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## COPLANAR PCB77 AND ANGIO INDUCED VASCULAR DISORDERS

Madhura Parulkar

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Madhura Parulkar, Student

Dr. Lisa Cassis, Major Professor

Dr. Howard Glauert, Director of Graduate Studies

COPLANAR PCB77 AND ANGIO INDUCED VASCULAR DISORDERS

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THESIS

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A thesis submitted in partial fulfillment of the  
requirements for the degree of Masters of Science in the  
Graduate Center for Nutritional Sciences  
at the University of Kentucky

By  
Madhura Parulkar  
Lexington, Kentucky

Director: Dr. Lisa Cassis, Professor of Nutritional Sciences  
Lexington, Kentucky

2011

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## ABSTRACT OF THESIS

### COPLANAR PCB77 AND ANGIO II INDUCED VASCULAR DISORDERS

Previous studies demonstrated that coplanar PCBs promote inflammation by release of pro-inflammatory cytokines like TNF, MCP-1, and VCAM-1 from endothelial cells as well as adipocytes. Also these PCBs at small doses may contribute to the development of obesity by inducing adipocyte differentiation. Obesity is a known risk factor that promotes cardiovascular disorders like atherosclerosis and AAAs. Evidence shows Ang II, a component of the RAS, leads to the formation of atherosclerosis and AAAs in both normal as well as hyperlipidemic mice. Earlier studies in our laboratory have also shown that coplanar PCB-77 promotes atherosclerotic lesion formation in ApoE<sup>-/-</sup> mice. The purpose of this study was to define the effects of PCB77 on Ang II induced vascular diseases like atherosclerosis and AAAs. Two different hyperlipidemic mouse models, which require different diets to get atherosclerosis, the ApoE deficient mice (ApoE<sup>-/-</sup>) requiring the normal mouse diet (Chow diet) and the Low Density Lipoprotein Receptor deficient mice (LDLr<sup>-/-</sup>) requiring the Western diet, were used for this study as both are susceptible to Ang II induced vascular disorders. The timing of PCB administration was also studied in LDLr<sup>-/-</sup> mice to see the profound effects of PCB77 on atherosclerosis and AAAs.

Keywords: Polychlorinated biphenyl; angiotensin II; hyperlipidemic mice; aneurysm; atherosclerosis

Madhura Parulkar

Students Name

December 08, 2011

Date

COPLANAR PCB77 AND ANGIO INDUCED VASCULAR DISORDERS

By

Madhura Parulkar

Dr. Lisa Cassis

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Director of Thesis

Dr. Howard Glauert

---

Director of Graduate Studies

December 08, 2011

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Date

## DEDICATION

I would like to dedicate this work to my husband Rajesh and darling daughter Reva for I would not have been where I am today without their support. I would also like to dedicate it to my mother, whose unrelenting and unconditional motivation I will cherish forever

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I would like to thank my parents and sister for making me the person that I am and for motivating me to aim higher and supporting me in every endeavor. A special thanks to my father-in-law and mother-in-law for their unstinting support and prayers. I am also very grateful to my husband and daughter and all my family members in US as well as back home in India who have also provided me with invaluable emotional and spiritual support throughout this journey.



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## CHAPTER 1: POLYCHLORINATED BIPHENYLS

### 1.1 INTRODUCTION

Polychlorinated Biphenyls (PCBs) (Figure 1.1) which are also known as Persistent Organic Pollutants are a major environmental and public health concern in the United States. Exposure to such hazardous chemicals can lead to compromised health and cause a number of pathologies like cardiovascular disorders, cancer, diabetes etc. PCBs were produced and sold in US as highly stable organic soluble industrial compounds and played a predominant role as dielectric, heat transfer fluids, plasticizers, wax extenders and flame retardants (Carpenter, 2006). They were highly functional due to their properties like high heat and electric resistance, non-solubility in water and high lipophilicity. Even though these compounds were banned in the US in 1977, due to their highly persistent nature they are yet found in all parts of the ecosystem. With an octanol: water coefficient of  $10^4$ , PCBs are considered highly hydrophobic and hence lipophilic. The lipophilicity increases with increasing degrees of



chlorination. Theoretically 209 congeners are possible but only 130 have been identified in commercial mixtures. Also being persistent they can be altered by physical and biological processes. They are vulnerable to both anaerobic and aerobic degradation and can be metabolized in the body (McFarland et al, 1989)

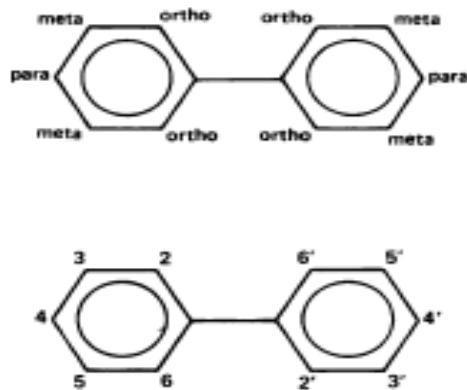


Figure 1.1 Structure and positional nomenclature (a) and numbering (b) for biphenyl.

(McFarland et al, 1989).

## 1.2 PCB TOXICITY

PCBs are lipophilic substances to which can enter the human body through ingesting animal fats, inhalation, or dermal contact. Exposure to PCBs can lead to suppression of the immune system, due to which there is increased risk of acquiring several human diseases (Smits et al, 2002). Both ortho-substituted and coplanar (dioxin-like) congeners act as tumor promoters that excentuate the effects of other carcinogenic substances. (Knerr et al, 2006)

PCB exposure during fetal and early life can cause reduction in IQ and behavior alteration and also cause developmental neurotoxicity (Seegal et al, 2005) The PCBs have also known to alter thyroid and reproductive function in both males and females. Also an increase in the risk of developing cardiovascular, liver disease and diabetes was seen. Women exposed to PCBs are at high risk of giving birth to infants of low birth weight (Baibergenova et al, 2003). These infants are at high lifetime risk for several diseases. As knowledge of their toxic effects has grown faster than

environmental levels have declined, PCBs remain dangerous contaminants

(Carpenter, 2006)

### 1.3 CLASSIFICATION OF PCBs

The chemical formula for PCBs is  $C_{12}H_{10-n}Cl_n$ , n ranges from 1-10. (Air Quality guidelines for Europe)

There are 2 types of classification-

- 1) Structural- Under the structural classification PCBs can be coplanar or non-coplanar. In coplanar PCBs the chlorine atoms are in the para or 0-4 meta positions. PCBs which are substituted in the para and meta positions will exhibit maximum coplanar conformational character. Figure 1.2 shows the structures of all the 4,4'-dichlorobiphenyl-substituted PCBs and represents a subset of the group of coplanar compounds.(Safe S et al, 1985)

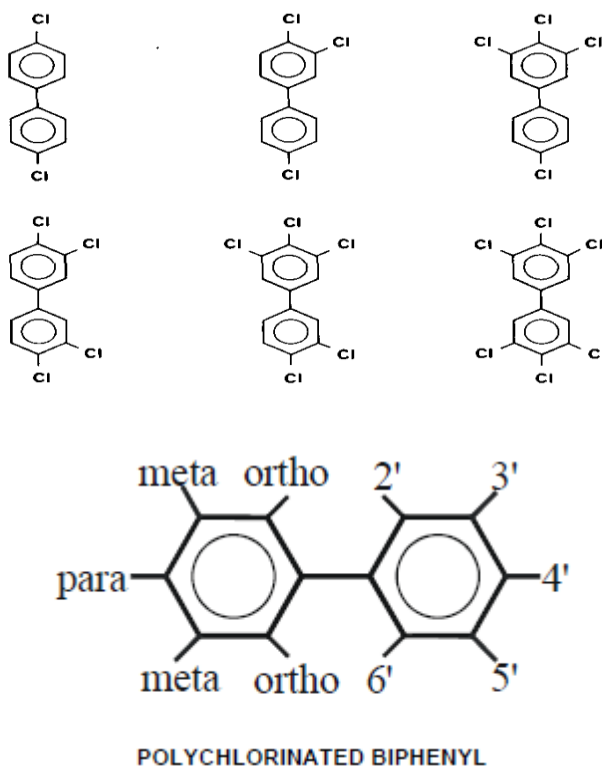


Figure 1.2 Coplanar PCB congeners substituted at both para and 0-4 meta positions

(Safe S et al, 1985; Carpenter, 2006).

In the non-coplanar PCBs the chlorine atoms are either mono-ortho substituted (Fig 1.3) or di-ortho substituted (Fig1.4). The chlorine in the ortho position causes steric differences between the chlorine and hydrogen atoms as a result of which there is a shift in the planar structure (Safe S et al, 1985)

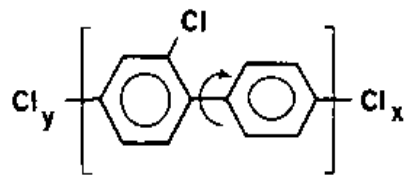


Figure 1.3 Mono-substituted PCB

(Safe S et al, 1985)

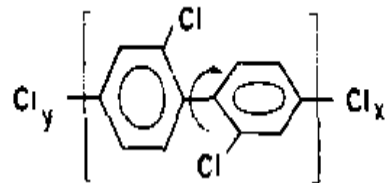


Figure 1.4 - Di-ortho substituted PCB

(Safe S et al, 1985)

Table 1.1 shows the various possible positions of chlorine atoms on the 209 congeners. ( Air Quality guidelines for Europe) The simplest structure being the presence of 1 chlorine atom at a given time and the most complex being the presence of 9 chlorine atoms.

Table 1.1 IUPAC numbers and chlorine atom positions of all PCB  
(Air Quality guidelines for Europe)

No.	Structure	No.	Structure	No.	Structure	No.	Structure
1	2	56	2,3,3',4'	111	2,3,3',5,5'	166	2,3,4,4',5,6
2	3	57	2,3,3',5	112	2,3,3',5,6	167**	2,3',4,4',5,5'
3	4	58	2,3,3',5'	113	2,3,3',5',6	168	2,3',4,4',5',6
4	2,2'	59	2,3,3',6	114**	2,3,4,4',5	169*	3,3',4,4',5,5'
5	2,3	60	2,3,4,4'	115	2,3,4,4',6	170***	2,2',3,3',4,4',5
6	2,3'	61	2,3,4,5	116	2,3,4,5,6	171	2,2',3,3',4,4',6
7	2,4	62	2,3,4,6	117	2,3,4',5,6	172	2,2',3,3',4,5,5'
8	2,4'	63	2,3,4',5	118**	2,3',4,4',5	173	2,2',3,3',4,5,6
9	2,5	64	2,3,4',6	119	2,3',4,4',6	174	2,2',3,3',4,5,6'
10	2,6	65	2,3,5,6	120	2,3',4,5,5'	175	2,2',3,3',4,5',6
11	3,3'	66	2,3',4,4'	121	2,3',4,5',6	176	2,2',3,3',4,6,6'
12	3,4	67	2,3',4,5	122	2',3,3',4,5	177	2,2',3,3',4',5,6
13	3,4'	68	2,3',4,5'	123**	2',3,4,4',5	178	2,2',3,3',5,5',6
14	3,5	69	2,3',4,6	124	2',3,4,5,5'	179	2,2',3,3',5,6,6'
15	4,4'	70	2,3',4',5	125	2',3,4,5,6'	180***	2,2',3,4,4',5,5'
16	2,2',3	71	2,3',4',6	126*	3,3',4,4',5	181	2,2',3,4,4',5,6
17	2,2',4	72	2,3',5,5'	127	3,3',4,5,5'	182	2,2',3,4,4',5,6'
18	2,2',5	73	2,3',5',6	128	2,2',3,3',4,4'	183	2,2',3,4,4',5',6
19	2,2',6	74	2,4,4',5	129	2,2',3,3',4,5	184	2,2',3,4,4',6,6'
20	2,3,3'	75	2,4,4',6	130	2,2',3,3',4,5'	185	2,2',3,4,5,5',6
21	2,3,4	76	2',3,4,5	131	2,2',3,3',4,6	186	2,2',3,4,5,6,6'
22	2,3,4'	77*	3,3',4,4'	132	2,2',3,3',4,6'	187	2,2',3,4',5,5',6
23	2,3,5	78	3,3',4,5	133	2,2',3,3',5,5'	188	2,2',3,4',5,6,6'
24	2,3,6	79	3,3',4,5'	134	2,2',3,3',5,6	189**	2,3,3',4,4',5,5'
25	2,3',4	80	3,3',5,5'	135	2,2',3,3',5,6'	190	2,3,3',4,4',5,6
26	2,3',5	81	3,4,4',5	136	2,2',3,3',6,6'	191	2,3,3',4,4',5',6
27	2,3',6	82	2,2',3,3',4	137	2,2',3,4,4',5	192	2,3,3',4,5,5',6
28	2,4,4'	83	2,2',3,3',5	138	2,2',3,4,4',5'	193	2,3,3',4',5,5',6
29	2,4,5	84	2,2',3,3',6	139	2,2',3,4,4',6	194	2,2',3,3',4,4',5,5'
30	2,4,6	85	2,2',3,4,4'	140	2,2',3,4,4',6'	195	2,2',3,3',4,4',5,6
31	2,4',5	86	2,2',3,4,5	141	2,2',3,4,5,5'	196	2,2',3,3',4,4',5,6'
32	2,4',6	87	2,2',3,4,5'	142	2,2',3,4,5,6	197	2,2',3,3',4,4',6,6'
33	2',3,4	88	2,2',3,4,6	143	2,2',3,4,5,6'	198	2,2',3,3',4,5,5',6
34	2',3,5	89	2,2',3,4,6'	144	2,2',3,4,5',6	199	2,2',3,3',4,5,6,6'
35	3,3',4	90	2,2',3,4',5	145	2,2',3,4,6,6'	200	2,2',3,3',4,5',6,6'
36	3,3',5	91	2,2',3,4',6	146	2,2',3,4',5,5'	201	2,2',3,3',4',5,5',6
37	3,4,4'	92	2,2',3,5,5'	147	2,2',3,4',5,6	202	2,2',3,3',5,5',6,6'
38	3,4,5	93	2,2',3,5,6	148	2,2',3,4',5,6'	203	2,2',3,4,4',5,5',6
39	3,4',5	94	2,2',3,5,6'	149	2,2',3,4',5',6	204	2,2',3,4,4',5,6,6'
40	2,2',3,3'	95	2,2',3,5',6	150	2,2',3,4',6,6'	205	2,3,3',4,4',5,5',6
41	2,2',3,4	96	2,2',3,6,6'	151	2,2',3,5,5',6	206	2,2',3,3',4,4',5,5',6
42	2,2',3,4'	97	2,2',3',4,5	152	2,2',3,5,6,6'	207	2,2',3,3',4,4',5,6,6'
43	2,2',3,5	98	2,2',3',4,6	153	2,2',4,4',5,5'	208	2,2',3,3',4,5,5',6,6'
44	2,2',3,5'	99	2,2',4,4',5	154	2,2',4,4',5,6'	209	2,2',3,3',4,4',5,5',6,6'
45	2,2',3,6	100	2,2',4,4',6	155	2,2',4,4',6,6'		
46	2,2',3,6'	101	2,2',4,5,5'	156**	2,3,3',4,4',5		
47	2,2',4,4'	102	2,2',4,5,6'	157**	2,3,3',4,4',5'		
48	2,2',4,5	103	2,2',4,5',6	158	2,3,3',4,4',6		
49	2,2',4,5'	104	2,2',4,6,6'	159	2,3,3',4,5,5'		
50	2,2',4,6	105**	2,3,3',4,4'	160	2,3,3',4,5,6		
51	2,2',4,6'	106	2,3,3',4,5	161	2,3,3',4,5',6		
52	2,2',5,5'	107	2,3,3',4',5	162	2,3,3',4',5,5'		
53	2,2',5,6'	108	2,3,3',4,5'	163	2,3,3',4',5,6		
54	2,2',6,6'	109	2,3,3',4,6	164	2,3,3',4',5',6		
55	2,3,3',4	110	2,3,3',4',6	165	2,3,3',5,5',6		

## 2) Functional Classification-

According to their functions PCBs can be classified as Dioxin like and Non Dioxin like PCBs.

Dioxin like PCBs resembles weak dioxins. 2,3,7,8 TCDD is the most toxic polychlorinated dioxin congener. They are usually coplanar and mono-ortho substituted in structure and are activators of the Aryl Hydrocarbon Receptor (AHR) similar to the dioxins. Many of the toxic effects are similar to TCDD. Non Dioxin like PCBs are either weak AHR ligands or they do not bind the AHR but to some other receptors like the CAR. These are usually the ortho-substituted PCB configurations (Safe S et al, 1985)

#### 1.4 TOXIC EQUIVALENT FACTORS- RISK ASSESSMENT OF PCBs

PCBs are usually present in mixtures along with Polychlorinated dibenzos-p-dioxins (PCDDs) and dibenzofurane (PCDFs) as persistent pollutants. PCDDs, PCDFs and PCBs have been shown to cause toxic responses similar to 2,3,7,8-tetrachlorodibenzo-pdioxin (TCDD), the most potent congener within these groups of compounds ( Van den Berg et al, 1998). All these compounds are present in environmental and biological samples in varying concentrations. These differences are caused by environmental degradation as a result of which these mixtures change spatially and temporally into the environment and are very different from the technical mixtures originally released into the environment. Because of the complex nature of these compounds, it is difficult to evaluate the risk to humans, fish, birds and wildlife. Hence for this purpose the concept of Toxic Equivalent Factors was introduced by the European Centre of Environmental Health of the World Health Organization (ECEHWHO) and the International Programme on Chemical Safety (IPCS) to assess the risk and for



regulatory control of exposure to these mixtures (Table 1.2) To apply the concept of TEF all compounds need to follow a common mechanism of action. A number of studies show that many of these compounds act via binding to the AHR as the initial step. When applying the TEF concept, the toxicity of these compounds relative to that of 2,3,7,8-TCDD is determined on the basis of available in vivo and in vitro data (Safe S et al,1985, Van den Berg et al, 1998)

Table 1.2 World Health Organization TEFs for humans, mammals and birds.

(Van den Berg et al, 1998)

Type	IUPAC No.	Congener structure	TEF
Non-ortho	77	3,3',4,4'-tetrachlorobiphenyls	0.0005
	126	3,3',4,4',5-pentachlorobiphenyls	0.1
	169	3,3',4,4',5,5'-hexachlorobiphenyls	0.01
Mono-ortho	105	2,3,3',4,4'- pentachlorobiphenyls	0.0001
	114	2,3,4,4',5- pentachlorobiphenyls	0.0005 <sup>a</sup>
	118	2,3',4,4',5- pentachlorobiphenyls	0.0001
	123	2',3,4,4',5- pentachlorobiphenyls	0.0001
	156	2,3,3',4,4',5- hexachlorobiphenyls	0.0005
	157	2,3,3',4,4',5'- hexachlorobiphenyls	0.0005
	167	2,3',4,4',5,5'- hexachlorobiphenyls	0.00001 <sup>a</sup>
	189	2,3,3',4,4',5,5'-heptachlorobiphenyls	0.0001 <sup>a</sup>
Di-ortho	170	2,2',3,3',4,4',5- heptachlorobiphenyls	0.0001 <sup>a</sup>
	180	2,2',3,4,4',5,5'- heptachlorobiphenyls	0.00001 <sup>a</sup>

<sup>a</sup>Based on very limited data.

## 1.5 TISSUE DISTRIBUTION OF PCBs

PCBs are fat soluble and highly lipophilic which is evident from their octanol: water coefficients of  $\geq 10^4$ . As a result of this high lipophilicity, PCBs are known

to accumulate more in lipid than non-lipid materials wherein their presence is almost negligible. In a study by Kodavanti and coworkers congener-specific distribution of PCBs was determined after chronic exposure in rats (Kodavanti et al, 1998). Rats were treated with a mixture of Aroclor 1254 in corn oil five times a week for 4 weeks and showed highest accumulation of PCBs (parts per million) in fat (551µg/g) followed by in liver (38.27 µg/g). Similar results have also been seen in other studies for specific PCB congeners like PCB 153 (Wyss et al, 1986) and PCB 180 (Koss et al, 1993). Also due to slightly different octanol: water coefficients of various congeners (Table 1.3) their degree of lipophilicity may differ.

Table 1.3 Partition Coefficients of Coplanar and Non-Coplanar PCBs

Kow > 6 describes an extremely hydrophobic compound

Congener	Coplanar/Noncoplanar	Log octanol: water coefficients
PCB77	Coplanar, Non-ortho	5.9
PCB153	Non-coplanar	7.0
TCDD		7.5

In a study previously conducted in our lab, Arsenescu et al. (Arsenescu et al, 2008) showed that adipocyte differentiation and proinflammatory adipokine expression were induced by PCB-77 but not by PCB-153 in 3T3-L1 adipocytes. A lack of effect of PCB-153 may have resulted from greater sequestration of this more-lipophilic PCB in the triacylglycerol droplet of the adipocyte, leaving less PCB-153 available to act at adipocyte target proteins and also due to other alternate mechanisms like differences in receptors for their action.

## CHAPTER 2: ARYL HYDROCARBON RECEPTOR

### 2.1 INTRODUCTION

PCBs are a huge family of chemicals, consisting of 209 possible congeners, only small parts of which resemble the dioxins. A number of multiple, overlapping structural classes of PCBs exist, but PCBs characteristically are present as mixtures, and never are present in the absence of PCBs which are dioxin like in the encompassing environment (Van den Berg et al, 1998). Likely, dioxins like TCDD and PCBs are rarely found in the absence of one another. Individual PCBs are known to have their own intrinsic toxicities, and can also interact with dioxins and other PCB congeners producing additive, synergistic, and/or antagonistic reactions, granting high variability to the activity of the PCB mixtures (White et al, 2009).

The mechanism of action by which dioxins and dioxin like PCBs produce their biochemical toxic effect is via the activation of the AHR.(Klinge et al, 1999) AHR is a basic helix-loop-helix transcription factor which is activated by ligands like

dioxins, PCBs etc. It belongs to the PER-ARNT-SIM (PAS) superfamily of transcription factors (Petrulis et al, 2002). The AHR is a highly conserved transcription factor across vertebrate species, and its presence has also been identified in invertebrates, such as *C. elegans* and *Drosophila* species. (Hahn et al, 1997)

## 2.2 MECHANISM OF ACTION

AHR is present in the cell in a non-ligand state. AHR exists as a complex in the cytosol along with chaperone proteins, which are two molecules of heat shock protein (Hsp) 90, and a molecule each of prostaglandin E synthase 3 (p23) and immunophilin-like protein hepatitis B virus X-associated protein 2 (XAP2, also known as AIP or ARA9) (Figure 2.1) (Petrulis et al, 2002). Toxins like dioxin and similar compounds enter the cell by diffusion. Activation of AHR by the ligand results in the disruption of the AHR complex in the cytosol causing dissociation of one Hsp 90 molecule and all the p23 and XAP2 molecules. The AHR-TCDD complex travels into the nucleus. Once in the nucleus, AHR must heterodimerize

with the AHR Nuclear Translocator (ARNT), at their respective PAS domains, releasing the remainder molecule of Hsp 90, after which transcription occurs. AHR-TCDD-ARNT complex then binds to the DNA at dioxin response elements (DRE; or xenobiotic response elements, XRE) in the promoter regions of target genes. This process depends upon the presence of co-activators or co-repressors and transcription may proceed. An assembly of coactivators and general transcription factors, including p300, SRC-1, p/CIP and transcription factor IIB, then interact with gene promoters and potentiate the expression of target loci (Harkinson, 2005). AHR is also believed to interact with other key regulatory molecules including nuclear steroid receptors and cell cycle control molecules. Many of the metabolic enzymes show altered expression after TCDD exposure. These are directly controlled by the actions of the AHR-TCDD-ARNT complex on upstream DREs (Puga et al, 2009)

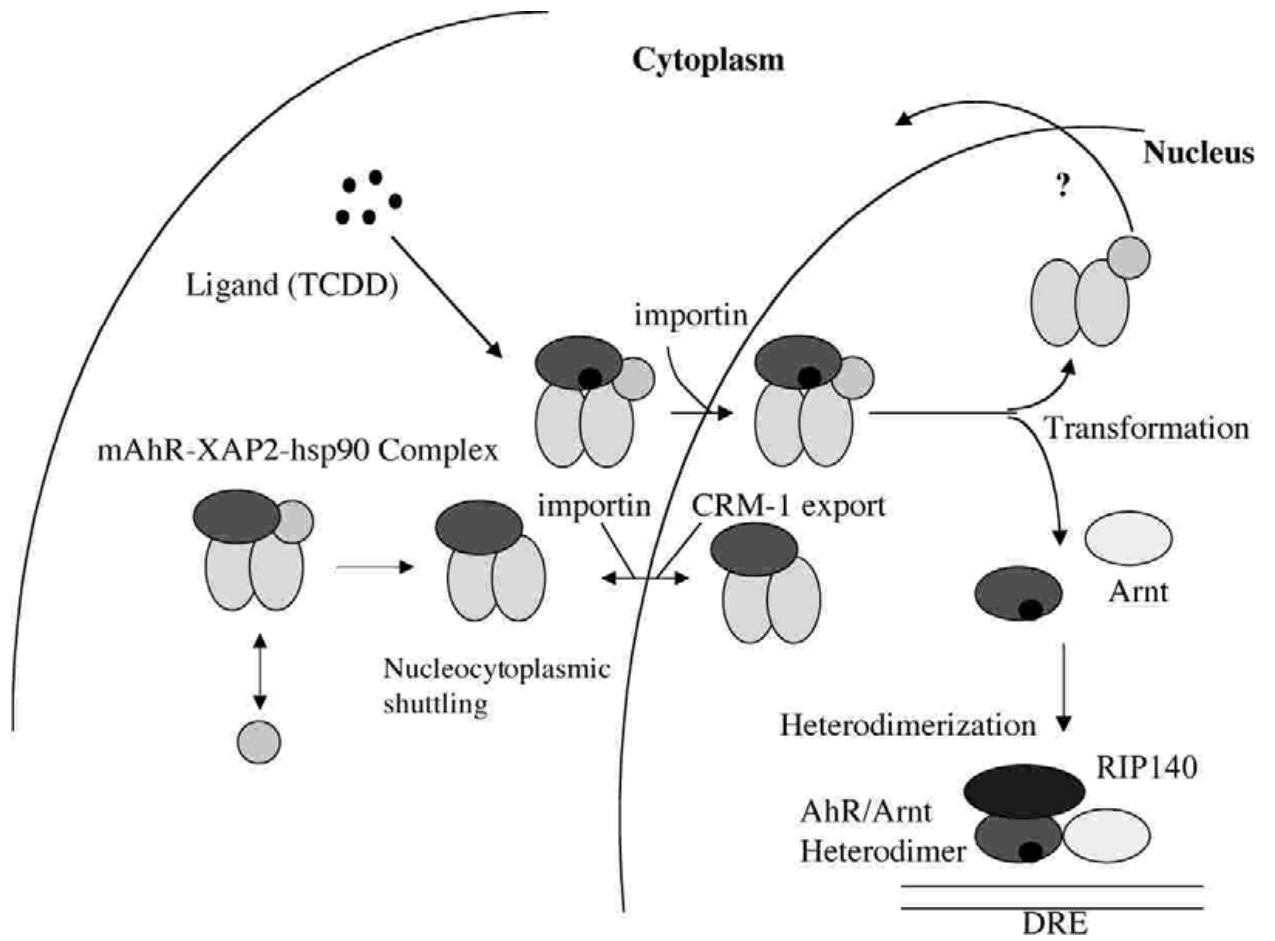


Figure 2.1 Model of ligand-dependent and -independent movement of the AHR between the cytoplasm and the nucleus

(Petrulis et al, 2002).

## 2.3 ROLES OF AHR

### 1. Physiological and Developmental Role of the AHR

A number of studies show evidence that strengthens the idea that AHR plays a dual role in normal biology as follows-

- a. Role as a mediator of an adaptive response to xenobiotics.
- b. Role as a mediator of normal embryonic development and adult physiology.

The AHR and all associated members of the signaling complex are expressed by both humans and the various laboratory species studied. The human cell and tissue culture studies have demonstrated human responsiveness to dioxins similar to that observed in other species (DeVito et al, 1995). In addition, exposed human populations have exhibited measurable biochemical responses, in regard to genes involved in drug metabolism as well as other known target genes for AHR and adverse health effects have been extensively observed in highly exposed populations.(White et al, 2009)



The PAS family of regulatory proteins are often known as “biological sensors” and being a part of the PAS superfamily the AHR is believed to play key roles in development, aging, hypoxia, and circadian rhythms ( Petruilis et al, 2002; Puga et al, 2009).

Different labs studying the physiology and development of AHR null or knockout mice show developmental irregularities and pathological endpoints that are induced when AHR signaling is interfered by receptor deletion. For example a 40-50% neonatal lethality rate, inflammation of the bile ducts and an 80% depletion of splenic lymphocytes is reported for 3-week old AHR null mice (Fernandez-Salguero et al, 1995).

The AHR knockout mice also present a phenotype which is vascular in nature, called patent Ductus Venosus (DV), which is a fetal porto-caval shunt of the developing liver that normally closes immediately after birth (Lahvis et al, 2000). Due to failure in closing of this shunt the mutant’s show an abnormal hepatic blood supply and altered arrangement of small molecules which then requires

hepatic clearance. These AHR mutant mice also demonstrate the presence of the hyaloid artery and an aberrant network of limbal vasculature in the developing eye. Similar to the DV, the hyaloid artery also exhibits a second fetal vascular structure that normally is absent after birth, yet is seen in the eyes of adult AHR null mice (Lahvis et al, 2000, Walliser et al, 2004).

Studies involving gene targeting, to generate AHR hypomorphic mice with decreased levels of AHR, had similar high frequency of DV as seen in the AHR null mice (Walliser et al, 2004). Exposure of these hypomorphic mice to a potent AHR ligand like TCDD between embryonic days E12.5-E18.5 led to the complete resolution of the DV and the maturation of a normal-sized liver by adulthood. From this study it could be concluded that endogenous agonist activates the AHR to regulate vascular development during normal ontogeny (Lahvis et al, 2000).

## 2. Toxic Response

Dioxin and dioxin like compounds bind the AHR, disrupting its endogenous function. That is, the physiological activator of the AHR likely induces rapid on/off signaling through the receptor. Dioxins, however, are believed to induce toxicity through the persistent activation of the AHR, thereby preventing the AHR from functioning in the maintenance of homeostasis.

Some toxic responses produced by TCDD causing inflammation appear to arise via a non-genomic pathway (Figure 2.2). This pathway is brought about by ligand-activation of AHR, but doesn't require nuclear translocation or for the dimerization partner of AHR, ARNT, thus it also doesn't require binding to the DRE followed by transcription, both very important components of the classical pathway (Li et al, 2008). In the ligand activated AHR non-genomic pathway TCDD elicits an inflammatory response by stimulating cascading events which involves rapid increase in intracellular Ca(2+) levels, activation of phospholipase A2 in the cytosol (cPLA2) and Cox-2 activation. (Matsumura et al, 2009).

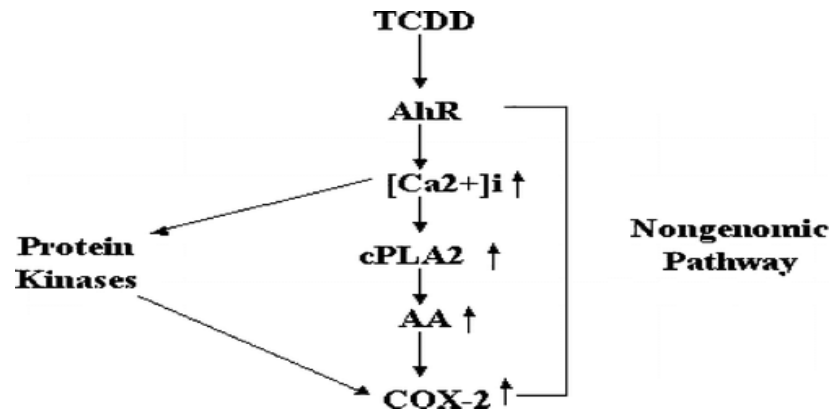


Figure 2.2: Toxic response by TCDD by non-genomic pathway

(Li et al, 2008)

## 2.4 ACTIVATORS OF AHR

Endogenous Ligands of particular interest are those that are known to be endogenously synthesized in higher organisms. Figure 2.3 enlists candidate endogenous AHR ligands that have been isolated from mammalian tissues.

Some of the dietary compounds have also been found to activate the AHR and play an important role as a physiologically relevant activator of this receptor (Figure 2.4).

Exogenous Ligands include the halogenated-dibenzo-*p*-dioxins, -dibenzofurans, -azo(xy)benzenes, -naphthalenes, polychlorinated biphenyls and polycyclic aromatic hydrocarbons comprise a family of important, structurally related AHR agonists.(Figure 2.5)

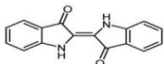
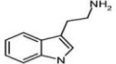
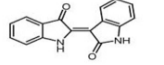
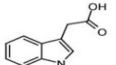
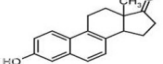
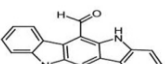
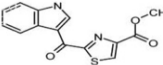
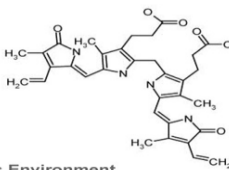
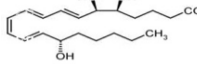
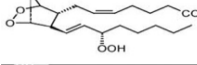
Compound structure	Name	AHR activation (EC <sub>50</sub> )	Compound structure	Name	AHR activation (EC <sub>50</sub> )
	Indigo	5 nM <sup>[101]</sup> 5 μM <sup>[102]</sup>		Tryptamine	0.2 mM <sup>[119]</sup>
	Indirubin	0.2 nM <sup>[101]</sup> 1.26 nM <sup>[103]</sup> 100 nM <sup>[101]</sup>		Indole Acetic Acid	0.5 mM <sup>[119]</sup>
	Equilenin	10 μM <sup>[108]</sup>		6-formylindolo- [3,2-b]carbazole (FICZ)	34-830 pM <sup>[127]</sup>
	2-(1'H-indole-3'- carbonyl)-thiazole-4- carboxylic acid methyl ester (ITE)	20 nM <sup>[107]</sup>		Bilirubin	30 μM <sup>[117]</sup>
	Lipoxin 4A	100 nM <sup>[114]</sup>			
	Prostaglandin G2	20 pM <sup>[105]</sup>			

Figure 2.3 Endogenous AHR ligands from mammalian tissues

(Denison et al, 2003)

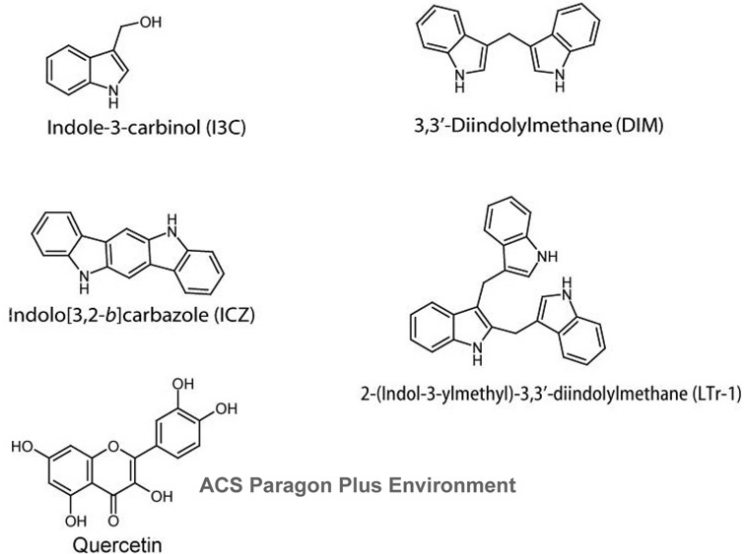


Figure 2.4 Dietary Compounds that activate AHR

(Denison et al, 2003)

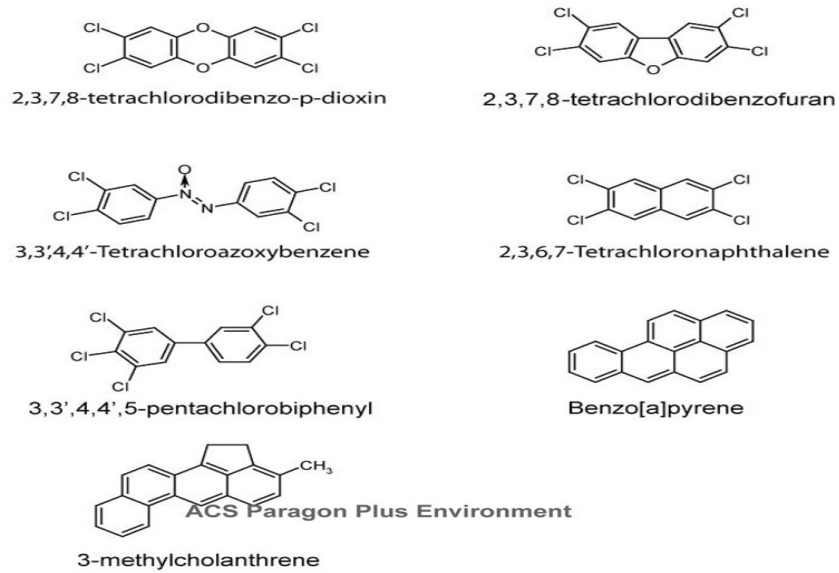


Figure 2.5 Exogenous AHR ligands

(Denison et al, 2003)

### CHAPTER 3: PCB AND INFLAMMATION

Several studies show that Persistent Organic Pollutants like dioxins and coplanar PCBs which are AHR agonists may promote inflammation and interrupt normal function of various cell types like endothelial cells (Toborek et al, 1995) and adipocytes (Arsenescu et al, 2008). Coplanar PCBs may affect the normal functions of vascular endothelial cells by inducing pathways signaling oxidative stress and initiating proinflammatory events such as increased expression of CYP1A1 gene, VCAM-1, NF-kappaB which play an important role in the pathology of atherosclerosis and cardiovascular disease (Toborek et al, 1995; Hennig et al, 2002). In separate experiments it was also seen that coplanar PCB 77 caused upregulation of MCP-1 expression by endothelial cells and that this effect is mediated by AHR, as well as p38 and JNK MAPK pathways (Choi et al, 2003).

Hennig and group also showed that dietary fat in combination with PCBs caused elicitation of aberrant gene patterns associated with dysregulated lipid metabolism (Arzuaga et al, 2009 ). The microarray data from this study showed



that coplanar PCB 77 caused disruption of dietary fatty acid induced changes in gene expression. In addition to the interactive effects of dietary fat and PCB exposure on genes associated with triacylglycerol synthesis and cholesterol/bile acid metabolism, dysfunction of several genes associated with fatty acid metabolism (both synthetic and oxidative pathways), i.e., genes that are also regulated by PPARs was also observed. For example, acyl-coenzyme A dehydrogenase-medium chain (Acadm) is a mitochondrial enzyme that catalyzes the initial step in fatty acid metabolism. All these altered gene findings provide an indication of the molecular mechanism supporting the experimental and epidemiological evidence that exposure to persistent organic pollutants are significant risk factors for hepatic and cardiovascular disease (Choi et al, 2003).

Coplanar PCBs which are AHR agonists are responsible for the observed increase in inflammation (Slim et al, 1999; Hennig et al, 2002; Arzuaga et al, 2007). Adipocytes also express AHR (Shimba et al, 1998). Hence if adipocytes release inflammatory cytokines it is possible that adipocytes would promote

obesity and obesity related cardiovascular disorders (Mullerova et al, 2006).

When 3T3-L1 adipocytes were treated with coplanar PCB 77, it increased the expression of proinflammatory adipokines like TNF- $\alpha$ , MCP-1, KC-1 and CD36 and also of adipocyte differentiation factors like PPAR $\gamma$ , aP2 and angiotensinogen (Arsenescu et al, 2008), suggesting that coplanar PCB77 promoted obesity and obesity related inflammatory disorders like cardiovascular diseases. In vivo experiments showed that when ApoE $^{-/-}$  mice were treated with either PCB 77 or vehicle, the treatment animals showed significant increase in total serum cholesterol especially the VLDL particle and increased Oil Red O staining in the aortic sections showing an increase in atherosclerosis in PCB treated animals (Arsenescu et al, 2008)

## CHAPTER 4: ANG II INDUCED VASCULAR DISORDERS

The Renin Angiotensin System (RAS) has been implicated in cardiovascular disorders like hypertension, atherosclerosis and aortic abdominal aneurysms (AAA) (Hollenberg, 2000; Grote et al, 2004; Lu et al, 2008). There is substantial evidence that suggests that Ang II plays a pivotal role in the atherogenic process.

### 4.1 ANGIO II AND ATHEROSCLEROSIS

Cardiovascular disorders are the most common cause of morbidity and mortality in developed countries (Grote et al, 2004). Atherosclerosis is the underlying cause for ischemic heart disease. Ang II may lead to the acceleration of the atherogenic process by a number of possible mechanisms (Singh et al, 2003).

Earlier studies showed Ang II caused elevations in blood pressure to indirectly lead to formation of atherosclerosis (Atlas, 2007), but current studies do not support this evidence (Gupte et al., 2009). Studies have shown profound increases in atherosclerotic lesion development despite no increase in arterial blood pressure measured by tail cuff and catheter measurements from the carotid artery on anesthetized mice (Daugherty et al, 2000). Norepinephrine

infusion also caused elevation in systolic blood pressure comparable to Ang II infusion in ApoE deficient mice, which had minimal effect on atherosclerosis (Weiss et al, 2001) (Cassis et al., 2009). Systolic blood pressure was measured by a computerized tail cuff system during treatment and at the end of each experiment. Similar findings were demonstrated in our lab earlier for Ang II-induced AAAs (Cassis et al, 2009). Infusion of Ang II and norepinephrine caused equivalent blood pressure increases in LDLR<sup>-/-</sup> and ApoE<sup>-/-</sup> mouse models, but only Ang II infusion caused AAA formation. Moreover, infusion of Ang II at a rate of 500 ng·kg<sup>-1</sup>·min<sup>-1</sup> did not significantly elevate blood pressure but resulted in a 50% AAA incidence. Also reduction of Ang II-induced increases in blood pressure by concurrent administration of hydralazine did not influence development of AAAs. Hence increases in blood pressure from infusion of Ang II do not significantly contribute to formation of AAAs.

In a pivotal study by Daugherty and group when Ang II was infused in LDLR<sup>-/-</sup> and ApoE<sup>-/-</sup> mice fed a high fat and cholesterol-containing diet; there was

accelerated formation of atherosclerotic lesions (Daugherty et al, 1999; 2000). An Ang II infusion of only 28 days led to an increase in the area of discernible lesions in the aortic intima. A more prolonged infusion of 8 weeks caused a more profound formation of atherosclerotic lesions in ApoE<sup>-/-</sup> mice fed on high fat, cholesterol and cholate diet (Weiss et al, 2001).

The infusion rate of Ang II is also very important. An infusion rate of 500ng/kg/min in C57BL/6 mice caused no observable changes in arterial blood pressure, body weight, and cardiac hypertrophy (Cassis et al, 2004). Similarly the same infusion rate as well as a higher infusion rate of 1000ng/kg/min in ApoE<sup>-/-</sup> mice did not produce any changes in arterial blood pressure, serum cholesterol concentrations or distribution of lipoprotein cholesterol (Daugherty et al, 2000), but these infusion rates promoted an increased severity of aortic atherosclerotic lesions.

Hypercholesterolemia caused by aberrant cholesterol metabolism has generally been considered as an independent mechanism in the development of severe

atherosclerosis. Some possible hypercholesterolemia-induced mechanisms are related to the RAS including increased AT1-receptor expression, increased responsiveness to Ang II and increased synthesis of angiotensin peptides (Daugherty et al, 2004).

A direct role of Ang II in atherosclerotic disease can be inferred from human trials (Heart Outcomes Prevention Evaluation Study Investigators, 2000) in which angiotensin converting enzyme I (ACE I) inhibition by ramipril reduced cardiovascular events in people with coronary artery disease without major changes on blood pressure. Patients treated with ramipril exhibited reduced rates of death, myocardial infarction, stroke, coronary revascularization, cardiac arrest, and heart failure. A very small part of the benefit could be credited to a reduction in blood pressure, as the mean reduction in blood pressure with treatment was extremely small (3/2 mm Hg).

## 4.2 ANG II INDUCED ABDOMINAL AORTIC ANEURYSMS.

Abdominal aortic aneurysms (AAAs) are progressive dilations of the infrarenal aorta that can rupture when increasing in size, commonly leading to death (Diehm *et al.*, 2005). AAAs affects 1.1 million American people (Lim *et al.*, 2011; Nordon *et al.*, 2011), being primarily present in males over 60 years of age. AAAs account for 2% of all deaths and are the tenth most common cause of mortality (Natl Vital Stat Rep). As AAAs progress in size, the risk of rupture increases. The only treatment option to prevent rupture of AAAs is surgical, either by open repair or endovascular approaches.

In addition to these well-described effects on blood pressure and atherosclerosis it is becoming increasingly evident that the RAS contributes to the development of AAAs. Pivotal studies demonstrated that a 1 month infusion of Ang II to hypercholesterolemic mice resulted in AAAs in 80-100% of male mice (Daugherty *et al.*, 2000). These aneurysms are observed in the suprarenal area of mouse aorta, whereas in humans AAAs form in the infrarenal region of abdominal aortas. The differing localization could reflect a discrepancy between

AAA formation in humans and mice because of hemodynamic differences. Humans being biped and mice are quadruped; it may be possible that hemodynamic forces would differ between these two species. This formation of Ang II induced AAA in the suprarenal region of the abdominal aorta is seen consistently in both LDLR<sup>-/-</sup> and ApoE<sup>-/-</sup> mice.

Saraff et al in 2003 conducted a study to find out the temporal sequence of events in Ang II-induced AAAs to provide mechanistic insight into AAA initiation and maturation. The earliest changes were noted at 1-4 days after Ang II infusion where there was medial accumulation of macrophages in the region that develops aneurysms along with disruption of elastin fibers. There is a clearly defined medial dissection and approximately 10% of male mice died between 4 and 10 days by a vascular hematoma that was grossly observed in the majority of the mice. In the mice that survived there was a development of thrombi which caused an inflammatory reaction that prominently involved infiltrating macrophages. These macrophages were present in the thrombi. After 14 days



the thrombi is replaced by extracellular matrix with increased macrophage deposition. T and B lymphocytes are also seen in this area. A remodeling of the artery follows and after a period of time the enlarged artery is completely re-endothelialized and the endothelium was demonstrable adjacent to the original medial layer. This remodeling is permanent and all three arterial layers are present. There was no evidence that atherosclerosis affects the formation of AAA's. Atherosclerotic lesions marked with lipid laden macrophages were detected after only 28 days of Ang II infusion. AAAs also form from Ang II infusion in normolipidemic C57BL/6 mice (Deng et al 2003), though the incidence is much less than in hyperlipidemic mice.

Gender also plays an important role in the incidence of AAA's. Males are more prone to get AAA's than females in both humans and mice. (Manning et al 2002, Henriques et al, 2004). The gender difference is due to endogenous steroid hormones like androgen. It is also seen that male sex hormones positively regulate AT1aR expression in a regional-specific manner to promote Ang II-

induced AAAs in both male and female ApoE<sup>-/-</sup> mice.(Henriques et al, 2008)

Another study also showed that estrogen administration reduced the incidence and severity of AAA. (Martin-McNulty et al 2003).

The receptor subtype also has a significant role to play for Ang II to exert its effect. Administration of an AT1 receptor antagonist, losartan, ablated the formation of Ang II induced AAAs proving that Ang II exerts its pathophysiological effects through AT1 receptors. (Daugherty et al 2001) Amongst all the RAS components like renin, prorenin/renin receptor, angiotensinogen, AT1- and AT2 receptors, cathepsin D, cathepsin G and chymase, the expression of all RAS components are upregulated except AT2 receptors in AAAs (Kaschina et al, 2009). Activation of AT2 receptors by agonists decreases the effects of AT1 receptor agonists. Coadministration of the AT2 receptor antagonist PD123319 greatly enhanced the development of AAAs (Daugherty et al. 2001).

The matrix metalloproteinase (MMPs) enzymes play a pivotal role in the pathogenesis of AAAs. The frequently detected MMPs in the aneurysmal tissue are MMP-2 and MMP-9 (Goodall et al. 2001; Longo et al. 2002; Pyo et al. 2000). Administration of doxycycline, an inhibitor of MMPs, reduced both the incidence and the severity of AAAs and are suggested to have anti AAA properties in humans (Manning et al, 2003; Thompson and Baxter 1999).

## STATEMENT OF PROBLEM

Obesity is a well-known risk factor that causes cardiovascular disorders like atherosclerosis and AAAs. PCBs because of their lipophilic nature are sequestered in the fat depots of obese people (Kodavanti et al,1998). PCBs are also implicated in various cardiovascular disorders like hypertension and atherosclerosis by producing inflammatory markers (Hennig et al, 2002). Ang II is also known to promote cardiovascular pathologies like atherosclerosis and AAAs (Singh et al, 2003, Daugherty et al,1999, 2000). Mouse models infused with Ang II are well known biological systems used for the study of cardiovascular disorders. Till date no studies have been conducted which link PCBs to AAAs. In this study, we are looking at the effects of PCBs on hyperlipidemic mice models like the LDLr<sup>-/-</sup> and ApoE<sup>-/-</sup>. The difference between the two models being the requirement of a western diet for the induction of the cardiovascular pathology in LDLr<sup>-/-</sup> mice.

Previously Arsenescu et al showed that PCBs promote obesity and increase inflammation by releasing cytokines like TNF $\alpha$ , MCP-1, etc (Arsenescu et al, 2008). In a separate study, Police et al showed that obesity increase in inflammatory markers in periaortic fat which promoted the formation of AAAs (Police et al, 2008). For this study, we hypothesized when hyperlipidemic mice on a western/normal diet were treated with PCBs it would lead to obesity which in turn would increase inflammation in the periaortic fat and enhance the formation of AAAs.

### **SPECIFIC AIMS**

**AIM 1** – PCB 77 promotes atherosclerosis and AAAs in hyperlipidemic mice like LDLr  $-/-$  and ApoE  $-/-$ .

**AIM 2** – the timing of dosage of PCBs would enhance the formation of AAAs in LDLr  $-/-$  receptor mice on a western diet.

## CHAPTER 5: MATERIAL AND METHODS

PCB 77 (3,3',4,4'-tetrachlorobiphenyl) was obtained from Accustandard Inc. (New Haven CT).

### 5.1 ANIMAL TREATMENT AND SAMPLE COLLECTION

Male LDLr<sup>-/-</sup> and ApoE<sup>-/-</sup> mice (The Jackson Laboratory, Bar Harbor, ME) at 2 months of age were housed in a pathogen-free environment. ApoE<sup>-/-</sup> mice were fed a standard diet (Chow) and LDLr<sup>-/-</sup> mice were fed a Western diet (Table 5.1; Table 5.2) supplemented with saturated fat (milk fat; 21% wt/wt) and cholesterol (0.15%; wt/wt, catalog no. TD88137; Harlan Teklad). Water was provided ad libitum. All procedures were approved by the Animal Care and Use Committee at the University of Kentucky.

Table 5.1 Composition of the Western Diet

(Catalog no. TD88137; Harlan Teklad)

Formula	g/kg
Casein	195
DL-Methionine	3
Sucrose	341.46
Corn Starch	150
Anhydrous Milkfat	210
Cholesterol	1.5
Cellulose	50
MineralMix,AIN-76 (170915)	35
Calcium Carbonate	4
Vitamin Mix, Teklad (40060)	10
Ethoxyquin, antioxidant	0.04

Table 5.2 Macronutrient content of the Western Diet

(Catalog no. TD88137; Harlan Teklad)

Nutrient	% by weight	% kcal
Protein	17.3	15.2
Carbohydrate	48.5	42.7
Fat	21.2	42

## 5.2 STUDY DESIGN

Study 1- In this study we contrasted effects of PCB77 on Ang II-induced vascular diseases in two different mouse models susceptible to atherosclerosis, namely ApoE<sup>-/-</sup> and LDLR<sup>-/-</sup> mice. We chose these two strains because they are both susceptible to Ang II-induced atherosclerosis and AAAs, but differ in the requirement for a high fat, western diet to induce atherosclerosis. ApoE<sup>-/-</sup> mice develop hypercholesterolemia and atherosclerosis when fed standard mouse diet, while LDLR<sup>-/-</sup> mice require a western, high fat diet to develop hypercholesterolemia and atherosclerosis. For both strains, we administered PCB77 prior to and towards the end of Ang II infusions. The ApoE<sup>-/-</sup> mice (n=10/group) and the LDLR<sup>-/-</sup> mice (n=10/group) were each divided equally into two groups (Vehicle vs. Treatment). Mice were started on the respective diets and after a week both groups were injected with PCB77 (170 $\mu$ M/kg; 49 mg/kg) or vehicle (safflower oil) in volumes corresponding to the weight of each mouse (10  $\mu$ L/g); the mice received PCB77 or vehicle injection prior to (2 doses), and during



week 3 (2 doses) of AngII infusion during the 6 week study duration. Body weight was measured weekly throughout the study. Mice from both groups were anesthetized (ketamine/xylazine 100/10mg.Kg. ip) on week 3 for ultrasound measurements and implantation of ALZET mini-osmotic AngII pump. Ultrasound measurements were taken in week 5 and 6 (weeks 3 and 4 of Ang II infusion) and blood pressure was measured during week 5 (Table 5.3). At the study end point, mice were anesthetized with ketamine/xylazine (100/10 mg/kg ip) for blood and tissue harvest.

Table 5.3 Experimental Design of Study 1

Week	1	2	3	4	5	6	7
Diet	Western Diet for LDLr <sup>-/-</sup> mice and Chow diet for ApoE <sup>-/-</sup> mice						
PCB/vehicle		↑				↑	
Ang II Infusion			↑				
Ultrasound		↑	↑		↑	↑	
Blood Pressure						↑	
Body Weight	Body Weights monitored throughout the study						

Table 5.4 Experimental Design of Study 2

Week	1	2	3	4	5	6	7
Diet	Western Diet for LDLr <sup>-/-</sup> mice						
PCB/vehicle		↑	↑				
Ang II Infusion			↑				
Ultrasound		↑	↑		↑	↑	
Blood Pressure						↑	
Body Weight	Body Weights monitored throughout the study						

Study 2 - This study follows the same protocol as above except this study was conducted only in the LDLr<sup>-/-</sup> mice. (Table 5.4) The mice received all PCB77 or vehicle injection prior to AngII infusion. This was done to see if the time of administration of the PCB/vehicle influenced the effects of PCB77 on AngII-induced vascular diseases. All mice were fed the western high fat diet for 1 week prior to and then throughout Ang II infusions.

### 5.3 INFUSION OF ANG II

Alzet osmotic minipumps (Model 2004; ALZA Scientific Products, Mountain View, California, USA) were implanted into LDLR<sup>-/-</sup> and ApoE<sup>-/-</sup> mice. Pumps were filled with saline solutions of Ang II (Sigma Chemical Co., St. Louis, Missouri, USA) that delivered (subcutaneously) 1,000 ng/min/kg of Ang II for 28 days. Mice were anesthetized with ketamine/xylazine (100/10 mg/kg ip). A small incision was made in the back of the neck and pumps were placed into the subcutaneous

space. The incision was closed with surgical staples which healed rapidly without the need for any medication. (Daugherty et al, 2000).

#### 5.4 DETERMINATION OF BLOOD PRESSURE

Systolic blood pressure was measured by volume pressure recording of the tail using the VISITECH noninvasive blood pressure system on five consecutive days in week 3 after Ang II infusion. Mice were conscious and restrained during the procedure as described earlier in a study. (Cassis et al, 2007). At least 5 measurements were recorded from each animal and the average of the measurements was considered.

#### 5.5 AAA QUANTIFICATION

AAAs were quantified in vivo by measurement of maximal lumen diameters of suprarenal abdominal aortas using high-frequency ultrasound (VisualSonics, Vevo 660, Toronto, CA) as described previously (Barisione et al., 2006) on anesthetized mice (ketamine/xylazine, 100/10 mg/kg, respectively, ip on day 0, 7,

14 and day 28 of Ang II infusion). AAAs were also quantified ex vivo by measurement of maximal width of suprarenal aortas dissected free from mice at 28 days and with extraneous tissues removed fixed in formalin by ImagePro 6 Plus 5.1 software (Media Cybernetics, Bethesda, MD). For determining the incidence of AAAs, three observers blinded to the experimental design evaluated the mouse AAAs in both the vehicle and treatment group.

## 5.6 ATHEROSCLEROSIS QUANTIFICATION

Atherosclerotic quantification was done by a procedure called en face carried out in the mouse aortic arch which is previously described. (Daugherty et al, 2005).

To measure the extent of intimal surface covered by grossly discernible lesions, image analysis was performed with SigmaScan (SPSS Inc., Chicago, Illinois, USA). The percent aortic arch lesion is quantified from 3 mm down from the subclavian arterial branch and the thoracic lesion area is quantified 9 mm further

down the length of the aorta. The atherosclerotic quantification is examined by two other observers blinded to the experimental design.

## 5.7 MEASUREMENT OF SERUM CHOLESTEROL AND PLASMA COMPONENTS

Serum total cholesterol and triglyceride concentrations were determined with enzymatic assay kits (Wako Chemical Co., Richmond, Virginia, USA) as described previously (Arsenescu et al., 2008).. At study endpoint, blood was obtained from anesthetized mice (ketamine/xylazine 100/10 mg/kg ip) by left ventricular puncture and collected in tubes containing EDTA (0.05 M). Plasma was obtained by centrifugation (5,000 rpm) at 4°C for 10 min.

## 5.8 STATISTICAL ANALYSIS

Data are represented as mean + SEM. Comparison of AAA incidence between groups was analyzed using Fisher's exact test. Unpaired Student's t test was used to compare parametric data between groups. One-way ANOVA was performed to compare body weight and blood pressure data with repeated measures on time. Analyses were performed using Graphpad Prism (Graphpad Software Inc., San Diego, CA, USA). Statistical significance was defined as  $P < 0.05$ .

## CHAPTER 6: RESULTS

### A) STUDY 1

In this study the ApoE<sup>-/-</sup> and LDLr<sup>-/-</sup> mice received PCB77 or vehicle injection prior to (2 doses), and during week 3 (2 doses) of AngII infusion during the 6 week study duration. ApoE<sup>-/-</sup> mice were fed standard mouse diet, while LDLR<sup>-/-</sup> mice were fed a western, high fat diet.

6.A.1 PCB77 had no effect on parameters like body weight and serum cholesterol but increased liver weights only in ApoE<sup>-/-</sup> mice and systolic blood pressure only in LDLr<sup>-/-</sup> mice.

Earlier studies in our laboratory showed that intraperitoneal injections of low doses of PCB77 promoted adipocyte differentiation and increased body weight as well as increased liver weights of ApoE<sup>-/-</sup> mice (Arsenescu et al, 2008). However in this study no difference in body weights was seen in ApoE<sup>-/-</sup> and



LDLr<sup>-/-</sup> administered PCB77 compared to vehicle control (Table 6.1; 6.2) but a significant increase in liver weights was seen only in ApoE<sup>-/-</sup> mice (Figure 6A.1).

Serum cholesterol concentrations were not significantly different between the vehicle and PCB77 groups for both ApoE<sup>-/-</sup> and LDLr<sup>-/-</sup> mice (Figure 6A.2).

However, systolic blood pressure was significantly increased in LDLr<sup>-/-</sup> administered PCB77 compared to vehicle, but not in ApoE<sup>-/-</sup> mice administered PCB77 (Figure 6A.3).

Table 6.1 Characteristics of Ang II-infused ApoE<sup>-/-</sup> mice administered vehicle or PCB77.

Parameter	PCB77	Control
Body weight (g)	29.75 $\pm$ 0.61	29.2 $\pm$ 0.75
Liver weight (g)	6.52 $\pm$ 0.19*	5.15 $\pm$ 0.09
Cholesterol (g/dL)	327.3 $\pm$ 32.8	337.9 $\pm$ 51.2
Systolic blood pressure mmHg	156 $\pm$ 6*	137 $\pm$ 5

Data are mean + SEM from n = 8-10/group. \*P<0.05 compared to control.

Table 6.2 Characteristics of Ang II-infused LDLr<sup>-/-</sup> mice administered vehicle or PCB77.

Parameter	PCB77	Control
Body weight (g)	26.95 $\pm$ 0.48	24.9 $\pm$ 0.62
Liver weight (g)	5.39 $\pm$ 0.26*	4.67 $\pm$ 0.20
Cholesterol (g/dL)	1580 $\pm$ 128	1461 $\pm$ 71
Systolic blood pressure mmHg	144 $\pm$ 3*	138 $\pm$ 2

Data are mean + SEM from n = 8-10/group. \*P<0.05 compared to control.

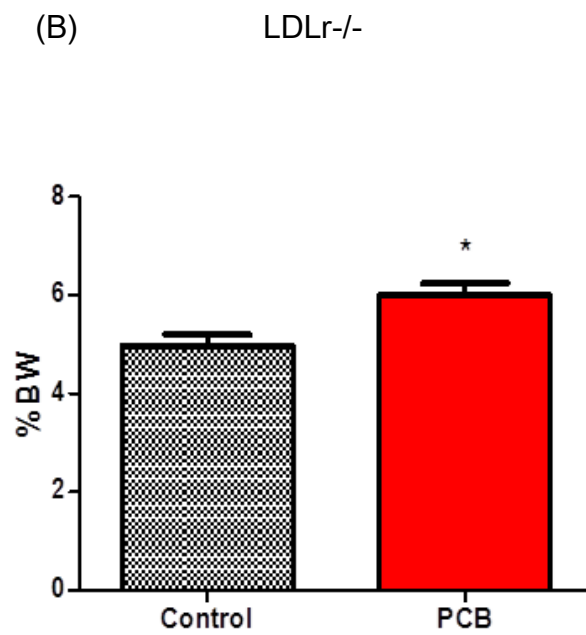
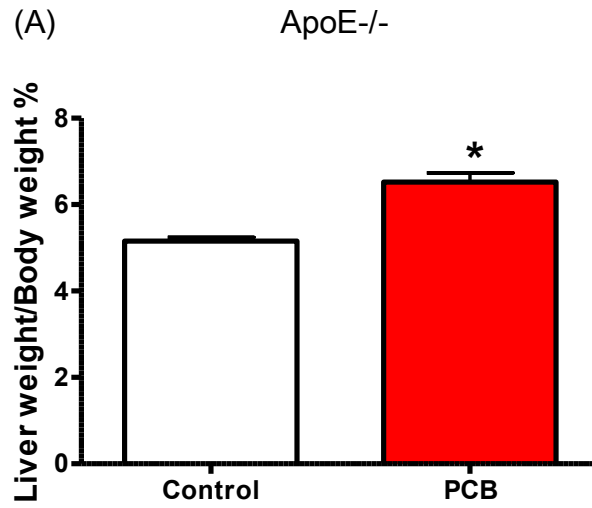


Figure 6A.1: Effect of PCB77 on liver wt/body wt % of ApoE<sup>-/-</sup> mice (A) and LDLr<sup>-/-</sup> mice (B). Data are mean  $\pm$  SE from 10 mice per group. \*P<0.05 compared to vehicle.

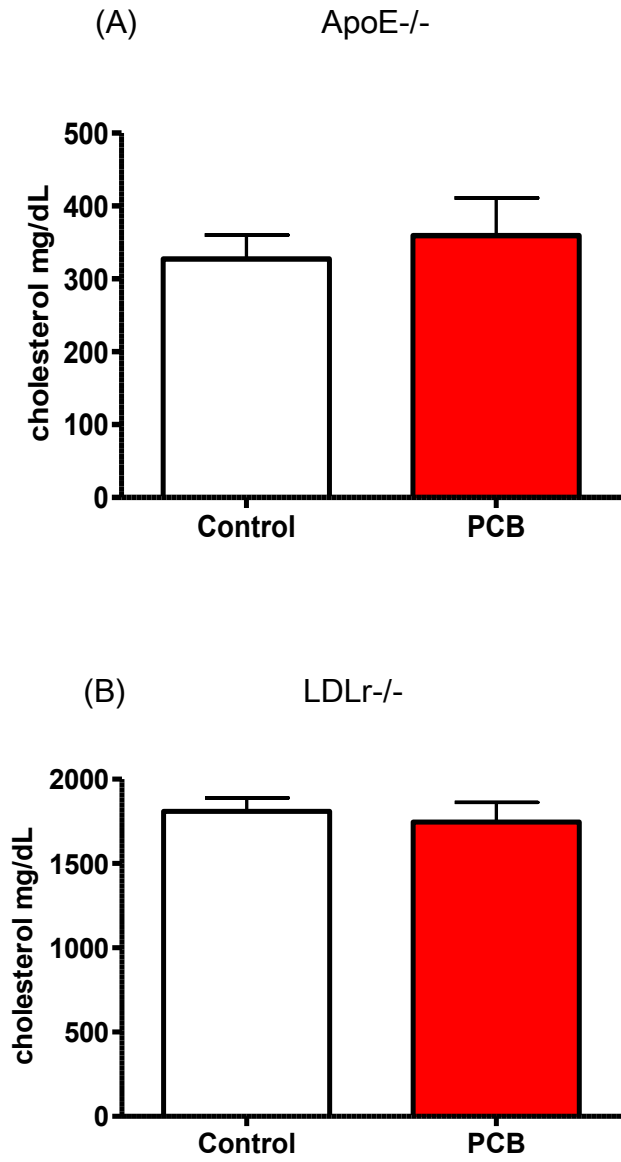


Figure 6A.2: Total Serum Cholesterol Concentrations in Control and Treated ApoE<sup>-/-</sup> (A) and LDLr<sup>-/-</sup> (B) mice.

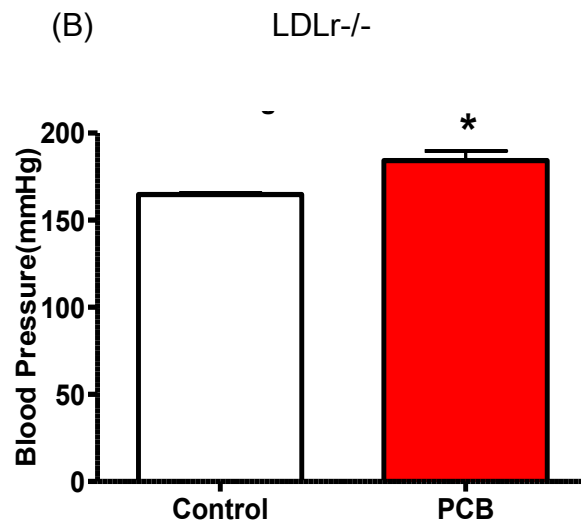
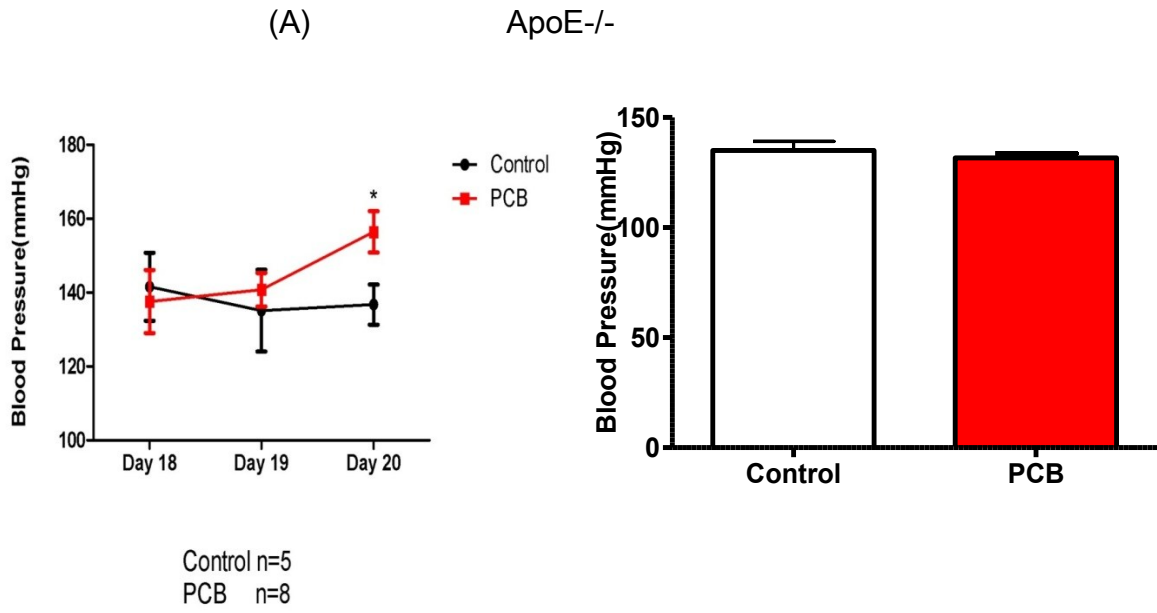
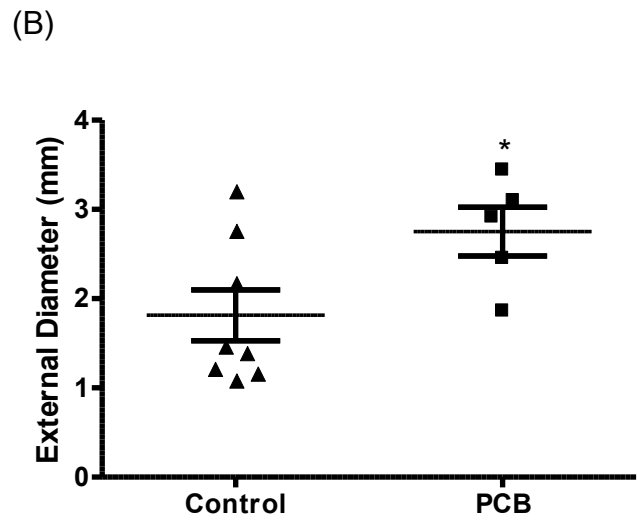
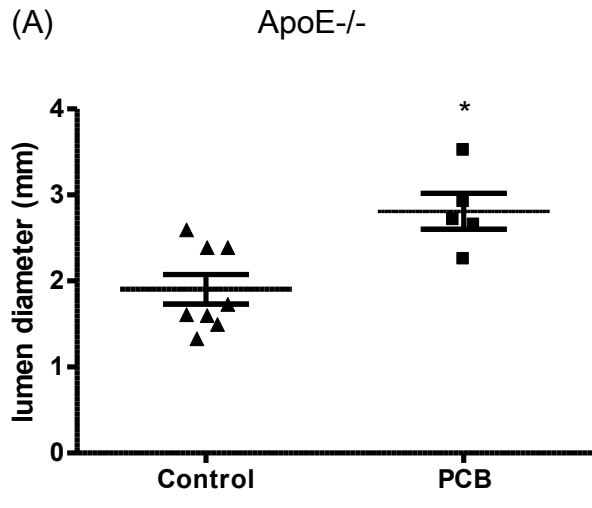


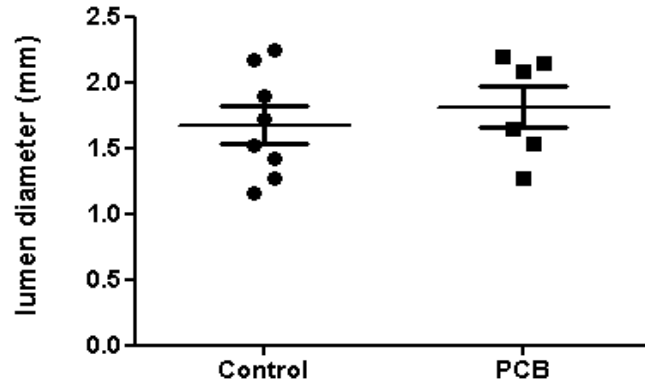
Figure 6A.3: Systolic blood pressure measurements in ApoE<sup>-/-</sup> (A) and LDLr<sup>-/-</sup> (B) mice administered vehicle or PCB77. Data are mean  $\pm$  SE from 10 mice per group. \*Significantly different from vehicle ( $p < 0.05$ ).

6.A.2 PCB77 increases lumen and external diameters of suprarenal aortas in ApoE<sup>-/-</sup> mice while it caused a reduction in external diameter of aortas from LDLr<sup>-/-</sup> mice.

Aortic lumen diameters of the suprarenal aorta were measured by high resolution ultrasound prior to Ang II infusion (baseline) and at regular intervals after Ang II infusion (day 7, 14 and 28). An increase of 50% above the baseline lumen diameter was considered as the formation of an aneurysm. Aortic lumen diameter was significantly increased in the Ang II infused ApoE<sup>-/-</sup> mice administered PCB77 (Figure 6A.4 (A), but there was no significant effect of PCB77 on the aortic lumen diameters of LDLr<sup>-/-</sup> mice (Figure 6A.4 (C). Maximal external diameters were measured on cleaned, excised suprarenal aortas. In ApoE<sup>-/-</sup> mice infused with Ang II, external diameters were significantly increased by PCB77 (Figure 6A.4 (B). In contrast, there was a decrease in external diameters of aortas from the LDLR<sup>-/-</sup> mice administered PCB77 (Figure 6A.4 (D).



(C)



(D)

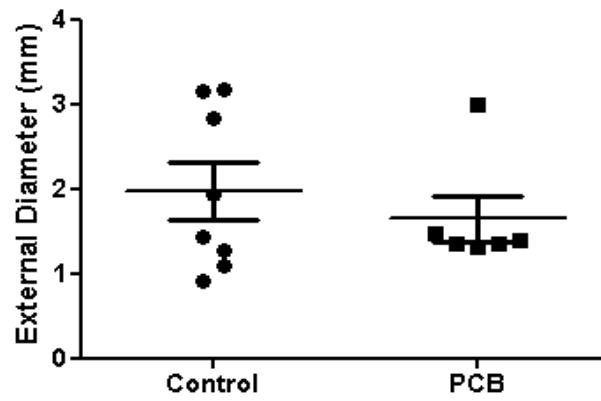


Figure 6A.4 Effect of PCB77 administration on lumen diameter in ApoE<sup>-/-</sup> (A) and LDLr<sup>-/-</sup> (C) and external diameter in ApoE<sup>-/-</sup> (B) and LDLr<sup>-/-</sup> (D). Data are mean  $\pm$  SE from 10 mice per group. \*P<0.05 compared to vehicle.



6.A.3 Significantly increased AAA severity, rupture, and decreased percent survival were observed for PCB77-treated ApoE<sup>-/-</sup> mice.

AAAs that formed in both strains of mice were examined by two observers blinded to the experimental design. The AAAs were categorized into 4 groups based on severity (Type I- less severe to Type IV- Rupture). PCB77 induced a higher AAA incidence and increased aortic rupture in ApoE<sup>-/-</sup> mice. Only 60% of mice in the PCB77 group survived while 80% of the control mice survived (Figure 6A.5).

An increase in the incidence and rupture was also observed in the PCB77 treated LDLr<sup>-/-</sup> mice but this difference was modest and not significant (Figure 6A.6).

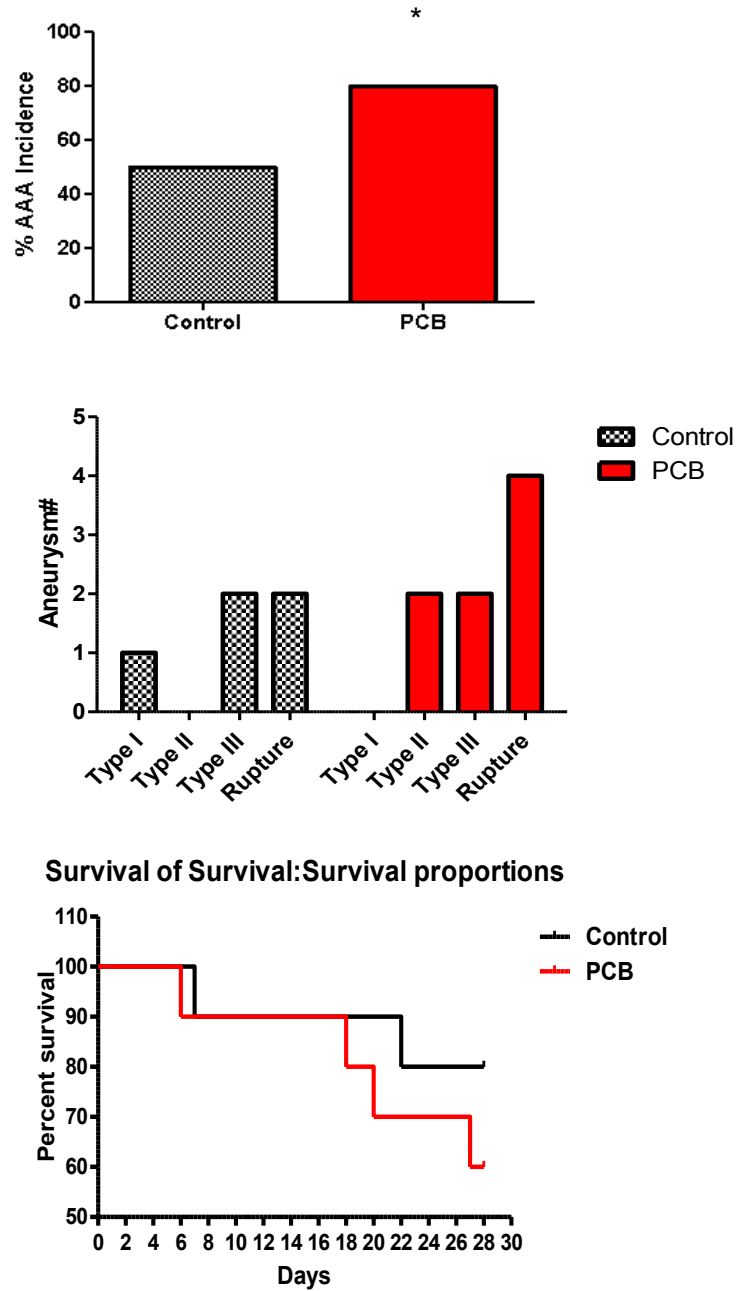


Figure 6A.5 AAA Incidence Severity, Rupture, and Percent Survival For PCB77-treated Ang II infused ApoE<sup>-/-</sup> mice.\*P<0.05 compared to vehicle

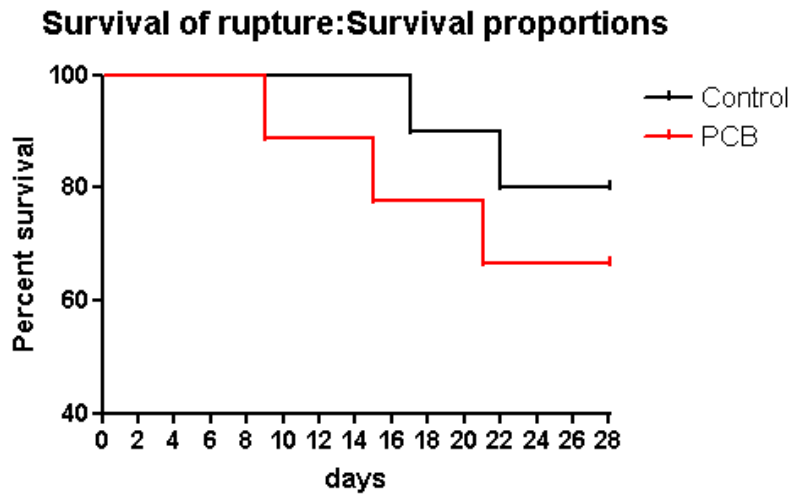
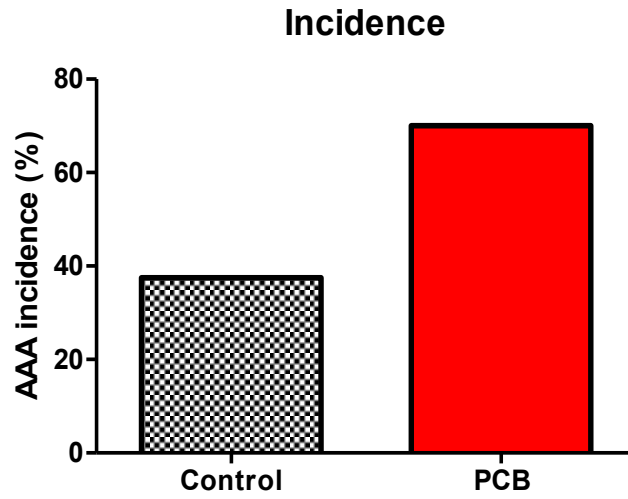


Figure 6A.6 AAA Incidence and Percent Survival for PCB77-treated Ang II infused LDLr<sup>-/-</sup> mice

6. A.4 Effect of PCB77 on AngII induced Atherosclerosis in ApoE<sup>-/-</sup> and LDLr<sup>-/-</sup> mice.

Ang II infusion has been described previously to augment atherosclerotic lesion development in male ApoE<sup>-/-</sup> mice (Daugherty et al, 2000). The atherosclerotic lesion area was determined by en face analysis of the aortic arch and thoracic aorta. An intimal surface covered by grossly discernible lesions was determined and is expressed as a percentage of the total intimal area. PCB77 administration in addition to Ang II infusion significantly increased atherosclerotic lesions in the aortic arch of ApoE<sup>-/-</sup> mice (Figure 6A.7,6A.8), but in contrast fewer atherosclerotic lesions were seen in the LDLr<sup>-/-</sup> mice (Figure 6A.9).

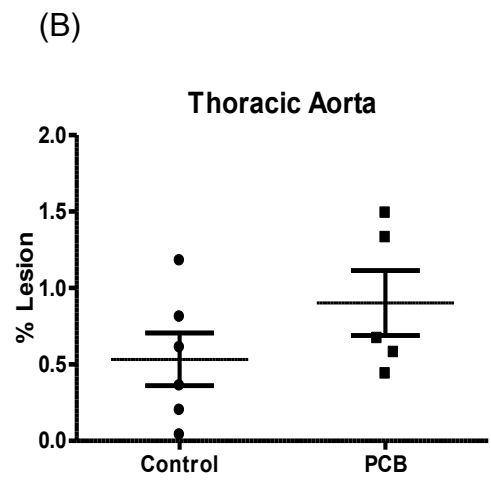
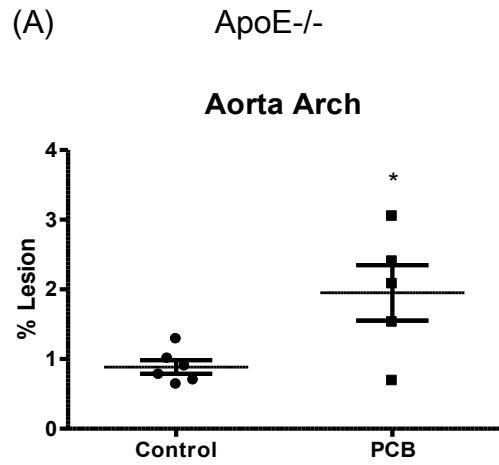


Figure 6A.7 Quantification of atherosclerosis in aortic arch (A) and thoracic aorta (B) in PCB77 treated male ApoE<sup>-/-</sup> mice. Data are mean ± SE from 10 mice per group. \*P<0.05 compared to vehicle.

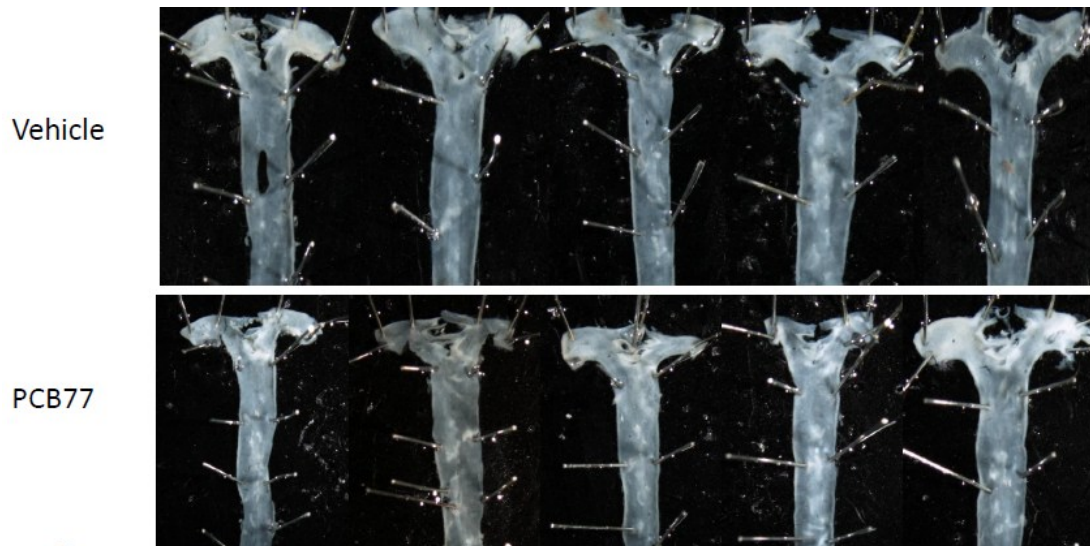


Figure 6A.8 Intimal surface areas of aortas from Ang II infused vehicle and PCB77 treated ApoE<sup>-/-</sup> mice. Lesions are opaque areas (white) on translucent intimal surface.

LDLr-/-

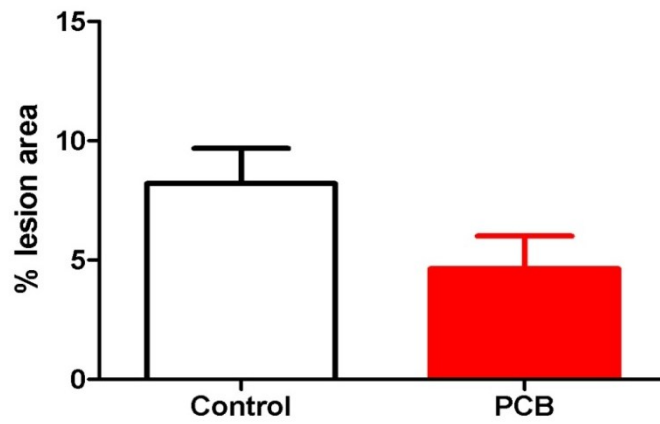


Figure 6A.9 Quantification of atherosclerosis in aortic arch (A) in vehicle and PCB77 treated male LDLr-/- mice. Data are mean  $\pm$  SE from 8 mice per group

## B) STUDY 2

This study was conducted only in the LDLr<sup>-/-</sup> mice. The mice received all PCB77 or vehicle injections prior to AngII infusion.

6.B.1 PCB77 had no effect on body weight and serum cholesterol but significantly increased systolic blood pressure in LDLr<sup>-/-</sup> mice infused with Ang II.

LDLr<sup>-/-</sup> mice treated with PCB77 prior to Ang II infusion did not show any effect on body weights and serum cholesterol (Figure 6B.1) similar to study 1. Also these PCB77 treated LDLr<sup>-/-</sup> mice had a significantly increased systolic blood pressure (Figure 6B.2) similar to findings in study 1. Hence the timing of the PCB injections had similar effects on the blood pressure in both the studies.



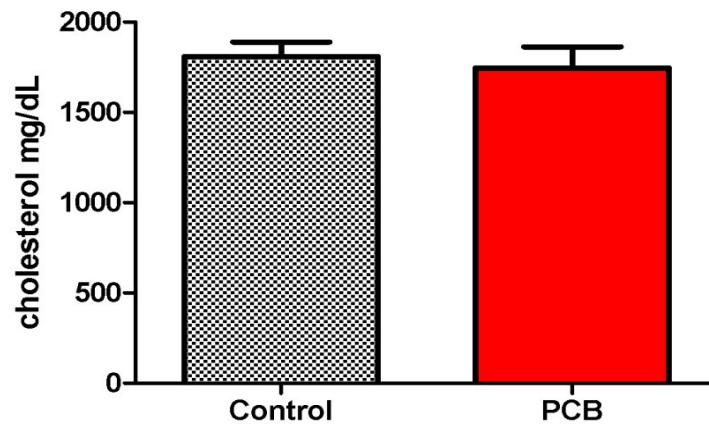


Figure 6B.1 Total serum cholesterol concentrations in control and PCB77-treated LDLr<sup>-/-</sup> mice.

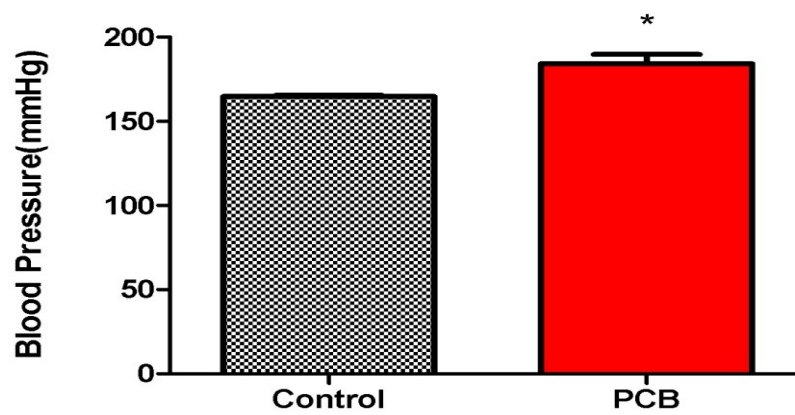


Figure 6B.2 Systolic blood pressure measurements of LDLr<sup>-/-</sup> mice administered vehicle or PCB77. Data are mean  $\pm$  SE from 10 mice per group. \*P<-0.05 compared to vehicle.

6. B.2 PCB77 significantly decreased the external diameter of suprarenal aortas from Ang II infused LDLr<sup>-/-</sup> mice.

Aortic lumen diameters remained unaffected when LDLr<sup>-/-</sup> mice were treated with PCB77 prior to Ang II infusion (Figure 6B.3A). Surprisingly, the external aortic diameter was significantly decreased in Ang II infused LDLr<sup>-/-</sup> administered PCB77 compared to control (Figure 6B.3B).

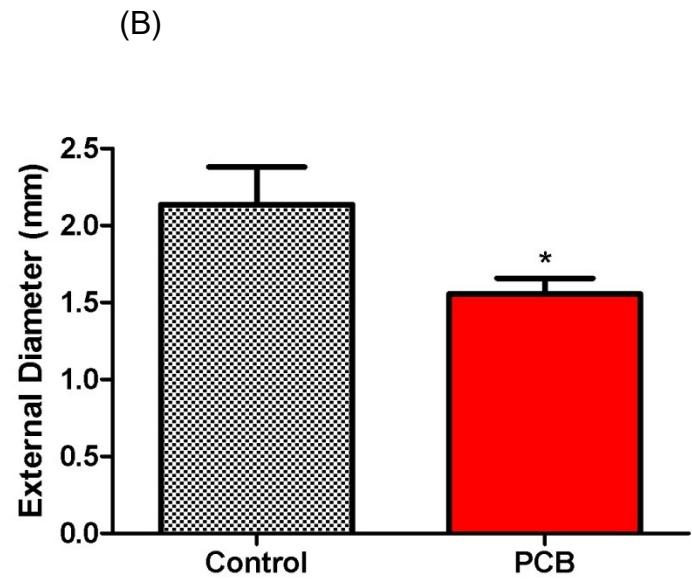
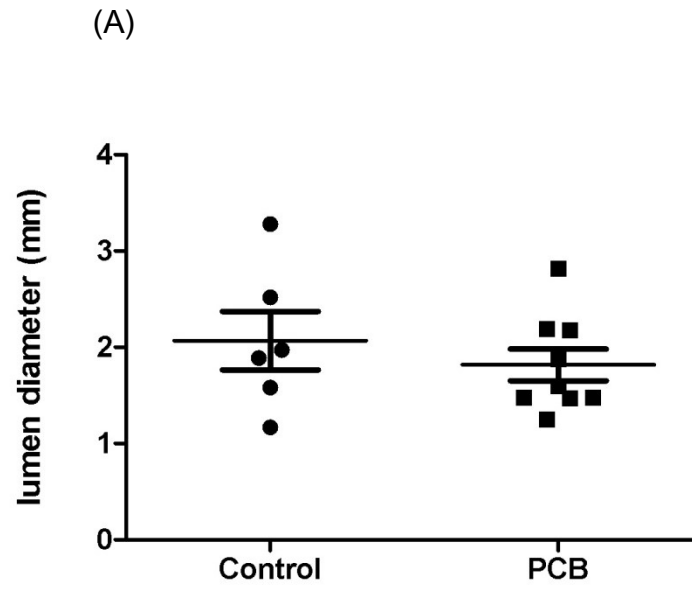
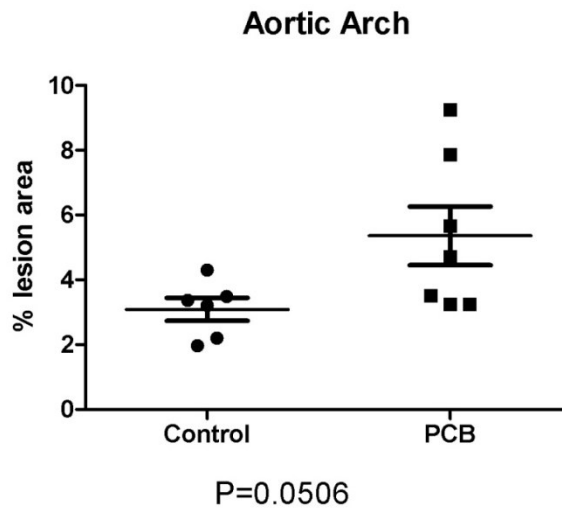


Figure 6B.3 Effect of PCB77 administration when given prior to Ang II infusions on lumen diameter (A) and external diameter (B) of suprarenal aortas from LDLr<sup>-/-</sup> mice. Data are mean  $\pm$  SE from 10 mice per group. \*P<0.05 compared to control.

6. B.3 Increase in atherosclerotic lesion formation was observed when compared to the previous study in PCB77 treated Ang II infused LDLr<sup>-/-</sup> mice.

In contrast to findings from study 1, when administered prior to infusions of Ang II, PCB77 resulted in a profound increase in atherosclerotic lesion formation in the aortic arch though the difference was not significant (Figure 6B.4).

(A)



(B)

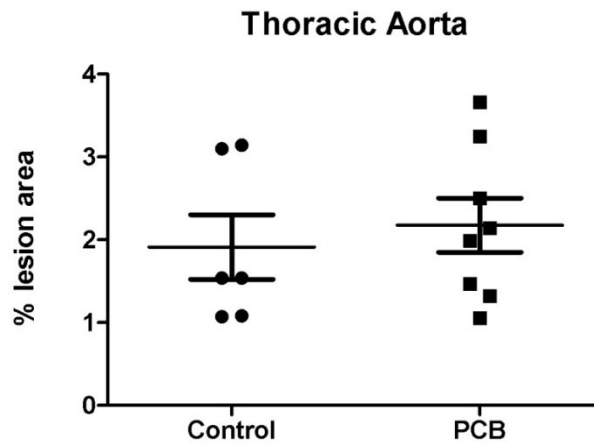


Figure 6B.4 Quantification of atherosclerosis in aortic arch (A) and thoracic aorta (B) in vehicle and PCB77 treated male LDLr<sup>-/-</sup> mice. Data are mean ± SE from 8 mice per group.

## CHAPTER 7: DISCUSSION

In this study we examined the effects of coplanar PCB77 on Ang II induced vascular diseases including atherosclerosis and AAAs in two different hyperlipidemic mouse strains, ApoE<sup>-/-</sup> and LDLr<sup>-/-</sup> mice. The timing interval of PCB77 administration was also examined in LDLr<sup>-/-</sup> mice. Results demonstrated that when PCB77 was administered prior to and during Ang II infusions in ApoE<sup>-/-</sup> mice it increased systolic blood pressure, aortic lumen diameters and external diameters of AAAs, and also increased the severity, rupture and incidence of AAAs. Also profound atherosclerotic lesions were seen in the ApoE<sup>-/-</sup> mice administered PCB77. No effect of PCB77 was observed on body weight and serum cholesterol in either mouse strain. In study 2, when PCB77 was administered prior to Ang II infusion in LDLr<sup>-/-</sup> mice, the results demonstrated increase in liver weights. Moreover, systolic blood pressure was increased in LDLr<sup>-/-</sup> mice regardless of the timing of PCB77 administration. However, in contrast to results from study 1, PCB77 administration prior to infusions of AngII

unexpectedly reduced AAA incidence and size. Our results suggest that background strain, use of high fat diets, and timing of PCB77 administration in reference to infusions of AngII influences the development of atherosclerosis and AAAs.

Ang II, the most important effector molecule of the RAS, plays a role in the pathophysiology of vascular disorders like atherosclerosis and AAAs. Previous studies show when Ang II is infused in hyperlipidemic mice like ApoE<sup>-/-</sup> and LDLr<sup>-/-</sup> mice at a higher dose of 1000ng/kg/min for 28days it leads to development of atherosclerosis and AAAs (Daugherty et al, 2000). Hence these mice serve as good models to study Ang II induced vascular diseases. A major difference between these mouse strains is the requirement of consumption of a western diet in LDLr<sup>-/-</sup> mice, but not in ApoE<sup>-/-</sup> mice, to induce atherosclerotic lesion formation. Hence nutrient composition of the diets used in this study was one variable under study in defining effects of PCB77. Also PCB77 being lipophilic in nature was probably getting sequestered in the adipose tissue in

mice that were fed with the western diet, which could be a possible reason for the varied results seen between the two mouse models based on the nutrient composition of the diets.

Coplanar PCBs also known as dioxin like compounds are AHR agonists. Due to being persistent in nature, they were banned in United States in 1977. These compounds are yet found in all parts of the global ecosystem along with substances like food, breast milk, oily fish etc. Evidence shows that PCBs are proinflammatory and are implicated in a number of cardiovascular diseases especially hypertension and atherosclerosis. Earlier experiments in our laboratory demonstrated that PCB77 caused notable atherosclerotic lesions in the aortic arch (Arsenescu et al 2008).

Evidence from previous studies show that PCBs at a higher dose mimic effects of the wasting syndrome and also cause weight loss. Hence for the present study PCB dosage was similar to the earlier studies in our laboratory (four injections of



49mg/kg per dose in 6 weeks of study duration) (Arsenescu et al, 2008). This dosage has shown to produce proinflammatory effects on endothelial cells (Toborek et al, 1995) and adipocytes (Arsenescu et al 2008) when injected in mice. Also this dose is considered as moderate exposure in experimental animals. (Sipka et al, 2008)

To the best of our knowledge this is the first study that examines the effect of coplanar PCBs on Ang II induced vascular disorders especially AAAs. PCBs are shown to promote adipocyte differentiation and inflammatory gene expression in adipocytes. (Arsenescu et al, 2008) In a separate independent study in our lab (Police et al, 2009) results showed that the type of adipocytes surrounding aortic regions and the ability of white adipocytes to recruit macrophages may localize AAAs to abdominal aortas of AngII-infused mice. Hence we hypothesized that PCBs promote obesity causing adipocyte differentiation which by recruitment of macrophages influence the formation of AAAs in Ang II infused mice.

PCB77 administration along with AngII infusion caused a significant increase in the lumen diameter and external aortic diameter in the ApoE<sup>-/-</sup> mice but not in the LDLr<sup>-/-</sup> mice. Surprisingly even when the timing interval of PCB77 administration was altered instead of an increase in AAA formation a significant decrease in external aortic diameter was observed. This may be due to the lipophilic nature of the PCB77. The western diet, which causes an increase in the adipose mass, maybe sequestering the PCBs and preventing them from further accentuating the AAA formation.

Surprisingly in the present study no effect was seen on body weight in both PCB treated ApoE<sup>-/-</sup> and LDLr<sup>-/-</sup> mice. No changes were seen on average body weights between control and PCB treated mice. Ang II causes weight loss in rats in a pressor independent mechanism (Brink et al, 1996). This weight loss is due to reduced caloric intake or maybe due to an added metabolic effect. We did not observe weight gain or loss in mice infused with AngII and administered PCB77,

suggesting that effects of PCB77 (increase body weight) and Ang II infusions (decrease body weight) may have been counterbalanced.

Previous investigators have shown that PCBs increase hepatic cholesterol synthesis which results in hypercholesterolemia in rats (Nakagawa et al, 1986).

In the present study, in the PCB treated ApoE<sup>-/-</sup> mice as well as LDLr<sup>-/-</sup> mice did not show any changes in total serum cholesterol concentrations despite of being on a high fat Western diet. This may be due to Ang II infusion influencing the effect of PCB77. Also in the LDLr<sup>-/-</sup> mice this lack of effect would be dietary interactions between the high fat diet and PCBs. PCBs being highly lipophilic and may get sequestered in the high fat diet, resulting in an absence of effect. It is also possible that LDLr<sup>-/-</sup> mice that are fed on a high fat diet have serum cholesterol concentrations that are very high producing a saturating effect for PCBs to induce any further changes in these levels.

Both Mouse models also exhibited modifications in liver weights in the PCB treated group. In the past Hennig and coworkers have shown that when wild type mice were fed a high fat diet (40% linoleic acid) and exposed to PCB77 there was a significant increase in liver-to-body weight ratio (Hennig et al, 2005). Earlier studies have also shown that rats fed a high fat diet can induce hepatotoxic responses to PCB77 and PCB153 including lipid peroxidation and hepatomegaly (Fadhel et al, 2002). Hence this study extends the findings of the previous studies.

PCB77 caused an increase in systolic blood pressure in LDLr<sup>-/-</sup> as well as ApoE<sup>-/-</sup> mice. AHR ligand 2,3,7,8-tetrachlorodibenzop-dioxin (TCDD) increased blood pressure in adult male C57BL/6 mice when dosed with vehicle or 300 ng TCDD/kg by oral gavage three times per week for 60 days (Kopf et al., 2008).

Earlier studies have also shown that AHR null mice developed cardiac hypertrophy and elevated mean arterial pressure due to increased levels of systemic Ang II and ET-1. (Zhang et al, 2010) Similar results were demonstrated

in studies by other investigators where AHR deficient mice at modest altitude (1632m) demonstrated increase in mean arterial pressure that was associated with increased systemic concentrations of AngII (Lund et al., 2003; Lund et al., 2008). However, in later studies by this group results indicated that AHR null mice are hypotensive at low altitude (225m) and blockage of the RAS showed minimal blood pressure control, suggesting normal blood pressure in heterozygous AHR (+/-) mice appears to be maintained by increased RAS, while hypotension in null AHR (-/-) mice may result from decreased RAS signaling (Zhang et al, 2010; Lund et al 2008). Result from this study supports these earlier studies indicating that exposure to an AHR ligand PCB77 caused elevations in systolic blood pressure after infusion with Ang II. Although earlier studies in our lab (Cassis et al, 2009) have demonstrated that Ang II mediated elevation in blood pressure is not implicated in Ang II induced atherosclerosis and AAAs. Hence it can be summarized that even though exposure to PCB 77 causes an

increase in systolic blood pressure that in turn would not induce Ang II mediated vascular disorders.

An increase in atherosclerotic lesion formation in the aortic arch was observed in ApoE<sup>-/-</sup> mice and LDLr<sup>-/-</sup> though more marked effects were observed in ApoE<sup>-/-</sup> mice. A number of studies indicate that coplanar PCBs play a role in atherosclerotic lesion formation either by endothelial dysfunction, oxidative stress, inflammatory cytokine expression (Toborek et al, 1995; Hennig et al, 2002) and increased lesion formation in ApoE<sup>-/-</sup> mice (Arsenescu et al, 2008). Coplanar PCBs (PCB 77 and PCB 114) significantly disrupt endothelial barrier function by allowing an increase in albumin transfer across endothelial monolayers and also cause cellular oxidative stress (Toborek et al 1995). Exposure to coplanar PCBs also increased the expression of inflammatory cytokines like cytochrome 450, VCAM-1, IL-6 etc. (Toborek et al, 1995; Hennig et al, 2002) and induced expression of VCAM in aortas of mice. (Hans et al, 2010). Ang II is also known to show similar effects and has been implicated in promoting

vascular disorders like atherosclerotic lesion formation. Hence the results of the present study indicates that exposure to PCB77 along with infusion of Ang II may have an additive effect to increase atherogenic lesion formation.

Also the lack of effect in the LDLR<sup>-/-</sup> mice after PCB injections were administered prior to Ang II infusion maybe due to feedback inhibition that may have occurred due to increased production of cyp1A1, cyp1A2 and cyp1B1. When PCBs were administered all together this increased the levels of these cytochrome P450's and they in turned inhibited their transcription blunting the effect of PCBs in these mice.

Exposure to PCB77 influenced the formation of AAAs increasing the severity and rupture in both mice models. The timing interval of PCB administration failed to see a profound effect on AAA development, surprisingly a significant decrease in external aortic diameter was seen but increased percent atherosclerotic lesion development in LDLr<sup>-/-</sup> mice. PCB77 primarily promoted more severe AAAs, with

increased aortic rupture in both mouse models. These effects of PCB77 were more pronounced when PCB77 was administered during AngII infusions



## CHAPTER 8: CONCLUSION

In conclusion, at low exposure levels coplanar PCB77 promoted the formation of Ang II induced vascular disorders namely atherosclerosis and abdominal aortic aneurysms in two hyperlipidemic mouse strains, ApoE<sup>-/-</sup> and LDLr<sup>-/-</sup> mice. The timing interval for PCB administration played a pivotal role in seeing profound effects on these vascular conditions. Peripheral parameters like body weights and serum cholesterol concentrations were not affected in ApoE<sup>-/-</sup> mice and LDLr<sup>-/-</sup> mice. In spite of no changes in cholesterol levels, systolic blood pressure was markedly increased in ApoE<sup>-/-</sup> mice as well as in LDLr<sup>-/-</sup> mice despite of the timing of PCB administration. Incidence of AAAs, severity and rupture of AAAs were evident in both mouse strains but were dependent on the timing of the PCB injections in LDLr<sup>-/-</sup> mice. These results suggest that the timing of PCB77 administration, in addition to dietary interactions, influence PCB77 augmentation of AngII-induced vascular diseases. Studies focusing on the mechanisms by which the timing of PCB77 administration influences the ability of PCB77 to

promote vascular diseases would be beneficial in shedding some light to these results. Also treatment that involves minimizing or limiting exposure to PCBs would be beneficial in reducing the incidence of these vascular disorders.

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## VITA

Madhura Parulkar was born in Bombay (now Mumbai), India on 4<sup>th</sup> September 1981. She received the degree for Bachelor of Science (Food, Nutrition and Dietetics) in Mumbai from Nirmala Niketan College of Homescience, affiliated to Mumbai University in May 2002. She has also completed a post graduate diploma in Dietetics from Shreemati Nathibai Damodar Thackersy College of Homescience Pune. She joined the Nutritional Sciences Master's program at the University of Kentucky in August 2007.

