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: sense, 5′-TCATCACCACCGAGACAG-3′; antisense, 5′-TTCTGTGGATGTCACGTCG-3′; MCP-1 sequences: 5′-CGGCTGATGCTACAGAGATT-3′; antisense, 5′-GCTTGGTCTGCAAGATCT-3′. For TaqMan reactions, TaqMan gene expression assays (Applied Biosystems) were used.

**MCP-1 and IL-6 protein level**

Cell culture media were harvested on ice and centrifuged at 2000 × g for 10 min at 4°C. The supernatants were collected and MCP-1 protein levels were measured using Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Mouse blood was collected in tubes containing EDTA, centrifuged at 1,500 g for 20 min at 4°C, and plasma was removed and stored at −80°C. Plasma levels of MCP-1 and IL-6 were measured using Mouse Adipokine LINCOplex kit (Millipore, St. Charles, MO) according to the manufacturer’s instructions. Luminex 100 (Luminex Corporation, Austin, TX) and Multiplex Data Analysis Software 1.0 (Upstate USA, Inc., Chicago, IL) were utilized for signal detection and data analysis, respectively.

**Cell viability**

Cell viability was evaluated using the commercially available MTS test (Sigma-Aldrich). Cells were plated into 96-well culture plates and treated with vehicle (DMSO) or increasing concentrations of PCB77 for 24 h. The MTS test was performed according to the manufacturer’s instructions. Absorbance at 490 nm was measured using a SpectraMax M2 (Molecular Devices, Sunnyvale, CA).

**Statistical analysis**

Values are reported as means ± SEM of at least three independent groups. Comparisons between two treatments were made by t-test; comparisons among three or more groups by one-way or two-way analysis of variance (ANOVA) followed by post-hoc Fisher’s LSD test using SigmaStat 2.0 software (Systat Software, Point Richmond, CA). Statistical probability of p < 0.05 was considered significant.
2.4 Results

*PCB77, as well as other AhR agonists, increase MCP-1 mRNA and protein levels in endothelial cells*

MCP-1 is a chemokine involved in recruitment of monocytes from blood stream to sub-endothelial space in early stages of atherosclerosis development. In order to test whether coplanar PCB77 can increase the expression of MCP-1 in endothelial cells, cells were treated with vehicle control or PCB77 (5 µM) for varying time intervals ranging from 2-24 h. Significant increases in both mRNA expression (measured by RT-PCR) (Figure 2.1) and protein levels released into culture media (measured by ELISA) (Figure 2.2) were observed only after 24 h. In order to find out the lowest concentration of PCB77 to achieve MCP-1 up-regulation, increasing concentrations of PCB77 ranging from 1-10 µM were used. A significant increase in MCP-1 mRNA and protein expression was observed at both 5 and 10 µM concentrations of PCB77 (Figures 2.2 and 2.4). Using the MTS test, cell viability was not affected at any of these concentrations. Since 5 µM was the lowest effective concentration of PCB77, this dose was used in the subsequent inhibitor studies. This concentration also was used in our previous work on endothelial dysfunction [160], and is similar to PCB levels reported in acutely exposed populations (3.4 µM, or 1 ppm). PCB77 is an example of a coplanar PCB with dioxin-like activity; thus, we also tested other AhR ligands, such as PCB126 and TCDD, on induction of MCP-1 expression. Both coplanar PCB126 (Figure 2.5) and TCDD (Figure 2.6) significantly induced MCP-1 mRNA levels after 24 h. Toxicant concentrations used in these experiments were normalized relative to their toxic equivalency factor (TEF). In contrast to the AhR ligands, non-coplanar PCB153 (2,2',4,4',5,5'-hexachlorobiphenyl) did not affect MCP-1 expression (data not shown).

*MCP-1 up-regulation is regulated through AhR*

Many endothelial responses to coplanar PCBs are mediated through their binding to AhR [18]. In order to test the role of AhR in MCP-1 up-regulation by PCB77, the AhR antagonist α-NF was used. Cells were pre-treated with α-NF (0.01 µM), followed by
vehicle (DMSO) or PCB77 treatment. MCP-1 mRNA expression was significantly increased by PCB77, but this was prevented by α-NF pre-treatment (Figure 2.7).

**MCP-1 up-regulation is oxidative stress-dependent**

PCB77 is known to cause AhR-mediated up-regulation of CYP1A1 in endothelial cells [157] and experiments with scup microsomes demonstrated that PCB77 binding to CYP1A causes its uncoupling and increased ROS production [275]. Oxidative stress can cause MCP-1 up-regulation in vascular endothelium [284]. Here, the glutathione precursor and antioxidant NAC was used to increase antioxidant potential of endothelial cells. Cells were pre-treated with 100 μM NAC for 1 h followed by vehicle (DMSO) or PCB77 (5 μM) treatment. PCB77-induced MCP-1 mRNA levels were blocked by NAC pre-treatment (Figure 2.8).

**MCP-1 up-regulation is mediated by p38 and JNK MAPKs**

MAPKs play a role in cellular responses to environmental stress, including endothelial activation. SB203580, a selective p38 kinase inhibitor, was used to examine the role of p38 kinase in MCP-1 expression. Pre-treatment for 1 h with 5 μM SB203580 blocked PCB77-induced MCP-1 mRNA expression (Figure 2.9). Similarly, SP600125, a selective inhibitor of JNK pretreatment (1 h, 20 μM) prevented PCB77-induced MCP-1 mRNA expression (Figure 2.10). In contrast, the ERK1/2 inhibitor, PD98029, at concentrations ranging from 1-20 μM had no effect on PCB77-induced over-expression of MCP-1 (data not shown).

**Cav-1 silencing prevents MCP-1 up-regulation by PCB77 in vitro and in vivo**

To investigate the role of caveolae in MCP-1 induction by PCB77, Cav-1 was silenced using the siRNA technique. Cav-1 is the major structural protein of caveolae that is required for caveolae formation in endothelial cells [66]. PCB77 induction of MCP-1 mRNA was abolished in the cells lacking Cav-1, suggesting that intact caveolae are required for this response (Figure 2.11). To confirm these findings in vivo, atherosclerosis-prone LDL-R⁻⁻⁻⁻Cav-1⁻⁻⁻⁻ mice were compared to LDL-R⁺⁻⁻⁻Cav-1⁻⁻⁻⁻ mice. PCB77 treatment increased both aortic mRNA expression levels of MCP-1 in LDL-R⁻⁻⁻⁻
Cav-1<sup>−/−</sup> mice, as measured by RT-PCR, and plasma protein levels of MCP-1 protein, assessed by LincoPLEX kit. However, no MCP-1 induction was detected in LDL-R<sup>−/−</sup> Cav-1<sup>−/−</sup> mice (Figures 2.12 and 2.13). Taken together, Cav-1 is required for MCP-1 up-regulation by PCB77 both in vitro and in vivo. In addition to MCP-1, interleukin-6 (IL-6) also is an important mediator of acute phase response and risk factor for cardiovascular disease [27, 285]. Up-regulation of aortic IL-6 mRNA (Figure 2.14) and plasma protein (Figure 2.15) levels by PCB77 followed a similar pattern as observed with MCP-1. These data suggest that Cav-1 is a common regulator of PCB77-induced vascular inflammatory response.

2.5 Discussion

Persistent organic pollutants [144] and in particular PCBs [143], were associated with an increased risk of cardiovascular disease. In this study, we have documented for the first time that coplanar PCBs, such as PCB77, can increase transcription and secretion of MCP-1 by endothelial cells. This is likely an important step in vascular inflammatory events involved in the promotion of atherosclerosis development by coplanar PCBs [155]. For example, MCP-1-mediated recruitment of monocytes is a critical event in atherosclerotic lesion formation [45, 286].

There is evidence that AhR may play a critical regulatory role in the induction of proatherogenic inflammatory markers. For example, MCP-1 was previously reported to be induced in various tissues by other environmental toxicants, such B[a]P [277], or TCDD [278]. AhR is a nuclear receptor that, after a ligand binding, translocates into the nucleus and initiates transcription through dioxin responsive elements (DREs) in regulatory regions of AhR-responsive genes. The majority of toxic effects of coplanar PCBs are initiated by their binding to AhR [131]. Our data demonstrate that coplanar PCB77 and PCB126, as well as TCDD, induce MCP-1 mRNA expression levels, suggesting AhR to be a common mediator of this pathway. In the present study, we used the AhR antagonist α-NF [287] to test the hypothesis that MCP-1 up-regulation by coplanar PCB77 is AhR-dependent, and we were able to abolish MCP-1 up-regulation by α-NF pre-treatment. We have previously demonstrated that coplanar PCBs trigger AhR-mediated up-regulation of CYP1A1 levels and activity in endothelial cells [159]. PCB-
induced up-regulation of CYP1A1 can cause overproduction of reactive oxygen species and activation of oxidative stress-sensitive transcription factors such as nuclear factor-κB (NF-κB) [18], a possible prerequisite for MCP-1 induction. Indeed, pre-treatment with the glutathione precursor and anti-oxidant NAC prevented MCP-1 induction, thus supporting a role of oxidative stress in this process.

MAPKs mediate cellular responses to various environmental stimuli and are important signaling components upstream of transcription factors that regulate an inflammatory response. There are three best characterized MAPKs sub-families, ERK, JNK, and p38 kinases [36], and all of them can be regulated by oxidative stress [37]. JNK and p38 in particular seem to be important mediators of pro-atherogenic events [38, 288] [288], and p38 activation can lead to endothelial MCP-1 up-regulation [289, 290]. Our data demonstrated that MCP-1 up-regulation by PCB77 could be diminished by both p38 and JNK inhibition.

We have evidence that caveolae may provide a critical signaling platform in the regulation of PCB-induced endothelial cell dysfunction and that Cav-1 can be a biomarker of PCB toxicity (Lim 2008). Caveolae are membrane domains enriched in cholesterol and sphingolipids, and the major structural proteins caveolins [291]. Caveolae are highly abundant in endothelial cells [279] where they are proposed to serve as platforms to compartmentalize and selectively modulate cell signaling events. Cav-1, a 22 kDa protein [53] is required for caveolae formation in endothelial cells [66], and mice which lack Cav-1 demonstrate increased severity of atherosclerosis [72]. In the current study we used Cav-1 siRNA to test the hypothesis that functional caveolae facilitate MCP-1 up-regulation by PCB77. Indeed, we found that in the absence of Cav-1, MCP-1 up-regulation was diminished. These results were confirmed in vivo following PCB77 treatment of mice lacking the Cav-1 gene. LDL-R−/− mice were used as a background control in this study as a model of atherosclerosis because their elevated LDL fraction resembles the lipoprotein profile of hypercholesterolemic humans [282]. We found that PCB77 increased aortic mRNA expression of both MCP-1 and IL-6 in control LDL-R−/− mice, which was accompanied by increased plasma MCP-1 and IL-6 protein levels. In contrast, Cav-1+/−LDL-R−/− mice were resistant to PCB77-induced MCP-1 and IL-6 up-
regulation, suggesting the physiological importance of caveolae in PCB-induced inflammation and atherosclerosis.

Our recent study showed that AhR binds Cav-1 in endothelial cells and that deletion of the Cav-1 gene partially decreased CYP1A1 induction by coplanar PCBs [96]. We also demonstrated that the production of reactive oxygen species induced by PCB77 was diminished in the absence of Cav-1 (Lim 2008). Since AhR activation and oxidative stress were required for MCP-1 up-regulation, our data suggest that PCB77-mediated AhR binding and activation and subsequent up-regulation of MCB-1 requires functional caveolae. In a related study we also found that Cav-1 was required for intercellular adhesion molecule-1 (ICAM-1) up-regulation by B[a]P in endothelial cells and that this effect was also mediated by p38 activation [183]. In addition, Cav-1 was demonstrated to bind p38 in endothelial cells and to facilitate its phosphorylation and activation of downstream targets [61]. PCB77 can also stimulate endothelial nitric oxide synthase (eNOS)-mediated nitric oxide (NO) production. This reaction requires Cav-1-dependent phosphorylation of eNOS upstream kinases, such as Src and phosphatidylinositol-3-kinase (PI3K)/Akt [163]. NO can combine with superoxide (O$_2^-$), to produce peroxynitrite (ONOO$^-$), thus further contributing to cellular oxidative stress. These mechanisms can contribute to caveolae-mediated regulation of inflammatory pathways by environmental toxicants that are AhR agonists (Figure 2.16).

In conclusion, we have demonstrated that coplanar PCB77 can up-regulate endothelial levels of MCP-1, a critical regulator of early stages of atherosclerosis. This process is regulated by caveolin-1 and caveolae-associated signaling pathways, including induction of AhR and reactive oxygen species, as well as p38 and JNK MAP kinases. It appears that functional caveolae are important for endothelial cell dysfunction, and caveolin-1 may emerge as a critical biomarker of cardiovascular toxicity by environmental contaminants. (The data presented in Chapter One were published [292].)

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Figure 2.1. PCB77 up-regulates MCP-1 mRNA expression in a time-dependent manner.

Primary porcine pulmonary artery endothelial cells were treated with vehicle control (DMSO) or PCB77 (5 µM) for indicated time periods. MCP-1 mRNA expression levels were measure using RT-PCR. Data represent mean ± SEM of 4 independent experiments. Two-way ANOVA revealed a statistically significant interaction between time and PCB77 treatment. *Significantly different compared to vehicle control (p<0.05).
Figure 2.2. PCB77 up-regulates MCP-1 protein expression in a time-dependent manner.

Cells were treated with vehicle control (DMSO) or PCB77 (5 µM) for indicated time periods. MCP-1 protein levels in cell culture media were measured using a human MCP-1 OptiEIA ELISA Kit. Data represent mean ± SEM of 4 independent experiments. Two-way ANOVA revealed a statistically significant interaction between time and PCB77 treatment. *Significantly different compared to vehicle control (p<0.05).
Figure 2.3. PCB77 up-regulates MCP-1 mRNA levels in a concentration-dependent manner.

Cells were treated with vehicle control (DMSO) or increasing concentrations of PCB77 for 24 h. MCP-1 mRNA expression levels were measured using RT-PCR. Data represent mean ± SEM of 4 independent experiments. One-way ANOVA revealed a statistically significant treatment effect. *Significantly different compared to vehicle control (p<0.05).
Figure 2.4. PCB77 up-regulates MCP-1 protein levels in a concentration-dependent manner.

Cells were treated with vehicle control (DMSO) or increasing concentrations of PCB77 for 24 h. MCP-1 protein levels in cell culture media were measured using a human MCP-1 OptiEIA ELISA Kit. Data represent mean ± SEM of 4 independent experiments. One-way ANOVA revealed a statistically significant treatment effect. *Significantly different compared to vehicle control (p<0.05).
Figure 2.5. PCB126 up-regulates MCP-1 mRNA expression.

Cells were treated with vehicle control (DMSO) or PCB126 (50 nM) for 24 h. MCP-1 mRNA expression levels were measured using RT-PCR. Data represent mean ± SEM of 3 independent experiments. *Significantly different compared to vehicle control (p<0.05).
Figure 2.6. TCDD up-regulates MCP-1 mRNA expression.

Cells were treated with vehicle control (DMSO) or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, 0.5 nM) for 24 h. MCP-1 mRNA expression levels were measured using RT-PCR. Data represent mean ± SEM of 3 independent experiments. *Significantly different compared to vehicle control (p<0.05).
Figure 2.7. Endothelial MCP-1 up-regulation by PCB77 is AhR-dependent.

Cells were pre-treated with the AhR antagonist α-naphthoflavone (NF), 0.01 µM for 1 h, followed by a vehicle control (DMSO) or PCB77 (5 µM) treatment for 24 h. MCP-1 mRNA expression levels were measured using RT-PCR. Data represent mean ± SEM of 4 independent experiments. Two-way ANOVA revealed a statistically significant interaction between NF and PCB77. *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control without NF (p<0.05).
Figure 2.8. Endothelial MCP-1 up-regulation by PCB77 is reactive oxygen species-dependent.

Cells were pre-treated with the glutathione precursor N-acetyl cysteine (NAC, 0.1 mM) for 1 h, followed by a vehicle control (DMSO) or PCB77 (5 µM) treatment for 24 h. MCP-1 mRNA expression levels were measured using RT-PCR. Data represent mean ± SEM of 4 independent experiments. Two-way ANOVA revealed a statistically significant interaction between NAC and PCB77. *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control without NAC (p<0.05).
Figure 2.9. MCP-1 up-regulation by PCB77 is regulated by p38 MAPK.

Cells were pre-treated with p38 inhibitor (SB203580, 5 µM) for 30 min, followed by vehicle control (DMSO) or PCB77 (5 µM) treatment for 24 h. MCP-1 mRNA expression levels were measured using RT-PCR. Data represent mean ± SEM of 4 independent experiments. Two-way ANOVA revealed a statistically significant interaction between SB203580 and PCB77. *Significantly different compared to vehicle control (p<0.05).

#Significantly different compared to PCB77-treated control without SB203580 (p<0.05).
Figure 2.10. MCP-1 up-regulation by PCB77 is regulated by JNK MAPK.

Cells were pre-treated with JNK inhibitor (SP600125, 20 µM) for 30 min, followed by vehicle (DMSO) or PCB77 (5 µM) treatment for 24 h. MCP-1 mRNA expression levels were measured using RT-PCR. Data represent mean ± SEM of 4 independent experiments. Two-way ANOVA revealed a statistically significant interaction between SP600125 and PCB77. *Significantly different compared to vehicle control (p<0.05).

#Significantly different compared to PCB77-treated control without SP600125 (p<0.05).
Figure 2.11. Cav-1 silencing prevents MCP-1 mRNA up-regulation by PCB77 in endothelial cells.

Cells were transfected with control or Cav-1 siRNAs for 48 h, and treated with vehicle control (DMSO) or PCB77 (5 µM) for 24 h. MCP-1 mRNA expression levels were measured using RT-PCR. Data represent mean ± SEM of 4 independent experiments. Two-way ANOVA revealed a statistically significant interaction between the presence of Cav-1 and PCB77. *Significantly different compared to vehicle control (p<0.05).
#Significantly different compared to PCB77-treated control cells (p<0.05).
Figure 2.12. Cav-1 knockout prevents MCP-1 mRNA up-regulation by PCB77 in mouse aorta.

LDL-R−/−/Cav-1+/+ (control) and LDL-R−/−/Cav-1−/− (Cav-1 deficient) mice were treated with vehicle control (olive oil) or PCB77 (170 µmole/kg). Aortic mRNA was isolated and MCP-1 mRNA expression levels were measured using RT-PCR. Data represent the mean ± SEM of 6 animals. Two-way ANOVA revealed a statistically significant interaction between the presence of Cav-1 and PCB77. *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control mice (p<0.05).
Figure 2.13. Cav-1 knockout prevents MCP-1 protein up-regulation by PCB77 in mouse plasma.

LDL-R−/−/Cav-1+/+ (control) and LDL-R−/−/Cav-1−/−(Cav-1-deficient) mice were treated with vehicle control (olive oil) or PCB77 (170 µmole/kg). Plasma samples were analyzed for MCP-1 levels using mouse adipokine LINCOplex kit. Data represent the mean ± SEM of 6 animals. Two-way ANOVA revealed a statistically significant interaction between the presence of Cav-1 and PCB77. *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control mice (p<0.05).
Figure 2.14. Cav-1 knockout prevents IL-6 mRNA up-regulation by PCB77 in mouse aorta.

LDL-R-/-/Cav-1+/+ (control) and LDL-R-/-/Cav-1-/- (Cav-1-deficient) mice were treated with vehicle control (olive oil) or PCB77 (170 µmole/kg). Aortic mRNA was isolated and IL-6 mRNA expression levels were measured using RT-PCR. Data represent the mean ± SEM of 6 animals. Two-way ANOVA revealed a statistically significant interaction between the presence of Cav-1 and PCB77. * Significantly different compared to vehicle control (p<0.05). # Significantly different compared to PCB77-treated control mice (p<0.05).
Figure 2.15. Cav-1 knockout prevents IL-6 protein up-regulation by PCB77 in mouse plasma.

LDL-R<sup>−/−</sup>/Cav-1<sup>+/+</sup> (control) and LDL-R<sup>−/−</sup>/Cav-1<sup>−/−</sup> (Cav-1-deficient) mice were treated with vehicle control (olive oil) or PCB77 (170 µmole/kg). Plasma samples were analyzed for IL-6 levels using mouse adipokine LINCOplex kit. Data represent the mean ± SEM of 6 animals. Two-way ANOVA revealed a statistically significant interaction between the presence of Cav-1 and PCB77. *Significantly different compared to vehicle control (p<0.05). †Significantly different compared to PCB77-treated control mice (p<0.05).
Figure 2.16. Caveolae-mediated up-regulation of MCP-1 and IL-6 by PCB77 in endothelial cells.

Interaction of PCB77 with endothelial caveolae results in stimulation of eNOS with subsequent production of peroxynitrite (ONOO⁻), and increased AhR transcriptional activity resulting in superoxide (O₂⁻) formation. This leads to an activation of oxidative stress-sensitive kinases (JNK and p38) and transcription factors (AP-1 and NF-κB) with subsequent up-regulation of MCP-1 and IL-6 expression. (Additional information obtained from [96, 163])
Chapter three: Omega-3 fatty acid oxidation products prevent vascular endothelial cell activation by coplanar polychlorinated biphenyls

3.1 Synopsis

Coplanar polychlorinated biphenyls (PCBs) may facilitate development of atherosclerosis by stimulating pro-inflammatory pathways in the vascular endothelium. Nutrition, including fish oil-derived long-chain omega-3 fatty acids, such as docosahexaenoic acid (DHA, 22:6ω-3), can reduce inflammation and thus the risk of atherosclerosis. We tested the hypothesis that cyclopentenone metabolites produced by oxidation of DHA can protect against PCB-induced endothelial cell dysfunction. Oxidized DHA (oxDHA) was prepared by incubation of the fatty acid with the free radical generator 2,2-azo-bis(2-amidinopropane) dihydrochloride (AAPH). Cellular pretreatment with oxDHA prevented production of superoxide induced by PCB77, and subsequent activation of nuclear factor-κB (NF-κB). A4/J4-neuroprostanes (NPs) were identified and quantitated using high performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS). Levels of these NPs were markedly increased after DHA oxidation with AAPH. The protective actions of oxDHA were reversed by treatment with NaBH4, which concurrently abrogated A4/J4-NP formation. Up-regulation of monocyte chemoattractant protein-1 (MCP-1) by PCB77 was markedly reduced by oxDHA, but not by unoxidized DHA. These protective effects were proportional to the abundance of A4/J4 NPs in the oxidized DHA sample. Treatment of cells with oxidized eicosapentaenoic acid (EPA, 20:5ω-3) also reduced MCP-1 expression, but less than oxDHA. Treatment with DHA-derived cyclopentenones also increased DNA binding of NF-E2-related factor-2 (Nrf2) and downstream expression of NAD(P)H:quinone oxidoreductase (NQO1), similarly to the Nrf-2 activator sulforaphane. Furthermore, sulforaphane prevented PCB77-induced MCP-1 expression, suggesting that activation of Nrf-2 mediates the observed protection against PCB77 toxicity. Our data implicate A4/J4-NPs as mediators of omega-3 fatty acid-mediated protection against the endothelial toxicity of coplanar PCBs.
3.2 Introduction

Chronic exposure to persistent organic pollutants, such as PCBs, contributes to the development of cardiovascular diseases in humans [293]. It has been well established that inflammation is an important mechanism contributing to the pathology of atherosclerosis, an underlying cause in the majority of cardiovascular deaths [10]. Coplanar PCBs can exacerbate early development of atherosclerosis by increasing production of inflammatory mediators, such as MCP-1, in the vascular endothelium [18, 292].

Changing the composition of dietary lipids is a promising strategy to prevent negative outcomes of exposure to environmental chemicals [158]. There is a substantial number of epidemiological studies demonstrating that fish-derived omega-3 polyunsaturated fatty acids (PUFAs) can reduce cardiovascular morbidity and mortality [294, 295]. DHA, and EPA are the major components of fish oil, and their anti-inflammatory properties contribute to the cardioprotective effects of fish oil [296].

Long-chain PUFAs in the body are subject to free radical-initiated oxidation, leading to the production of prostaglandin-like compounds called isoprostanes (IsoPs)[242]. This reaction proceeds through the formation of an unstable endoperoxide intermediate, which can then be reduced to generate IsoPs containing F-type prostane rings (F-IsoPs) [240]. Alternatively these intermediates can undergo isomerization to form molecules with E-type and D-type prostane rings (E/D-IsoPs) [297]. E/D-IsoPs are subsequently dehydrated resulting in A-type and J-type compounds (A/J-IsoPs) [239]. Oxidation of DHA specifically leads to formation of neuroprostanes (NPs) which are IsoP-like compounds found commonly in DHA-rich tissues, in particular brain [240, 247]. A_{4}/J_{4}-NPs are cyclopentenone metabolites of DHA, that contain electrophilic α,β-unsaturated carbonyl moieties, which allow them to form Michael adducts with nucleophiles, including thiol groups in signaling proteins [247]. As a result, they can inhibit inflammatory responses, for example by binding to IκB kinase β (IKKβ), thus inhibiting transcription factor nuclear factor-κB (NF-κB) [247].
Reactive oxygen species (ROSs) are critical mediators of PCB-induced endothelial inflammation [160]. Redox imbalance leads to activation of oxidative stress-sensitive kinases and transcription factors, including NF-κB, and increased production of inflammatory cytokines and adhesion molecules [298]. Nrf2 plays a major role in cellular response to oxidative stress by binding to its cognate antioxidant response element (ARE) in promoters of genes encoding cytoprotective proteins, including enzymes involved in replenishing cellular anti-oxidants, enzymes facilitating xenobiotic detoxification, and other pro-survival genes, for example proteasome sub-units [252]. NAD(P)H:quinone oxidoreductase (NQO1) is a Nrf2-regulated enzyme that maintains levels of lipid-soluble antioxidants and also detoxifies toxic quinones [268]. Nrf2 is present in aortic endothelial cells, where its activation inhibits inflammatory signaling [269]. Several naturally occurring chemoprotective compounds can activate Nrf-2 and stimulate antioxidant responses [299]. Interestingly, DHA-derived cyclopentenones increased Nrf-2 transcriptional activity by direct binding to sulfhydryl groups on Keap1, a negative regulator of Nrf2 [248]. In this report we test the hypothesis that cyclopentenone products of DHA oxidation prevent PCB toxicity in endothelial cells by activation of antioxidant responses and inhibition of PCB-induced oxidative stress.

3.3 Materials and Methods

Materials and chemicals

PCB77 was a generous gift from Dr. Larry W. Robertson, University of Iowa, Iowa City, IA. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DHA and EPA (>99% pure by gas-liquid chromatography) were obtained from Nu-Chek Prep (Elysian, MN). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA), and all other chemicals from Sigma-Aldrich Corporation (St. Louis, MO), unless otherwise specified.

Cell culture

Primary endothelial cells were isolated from porcine pulmonary arteries as described previously [281]. The basic culture media consisted of medium 199 (M199) containing 10% fetal bovine serum (FBS). At confluence, cells were incubated overnight
with treatment media, followed by an exposure to tested compounds in treatment media (M199 with 0.5% FBS for parent fatty acids, and M199 with 5% FBS for oxidized fatty acids, respectively). PCB77 was solubilized in dimethyl sulfoxide (DMSO), and subsequently diluted in cell culture media to 5 μM. Similar PCB levels were found in human serum after acute exposure to PCBs [119, 300, 301].

**Fatty acid treatments**

Fatty acids were diluted in EtOH (50 mg/ml), aliquoted, and stored at −80°C. Treatment with parent fatty acids was performed as described previously [302]. Briefly, the ethanol was evaporated with nitrogen gas, and the fatty acids were diluted to 1 mM in M199 cell culture medium containing 33 mg/ml of fatty acid-free bovine serum albumin (BSA) to achieve a molar fatty acid to BSA ratio of 2:1. This solution was incubated for 2 h at 37°C to allow binding of the FA to BSA, and then further diluted in treatment media to final treatment concentrations. For the experiments with oxidized fatty acids, ethanol stock solutions were diluted to 1 mM in phosphate-buffered saline (PBS), containing 2 mM of a free radical generator free radical generator AAPH (Cayman Chemical, Ann Arbor, MI), a method that was reported to produce cyclopentenone IsoPs [247]. The solutions were incubated at 37°C for 16 h, unless otherwise indicated, diluted in treatment media, filtered, and exposed to the cells. NaBH$_4$-mediated reduction of oxDHA was performed as before [247] with minor modifications. One ml of 18% (w/w) NaBH$_4$ in water was added to 0.66 mg of previously oxidized DHA in 2 mM AAPH/PBS, vortexed, and incubated on ice for 30 min. Then, a molar excess of HCl was added to neutralize NaBH$_4$, and lipids were extracted into the chloroform phase by the addition of chloroform and methanol (a final ratio of 1:1:0.9 of chloroform:methanol:acidic aqueous phase), followed by vortexing and centrifugation for 5 min each. The lower phase was dried under nitrogen, and the lipid extracts were re-dissolved in EtOH/PBS/treatment media, and exposed to the cells.

**Assessment of superoxide (O$_2^-$) levels**

Cells were grown to confluence in 4-chamber culture slides (BD Biosciences, Bedford, MA). After treatments, the cells were rinsed 2x with Krebs-Ringer buffer
(KRB; 118 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl$_2$, 12 mM MgCl$_2$, 1 mM NaH$_2$PO$_4$, 25 mM NaHCO$_3$, and 11 mM glucose, pH = 7.4), followed by incubation with 5 µM dihydroethidium (DHE) or KRB (blank) at 37°C for 30 min. Cells were then rinsed with KRB, fixed with 10% buffered formalin, and washed with PBS. Slides were mounted with ProLong Gold Antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA) to visualize the nuclei. The slides were evaluated under an Olympus BX61W1 fluorescence microscope and the images were captured digitally using a Retiga-EXi camera and QCapture Pro 5.1.1.14 sotware (QImaging, Surrey, BC, Canada). Mean fluorescence intensity was quantified using ImageJ 1.42q (NIH, Bethesda, MD).

**Electrophoretic mobility shift assay (EMSA)**

After treatments, nuclear extracts were prepared as described previously [183]. DNA-binding activities of NF-κB and Nrf2 were assessed using LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL) and binding reactions were carried out as published before [303], with 8 µg of antibodies against p65 (NF-κB subunit) or Nrf2 used to confirm band specificity. Synthetic 5’-biotinylated complementary oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). The cognate DNA sequence for NF-κB (5’-AGTTGAGGGGACTTTCCCAGGC-3’) was described previously [163], and antioxidant response element (ARE) from porcine NQO1 promoter (5’-TAGTCACAGTGACTCGGAGTTCCAGGC-3’) was identified based on conserved ARE sequence [251].

**Analysis of A$_4$/J$_4$-NPs**

Analysis of NPs and DHA was carried out using a Shimadzu UFLC coupled with an ABI 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode. DHA and NPs were separated using a Zorbax Eclipse XDB C8 column, 5 um, 4.6 X 150 mm (Agilent Technologies, Santa Clara, CA). The mobile phase consisted of 63/37/0.5 v/v/v: water/acetonitrile/formic acid as solvent A and 50/50 v/v: acetonitrile/ IPA as solvent B. For the analysis of DHA and NPs the separation was achieved using a gradient of 100 to 0 % solvent B in 6 min and maintaining at 0 % B
for the next 9 min and equilibrated back to the initial conditions in 3 min. The flow rate was 0.5 mL/min with a column temperature of 30°C. The sample injection volume was 10 µL. The mass spectrometer was operated in the negative ESI mode with optimal ion source settings with a declustering potential of -80 V, entrance potential of -10 V, collision energy of -14 V, collision cell exit potential of -11 V, curtain gas of 20 psi, ion spray voltage of -4500 V, ion source gas1/gas2 of 40 psi and temperature of 550°C. MRM transitions monitored were as follows: NPs - m/z 357/339, m/z 357/295, m/z 357/313, m/z 357/161, m/z 357/175, and DHA – m/z 327/283, m/z 327/229, m/z 327/177, m/z 327/191, m/z 327/249. D4- isoprostane was used as an internal recovery standard and a surrogate calibrator to quantitate NPs and DHA. The MRM transitions used to quantitate d4-isoprostane were m/z 333.5/314.6, m/z 333.5/296.6.

**Measurement of MCP-1 mRNA and protein levels**

MCP-1 mRNA expression was assessed using real-time PCR (RT-PCR), and MCP-1 protein levels were measured in cell culture media using Quantikine ELISA kit (R&D Systems, Minneapolis, MN) as described previously [292].

**Measurements of thiobarbituric acid reactive substances (TBARS)**

TBARS formation in oxidized fatty acid solutions before cell treatments was assessed using the TBARS Assay Kit (Cayman Chemical) according to manufacturer’s instruction.

**Western blotting**

NQO1 protein levels were assessed by Western Blotting as described previously [304]. Rabbit polyclonal anti NQO1 antibody was incubated overnight at 4 °C in blocking buffer (5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20).

**Statistical analysis**

Values are reported as means ± SEM obtained from of at least three independent experiments. Comparisons were made by one-way or two-way analysis of variance (ANOVA), followed by post-hoc Fisher’s least significant difference (LSD) test, using
SigmaStat 2.0 software (Systat Software, Point Richmond, CA). Statistical probability of p < 0.05 was considered significant.

3.4 Results

Oxidized DHA prevents up-regulation of superoxide by PCB77 in cultured endothelial cells.

Oxidative stress is a key component of endothelial activation by coplanar PCBs [160]. Cyclopentenone metabolites of omega-3 PUFAs can activate antioxidant defenses in the cell [248], which could provide a protection from PCB toxicity. To test this hypothesis, endothelial cells were pretreated with oxDHA, produced by free radical-initiated oxidation with AAPH, followed by exposure to vehicle or PCB77. Production of superoxide was assessed using DHE, a cell-permeable compound that can be oxidized by \( \text{O}_2^- \) into a fluorescent product [305]. Antimycin A, a mitochondrial electron transport inhibitor [306], was used as a positive control. \( \text{O}_2^- \) levels were assessed by fluorescent microscopy (Figure 3.1A) and fluorescence intensity was quantified by ImageJ 1.42q software (Figure 3.1B). Under these experimental conditions, PCB77 increased superoxide production and pre-treatment with oxDHA prevented this, demonstrating that oxDHA can protect endothelial cells from PCB-induced oxidative stress.

Activation of NF-\( \kappa \)B by PCB77 can be inhibited by oxidized DHA

ROS production is known to enhance nuclear translocation and transcriptional activity of NF-\( \kappa \)B [298] a central regulator of endothelial inflammatory responses [307]. DNA-binding activity of NF-\( \kappa \)B after exposure to PCB77 was measured by EMSA. Tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) was used as a positive control, and supershift with antibody against NF-\( \kappa \)B subunit p65 confirmed the band identity. In agreement with our previous studies [163], PCB77 increased NF-\( \kappa \)B activity after 6 h. Pre-treatment with oxDHA completely abolished NF-\( \kappa \)B activation (Figure 3.2), a likely result of a decreased inflammatory ROS production in the vascular endothelium to PCB77 after exposure to oxDHA.
Oxidation of DHA in vitro leads to the production of A₄/J₄-NPs

Cyclopentenone metabolites of DHA, i.e. A₄/J₄-NPs, are uniquely active due to their ability to interact with sulfhydryl groups of signaling proteins [247, 248]. We examined our oxDHA preparations, generated by treatment with AAPH, for the presence of A₄/J₄-NPs by infusion mode tandem mass spectrometry. We identified an ion with the predicted m/z ratio for the M-H- ion of J₄/A₄ NPs (m/z 357) in these preparations. A product ion spectrum obtained after collisional dissociation of this m/z 357 species correlated well with that reported previously for J₄/A₄-NP [239] (Figure 3.3). Although the unavailability of synthetic standards for these molecules makes unambiguous assignment of the product ion spectrum challenging as observed by [239], some of the abundant product ions derived from the m/z 357 species, for example m/z 339 ([M − H] − H₂O)⁻, m/z 313 ([M − H] − CO₂)⁻, and m/z 295 ([M − H] − H₂O − CO₂)⁻, represent commonly observed transitions for this category of oxidized fatty acids. We used several of these precursor product ion pairs to establish selective reaction monitoring mode HPLC ESI MS/MS methods for quantitation of A₄/J₄-NPs in our oxDHA samples. We determined that, as also observed previously, untreated DHA contains low levels of A₄/J₄-NPs (presumably the result of auto oxidation) but levels of these compounds were increased markedly by AAPH-initiated oxidation (Figure 3.4), and abrogated after reduction with NaBH₄ (Figure 3.4). These data demonstrate that oxDHA contains substantially elevated levels of A₄/J₄-NPs, which can be reduced using NaBH₄.

Oxidation of omega-3 PUFAs is associated with the protection from PCB toxicity

The capacity of parent DHA and EPA to prevent PCB-induced inflammation was tested by measuring the expression levels of MCP-1, a cytokine mediator of monocyte recruitment into endothelium in early stages of atherosclerosis [307]. DHA and EPA were delivered using fatty acid-free BSA as a vehicle. After pre-treatment with control or fatty acids, cells were treated with PCB77, and mRNA levels of MCP-1 were assessed using RT-PCR. Neither DHA, nor EPA, had any effect on MCP-1 up-regulation by PCB77 (data not shown). By contrast, oxDHA decreased up-regulation of MCP-1 by PCB77 in a dose-dependent manner, with 40 µM being the most effective concentration (Figure 3.5). In order to further demonstrate that only the oxidized metabolites are
protective, the same concentrations of parent DHA were oxidized for increasing periods of time before treatments. The levels of oxidation are expressed as malondialdehyde (MDA) equivalents using TBARS assay and increased gradually over 24 hours (dashed line in Figure 3.6). There was a direct correlation between the level of DHA oxidation measured using this assay and the effectiveness of preventing MCP-1 up-regulation by PCB77 (Figure 3.6), supporting the hypothesis that DHA oxidation can result in production of anti-inflammatory compounds.

**Cyclopenenone metabolites are likely the anti-inflammatory component of oxDHA**

To test the hypothesis that A₄/J₄-NPs are the active compounds that prevent PCB toxicity, oxDHA was subjected to chemical reduction with NaBH₄ in order to reduce the carbonyl moiety on the cyclopentenone ring to a non-reactive alcohol [247, 308]. Residual NaBH₄ was removed by using chloroform-methanol extraction. This procedure decreased the concentration of A₄/J₄-NPs to the baseline (Figure 3.4). As presented in Figure 3.7, oxDHA prevented MCP-1 up-regulation by PCB77, but after reduction with NaBH₄ (red DHA), it had no effect. This demonstrates that NaBH₄-sensitive components of our oxDHA preparations which clearly include A₄/J₄-NPs are responsible for prevention of the PCB-induced inflammatory response.

**Oxidized DHA is more protective than oxidized EPA (oxEPA)**

The relative potency of omega-3 PUFAs in cardiovascular prevention varies [309, 310]. Free radical-induced oxidation of both DHA and EPA yields cyclopentenone metabolites that can exert anti-inflammatory responses [247, 311]. Since DHA is more susceptible to oxidation due to higher number of double bonds [312], we tested the hypothesis that oxDHA is more protective than oxEPA. DHA and EPA were oxidized with 2 mM AAPH for 16 h. Under these conditions, DHA was oxidized to a greater extent than EPA measured using the TBARS assay (1.73±0.02 µM MDA per 40 µM of DHA) than EPA (1.22±0.02 µM MDA per 40 µM of EPA), which is consistent with previous reports [312]. Correspondingly, oxEPA significantly inhibited PCB-mediated up-regulation of MCP-1 mRNA (Figure 3.8) and protein (Figure 3.9), but oxDHA had a more pronounced inhibitory effect (Figures 3.8 and 3.9). This suggests that oxDHA is
more protective than oxEPA against PCB toxicity which likely relates to its greater susceptibility to free radical-initiated oxidation and IsoP formation.

**Nrf2 activation plays a role in protection against PCB77 toxicity**

Nrf2 is a key regulator of antioxidant defenses in cells [252], and an important anti-inflammatory mediator in vascular endothelium [269]. Previous studies suggested that A₄/J₄-NPs can activate Nrf2 by binding to sulfhydryl groups in Keap1 [248]. Since coplanar PCBs cause endothelial dysfunction by increasing oxidative stress, an increase in Nrf2 transcriptional could prevent PCB toxicity. Nrf2 activity was assessed by EMSA using DNA sequence corresponding to Nrf2-binding site in the promoter of NQO1, a Nrf2-responsive antioxidant enzyme [268]. Nrf2 activity was significantly induced by cell exposure to oxDHA (Figure 3.10). OxEPA tended to increase Nrf2 activity, but this increase was not significant. The Nrf2 data corresponded to a lesser degree of oxidation of EPA compared with DHA (1.18±0.03 µM MDA per 40 µM of EPA versus 1.93±0.07 µM MDA for DHA, respectively). Nrf2 stimulation by oxDHA was comparable to that observed in cells treated with sulforaphane (Figure 3.10), a dietary isothiocyanate known to stimulate Nrf2 transcriptional activity [255]. The ability of oxDHA to increase protein levels of NQO1 was tested by Western Blot (Figure 3.11). NQO1 was up-regulated markedly by oxDHA, and similar but less pronounced effects were obtained by exposure to sulforaphane (Figure 3.11). In order to confirm that up-regulation of Nrf2 activity can lead to protection against PCB toxicity, the ability of sulforaphane to prevent MCP-1 up-regulation by PCB77 was tested. Cells were pretreated with sulforaphane, followed by exposure to PCB77. MCP-1 mRNA expression induced by PCB77 was prevented by sulforaphane treatment (Figure 3.12), suggesting that induction of Nrf2 can protect against PCB-mediated endothelial cell activation.

### 3.5 Discussion

Humans are constantly exposed to complex mixtures of environmental chemicals with potentially deleterious effects. Diet modifications are viable means for preventing adverse outcomes of these exposures. This current work demonstrates that oxidized metabolites of long chain omega-3 polyunsaturated fatty acids, and in particular
cyclopentenone NPs formed by free radical-initiated oxidation of DHA, can prevent endothelial dysfunction induced by coplanar PCBs. PCBs are ubiquitous environmental pollutants, and significant levels are present in human tissues [313]. Furthermore, increased exposure to PCBs can contribute to cardiovascular mortality [143, 314], and coplanar PCBs facilitate atherosclerotic lesion formation in vivo [155]. Increased endothelial expression of adhesion molecules and cytokines, such as MCP-1, augments monocyte recruitment in early stages of atherosclerosis, and contributes to plaque formation [44]. We have previously demonstrated that coplanar PCBs can induce MCP-1 expression in endothelial cells and that this effect is mediated via AhR [292].

Oxidative stress is a central mediator of PCB-induced endothelial dysfunction. Coplanar PCBs increase ROS production by up-regulation and uncoupling of cytochrome P450 monooxygenases [275] and/or activation of endothelial nitric oxide synthase [163], while non-coplanar PCBs can affect endothelial NADPH oxidase [166]. PCB-induced ROS can activate the transcription factor NF-κB [18, 315], an integral mediator of inflammatory responses in the vascular endothelium [316]. In particular, ROS stimulate IKKβ-independent phosphorylation of IkB and nuclear translocation NF-κB [298], resulting in an increased production of inflammatory mediators, including MCP-1 [307]. Coplanar PCBs and other dioxin-like chemicals up-regulate MCP-1 release by endothelial cells, thus contributing to monocyte recruitment and plaque formation [292].

Diets rich in fish oil-derived omega-3 PUFAs are associated with lower rates of cardiovascular mortality [295], partially due to the anti-inflammatory properties of DHA and EPA [296]. Dietary intervention with omega-3 PUFAs also leads to reduced oxidative stress in vivo [317]. Recent evidence suggests that metabolites of omega-3 PUFAs contribute to the inhibition of an inflammatory response [247, 318]. Most importantly, free radical-mediated oxidation of DHA and EPA in tissues has been reported to result in production of biologically active IsoPs [247, 317]. To test the hypothesis that omega-3 PUFAs-derived IsoPs can alleviate PCB toxicity in endothelial cells, oxidized metabolites were produced by incubation of fatty acids with the free radical generator AAPH. Since increased ROS production is required for endothelial toxicity of coplanar PCBs [160], the capacity of oxDHA to prevent PCB77-induced rise
in $O_2^-$ was assessed using DHE fluorescence. Pre-treatment with oxDHA abolished PCB77-induced ROS production. The observed protection could be caused by activation of Nrf2, a transcription factor that regulates a variety of genes involved in antioxidant defenses [252]. Indeed, IsoPs produced by oxidation of omega-3 PUFAs can specifically activate Nrf2 by covalently binding its regulator protein Keap1 [248], and Nrf2 activation has recently been implicated in prevention of PCB toxicity [266].

Subsequent experiments from this study showed that oxDHA increases Nrf2 DNA-binding activity, and also protein levels of NQO1, a Nrf2-regulated enzyme involved in the detoxification of reactive quinones and replenishing antioxidants [268]. Because PCB metabolism results in the formation of toxic quinones [267], Nrf2 activation and NQO1 induction could explain the decrease in PCB77-induced superoxide formation observed after oxDHA pre-treatment. As a result of the decreased ROS formation, oxDHA prevented PCB77-induced activation of NF-κB. It has been shown that DHA-derived A$_4$/J$_4$-NPs can induce NF-κB directly by binding to IKKβ [247]. This mechanism, however, is not likely to counteract PCB toxicity, because coplanar PCBs induce NF-κB through oxidative stress signaling, i.e. downstream from IKKβ [298]. Rather, Nrf2-mediated induction of antioxidant enzymes probably resulted in rapid detoxification of PCB77, and prevented the ROS build-up and NF-κB activation. Another possibility would be NF-κB inhibition by Nrf2 cross-talk [319]. In our study, a well established Nrf2 inducer, dietary isothiocyanate sulforaphane [255], also activated Nrf2 and NQO1 expression, and subsequently prevented MCP-1 up-regulation by PCB77. This suggests that Nrf2 activation by dietary compounds can prevent environmental insult caused by coplanar PCBs in the vascular endothelium.

Oxidized DHA prevented MCP-1 up-regulation by PCB77 in a dose-dependent manner. Both oxDHA and oxEPA decreased MCP-1 up-regulation, but oxDHA was more potent. This was associated with a more pronounced oxidation of DHA (assessed using the TBARS assay), potentially resulting in a higher concentration of the active metabolites. Oxidation of DHA for increasing periods of time led to larger TBARS levels, and more effective inhibition of MCP-1 up-regulation. However, parent long-chain fatty acids (DHA and EPA) had no effect on MCP-1 up-regulation by PCB77.
These data support the notion that oxidized omega-3 fatty acid metabolites are uniquely protective in endothelial cells, findings consistent with a previous report where only oxidized omega-3 PUFAs prevented cytokine production [245].

Oxidation of long-chain PUFAs leads to formation of a complex mixture of metabolites. Prostaglandin-like products of free radical-mediated oxidation include F-type IsoPs with hydroxylated cyclopentane ring, or carbonyl-containing D/E-IsoPs that subsequently get dehydrated to form cyclopentenone-containing compounds (A/J-IsoPs) [320]. Certain factors, including oxygen tension and glutathione concentrations, affect the relative levels of different IsoPs [321]. Prostaglandins and IsoPs containing cyclopentenone rings are particularly effective in reducing an inflammatory response [247]. For example, arachidonic acid-derived 15-deoxyΔ12,14-PGJ2 (15d-PGJ2) inhibited NF-κB activation [246] and MCP-1 production [322], while prostaglandins lacking a cyclopentenone group were ineffective in these studies. Also, DHA-and EPA-derived cyclopentenones specifically activated Nrf2 [248]. Omega-3 PUFAs-derived IsoPs (NPs) are present in various tissues [239]; and their concentrations increase after dietary supplementation with fish oil [317]. In order to find out whether cyclopentenone NPs are the protective metabolites of oxDHA, A4/J4-NPs in oxDHA were identified using tandem mass spectrometry approaches. A species of m/z 357, corresponding to the predicted m/z of the M-H- ion of A4/J4 NPs was detected and its product ion spectrum revealed the presence of daughter ions consistent with the behavior of A4/J4-NPs reported previously [239]. Relative levels of A4/J4-NPs increased markedly after oxidation by AAPH, consistent with the observation that parent omega-3 PUFAs were ineffective in preventing MCP-1 up-regulation. Subsequently, cyclopentenone groups were reduced using NaBH4, which resulted in loss of protection against PCB-induced MCP-1 up-regulation. Taken together, our data show for the first time that only oxidized DHA can counteract PCB toxicity and that cyclopentenone NPs are likely the major active DHA oxidation metabolites.

Our study shows that components of oxidized DHA, most likely A4/J4-NPs, can alleviate endothelial dysfunction caused by coplanar PCB77. This is likely mediated by activation of Nrf2 and and cellular antioxidant defenses, resulting in reduced ROS.
formation and decreased production of inflammatory chemokine MCP-1 (Figure 3.13). These data imply that dietary supplementation with omega-3 PUFAs, and in particular DHA, might prevent toxicity resulting from environmental exposure to PCBs.
Figure 3.1. Oxidized DHA prevents PCB77-induced superoxide production.

Oxidized DHA (oxDHA) was generated via oxidation for 16 h in 2mM AAPH. Cells were pre-treated with control or oxDHA (40 μM) for 4 h; followed by exposure to vehicle control (DMSO) or PCB77 (5 μM) for 8 h, or antimycin A (50 μM) for 6 h. Cells were then stained with DHE, and red fluorescence was assessed using microscope (A) and quantified by ImageJ 1.42q (B). Data represent mean ± SEM of 3-4 independent experiments. Two-way ANOVA revealed a statistically significant interaction between oxDHA and PCB77. *Significantly different compared to vehicle control (p<0.05).

#Significantly different compared to PCB77-treated control without oxDHA (p<0.05).
Figure 3.2. Oxidized DHA prevents activation of NF-κB.

Oxidized DHA (oxDHA) was generated via oxidation for 16 h in 2mM AAPH. Cells were pre-treated with control or oxDHA (40 μM) for 4 h, followed by exposure to control or PCB77 (5 μM) for 6 h. TNF-α (5 ng/mL) treatment for 2 h was used as a positive control. NF-κB DNA-binding was assessed by EMSA (A) and quantified by UN-SCAN-IT gel 5.1 (B). 1, control; 2, PCB77; 3, oxDHA; 4, oxDHA + PCB77; 5, TNF-α; 6, p65 supershift. Data represent mean ± SEM of 3-4 independent experiments. Two-way ANOVA revealed a statistically significant interaction between oxDHA and PCB77. *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control without oxDHA (p<0.05).
Figure 3.3. Analysis of \( \text{A}_4 / \text{J}_4 \)-NPs by HPLC-ESI-MS/MS.

Oxidized DHA sample was infused into the ion source of our instrument, a species of \( m/z \) 357 generated in negative mode ESI was subjected to CID, and daughter ions identified from \( m/z \) 50 to 400. (The analysis was performed by Dr. Manjula Sunkara and Dr. Andrew Morris, Division of Cardiovascular Medicine, The Gill Heart Institute, University of Kentucky)
Figure 3.4. A$_4$/J$_4$-NP levels in the treatment samples.

A$_4$/J$_4$-NP levels in 40 µM of controls, unoxidized DHA, AAPH-oxidized DHA (oxDHA), and NaBH$_4$-reduced DHA (redDHA) were quantitated by HPLC-ESI-MS/MS using d4-isoprostane as a surrogate calibration standard. (The analysis was performed by Dr. Manjula Sunkara and Dr. Andrew Morris, Division of Cardiovascular Medicine, The Gill Heart Institute, University of Kentucky)
Figure 3.5. Oxidized DHA prevents MCP-1 mRNA up-regulation by PCB77.

Cells were pre-treated with control or oxDHA (0 – 40 µM) for 4 h. After exposure to control or PCB77 (5 µM) for 24 hours, MCP-1 mRNA expression was assessed using RT-PCR. Data represent mean ± SEM of 3-4 independent experiments. Two-way ANOVA revealed a statistically significant interaction between oxDHA and PCB77. *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control without oxDHA (p<0.05).
Figure 3.6. Inhibition of PCB77-induced MCP-1 mRNA expression is proportional to DHA oxidation.

DHA was oxidized for increasing periods of time, and levels of malondialdehyde (MDA) were assessed using TBARS assay (dashed line). After exposure to control or PCB77 (5 µM) for 24 hours, MCP-1 mRNA expression was assessed using RT-PCR. Data represent mean ± SEM of 6 independent experiments. Two-way ANOVA revealed a statistically significant interaction between oxDH and PCB77. *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control without oxDHA (p<0.05).
Figure 3.7. Reduced DHA is ineffective in preventing MCP-1 mRNA up-regulation by PCB77.

DHA was oxidized by AAPH (oxDHA) and/or reduced using NaBH₄ (redDHA). Cells were pre-treated with respective controls, oxDHA, or redDHA for 4 h. After exposure to control or PCB77 (5 µM) for 24 hours, MCP-1 mRNA expression was assessed using RT-PCR. Data represent mean ± SEM of 4 independent experiments. Two-way ANOVA revealed a statistically significant interaction between oxDHA and PCB77. * Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control without oxDHA (p<0.05).
Figure 3.8. Inhibition of PCB77-induced MCP-1 mRNA up-regulation by oxDHA and oxEPA.

Cells were pre-exposed to control, oxidized DHA (oxDHA, 40 µM), or oxidized EPA (oxEPA, 40 µM) for 4 h, followed by exposure to control or PCB77 (5 µM) for 24 hours. MCP-1 mRNA expression was assessed using RT-PCR. Data represent mean ± SEM of 4 independent experiments. Two-way ANOVA revealed a significant interaction between oxidized fatty acids and PCB77. *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control without oxidized fatty acid (p<0.05).
Figure 3.9. Inhibition of PCB77-induced MCP-1 protein up-regulation by oxDHA and oxEPA.

Cells were pre-exposed to control, oxidized DHA (oxDHA, 40 µM), or oxidized EPA (oxEPA, 40 µM) for 4 h, followed by exposure to control or PCB77 (5 µM) for 24 hours. MCP-1 protein levels in cell culture media were assessed using ELISA. Data represent mean ± SEM of 4 independent experiments. Two-way ANOVA revealed a significant interaction between oxidized fatty acids and PCB77. *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control without oxidized fatty acid (p<0.05).
Figure 3.10. Oxidized omega-3 fatty acids up-regulate Nrf2 DNA-binding activity.

Cells were treated with control, oxidized DHA (oxDHA, 40 µM), oxidized EPA (oxEPA, 40 µM), or sulforaphane (SR, 10 µM), for 4 h. Nrf2 DNA-binding was assessed by EMSA (A) and quantified by UN-SCAN-IT gel 5.1 (B). 1, control; 2, oxDHA; 3. oxEPA; 4, SR; 5, Nrf2 supershift. Data represent mean ± SEM of 3 independent experiments. One-way ANOVA revealed a statistically significant treatment effect. *Significantly different compared to vehicle control (p<0.05).
Figure 3.11. Oxidized DHA up-regulates NQO1 protein expression.

Cells were treated with control, oxidized DHA (oxDHA, 40 µM), or sulforaphane (SR, 10 µM), for 24 h. Protein levels of NQO1 were measured by Western Blot and quantified by UN-SCAN-IT gel 5.1. Data represent mean ± SEM of 4 independent experiments. One-way ANOVA revealed a statistically significant treatment effect. *Significantly different compared to vehicle control (p<0.05).
Figure 3.12. Sulforaphane prevents PCB77-induced MCP-1 mRNA expression.

Cells were pre-treated with sulforaphane (SR, 5 µM) for 4 h, followed by exposure to control or PCB77 (5 µM) for 24 hours. MCP-1 mRNA expression was assessed using RT-PCR. Data represent mean ± SEM of 3 independent experiments. Two-way ANOVA revealed a statistically significant interaction between SR and PCB77 (C). *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control without SR (p<0.05).
A4/J4-NPs bind Keap1 in the cytoplasm thus increasing Nrf2 nuclear translocation and DNA-binding activity. This leads to induction of antioxidant defense genes, including NQO1. Antioxidant enzymes (NQO1) inhibit ROS production by PCB77, leading to inhibition of PCB77-induced NF-κB activation and expression of pro-inflammatory genes, such as MCP-1.
Chapter Four: Overall discussion

4.1. Discussion

4.1.1 Summary

The work presented in this dissertation provides new mechanistic insights into cardiovascular toxicity of coplanar polychlorinated biphenyls (PCBs), and offers a novel approach for prevention of the negative outcomes of PCB exposure using dietary omega-3 polyunsaturated fatty acids (PUFA) (Figure 4.1). Monocyte chemoattractant protein-1 (MCP-1) was identified as an important mediator released in vascular endothelium in response to coplanar PCBs, as well as other dioxin-like chemicals. This could be a key event in atherosclerosis induced by coplanar PCBs, because MCP-1 mediates monocyte transmigration leading to development of fatty streaks, an early atherosclerotic lesion. Caveolin-1, a major structural component of caveolae, is required for the process of MCP-1 up-regulation, and caveolae have a potential to be an important target for both nutritional and pharmacological intervention against PCB toxicity. Dietary modulation of the toxicity of environmental chemicals presents a safe and effective means for the prevention of chronic pathologies, such as atherosclerosis. We demonstrated that oxidized metabolites of fish oil-derived docosahexaenoic acid (C22:6ω-3, DHA), are effective in up-regulation of antioxidant responses in the cell and prevention of PCB-induced expression of inflammatory mediators.

4.1.2 Monocyte chemoattractant protein-1 up-regulation by coplanar polychlorinated biphenyls

Atherosclerosis is an underlying cause of death in cardiovascular events such as myocardial infarction and cerebral infarction, and thus contributes to the majority of deaths in the U.S. [1]. The endothelium is involved primarily in the early stages of atherosclerosis, when adhesion and transmigration of monocytes leads to their deposition and differentiation to macrophages in the sub-endothelial space. The macrophages then engulf deposited oxidized low-density lipoprotein (oxLDL) particles, leading to accumulation of foam cells and development of early atherosclerotic lesions [2]. MCP-1 is an integral mediator in this process. It is a 13 kDa C-C chemokine produced by a
variety of cell types, including endothelial cells, monocytes, fibroblasts, vascular smooth muscle cells (VSMCs), and adipocytes [323]. MCP-1 binds its cognate receptor C-C chemokine receptor 2 (CCR2) on the surface of monocytes and promotes chemotaxis [324]. In the context of atherosclerosis, MCP-1 is required for plaque formation in vivo [45]. Also, MCP-1 was found in human atherosclerotic lesions [42] and high levels of circulating MCP-1 were associated with an increased risk for coronary heart disease (CHD) [43].

Environmental contaminants, in particular persistent organic pollutants, increase the incidence of cardiovascular disease [8]. Specifically, increased exposure to PCBs can contribute to cardiovascular deaths [143, 144], as well as facilitate atherosclerosis formation in vivo [155]. Recently, several mechanisms have been proposed to elucidate this process. In this study, we have demonstrated that coplanar PCB77 increases production of MCP-1 by primary endothelial cells (Figures 2.1–2.4), and also up-regulates circulating levels of this chemokine in vivo (Figure 2.13). This MCP-1 increase is likely to facilitate the formation of atherosclerotic plaque [45].

The majority of toxic effects caused by coplanar PCBs are mediated via their specific binding to the aryl hydrocarbon receptor (AhR) and up-regulation of downstream genes containing the dioxin response element (DRE) in their promoters [325]. DRE-regulated genes range from cytochrome P450 (CYP) monoxygenases, largely involved in the first step of detoxification/metabolism of PCBs [275], to a variety of DRE-containing inflammatory genes [326]. MCP-1 up-regulation in endothelial cells could be prevented by co-treatment with the AhR antagonist α-naphthoflavone (Figure 2.7), and MCP-1 was also up-regulated by other AhR agonists, including PCB126 and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Figures 2.5 and 2.6, respectively). The effective concentrations of these compounds were selected based on their relative potency for AhR binding, i.e. proportional to 5 µM of PCB77 by their toxic equivalency factors (TEFs) [132]. MCP-1 up-regulation by these compounds was significant, however, relatively less pronounced than in the case of PCB77. It is possible that PCB77 induces higher levels of ROS by some specific mechanisms, potentially because of its activation of eNOS [163]. Also, PCB77 is metabolized more easily than the other AhR agonists used, and this can
lead to production of biologically active metabolites. A specific contribution of other, non-AhR-mediated mechanisms to MCP-1 up-regulation should be considered in further studies.

Some of the previous work supports this notion that MCP-1 up-regulation is an inflammatory response shared by AhR agonists. AhR activation by benzo[a]pyrene (B[a]P) led to MCP-1 induction and increased plaque formation in mice [277]. B[a]P is a major toxic component of fine particulate matter that is thought to contribute to an increase in cardiovascular risk caused by air pollution [327]. Endothelial dysfunction by B[a]P was described previously [183], and is also mediated by AhR binding, as well as by augmented production of reactive oxygen species (ROS) [183, 292]. Increased levels of MCP-1 were also associated with macrophage accumulation and increased atheroma formation after arsenic exposure [328]. Taken together, this evidence suggests that MCP-1 is a common pro-inflammatory response leading to atherosclerosis in response to toxic chemicals, in particular AhR agonists.

Pretreatment of endothelial cells with N-acetyl-L-cysteine (NAC), a precursor of glutathione (GSH), was effective in preventing MCP-1 up-regulation by PCB77 (Figure 2.8). This is not surprising, because a majority of the cellular responses to coplanar PCBs in endothelial cells are mediated through oxidative stress [160]. PCB77 can deplete the levels of cellular antioxidant GSH, and conversely, NAC was previously found to be protective against the activation of pro-inflammatory transcription factor activator protein-1 (AP-1) by PCB77 [162]. Coplanar PCBs probably increase oxidative stress by a combination of several mechanisms; first, they bind to AhR and up-regulate CYP levels, predominantly CYP1A1. Subsequent processing of coplanar PCBs by CYP1A1 results in its uncoupling and an increased production of ROS [275]. Furthermore, PCB77 can stimulate endothelial nitric oxide synthase (eNOS)-mediated nitric oxide (NO) production. NO subsequently combines with CYP-derived ROS, in particular superoxide (O$_2^-$), to produce peroxynitrite (ONOO$^-$), and this can lead to damage to cellular proteins [163].

Changes in a cellular redox status can be translated into signaling events by chemical modifications of protein structures. Cysteine sulfhydryl groups are particularly
susceptible to such modifications, and can result in formation of disulfide bonds [329], sulfenic acid (Cys-SOH) [330] or other substituent; thus changing secondary structures, reactivity, and signaling properties of the protein [35]. A number of proteins that play a key role in the regulation of endothelial inflammatory signaling are susceptible to regulation by this mechanism, including AP-1 and nuclear factor-κB (NF-κB) [35]. Since NF-κB is a central mediator of endothelial toxicity of PCBs [18], and it also regulates MCP-1 transcription in endothelial cells [40], NF-κB is potentially the adaptor protein needed to increase MCP-1 expression in response to PCB77-induced oxidative stress.

Here we also showed that c-Jun N-terminal kinase (JNK) is involved in up-regulation of MCP-1 by PCB77 (Figure 2.10), using a pharmacological inhibitor of JNK SP600125. JNK is activated by up-stream mitogen-activated protein kinase (MAPK) kinases MKK4 and MKK7. In turn, JNK phosphorylates c-Jun, a subunit of transcription factor AP-1, thus increasing its transcriptional activity [39]. It has been firmly established that AP-1, together with NF-κB, regulates MCP-1 gene expression in endothelial cells [33]. JNK also responds to cellular oxidative stress, because apoptosis signal-regulating kinase 1 (ASK1), a MAPK kinase kinase upstream of MEK4/7, is inhibited by thioredoxin, a redox sensor protein that is subject to thiol-disulfide exchange reactions [331]. Sustained activation of JNK under intense oxidative stress can lead to apoptosis [332] and correspondingly, PCB77 exposure resulted in apoptosis when GSH synthesis was inhibited [162]. These data support the notion that the JNK/AP-1 pathway is an important mediator of endothelial dysfunction induced by environmental pollutants, as suggested previously [183].

In addition, a pharmacological inhibitor of p38 MAPK, SB203580, abolished completely MCP-1 up-regulation by PCB77 (Figure 2.9). MAPK kinases MKK3 and MKK6 are responsible for p38 activation by phosphorylation [37]. On the other hand, MAPK phosphatase-1 (MKP-1) is a negative regulator of p38 [288]. Activity of p38 can be affected by cellular levels of oxidative stress, specifically H₂O₂ binding to MKP-1 resulting in formation of sulfenic acid and inhibition of the enzyme [333]. There are some reports demonstrating that p38 can up-regulate NF-κB transcriptional activity, which
could explain MCP-1 up-regulation through p38, but specific mechanisms are under study [40].

Taken together, the inhibitory studies demonstrate that JNK and p38 MAPK pathways are involved in the up-regulation of MCP-1 by PCB77. Both of these MAPKs respond to upstream redox-sensitive signaling proteins, and thus their activation is likely a response to PCB77-induced rise in ROS production. Downstream targets of JNK and p38 are transcription factors AP-1 and NF-κB, respectively. These pathways converge at the level of MCP-1 promoter and contribute to PCB-induced endothelial inflammation.

4.1.3 Caveolae-mediated regulation of polychlorinated biphenyl toxicity

Caveolae are membrane microdomains that play a role in cellular signaling by compartmentalization of signaling molecules and facilitating their interactions [65]. Because caveolae regulate a number of pathways significant in the etiology of human pathologies, they have been explored for potential therapeutic use; in particular in cardiovascular diseases, carcinogenesis, and muscular dystrophy [49, 57]. In the development of atherosclerosis, the overall levels of caveolin-1 (Cav-1), a major structural protein of caveolae, correlate with the development of atherosclerotic plaque [72]; and specifically the presence of Cav-1 in endothelial cells facilitates atherosclerosis [46].

Currently, the evidence shows that coplanar PCBs can increase Cav-1 levels in endothelial cells [96]. This could be a contributing factor in PCB-induced endothelial cell dysfunction and atherosclerosis, since endothelial Cav-1 increases expression of pro-inflammatory mediators and LDL transmigration [46]. Interestingly, after exposure to endothelial cells PCB77 accumulated mainly in the caveolae fraction [96]. That is conceivable, since lipophilic substances, such as PCBs, tend to associate with albumin and lipoproteins in plasma and cell culture media [106, 107], and the albumin receptor gp60 is localized to caveolae [167]. In order to decipher whether caveolae play a role in cytokine production induced by PCB77, Cav-1 protein levels in primary endothelial cells were reduced using the small inhibitory RNA (siRNA) technique. In the absence of Cav-
1, MCP-1 up-regulation by PCB77 was diminished (Figure 2.11), suggesting that Cav-1 is indeed required for PCB-induced pro-inflammatory signaling.

In order to confirm that caveolae play a role in PCB toxicity in vivo, Cav-1-deficient (Cav-1−/−) mice were utilized. Cav-1−/− mice do not form morphologically detectable caveolae, and exhibit a characteristic vascular phenotype, including aberrant NO production and pulmonary hypertension [66, 70]. In order to study pro-atherogenic endpoints induced by PCB77, LDL receptor-deficient (LDL-R−/−) mice were used as a background genotype. Inbred mouse strains do not spontaneously develop atherosclerosis, and therefore dyslipidemic genetically modified mice, in particular apolipoprotein E-deficient (Apo-E−/−) and LDL-R−/− mice, are the most commonly used in vivo models to study plaque development [282]. After treatment with PCB77 for 8 days, control mice had significantly elevated plasma levels of MCP-1 (Figure 2.13), and this was accompanied by an increase in aortic production of MCP-1 mRNA (Figure 2.12). While baseline levels of MCP-1 were not different between Cav-1−/− and control mice, Cav-1 knockout completely abolished up-regulation of MCP-1 by PCB77.

Several mechanisms should be considered in order to explain this observation. Caveolae can modulate interactions among signaling proteins, either by localization of acyl-modified proteins into liquid ordered caveolae membranes, or by their direct binding to caveolin’s caveolin scaffolding domain (CSD) [64]. In a recent study, it was demonstrated that Cav-1 can directly bind AhR [96]. Other nuclear receptors, and specifically the androgen receptor (AR) [334] and estrogen receptor [335], can interact with Cav-1 too, which unravels an interesting new mechanism in a regulation of these transcription factors. Similar to the studies with AR, AhR activation and induction of its gene targets was reduced in the absence if Cav-1 [96]. This decreased CYP1A1 expression was accompanied by a reduced ROS production by PCB77 in the endothelium, which could present a key mechanism for Cav-1 in the regulation of PCB-mediated pro-inflammatory signaling. In upport of this hypothesis, caveolae also seem to play a role in endothelial activation by B[a]P, which is also an AhR agonist. Up-regulation of the intercellular adhesion molecule-1 (ICAM-1) by B[a]P was dependent on
the presence of Cav-1 [183]. Also, similarly to the data presented here, exposure to B[a]P involved the oxidative stress-sensitive kinases p38 and JNK [183].

As mentioned previously, eNOS activity is regulated by binding to Cav-1’s CSD [73]. Exposure to PCB77 stimulated eNOS phosphorylation and increased NO production in endothelial cells [163]. This occurred as early as 5 min after PCB exposure, suggesting that it was not a transcription-mediated event. Cav-1-dependent phosphorylation of kinases upstream of eNOS, such as Src and phosphatidylinositol-3-kinase (PI3K)/Akt, was involved, and Cav-1 silencing prevented eNOS up-regulation and NO production. A similar caveolae-dependent signaling cascade was described previously, when activation of eNOS by albumin binding to the gp60 receptor was mediated by Src and PI3K/Akt [336]. PCB77-induced eNOS activation resulted in NO combining with superoxide, presumably from other sources, such as CYP1A1 uncoupling, leading to production of ONOO\(^-\) and subsequent NF-κB activation [163]. Increased levels of oxidative stress resulting from eNOS phosphorylation by PCB77 might contribute to Cav-1-dependent production of ROS and MCP-1 up-regulation.

In addition, we were able to demonstrate that PCB77 can up-regulate interleukin-6 (IL-6) in a Cav-1-dependent manner, similar to MCP-1. IL-6 is a pro-inflammatory cytokine produced by macrophages, endothelial cells, adipocytes, and VSMCs [285, 337]. Increased levels of circulating IL-6 stimulate production of acute phase mediators, such as C-reactive protein (CRP), in the liver. Circulating levels of both IL-6 [338] and CRP [339] are positively correlated with cardiovascular risk and endothelial dysfunction. CRP is not only a biomarker for heart disease, but it is actively involved in the endothelial inflammatory reaction [340]. In the present study, PCB77 increased IL-6 levels in mouse plasma, as well as aortic mRNA expression, and this was abolished in Cav-1\(^-\) mice (Figures 2.14 and 2.15). Since endothelial expression of IL-6 is under regulation of some of the same transcription factors as MCP-1, such as AP-1 and NF-κB [341], the same considerations for its caveolae-mediated regulation apply. Taken together, these data provide compelling evidence that inflammatory reactions induced by PCB77 in vivo can be regulated at the level Cav-1, potentially using dietary compounds and/or pharmaceutical approaches.
4.1.4 Oxidized DHA metabolites prevent PCB toxicity

Diets enriched in omega-3 PUFAs can provide a remarkable degree of protection from cardiovascular diseases [295]. Long-chain PUFAs in the body can undergo conversion into a wide variety of active metabolites, formed by both enzymatic [212] and non-enzymatic [238, 239] reactions. Metabolites of omega-6 PUFA arachidonic acid (AA, 20:3ω-6) have been widely studied, and are actively involved in the regulation of cardiovascular physiology, as well as in the pathology of atherosclerosis [203]. However, in the case of DHA and eicosapentaenoic acid (C20:5ω-3, EPA), the most common very long chain omega-3 PUFAs in humans, as well as the biological activities and a rate of formation of these metabolites, are far less understood. Omega-3 PUFAs can be processed enzymatically into protectins and resolvins [212], or non-enzymatically into isoprostanes (IsoP) [247]; and both of these groups show some promise in the prevention of inflammatory signaling, and potentially cardiovascular diseases. In the present study, we hypothesized that the products of free radical-initiated oxidation of DHA can prevent endothelial inflammation induced by coplanar PCB77.

We utilized omega-3 PUFAs oxidized in vitro by 2,2′-azobis-2-amidinopropane hydrochloride (AAPH), a method that was previously found to produce substantial levels of cyclopentenone IsoPs [247]. While non-enzymatic oxidation of long-chain PUFAs yields a variety of products, it was suggested that the IsoPs containing cyclopentenone rings, i.e., A/J-type IsoPs, are particularly biologically active [342]. Cyclopentenones contain electrophilic α,β-unsaturated carbonyl moieties that allow them to undergo Michael addition with cellular nucleophiles, in particular protein thiol groups; and thus cause potent changes in cell signaling and inflammatory responses [247]. Here we were able to demonstrate that AAPH-induced oxidation of DHA produces substantially increased levels of A₄/J₄-neuroprostanes (A₄/J₄-NPs), i.e., DHA-derived cyclopentenone IsoPs.

High performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) demonstrated a presence of [M - H]⁻ ion of m/z 357, corresponding to the previously described A₄/J₄-NPs. A number of products of collisional dissociation, such as m/z 339 ([M – H] - H₂O)⁻ and m/z 313 ([M – H] – CO₂)⁻, confirmed
the molecule identity, [239] (Figure 3.3). Due to an unavailability of analytical standards for A4/J4-NPs, the levels of these compounds in our preparations were estimated using a surrogate standard, d4-isoprostane. This way, we found that levels of A4/J4-NPs present in the preparations that were effective in preventing PCB toxicity were ~ 300 pM (Figure 3.4). Interestingly, these levels are significantly lower than those previously required for inhibition of NF-κB activation by lipopolysaccharide (LPS) [247].

Pre-treatment of primary endothelial cells with these levels of oxidized DHA (oxDHA) abolished completely PCB77-induced production of O2- (Figure 3.1), NF-κB binding (Figure 3.2), and MCP-1 up-regulation (Figure 3.5-3.9). Unoxidized DHA was ineffective in preventing PCB toxicity (data not shown), and the degree of the inhibition was proportional to the extent of DHA oxidation (Figure 3.6), produced by exposure of DHA to AAPH for increasing periods of time. In order to support the notion that specifically cyclopentenone A4/J4-NPs cause the protective effect, oxDHA was treated with sodium borohydride (NaBH4), an agent that can reduce the α,β-carbonyl to a non-reactive alcohol, i.e. F4-NP [247, 308]. The reduction abolished completely the protection against PCB77 toxicity (Figure 3.7), suggesting that cyclopentenones are indeed the active components.

DHA and EPA vary slightly in their biological activities, and their relative contribution to cardioprotective properties of fish oil has been studied extensively [310]. In the present study, oxidation of the same concentrations of DHA and EPA resulted in relative more protection by oxDHA (Figures 3.8-3.9). This could be caused by the fact that DHA is more susceptible to peroxidation due to a higher number of double bonds [312], an observation that was confirmed in our study by an assessment of thiobarbituric acid reactive substances (TBARS) in the oxidized fatty acid preparations. Subsequent studies, using specific products of both enzymatic and non-enzymatic peroxidation of omega-3 PUFAs, should further clarify this issue; but the current data suggest that dietary supplementation with DHA could be more beneficial than an intake of similar concentrations of EPA. This can be addressed by either dietary supplements, or consumption of specific fish species, for example Atlantic salmon and bluefin tuna are particularly good sources of DHA [220].
It is not uncommon that only oxidized omega-3 PUFAs prevent endothelial inflammation. It was observed previously that oxidized EPA, but not the unoxidized fatty acid, prevented leukocyte adhesion to the endothelium [343, 344]. Furthermore, only oxidized DHA and EPA prevented MCP-1 up-regulation by cytokines in endothelial cells [245]. In both cases, inhibition of NF-κB by these oxidized fatty acids was involved, and it is possible that the binding of cyclopentenone IsoPs to I-κB kinase β (IKKβ) [247] was responsible for this effect. Furthermore, binding these oxidized metabolites to peroxisome proliferator-activated receptor α (PPARα) was required for this protection [245, 343]. PPARs are major targets for drug-mediated prevention of cardiovascular diseases [9], and they are also anti-inflammatory [345, 346], partially due to negative cross-talk with AP-1 and NF-κB [347]. Considering a recently identified role for PPARα in the prevention of endothelial toxicity of PCB77 [157], PPARα binding by oxDHA could contribute to the prevention of PCB toxicity observed in the present study.

Oxidative stress is a key mediator of PCB-induced endothelial inflammation [162, 348]. Here we observed that oxDHA at concentrations as low as 40 µM (corresponding to 300 pM of A₄/J₄-NPs) was a potent inducer of a transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) (Figure 3.10). Inducers of Nrf2-mediated adaptive antioxidant response have been considered in chemoprevention of chronic diseases, such as atherosclerosis [269] and Nrf2 activation mediates a reduced plaque formation in protected regions of aorta induced by shear stress [349]. Nrf2 stimulation could be an explanation for the inhibition of PCB77-induced NF-κB DNA binding (Figure 3.2) and MCP-1 up-regulation (Figure 3.5) observed after oxDHA pre-treatment.

Nrf2 is involved in up-regulation of a variety of antioxidant enzymes [319]. Many of them are involved in replenishing cellular antioxidants, for example gamma-glutamylcysteine synthetase, a rate-limiting enzyme in GSH synthesis [350], and heme oxygenase-1, a cellular source of an antioxidant bilirubin [351]. Since the lack of GSH exacerbates endothelial PCB toxicity [162], Nrf2 activation could be protective. PCB metabolism results in the formation of highly reactive toxic quinones [267]. Another Nrf2 target, NAD(P)H:quinone oxidoreductase 1 (NQO1), can detoxify quinones [268], and thus potentially facilitate PCB detoxification. Since oxDHA up-regulated NQO1 protein
levels in our system (Figure 3.11), this could contribute to reduced PCB toxicity after oxDHA pre-treatment. And finally, Nrf2 up-regulates GSH S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs)[252], enzymes that are involved in phase II metabolism of PCBs and can facilitate detoxification and removal of these compounds [352].

It is therefore not surprising that up-regulation of Nrf2 DHA-binding activity (Figure 3.10) and NQO1 levels (Figure 3.11) by oxDHA resulted in decreased PCB77-induced production of ROS (Figure 3.1) and NF-κB activation (Figure 3.2), as well as MCP-1 up-regulation (Figure 3.5). In addition, we were able to reduce PCB toxicity using dietary isothiocyanate sulforaphane, a well established Nrf2 inducer [255] (Figure 3.12). This further supports the notion that Nrf2 activation prevents endothelial dysfunction induced by coplanar PCBs and dietary Nrf2 inducers have a potential for cardiovascular protection in PCB-exposed populations.

Activation of the Nrf2 pathway in response to oxidized omega-3s [248], as well as other lipids [244], was described previously. Specifically, cyclopentenone A₃/J₃-IsoPs and A₄/J₄-NPs derived by free-radical-initiated oxidation of EPA and DHA, respectively, were potent Nrf2 inducers [248]. A key structural determinant for Nrf2 activation was an unsaturated α,β-carbonyl on the cyclopentenone group that bound sulfhydryls on Keap-1, a negative regulator of Nrf2. This prevented Keap-1 mediated ubiquitination and proteosomal degradation of Nrf2, and resulted in increased transcriptional activity of Nrf2 [248]. Interestingly, we observed that cyclopentenones were required for the prevention of PCB toxicity in our study, because NaBH₄-reduced oxDHA was ineffective (Figure 3.7). This suggests that IsoPs containing a cyclopentenone ring inhibit PCB toxicity by Keap-1 binding and Nrf2 activation.

There is substantial evidence that the composition of dietary lipids affects the toxic outcomes of PCB exposures. Higher levels of dietary fat exacerbate PCB-induced oxidative stress in tissues, and linoleic acid (C18:2ω-6, LA) is particularly damaging [353]. Its metabolites produced by CYP-catalyzed metabolism, leukotoxins and their diol derivatives, accentuate these toxic effects [161]. On the other hand, a diet enriched in oleic acid (C18:1ω-9, OA) decreased endothelial expression of adhesion molecules.
induced by PCB77 in vivo [180], and omega-3 PUFA α-linolenic acid (C18:3ω-3, ALA) can prevent PCB-induced inflammation in endothelial cells [158]. There is currently a lack of information on the interaction between very long-chain omega-3 PUFAs and PCBs, and our current study shows that dietary supplementation with DHA could offer some protection by the formation of its oxidized metabolites.

It is becoming increasingly obvious that metabolites of different PUFAs have specific biological activities. For example, it was demonstrated repeatedly that omega-3 PUFA supplementation decreases whole-body levels of AA-derived F2-IsoPs, a biomarker of oxidative stress in vivo and a predictor of cardiovascular diseases [317] [354]. While dietary supplementation with omega-3s PUFAs leads to increased formation of their respective IsoPs [317], this intervention also leads to reduced oxidative stress and inflammatory responses [296]. The explanation for this could be an inhibition of NF-κB-mediated transcription by EPA- and DHA-derived cyclopentenone IsoPs [247], as well as activation of Nrf2 pathway by oxidized DHA and EPA [248]. Taken together, this evidence suggests that metabolites of various PUFAs differ in their specific biological properties and their potential for the prevention of PCB toxicity, with omega-3-derived IsoPs being uniquely protective.

4.2. Future directions

The work presented here offers intriguing new mechanisms involved in the toxicity of coplanar PCBs and identifies new targets for dietary prevention of these adverse events. However, in order to be able to present clear dietary guidelines for populations exposed to PCBs and other persistent environmental chemicals, it would be beneficial to address some of the following issues.

Data produced by our laboratory and others [277] argue that endothelial MCP-1 expression is a marker of pro-inflammatory reaction induced by AhR agonists, while MCP-1 is also a key mediator of monocyte infiltration in the formation of atheroma [42]. In a future study, it would be beneficial to explore the idea that MCP-1 up-regulation is a key event in atherosclerosis promotion by AhR agonists, such as coplanar PCBs. An assessment of PCB-induced atherosclerosis [155] in control and MCP-1-deficient mice
[45] would allow to draw such conclusion. Once it is confirmed that MCP-1 is required for PCB-induced atherosclerosis, in vitro up-regulation of MCP-1 in endothelial cells could be used as a surrogate test for screening of pro-atherogenic potential of environmental chemicals.

The data in this dissertation, as well as other studies [163], demonstrate that Cav-1 is required for PCB-induced inflammation. It was also observed that PCB77 after an exposure to endothelial cells accumulated predominantly in the caveolae fraction [96]. However, it has not been addressed directly whether Cav-1 is required for PCB uptake in the cells and whether this uptake depends on Cav-1 levels in particular tissues. Some studies suggest that other small lipophilic molecules, for example the flavonoid resveratrol, depend in their uptake on cellular Cav-1 levels [95]. Considering a wide availability of analytical techniques for PCB measurements, and also molecular biology methods for modulation of Cav-1 protein levels; it would be useful to evaluate the role of uptake in caveolae-dependent toxicity of PCBs.

Regardless, it was shown convincingly that the presence of Cav-1 correlated with PCB toxicity. Because of the many functions caveolae play in the development of vascular pathologies and other diseases [57], modulating caveolae composition and Cav-1 levels by pharmacological and dietary means has been an attractive area of research. Dietary phenolics have been particularly effective in decreasing Cav-1 levels. Data from our laboratory showed that green tea-derived (-)-epigallocatechin-3-gallate (EGCG) can down-regulate Cav-1 levels in endothelial cells [194], which was associated with reduced endothelial inflammation. Other dietary flavonoids, such as daidzein [355, 356] and quercetin [357], can also decrease Cav-1 levels in the vasculature. Interestingly, several of these polyphenolics, and specifically quercetin [357] and EGCG [159], are known to reduce PCB toxicity as well. Some of the evidence even suggests that decreased PCB77-induced expression of adhesion molecules after quercetin treatment was caused by decreased Cav-1 protein levels [357]. A targeted study using these protective flavonoids while over-expressing Cav-1 could clarify whether reduction of Cav-1 levels is indeed the protective mechanism. This would be useful in future to identify dietary compounds that decrease Cav-1 levels as effective in chemoprevention from PCB toxicity.
Here we showed that PCB77 can up-regulate endothelial expression of MCP-1 and IL-6 and this effect was Cav-1-dependent. Other cell types can express both MCP-1 and IL-6, as well as contribute to the development of atherosclerotic plaque, for example adipocytes [26], VSMCs [358], and macrophages [359]. Our data documented up-regulation of MCP-1 mRNA expression in the aorta and cultured endothelial cells, suggesting that the endothelium contributes substantially to circulating plasma levels of this cytokine. However, it would be interesting to evaluate how expression of MCP-1 and IL-6 by other cell types contribute to the circulating levels of these cytokines. PCB77 can increase MCP-1 expression in cultured 3T3-L1 adipocytes [155]. Interestingly, adipocytes [63], VSMCs [360], and most macrophage types [361] express substantial levels of Cav-1 and form caveolae. Information about potential caveolae-mediated regulation of MCP-1 and IL-6 expression in any of these tissues is lacking at this point and presents an interesting area of future research.

Cellular enrichment with long-chain omega-3 PUFAs leads to their incorporation both into the bulk of membranes as well as the caveolae/lipid raft fraction and increases their unsaturation index. This unsaturation can interfere with signaling proteins that are normally resident in rafts, such as Src kinases [362]. Dietary supplementation with omega-3 PUFAs caused Cav-1 displacement from caveolae and decreased raft cholesterol content [187]. It was therefore surprising that parent EPA and DHA did not prevent PCB77 toxicity, particularly because of the previously reported effects of these fatty acids on caveolae structure [191, 363]. Since Cav-1 was required for MCP-1 up-regulation by PCB77, it would be reasonable to expect that changes in caveolae composition caused by the incorporation of omega-3 PUFAs, including Cav-1 displacement from caveolae [191], would prevent MCP-1 up-regulation. This was not found in our studies.

A possible explanation is that while the aforementioned studies showed a displacement of Cav-1 after treatment with omega-3 PUFAs, the total protein level of Cav-1 was unaltered. Cav-1 protein has signaling properties independent of its incorporation to caveolae determined by its domains with specific functions [364]. For example a delivery of CSD peptide, in the absence of other portions of Cav-1, such as an
oligomerization domain that allows for caveolae assembly [364], induced significant changes after in vivo delivery [74]. This suggests that while the presence of Cav-1 is required for MCP-1 up-regulation by PCB77, a formation of intact caveolae might not be necessary. We identified that AhR binding to Cav-1 was probably a key event in Cav-1-dependent regulation of coplanar PCB toxicity. Further studies using deletion mutants of Cav-1 targeting specific functional domains might unravel the mechanism underlying this interaction and optimized means for a regulation of this pathway.

The ultimate goal of our studies is to identify specific nutrients that can alleviate PCB toxicity in already exposed populations. We have been able to demonstrate that IsoPs derived from omega-3 PUFAs prevent completely endothelial activation by PCB77 in vitro. The next step should be to confirm that dietary intake of EPA and DHA can prevent PCB toxicity in vivo as well, as a result of formation of these reactive metabolites.

Many of the previously published studies provided clues about the levels of various IsoPs in tissues, as well as protocols for modulating their concentrations. The levels of non-enzymatically produced F₂-IsoPs in normal human plasma and urine are ~1.5 orders of magnitude higher than those formed by cyclooxygenase (COX) enzymes [234], suggesting that the contribution of non-enzymatic pathways to the formation of bioactive metabolites is substantial. Levels of various types of IsoPs tend to be comparable; however, oxygen tension can facilitate E/D-IsoPs formation, while high tissue levels of GSH increase relative F-IsoPs concentrations [321]. A/J IsoPs are formed in vivo by dehydration of E/D-IsoPs. Levels of A₂/J₂-IsoPs in rat livers were ~5 ng/g and one-third of E₂/D₂-IsoP concentrations, demonstrating that the rate of dehydration from E/D-IsoPs to A/J-IsoPs in vivo is substantial [365]. We are specifically interested in A₄/J₄-NP, which were proven to be the protective metabolites in our study. Reported levels of A₄/J₄-NPs in human brain were ~ 98 ng/g [239]. This is approximately 1000-fold higher than the concentration we found to be effective against PCB77 toxicity (300 pM). Although brain has approximately 10 times higher DHA levels that heart tissue [366], it is still conceivable that sufficient levels of A₄/J₄-NPs are formed in the vasculature.
In vivo, the majority of fatty acids are present in an esterified form. Because unlike the enzymatic metabolism, free radical-initiated isoprostane formation does not require fatty acids to be hydrolyzed first, the majority of isoprostanes in vivo are present in their phospholipid-bound form [242]. They can be subsequently released by an action of phospholipases. Platelet-activating factor (PAF) acetylhydrolase can catalyze cleavage of F₂-IsoPs [243], while phospholipases responsible for release of other IsoPs are currently under study. Interestingly, it seems that some IsoPs can exhibit their biological activity, including Nrf2 activation, in their conjugated form; as it was demonstrated in a case of E₂-IsoP conjugated to glycerophosphocholine [244]. This again supports the notion that sufficient levels of active IsoPs are likely available in vivo.

In mouse models, coplanar PCBs increase expression of adhesion molecules [18] and also the pro-inflammatory cytokines MCP-1 (Figures 2.12 and 2.13) and IL-6 (Figures 2.14 and 2.15). Furthermore, PCB77 exposure exacerbates atherosclerosis development in Apo-E<sup>−/−</sup> mice [155]. In order to assess the protection by dietary omega-3 PUFAs, these PCB exposures could be accompanied for example by 4% menhaden fish-oil-feeding protocol, which successfully increased a formation of F₃-IsoPs and F₄-IsoPs in mouse lung tissue after 10 weeks [317]. The same amount of fish oil after 4 weeks resulted in significantly increased A₃/J₃-IsoP levels in rat liver tissue [238]. A use of this treatment regime would allow assessing A₃/J₃-IsoPs and A₄/J₄-NPs in vascular tissues and potentially demonstrating a protection against PCB toxicity. Ultimately, this effort to identify bioactive nutrients that can counteract PCB toxicity should result in epidemiological studies that allow assessment of dietary modulation of persistent organic pollutants toxicity. For example National Health and Nutrition Examination Survey (NHANES) already identifies risks associated with intake of certain pollutants [367], and it could include also information on how this risk is modified by diet.

An in vivo study also would be useful to identify a full range of DHA and EPA metabolites being formed after omega-3 PUFA supplementation. In this dissertation, we focused on cyclopentenone IsoPs; based on their previously identified biological activities. They were also substantially enriched in our oxDHA preparations (Figure 3.4), and the biological effects could be removed by NaBH₄ reduction (Figure 3.7). However
without a doubt, omega-3 PUFAs can be processed by the body into a variety of metabolites. Recently identified products of free radical-mediated peroxidation of EPA in vivo included monocyclic peroxides, serial cyclic peroxides, bicyclic endoperoxides, and dioxolane-endoperoxides, and their biological activities are largely unknown [368]. EPA is a substrate for some of the AA-processing enzymes involved in prostaglandin synthesis, and can prevent production of AA-derived eicosanoids, while forming its own unique metabolites [202]. Protectins and resolvins, hydroxylated products of CYP-mediated metabolism of DHA and EPA, have clearly anti-inflammatory properties [212], and their tissue levels can be increased by fish oil supplementation [369]. All of these compounds have a potential to modulate vascular inflammation and to interfere with toxicity of environmental contaminants and should be a subject of further studies.

4.3 Conclusions

In conclusion, we were able to demonstrate that coplanar PCBs up-regulate MCP-1. This response is mediated by AhR binding and seems to be shared by a range of dioxin-like chemicals. Because MCP-1 is an important mediator in the process of atherosclerosis development, MCP-1 up-regulation by environmental chemicals could be assayed using an in vitro model and used as an indicator of cardiovascular toxicity of these toxicants.

Cav-1 is a regulator of many pro-inflammatory pathways in endothelial cells. Here we show that it is required for PCB-induced up-regulation of pro-inflammatory cytokines. This finding could be valuable for dietary prevention of PCB toxicity, because several dietary phenolics were found to be effective in reducing Cav-1 levels in vasculature. The mechanisms involved in Cav-1-mediated regulation of PCB-induced endothelial cell dysfunction should be studied in future in greater detail. However, they likely involve modulation of AhR and eNOS activity by Cav-1, and uptake of PCBs into endothelial caveolae.

Dietary modulation of pro-inflammatory signaling involved in cardiovascular toxicity of PCBs presents a safe and effective method for improving life quality in exposed populations. In this study, we demonstrated that oxidized metabolites of DHA
are potent stimulators of antioxidant responses in endothelial cells, and can therefore alleviate toxic effects induced by PCB77. This suggests that omega-3 PUFA supplementation could help lessen the adverse outcomes of PCB exposure.
Figure 4.1. A summary figure describing toxic effects of PCB exposure with a focus on endothelial dysfunction.

PCBs in humans elicit adverse effects in various organs and tissues, including increased risk of cardiovascular diseases. A hallmark of cardiovascular toxicity of PCBs is increased production of inflammatory mediators by vascular endothelial cells. Coplanar PCB77 enters endothelial cells in caveolin-1 (Cav-1)-dependent manner and increases production of reactive oxygen species (ROS). This results in activation of oxidative stress-sensitive transcription factors and increased expression of monocyte chemoattractant protein-1 (MCP-1), a chemokine that facilitates monocyte recruitment into sub-endothelial space. Free radical-initiated oxidation of docosahexaenoic acid yields A4/J4-neuroprostanes (A4/J4-NPs) that activate nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2, an antioxidant response transcription factor decreases PCB77-induced oxidative stress. (Additional details are available in Figures 2.16 and 3.13.)
Appendices

Appendix A. Methods

1. Whole cell protein extraction and Western Blot (courtesy of Dr. Sung Gu Han)
   a. Whole cell protein extraction
      - Perform all steps on ice
      - Rinse the cell monolayer 2x with ice-cold phosphate buffered saline (PBS)
      - Remove the remaining PBS completely
      - Add 500 µL/100 mm cell culture plate of ice-cold lysis buffer containing: 1x RIPA buffer (Cell Signaling Technology #9806), 1x Halt protease inhibitor cocktail (Thermo Scientific #78429), and 1% sodium dodecyl sulfate (SDS)
      - Scrape and collect the cells in a 1.5mL eppendorf tube
      - Homogenize by pipetting up and down 20x
      - Sonicate for 10 s
      - Centrifuge at 20,000 g, 4°C, 15 min
      - Collect the supernatant, freeze using dry ice, and store at -80°C
   b. Protein concentration measurement and sample preparation
      - Assess protein concentration in the lysates using bicinchoninic acid (BCA) assay (Thermo Scientific #23225) according to manufacturer’s instructions
      - Combine cell lysate containing 50 µg of protein with an appropriate amount of 6x Laemmli buffer containing: 375 mM Tris HCl (pH 6.8), 12% (w/v) SDS, 60% (v/v) glycerol, 0.012% (w/v) bromophenol blue, and 125 mM dithiothreitol (DTT), and heat at 95°C for 5 min
   c. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
      - Load the samples on 10% polyacrylamide gel and run for 2 h at 100 V using running buffer containing: 25 mM Tris HCl (pH 8.3), 192 mM glycine, and 0.1% SDS
      - Transfer proteins onto nitrocellulose membrane with Trans-Blot Semi-Dry Transfer Cell (Bio-Rad Laboratories) for 60 min at 300 mA using transfer
buffer containing 25 mM Tris HCl (pH 8.3), 192 mM glycine, and 20% (v/v) methanol

- Confirm transfer and equal loading using Ponceau S solution (Sigma-Aldrich #P7170) according to manufacturer’s instructions

d. Western Blot

- Rinse the membrane 1x for 10 min on shaker at room temperature (RT) using tris-buffered saline with tween 20 (TBST) containing 50 mM Tris HCl (pH 7.4), 150 mM NaCl, and 0.05% (v/v) tween 20
- Block the membrane for 2 h on shaker at RT with TBST containing 5% (w/v) nonfat milk
- Rinse the membrane 1x for 10 min on shaker at RT with TBST
- Bind the primary antibody (typically 1:1000 v/v dilution) on shaker overnight at 4°C with TBST containing 5% nonfat milk
- Rinse the membrane 3x for 10 min on shaker at RT with TBST
- Bind the secondary antibody (typically 1:4000 v/v dilution) for 2 h on shaker at RT with TBST containing 5% nonfat milk
- Rinse the membrane 4x for 10 min on shaker at RT with TBST
- Visualize the target protein with autoradiography using Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare Life Sciences #RPN2109) according to manufacturer’s instructions

2. Treatment of cells with fatty acids using fatty acid-free bovine serum albumin delivery (courtesy of Dr. Kevin Fritsche)
a. Preparation of fatty acid aliquots in EtOH

- Purchase pure fatty acids from Nu-Chek Prep, Inc.
- Prepare 50 mg/mL solution of fatty acid in filtered EtOH (work quickly, because polyunsaturated fatty acids oxidize easily)
- Prepare aliquots of the 50 mg/mL solution in polypropylene conical tubes corresponding to 2 mL of 1mM solution of the fatty acid (for example 13.2228 µL for docosahexaenoic acid (DHA, 22:6ω-3))
• Add some N₂ gas, close, seal with parafilm, and keep at -80°C for no longer than 2 months

b. Preparation of bovine serum albumin (BSA)-fatty acid complexes

• Purchase fatty acid-free BSA (10% w/v in PBS) from Sigma-Aldrich (#A1595)
• Dilute 3x in serum-free cell culture media to achieve 33 mg/mL (0.5 mM) of BSA and warm it up to 37°C
• Add 2 mL of the BSA solution to the previously prepared fatty acid aliquot to achieve 1 mM concentration of fatty acid and 2:1 fatty acid to BSA ratio
• Prepare control samples containing equal concentrations of EtOH and BSA as treatment solutions
• Mix gently, add some N₂ gas, close, and seal with parafilm
• Shake at 37°C for 2 h using Amersham Hybridization Oven/Shaker (GE Healthcare Life Sciences)

c. Cellular treatment with fatty acids

• Warm up treatment media to 37°C (for primary porcine pulmonary artery endothelial cells use M199 enriched with 0.5% fetal bovine serum (FBS))
• Dilute the fatty acid-BSA complexes in the media to desired concentrations
• Filter the solutions using 0.2 µm pore size syringe filters
• Treat the cells with control samples or fatty acid solutions

3. Treatment of cells with oxidized fatty acids (adapted from Joseph Layne)

a. Preparation of fatty acid aliquots in EtOH

• As in 2a

b. Oxidation of fatty acids

• Prepare 2 mM solution of 2,2-azo-bis(2-amidinopropane) dihydrochloride (AAPH) (Sigma-Aldrich #440914) in PBS
• Add 2 mL of the AAPH solution to the previously prepared fatty acid aliquot to achieve 1 mM concentration of fatty acid and 2 mM concentration of AAPH
• Prepare control samples containing equal concentrations of EtOH and AAPH as treatment solutions
• Vortex vigorously, close, and seal with parafilm
• Shake at 37°C for 16 h using Amersham Hybridization Oven/Shaker

c. Cellular treatment with oxidized fatty acids
• Warm up treatment media to 37°C (for primary porcine pulmonary artery endothelial cells use M199 enriched with 5% FBS)
• Dilute the fatty acid/AAPH solutions in the media to desired concentrations
• Filter the solutions using 0.2 µm pore size syringe filters
• Treat the cells with control samples or oxidized fatty acid solutions

4. Measurement of superoxide (O$_2^-$) production in endothelial cells using dihydroethidium (courtesy of Dr. Sung Gu Han)
a. Cell culture and treatment
• Plate the cells in 4-chamber polystyrene culture slides (BD Biosciences #354104) and grow till confluence
• Treat as desired
b. Staining with dihydroethidium
• Prepare fresh sterile Krebs-Ringer buffer (KRB), containing: 118 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl$_2$, 12 mM MgCl$_2$, 1 mM NaH$_2$PO$_4$, 25 mM NaHCO$_3$, and 11 mM glucose, pH 7.4; and warm it up to 37°C
• Prepare 20 mM stock solution of dihydroethidium (DHE) (Sigma-Aldrich # 37291) in dimethyl sulfoxide (DMSO) in amber glass vial and store at -20°C (when handling the reagent, avoid exposure to light)
• Rinse the cells 2x with KRB
• Incubate the cells with 5 µM DHE/KRB or KRB (blank) at 37°C for 30 min
• Rinse the cells 2x with KRB
• Fix the cells with 10% buffered formalin for 10 min at RT on rotator
• Rinse the cells 1x with PBS
c. **Mounting and epifluorescence microscopy**
  • Remove the glass chambers using the removal key
  • Shake off the rest of the liquid
  • Mount with ProLong Gold Antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen # P-36931) according to manufacturer’s instructions
  • View the slides under an Olympus BX61W1 fluorescence microscope and capture the images digitally using QCapture Pro 5.1.1.14 software (QImaging)
  • Set up the shutter for 50 ms (DAPI) or 150 ms (DHE)
  • Measure mean fluorescent intensity of the images using ImageJ 1.42q (NIH, freeware)

5. **Chloroform-methanol extraction of fatty acids**
a. **Extraction of an oxidized fatty acid sample**
  • The final ratio of volumes is CHCl₃:MeOH:HCl equals 1:1:0.9 (the 0.9 of HCl is made by addition of concentrated (10 M) HCl to lipid solution)
  • Add 0.1M HCl (for example 20 µL of 10 M HCl to 2 mL of lipid solution) and mix
  • Add MeOH and CCl₃ (for example 2.2 mL of MeOH and CCl₃ each for 2 mL of lipid/HCl)
  • Vortex for 5 min
  • Centrifuge at 4,000 rpm, RT, 5 min
  • Remove the upper phase, and transfer the lower phase to a screw-cap borosilicate glass tube using a glass Pasteur pipette
  • Evaporate the solvent using N₂ gas
  • Re-suspend in a suitable solvent
b. **Extraction of a fatty acid sample reduced by NaBH₄**
   - For reduction, use 6% (1.586 M) NaBH₄ for 30 min on ice
   - To neutralize NaBH₄, first add a molar excess of HCl, i.e., 1.6666 M (0.6 mL 10 M HCl to 3 mL of lipid solution)
   - Add 4 mL of each chloroform and methanol (keep the 1:1:0.9 ratio)
   - Continue as in 5a
Figure I. Caveolin-1 knockout prevents TNF-α protein up-regulation by PCB77 in mouse plasma.

LDL-R⁻/⁻/Cav-1⁺/+ (control) and LDL-R⁻/⁻/Cav-1⁻/⁻ (caveolin-1-deficient) mice were treated with vehicle control (olive oil) or PCB77 (170 µmole/kg). Plasma samples were analyzed for TNF-α levels using mouse adipokine LINCOplex kit. Data represent the mean ± SEM of 6 animals. Two-way ANOVA revealed a statistically significant interaction between the presence of caveolin-1 and PCB77. *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control mice (p<0.05).
Figure II. Caveolin-1 knockout does not affect plasminogen activator inhibitor-1 protein up-regulation by PCB77 in mouse plasma.

LDL-R^{-/-}/Cav-1^{+/+} (control) and LDL-R^{-/-}/Cav-1^{-/-} (caveolin-1-deficient) mice were treated with vehicle control (olive oil) or PCB77 (170 µmole/kg). Plasma samples were analyzed for plasminogen activator inhibitor-1 (PAI-1) levels using mouse adipokine LINCOplex kit. Data represent the mean ± SEM of 6 animals. Two-way ANOVA did not reveal a statistically significant interaction between the presence of caveolin-1 and PCB77. *Significantly different compared to vehicle control (p<0.05).
Figure III. Caveolin-1 knockout increases resistin protein levels in mouse plasma, but PCB77 does not have any effect.

LDL-R−/−/Cav-1+/+ (control) and LDL-R−/−/Cav-1−/− (caveolin-1-deficient) mice were treated with vehicle control (olive oil) or PCB77 (170 μmole/kg). Plasma samples were analyzed for resistin levels using mouse adipokine LINCOpex kit. Data represent the mean ± SEM of 6 animals. Two-way ANOVA did not reveal a statistically significant interaction between the presence of caveolin-1 and PCB77. *Significantly different from control genotype mice (p<0.05).
Figure IV. PCB77 up-regulates leptin protein levels only in plasma of caveolin-1\(^{-/-}\) mice.

LDL-R\(^{-/-}/\)Cav-1\(^{+/+}\) (control) and LDL-R\(^{-/-}/\)Cav-1\(^{-/-}\) (caveolin-1-deficient) mice were treated with vehicle control (olive oil) or PCB77 (170 \(\mu\)mole/kg). Plasma samples were analyzed for leptin levels using mouse adipokine LINCOplex kit. Data represent the mean ± SEM of 6 animals. Two-way ANOVA did not reveal a statistically significant interaction between the presence of caveolin-1 and PCB77. *Significantly different compared to PCB77-treated control mice (p<0.05).
Figure V. Neither PCB77 nor Cav-1 affects plasma levels of adiponectin.

LDL-R^{-/-}/Cav-1^{+/+} (control) and LDL-R^{-/-}/Cav-1^{-/-} (caveolin-1-deficient) mice were treated with vehicle control (olive oil) or PCB77 (170 µmole/kg). Plasma samples were analyzed for adiponectin levels using mouse adipokine LINCOplex kit. Data represent the mean ± SEM of 6 animals. Two-way ANOVA did not reveal a statistically significant interaction between the presence of caveolin-1 and PCB77.
Figure VI. Unoxidized DHA and EPA do not affect MCP-1 mRNA up-regulation by PCB77.

DHA and EPA (40 µM each) were delivered using fatty acid-free bovine serum albumin. Cells were pre-treated for 4 h, followed by exposure to vehicle control (DMSO) or PCB77 (5 µM) for 24 hours. MCP-1 mRNA expression was assessed using RT-PCR. Data represent the mean ± SEM of 4 independent experiments. Two-way ANOVA revealed no interaction between unoxidized fatty acids and PCB77. *Significantly different compared to vehicle control (p<0.05).
Figure VII. Unoxidized DHA and EPA do not affect vascular cell adhesion molecule-1 (VCAM-1) mRNA up-regulation by PCB77.

DHA and EPA (40 µM each) were delivered using fatty acid-free bovine serum albumin. Cells were pre-treated for 4 h, followed by exposure to vehicle control (DMSO) or PCB77 (5 µM) for 24 hours. VCAM-1 mRNA expression was assessed using RT-PCR. Data represent the mean ± SEM of 4 independent experiments. Two-way ANOVA revealed no interaction between unoxidized fatty acids and PCB77. *Significantly different compared to vehicle control (p<0.05).
Figure VIII. Inhibition of PCB77-induced VCAM-1 mRNA expression is proportional to DHA oxidation.

DHA was oxidized for increasing periods of time, and levels of malondialdehyde (MDA) were assessed using TBARS assay (dashed line). After exposure to control or PCB77 (5 µM) for 24 hours, VCAM-1 mRNA expression was assessed using RT-PCR. Data represent mean ± SEM of 3 independent experiments. Two-way ANOVA revealed a statistically significant interaction between oxDHA and PCB77. *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control without oxidized DHA (p<0.05).
Figure IX. Reduced DHA is ineffective in preventing VCAM-1 mRNA up-regulation by PCB77.

DHA was oxidized by AAPH (oxDHA) and/or reduced using NaBH₄ (redDHA). Cells were pre-treated with respective controls, oxDHA, or redDHA for 4 h. After exposure to control or PCB77 (5 µM) for 24 hours, VCAM-1 mRNA expression was assessed using RT-PCR. Data represent mean ± SEM of 4 independent experiments. Two-way ANOVA revealed a statistically significant interaction between oxDHA and PCB77. *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control without oxDHA (p<0.05).
**Figure X. Sulforaphane prevents PCB77-induced VCAM-1 mRNA expression**

Cells were pre-treated with sulforaphane (5 µM) for 4 h, followed by exposure to control or PCB77 (5 µM) for 24 hours. VCAM-1 mRNA expression was assessed using RT-PCR. Data represent mean ± SEM of 3 independent experiments. Two-way ANOVA revealed an interaction between sulforaphane and PCB77. *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control without sulforaphane (p<0.05).
Figure XI. Oxidized DHA and EPA do not decrease CYP1A1 protein expression induced by PCB77.

Cells were pre-exposed to control, oxidized DHA (oxDHA, 40 µM), or oxidized EPA (oxEPA, 40 µM) for 4 h, followed by exposure to control or PCB77 (5 µM) for 24 hours. CYP1A1 protein levels in the cells were assessed using Western Blot. Data represent mean ± SEM of 4 independent experiments. Two-way ANOVA did not reveal a significant interaction between oxidized fatty acids and PCB77. *Significantly different compared to vehicle control (p<0.05). #Significantly different compared to PCB77-treated control without oxidized fatty acid (p<0.05).
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BOOK CHAPTERS:
