MODULATION OF ENDOTHELIAL CELL ACTIVATION BY OMEGA-6 AND OMEGA-3 FATTY ACIDS

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MODULATION OF ENDOTHELIAL CELL ACTIVATION
BY OMEGA-6 AND OMEGA-3 FATTY ACIDS

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate Center for Nutritional Sciences at the University of Kentucky

By
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Lexington, Kentucky
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Lexington, Kentucky
2007

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Endothelial activation is considered to be an early and critical event in the pathology of atherogenesis which can be modified by environmental factors such as diet, pollutants, and lifestyle habits. Dietary ω-6 and ω-3 fatty acids have been reported to either amplify or diminish inflammatory responses related to atherosclerosis development. However, the interactions of ω-6 and ω-3 fatty acids with inflammatory cytokines or organic pollutants on endothelial cell activation are not well understood. The studies presented in this dissertation tested the hypothesis that ω-6 and ω-3 fatty acids alone, or in varying ratios can differently modulate pro-atherogenic mediators and inflammatory responses that are initiated by tumor necrosis factor-α (TNF-α) or polychlorinated biphenyls (PCBs) in endothelial cells. Exposure to TNF-α induced oxidative stress, p38 MAPK, NF-κB, COX-2 and PGE2, which was amplified by pre-enrichment with linoleic acid but blocked or reduced by α-linolenic acid. Furthermore, TNF-α-induced caveolin-1 up-regulation and the co-localization of TNF receptor-1 with caveolin-1 was markedly increased in the presence of linoleic acid and diminished by α-linolenic acid. Silencing of the caveolin-1 gene completely blocked TNF-α-induced production of COX-2 and PGE2 and significantly reduced the amplified response of linoleic acid plus TNF-α. These data suggest that omega-6 and omega-3 fatty acids can differentially modulate TNF-α-induced inflammatory stimuli and that caveolae and its fatty acid composition play a regulatory role in these observed metabolic events. Besides cytokines, lipophilic environmental contaminants such as PCBs can also trigger inflammatory events in endothelial cells. Our data suggest that increasing the relative amount of α-linolenic acid to linoleic acid can markedly decrease oxidative stress and NF-κB-responsive genes. The inhibitor study revealed that the modulation effect of ω-6 and ω-3 fatty acids on PCB toxicity was mainly through the oxidative stress sensitive transcription factor, NF-κB. In conclusion, our studies demonstrate that different dietary fats can selectively modulate vascular cytotoxicity caused by TNF-α as well as by persistent organic pollutants such as PCBs. We also demonstrated the important relevance of substituting dietary ω-3 fatty acids with ω-3 fatty acids.
acids such as α-linolenic acid for ω-6 fatty acid such as linoleic acid in reducing cardiovascular diseases.

KEYWORDS: Atherosclerosis, fatty acids, inflammation, TNF-α, PCB
MODULATION OF ENDOTHELIAL CELL ACTIVATION BY OMEGA-6 AND OMEGA-3 FATTY ACIDS

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## TABLE OF CONTENTS

Acknowledgments.............................................................................................................. iii
List of Tables ..................................................................................................................... vi
List of Figures ................................................................................................................... vii

**Chapter One : Introduction**
- Endothelial Cell Activation and Atherosclerosis........................................................ 1
- Dietary Fatty Acids and Atherosclerosis ...................................................................... 1
- Pro-Inflammatory Cytokine TNF-α-Stimulated Endothelial Activation ..................... 3
- Significance of Caveolae in Fatty acids and TNF-α Mediated Endothelial Cell Activation.................................................................................................................... 6
- PCB-Induced Endothelial Cell Activation.................................................................. 9
- Hypothesis and Significance of the Current Study................................................... 10

**Chapter Two : Effects of ω-6 and ω-3 Fatty Acids on TNF-α-Induced Endothelial Cell Activation**.......................................................................................................................... 15
- Synopsis .................................................................................................................... 15
- Introduction ............................................................................................................... 16
- Materials and Methods ............................................................................................ 19
- Results ....................................................................................................................... 23
- Discussion ................................................................................................................. 44

**Chapter Three : Involvement of Caveolae in Regulation of Endothelial Cell Activation Mediated by Fatty Acids and TNF-α**.......................................................................................................................... 47
- Synopsis .................................................................................................................... 47
- Introduction ............................................................................................................... 48
- Materials and Methods ............................................................................................ 49
- Results ....................................................................................................................... 52
- Discussion ................................................................................................................. 65

**Chapter Four : Changing Ratios of ω-6 to ω-3 Fatty Acids Can Differentially Modulate Polychlorinated Biphenyl Toxicity in Endothelial Cells** .................................................. 68
- Synopsis .................................................................................................................... 68
- Introduction ............................................................................................................... 68
- Materials and Methods ............................................................................................ 71
- Results ....................................................................................................................... 72
- Discussion ................................................................................................................. 90

**Chapter Five : Summary** ........................................................................................... 94
- Conclusion ................................................................................................................ 94
- Future Directions ...................................................................................................... 97

**Appendix: Protocols**........................................................................................................ 100
- Primary Culture of Porcine Pulmonary Artery Endothelial Cells .......................... 100
- Freeze Endothelial Cells ....................................................................................... 101
- Start a Frozen Endothelial Cell Line ....................................................................... 102
- Cell Culture and Experimental Media ..................................................................... 102
- Preparation of Fatty Acid Enriched Media ............................................................. 103
- Oxidative Stress Measurement .............................................................................. 104
- PGE₂ EIA kit ........................................................................................................... 105
- Immunofluorescence .............................................................................................. 108
- Confocal Microscopy .............................................................................................. 110
List of Tables

Table 1.1  Major dietary polyunsaturated fatty acids and sources............................ 12
List of Figures

Figure 1.1 Major TNF receptor-mediated cellular responses ........................................ 13
Figure 1.2 Organization of lipid rafts and caveolae membranes ............................ 14
Figure 2.1 Optimal TNF-α concentration and incubation time for E-selectin expression ........................................................................................................ 28
Figure 2.2 Immunofluorescence microscopy study of TNF-α-induced E-selectin expression ......................................................................................................... 28
Figure 2.3 TNF-α-induced VCAM-1 and COX-2 expression .................................. 29
Figure 2.4 Optimal linoleic acid incubation time for VCAM-1 gene expression... 30
Figure 2.5 Effect of linoleic acid and α-linolenic acid on TNF-α-induced E-selectin gene expression .................................................................................................. 31
Figure 2.6 Effect of linoleic acid and α-linolenic acid on TNF-α-induced COX-2 gene expression ................................................................................................. 32
Figure 2.7 Effect of linoleic acid and α-linolenic acid on TNF-α-induced COX-2 protein expression ................................................................................................. 33
Figure 2.8 Effect of linoleic acid and α-linolenic acid on TNF-α-induced oxidative stress ................................................................................................................. 34
Figure 2.9 Effect of linoleic acid and α-linolenic acid on TNF-α-induced activation of NF-κB ........................................................................................................... 35
Figure 2.10 Effect of linoleic acid and α-linolenic acid on TNF-α-induced p38 MAPK activation ......................................................................................................... 36
Figure 2.11 Effect of p38 MAPK inhibitor on TNF-α-induced COX-2 up-regulation ......................................................................................................................... 37
Figure 2.12 Effect of p38 MAPK inhibitor on TNF-α- and linoleic acid-induced VCAM-1 up-regulation ......................................................................................... 38
Figure 2.13 Effect of TNF-α on cPLA2 activation ...................................................... 39
Figure 2.14 Effect of linoleic acid and α-linolenic acid on PGE2 production ........ 40
Figure 2.15 Optimal TNF-α incubation time for PGE2 production ......................... 41
Figure 2.16 Effect of linoleic acid and α-linolenic acid on TNF-α-induced PGE2 production ................................................................................................................. 42
Figure 2.17 Proposed mechanism for fatty acid-mediated modulation of endothelial cell activation induced by TNF-α ......................................................... 43
Figure 3.1 Effect of linoleic acid and α-linolenic acid on caveolin-1 expression ... 56
Figure 3.2 Immunofluorescence microscopy study of TNF-α-induced caveolin-1 up-regulation ................................................................................................. 57
Figure 3.3 Effect of linoleic acid and α-linolenic acid on TNF-α-induced TNFR-1 and caveolin-1 localization ............................................................................. 58
Figure 3.4 Effect of linoleic acid and α-linolenic acid on TNF-α-induced caveolin-1 expression ......................................................................................................... 59
Figure 3.5 Effects of fatty acids on TNF-α-induced caveolin-1 redistribution ...... 60
Figure 3.6 Effects of fatty acids on TNF-α-induced caveolin-1 redistribution ...... 61
Figure 3.7 Effect of caveolin-1 silencing on TNF-α-induced caveolin-1 up-regulation ....................................................................................................................... 62
Figure 3.8  Effect of caveolin-1 silencing on linoleic acid and TNF-α-induced caveolin-1 expression ................................................................. 62
Figure 3.9  Effect of caveolin-1 silencing on linoleic acid and TNF-α-induced COX-2 expression ................................................................. 63
Figure 3.10 Effect of caveolin-1 silencing on linoleic acid and TNF-α-induced PGE2 production ................................................................. 64
Figure 4.1  Effect of different ratios of linoleic acid to α-linolenic acid on PCB77-induced oxidative stress ...................................................... 76
Figure 4.2  Effect of different ratios of linoleic acid to α-linolenic acid on the activation of NF-κB ................................................................. 77
Figure 4.3  Effect of different ratios of linoleic acid to α-linolenic acid on PCB77-induced activation of NF-κB ...................................................... 78
Figure 4.4  Effect of different ratios of linoleic acid to α-linolenic acid on PCB77-induced VCAM-1 gene expression ........................................ 79
Figure 4.5  Effect of different ratios of linoleic acid to α-linolenic acid on PCB77-induced VCAM-1 protein expression ........................................ 80
Figure 4.6  Effect of PCB77 on cPLA2 phosphorylation ........................................ 81
Figure 4.7  Effect of PCB77 on COX-2 expression .......................................... 81
Figure 4.8  Effect of PCB77 on PGE2 synthase expression ................................ 82
Figure 4.9  Effect of different ratios of linoleic acid to α-linolenic acid on PCB77-induced COX-2 gene expression .......................................... 83
Figure 4.10 Effect of different ratios of linoleic acid to α-linolenic acid on PCB77-induced COX-2 protein expression .......................................... 84
Figure 4.11 Optimal PCB77 incubation time for PGE2 production .................. 85
Figure 4.12 Effect of different ratios of linoleic acid to α-linolenic acid on PCB77-stimulated release of PGE2 ..................................................... 86
Figure 4.13 Effect of NF-κB inhibitor on PCB77- or linoleic acid-induced VCAM-1 expression ................................................................. 87
Figure 4.14 Effect of NF-κB inhibitor on PCB77- or linoleic acid-induced COX-2 expression ................................................................. 88
Figure 4.15 Proposed mechanism for fatty acid-mediated modulation of endothelial cell activation induced by PCB77 ...................................... 89
Figure 5.1 Summery .................................................................................. 99
Figure 5.2 Future direction ...................................................................... 99
Chapter One: Introduction

Endothelial Cell Activation and Atherosclerosis

Cardiovascular disease is the number one killer worldwide, including in the United States. For example, from the United States Chronic Disease Overview (1999), cardiovascular disease is responsible for more than 30 percent of all the deaths in the United States, which is more than all forms of cancer which is 23 percent of deaths. Cardiovascular disease can refer to many different types of heart or blood vessel problems and atherosclerosis is the generally the cause of all these problems. Atherosclerosis is an inflammatory disease of the vessel wall which predominantly involves endothelium and vascular smooth muscle layers of the arterial wall. Vascular inflammation is a critical event in the pathogenesis of many human diseases, including atherosclerosis, hypertension, autoimmune diseases [1-3]. Progression of atherosclerosis can lead to the development of a plaque that is vulnerable to rupture and would then produce acute coronary syndromes, such as arterial thrombosis and myocardial infarction or stroke [4].

Numerous risk factors associated with atherosclerosis have been identified, such as hypertension, diabetes mellitus, and dyslipidemia. These particular cardiovascular risk factors commonly occur in obese individuals as components of the metabolic syndrome [5]. Furthermore, smoking, obesity, insulin resistance, hypertriglyceridemia, elevated levels of plasma cholesterol, low high-density lipoprotein, shear stress, and oxidative stress can also increase the risk of atherosclerosis [6, 7]. A growing body of literature also indicates that environmental factors such as diet, lifestyle habit, and nutrition factors can play an important role in the development of atherosclerosis. Substantial evidence from epidemiological studies suggests that cardiovascular diseases are linked to environmental pollution. For example, there was a significant increase in mortality from cardiovascular diseases among Swedish capacitor manufacturing workers exposed to polychlorinated biphenyls (PCBs) for at least five years [8], and most excess deaths were
due to cardiovascular disease in power workers exposed to phenoxy herbicides and PCBs in waste transformer oil [9].

Atherosclerosis depends critically on altered behavior of the intrinsic cells of the artery wall, in particular the endothelial cells. Endothelial cells are constantly exposed to blood flow and are directly in contact with blood components. These components include various kinds of cells, cytokines, toxic wastes, and dietary nutrients which might be accompanied with environmental pollutant. In healthy condition, endothelial cell surface of the lumen is non-adhesive and anti-thrombogenic. But in certain diseases, endothelial cell activation has been considered to trigger the initial development of atherosclerosis which involves the development of numerous oxidative and inflammatory reactions and will result in lymphocyte and monocyte adhesion to the vascular endothelium [10]. Oxidized LDL is one of the important triggers for the formation of the atherosclerotic lesion. It may enhance migration and localization of inflammatory cells including T lymphocytes, monocytes, and macrophages [11]. Up-regulation of adhesion molecules on the surface of endothelial cells is prominent when they are exposed to pro-inflammatory molecules such as tumor necroses factor-α (TNF-α), interleukin-1β (IL-1β), interferon-γ (IFN-γ), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) [12, 13]. In sites of inflammation, leukocytes will migrate across activated endothelium. This is a three-step process: the first step is the rolling of leukocytes on the endothelium which is mediated by selectins and addressins [14]; the second step is the attachment of leukocytes to the endothelium which is mediated by vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1); the third step is the transmigration of leukocytes across the thin layer of endothelial cells [15, 16]. Accumulation of monocytes in vascular subendothelial spaces and their conversion into lipid-laden “foam cells” is an early and important event in atherosclerosis [17]. Severe endothelial cell activation and injury can lead to necrotic and apoptotic cytotoxicity, and ultimately to disruption of endothelial integrity.

Overall, the inflammatory reaction in endothelial cells is mediated by complex interactions between circulating nutrients, cytokines and environmental pollutants. Research in the current dissertation is trying to address the effects of dietary essential
omega-6 or omega-3 fatty acids on pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) and environmental contaminant PCB77 on the activation of endothelial cells.

Dietary Fatty Acids and Atherosclerosis

Prospective and cross-sectional studies indicate that the dyslipidemia, insulin resistance and type 2 diabetes is an important contributor to atherosclerotic disease [18]. The chronic elevation of serum free fatty acids, also referred as albumin-bound non-esterified fatty acids is important component of the dyslipidemia and have been found to be associated with metabolic risk markers for coronary heart disease [19] and increased risk of cardiovascular diseases [20]. The composition of intracellular non-esterified fatty acids pool is affected by the concentration of exogenous fatty acids. The albumin-bound fatty acid can originate from distal lipolysis of triglycerides from adipose tissue and focal lipolysis of the triglycerides of VLDL and chylomicrons. During the postprandial period albumin-bound free fatty acids mainly come from hydrolysis of triglyceride-rich lipoproteins of intestinal origin by the lipoprotein lipase.

Diets high in omega-6 fatty acids have been shown to increase the risk of cardiovascular diseases. In contrast, due to the low rates of coronary heart disease in Greenland Eskimos and Japanese who are exposed to a diet rich in fish oil, the beneficial effects of omega-3 fatty acids on cardiovascular health have been well documented. The major dietary sources of omega-6 and omega-3 fatty acids are listed in Table 1. Linoleic acid, the major omega-6 fatty acids in the American diet, is considered to be pro-inflammatory and pro-atherogenic as it favors oxidative modification of LDL cholesterol, increases platelet response to aggregation, and suppresses the immune system [21]. While, omega-3 fatty acids from fish oil have been shown to markedly reduce mortality from cardiovascular diseases [22-24]. Furthermore, a higher intake of α-linolenic acid which is enriched in vegetable oil has also been shown to inversely associate with risk of myocardial infarction [25] and carotid atherosclerotic disease [26]. In vitro studies also have shown that omega-3 fatty acids can down-regulate the expression of vascular adhesion molecules in endothelial cells [27]. There is evidence that intake of omega-3 fatty acids are reflected in the concentration of these fatty acids not only in serum
phospholipids [28], but also in serum non-esterified fatty acids [28, 29]. In addition, accumulation of linoleic acid can affect endothelial cell lipid pools [30, 31]. However the primary mechanisms of the beneficial effects of dietary omega-3 especially \( \alpha \)-linoleic acid on endothelial cell activation and the interaction of omega-3 with omega-6 fatty acids have not been well studied yet. The human body can convert 18 carbon fatty acids into their 20 or longer chain derivatives via elongation and desaturation reactions. In the current study, we focused on the 18-carbon linoleic acid and \( \alpha \)-linolenic acid because they are the major parental essential fatty acids for the omega-6 and omega-3 fatty acid families, respectively, and are more sufficient in the human diet than their longer chain derivatives.

**Pro-Inflammatory Cytokine TNF-\( \alpha \)-Stimulated Endothelial Activation**

Tumor necrosis factor (TNF) was isolated in 1984 and was named after its ability to cause tumor necrosis or regression in patients whose tumor has become infected with a bacterial infection [32, 33]. TNF is also referred to as TNF-\( \alpha \), cachectin, or differentiation-inducing factor (DIF) [34]. TNF-\( \alpha \) can cause necrotic cell death, and may also cause apoptotic cell death, cellular proliferation, differentiation and inflammation. TNF-\( \alpha \) is produced mostly by activated macrophages and monocytes, but also by many other cell types including B lymphocytes, T lymphocytes and fibroblasts [35]. Fig.1.1 adapted from MacEwan et al [35], shows TNF mediated cellular responses.

TNF-\( \alpha \), a trimeric 17-kd cytokine, is one of the most potent pro-inflammatory cytokines [36, 37] which is highly expressed throughout the full spectrum of atherosclerotic development [38]. TNF-\( \alpha \) has numerous pro-inflammatory and procoagulant effects on endothelial cells [39]. It can stimulate production of reactive oxygen species (ROS) through induction of enzymes such as xanthine oxidase, COX, and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase as well as other sources [40]. TNF-\( \alpha \) increases recruitment of inflammatory cells to vascular lesion sites [39], and promotes vascular smooth muscle cell adverse remodeling and plaque
rupture [41]. Further, it promotes matrix degradation [42], thus facilitate influx of inflammatory cells to the vessel wall.

TNF-α effects on endothelial cells include protein synthesis-independent changes in cell shape and motility that may contribute to vascular leakage at sites of inflammation as well as expression of new proteins that may regulate other parameters of the inflammatory response such as vasoregulation (e.g., COX-2 and GTP cyclohydrolase I), leukocyte adhesion (e.g., E-selectin, ICAM-1, VCAM-1), leukocyte activation (chemokines such as IL-8 or MCP-1), and coagulation (tissue factor and PAI-1). TNF-α may also selectively turn off synthesis and eventually expression of some proteins such as thrombomodulin or eNOS.

TNF-α engagement of TNF receptor-1 (TNFR-1) recruits the adaptor proteins TRADD, TRAF-2 into lipid rafts and activates NF-κB and MAPK pathways [43, 44]. Two specific transcription factors, NF-κB and activator protein-1 (AP-1) are essential for TNF-α induction of adhesion molecules. E-selectin, ICAM-1, and VCAM-1 genes each contain DNA sequences in their 5’ flanking regions that bind various forms of NF-κB and AP-1 and that mutations of these sequences reduce TNF-caused these adhesion molecule up-regulation [45]. NF-κB is a DNA binding factor that can be activated by several inflammatory mediators such as TNF-α, interferon (IFN)-β, interleukin (IL)-8, IL-1β, IL-2, and IL-6 [46, 47]. The NF-κB complex consists of two heterodimers, p50 and p65. The activation of NF-κB requires the phosphorylation of NF-κB-inhibitor IκBα and then free NF-κB dimmers can translocate into nucleus, bind to the promoter regions of target genes, and induce transcription [47].

Besides the induction of adhesion molecules, most of cellular, tissue, and systemic responses to TNF-α can be ascribed to TNF-α-induced PGE2 release. Prostanoids are lipid-derived mediators involved in various homeostatic and inflammatory processes through out the body. The biosynthesis of prostanoids involves three enzymatic reactions including phospholipase A2 (PLA2) which liberates arachidonic acid (AA) from the sn-2 position of cellular membrane phospholipids, cyclooxygenase (COX) which converts AA to the endoperoxide intermediate Prostaglandin H2 (PGH2). PGH2 is subsequently converted to prostanoids by the action of cell-specific synthases
which are also called terminal prostaglandin synthases [48]. After the formation, prostanoids are released from the cells and act on their cognate receptors on cell surfaces to exert their biological actions. Prostaglandin E\(_2\) (PGE\(_2\)) which PGE synthase is the most common prostanoids that can be produced by a wide variety of cells and tissues and has a broad range of bioactivity. There are four types of PGE receptor, EP1, EP2, EP3, and EP4 [49].

In the current study, we tested the hypothesis that omega-6 and omega-3 fatty acids can differentially modulate TNF-\(\alpha\)-induced endothelial cell activation via oxidative stress-sensitive transcription factor NF-\(\kappa\)B signaling pathway, thus influence the inflammatory mediator PGE\(_2\) synthesis.

Significance of Caveolae in Fatty acids and TNF-\(\alpha\) Mediated Endothelial Cell Activation

In the 1950s the membrane invaginated vesicles of 50 to 100 nm were observed by electron microscopy, which were given the name of caveolae due to their cave-like appearance [50]. After the identification of caveolin, the signature protein of caveolae, caveolae have been implicated to be important in a variety of cellular functions including endocytic processes, cholesterol and lipid homeostasis, signal transduction, and tumor suppression [51]. Fig.1.2 shows the organization of caveolae membranes as adapted from Razani et al [51].

Caveolae have been considered a specialized form of lipid raft [52]. But certain proteins have been found to preferentially partition into lipid rafts or caveolae but not both [53]. Furthermore, the localization caveolin proteins to caveolae distinguishes these membrane domains from lipid rafts [51]. Forty years after the definition of caveolae, the 22-kDa protein caveolin was isolated and subsequently cloned [54, 55]. There are three gene family members of caveolin: caveolin-1, -2, and -3. Adipocytes, endothelial cells, pneumocytes, and fibroblasts have the highest levels of caveolin-1 and -2 [56, 57], whereas caveolin-3 expression is limited to muscle cell types [58].

Numerous roles have been attributed to caveolae proteins: vesicular transport [59], cellular cholesterol homeostasis [60], oncogenes and tumorigenesis [61], and signal
transduction mechanisms [62, 63]. Several lines of research suggest that caveolin family members function as scaffolding proteins [64] to concentrate and organize specific lipids, such as cholesterol and sphingolipids [60, 65] and lipid-modified signaling molecules within caveolae membranes [66-68].

Cholesterol and glycosphingolipids are essential components of caveolae. Sterol-binding agents can bind to cholesterol and disrupt caveolar structure [69]. Also, utilize cholesterol oxidase which reduces cellular cholesterol can cause the translocation of caveolin-1 from the plasma membrane to intracellular membrane compartments [70]. Consequently, changes in the lipid component of caveolae membranes can potentially result in profound changes in caveolar functioning. Thus, caveolae lipids are important for organizing and regulating the molecular interactions of multiple signaling pathways.

Gafencu et al [71] investigated fatty acid composition of caveolae from endothelial cells and indicated that palmitic acid (16:0), palmitoleic (16:1), stearic acid (18:0), and oleic acid (18:1) were most prevalent which compose the 80% of total fatty acids, linoleic acid (18:2) was about 8 to 10% of total fatty acids. Omega-3 fatty acids have been shown to alter caveolae microenvironment, thereby modifying location and function of proteins in caveolae. Eicosapentaenoic acid (EPA) induces displace caveolin-1 from caveolae in endothelial cells [72] and omega-3 fatty acid feeding reduces both cholesterol and caveolin-1 in mouse colon [73]. EPA and docosahexaenoic acid (DHA) have also been shown to decrease lipid raft sphingomyelin, cholesterol content [74] and to selectively alter subcellular distribution of cytosolic proteins, including Ras isoforms, by modifying membrane lipid composition [75]. Furthermore, H-Ras has been shown to target to the plasma membrane and caveolin is crucial for the function of H-Ras [76].

Caveolin-1 can bind to many types of plasma membrane receptor proteins or membrane associated enzymes and concentrate these molecules within caveolae [51]. Fatty acid translocase (FAT/CD36), a major protein associated with fatty acid uptake [77], is preferentially located in the caveolae at the plasma membrane level [78]. TNFR-1 has been reported to concentrated within the caveolae of a human endothelial cell line [79]. Interleukin-1β-induced COX-2 in human fibroblasts is colocalized with caveolin-1 [80]. Previous studies demonstrated that in the absence of ligand, PDGF is associated with rafts, and in the presence of ligand, it transiently associates with caveolae [81].
It has been widely accepted that binding of TNF-α to monomeric receptors results in receptor trimerization and that this ligand-induced receptor clustering causes recruitment of specific adapter signaling proteins to intracellular domains of receptors that have been incorporated into these ligand-receptor complexes [82]. There are two types of TNF receptor: TNFR-1 and TNFR-2. The TNFR1 is expressed on all cell types including endothelial cells and is better understood, while the TNFR2 is mainly expressed on cells of the immune system. TNFR-1 contains a death domain, termed TNFR-associated death domain (TRADD) which is recruitment at the plasma membrane, and this region is required for TNF-α-induced pro-inflammatory cellular responses, such as activation of nuclear factor κB (NF-κB) [34]. In promonocyte cell line U937, if the caveolae like domains are disrupted, the surface expression of TNFR-1 and CD36 are significantly reduced [83]. The p38 MAPK pathway is activated by hydrogen peroxide and UV light [84, 85]. p38MAPK, but not p42/44 is a critical component of the oxidant stress (H2O2)-sensitive signaling pathways [85]. Phosphorylation of caveolin-1 occurs specifically through activation of the p38 MAPK [86]. Activation of MAPKs has also been shown to associate with subcellular redistribution of caveolin and cholesterol [87].

Caveolin-1 has been proposed to be essential in a variety of cellular functions. Caveolin-1 knockout mice revealed that caveolin-1 is not essential for maintaining life but plays an important role in vascular functions [88, 89]. Both in vivo and in vitro experiments demonstrated that caveolin-1 is essential for caveolae formation and interacts with a variety of signaling molecules in endothelial cells, suggesting that caveolin-1 may be an essential signaling platform. In the current study, we tested the hypothesis that omega-6 and omega-3 fatty acids can differentially modulate TNF-α-induced endothelial cell activation and that functional caveolae are required for endothelial cell activation. TNF-α-induced proinflammatory signaling pathways were analyzed under experimental conditions with intact caveolae and with cells in which caveolin-1 was silenced by siRNA.
PCB-Induced Endothelial Cell Activation

Polychlorinated biphenyls (PCB) are produced by chlorination of biphenyl and comprise a class of 209 individual compounds, depending on the position of chlorine atom substituents. The commercial use of PCBs started in the late 1920s including fluids in transformers, capacitors and hydraulic transfer systems, plasticizers in paints, dyes, plastics and rubber products etc. [90]. The total amount produced world-wide is estimated at 1.5 million tons. PCBs have non-flammability, chemical stability and high dielectric constants.

More than 90% of human PCBs exposure derives from food and PCBs are poorly metabolized and tend to accumulate in animal tissues, including humans. The accumulation particularly in tissues and organs rich in lipids appears to be higher in the case of penta and more highly chlorinated biphenyls. Studies have revealed PCBs in human fat tissue and blood plasma. [91-93]. Scientific Cooperation (EU SCOOP Task 3.2.5 June 2000) reported that the main contributors to the daily dietary exposure of dioxins appear to be fish and fish products (11-63%); meat and meat products (6-32%), milk and diary products (16-39%). Following exposure to animals, PCB will accumulate in meat, liver and particularly in fat tissues. PCBs build up (bioaccumulation) in the environment, increasing in concentration as you move up the food chain. This is of special concern in areas where fish are exposed to PCB contamination and may be consumed by humans (as in the Hudson River).

PCBs were banned by the EPA in 1979, and are classified as a probable human carcinogen by numerous national and international health-protective organizations, such as the EPA, The Agency for Toxic Substances and Disease Registry (an arm of the U.S Public Health Service) and the World Health Organization. Research also links PCB exposure to developmental problems.

Numerous experimental and epidemiological studies have demonstrated that environmental pollution is strongly involved in the pathogenesis of atherosclerosis. Endothelial cells directly contact with blood substances which include environmental pollutant. Certain polyhalogenated aromatic hydrocarbons can cause endothelial cell activation, thus can be implicated in atherosclerotic lesion formation [94, 95]. Coplanar PCBs, such as PCB77 can induce oxidative stress through the aryl hydrocarbon receptor.
(AhR)-cytochrome P450 1A1 (CYP1A1) pathway [96]. Induction of CYP1A1 is considered cardiotoxic through generating reactive oxygen species (ROS).

Previous studies have shown that environmental contaminants such as PCBs can be implicated in atherosclerotic lesion formation [97, 98]. We have previously reported that coplanar polychlorinated biphenyl (PCBs) such as PCB77 can induce inflammatory genes VCAM-1 and IL-6 in vascular endothelial cells [96, 99]. PCBs can significantly activate CYP1A1 enzyme [100]. PCB77, a coplanar PCB, is a 3-methylcholanthrene-type cytochrome P450 inducer and an aryl hydrocarbon receptor (AhR) ligand.

Mechanisms of the interaction of ω-6 or ω-3 fatty acids and PCBs in endothelial cell activation are not clear. Exposure to PCBs can change fatty acid composition of tissues like the liver with a proportional decrease in arachidonic acid and increase in oleic and linoleic acid [101, 102]. Long term exposure to PCBs have been shown to increase plasma triglycerides and decrease HDL-cholesterol [103]. In the current study, we tested the hypothesis that different types of dietary polyunsaturated fatty acids can selectively modify PCB-induced vascular endothelial cell activation and the mechanisms of PCB77-induced inflammation can be modified by different ratios of omega-6 to omega-3 fatty acids.

**Hypothesis and Significance of the Current Study**

Previous studies have demonstrated that increasing levels of circulating cytokines such as TNF-α, environmental contaminants such as PCBs, or certain dietary nutrients such as omega-6 fatty acids can cause endothelial activation and promote an inflammatory response [6, 104, 105]. Furthermore, our previous data have shown that linoleic acid could potentiate inflammatory events induced by both TNF-α and PCBs [106, 107]. Most of these inflammatory events involve oxidative stress and result in the activation of oxidative stress sensitive transcription factors NF-κB or AP-1 which can consequently stimulate the expression of cytokines and adhesion molecules [108, 109]. But the anti-atherogenic and anti-inflammatory effects of ω-3 fatty acids, in particular α-linolenic acid, have not been investigated under these stimulations. And the modulation effect of changing the relative amounts of omega-6 and omega-3 fatty acids on
environmental pollutant-induced endothelial cell activation has not been well elucidated yet. It is possible that changes in the lipid (fatty acid) environment can alter the membrane lipid rafts, inflammatory mediators, cell signal transduction, and gene expression.

This study investigated the effects of omega-6 and omega-3 fatty acids on TNF-\(\alpha\) and PCB77-induced PGE\(_2\) release in endothelial cells. This was accomplished by examining points of potential intervention in the cellular signaling cascades. We explored the impact of these fatty acids alone, or at different ratios on TNF-\(\alpha\) binding to TNFR-1, cytoplasmic phospholipase A2 activation, and COX-2 expression. In addition, we explored their impact on the binding activity of NF-\(\kappa\)B, and signaling pathways such as p38 MAPK.

Research described in this dissertation provides a better understanding of the differential modulation effects by omega-6 and omega-3 fatty acids alone, or by changing the ratios of omega-6 to omega-3 fatty acids on endothelial cell activation induced by pro-inflammatory cytokine TNF-\(\alpha\) or PCB77 and the underlying molecular mechanisms. There is a great need to further explore this nutritional paradigm in environmental toxicology and to improve our understanding of the relationship between nutrition and lifestyle, exposure to environmental toxins and disease. Nutrition may provide the most sensible means to develop primary intervention and prevention strategies of diseases associated with many inflammatory and environmental toxic insults.
Table 1.1 Major dietary polyunsaturated fatty acids

<table>
<thead>
<tr>
<th>Dietary Fatty acids</th>
<th>ω-6 or ω-3 families</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>linoleic acid (LA; 18:2)</td>
<td>ω-6 fatty acid</td>
<td>soy, corn, safflower, and sunflower oils</td>
</tr>
<tr>
<td>arachidonic acid (AA; 20:4)</td>
<td>ω-6 fatty acid</td>
<td>animal products</td>
</tr>
<tr>
<td>α-linolenic acid (ALA; 18:3)</td>
<td>ω-3 fatty acid</td>
<td>canola, walnut, and flaxseed oils</td>
</tr>
<tr>
<td>eicosapentaenoic acid (EPA; 20:5)</td>
<td>ω-3 fatty acid</td>
<td>fish oil</td>
</tr>
<tr>
<td>docosahexaenoic acid (DHA; 22:6)</td>
<td>ω-3 fatty acid</td>
<td>fish oil</td>
</tr>
</tbody>
</table>
Figure 1.1 Major TNF receptor-mediated cellular responses
Figure 1.2 Organization of lipid rafts and caveolae membranes

A, lipid rafts: the liquid-ordered phase is dramatically enriched in cholesterol and exoplasmic oriented sphingolipids (sphingomyelin and glycosphingolipids). The lipid-disordered phase is composed essentially of phospholipids (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine). B, caveolae: upon integration of the caveolin-1 protein, liquid-ordered domains form small flask-shaped invaginations called caveolae. Caveolin-1 monomers assemble into discrete homo-oligomers containing ~14 to 16 individual caveolin molecules. Adjacent homo-oligomers are thought to pack side-by-side within caveolae membranes thereby providing the structural meshwork for caveolae invagination. Adapted from Razani et al. [51].
Chapter Two: Effects of ω-6 and ω-3 Fatty Acids on TNF-α-Induced Endothelial Cell Activation

Synopsis

TNF-α, an important pro-inflammatory mediator of endothelial cell activation, can lead to the formation of lipid-derived prostaglandins. Different ω-6 and ω-3 fatty acids have been implicated to have pro- or anti-inflammatory actions, respectively. However, the mechanisms of ω-6 and ω-3 fatty acids mediated TNF-α-induced production of the major inflammatory mediator prostaglandin E2 (PGE2) and the underlying signaling mechanism remains unclear. The present study focused on the effects of ω-6 and ω-3 fatty acids on TNF-α-induced oxidative stress and the inflammatory signaling that involves in PGE2 production. Porcine pulmonary-arterial endothelial cells were first enriched with linoleic acid (LA; 18:2 ω-6) or α-linolenic acid (ALA; 18:3 ω-3), followed by exposure to TNF-α. TNF-α-induced oxidative stress, p38 MAPK activation and activation of nuclear factor κB (NF-κB) were markedly increased in the presence of linoleic acid and diminished by pretreatment with α-linolenic acid. Similar protective effects of α-linolenic acid were observed by measuring COX-2 expression and the production of PGE2. Treatment with p38 MAPK specific inhibitor significantly decreased TNF-α-induced COX-2 and VCAM-1 protein expression. Taken together, our data suggest that in endothelial cells, linoleic acid and α-linolenic acid can generate distinct signaling outcomes in response to inflammatory stimuli caused by TNF-α and p38 MAPK plays an important role in fatty acid mediated TNF-α-induced inflammatory response.
Introduction

Vascular endothelial dysfunction is thought to be involved in the initiation of atherosclerotic lesions. Endothelial cells lying at the blood-tissue interface play an essential role in homeostasis of the circulation and vessels. At normal state, endothelial cells have antithrombotic and anticoagulant activities [110]. But the “activation” of endothelial cells can cause changes to the hemodynamic equilibrium and the development of numerous oxidative and inflammatory reactions, which result in lymphocyte and monocyte adhesion to the vascular endothelium [10, 111]. This is considered to involve in the very early stage of atherogenesis. Leukocyte adhesion to the endothelium is facilitated by the appearance of adhesion molecules at the surface of lymphocytes, monocytes, and endothelial cells. These molecules are mainly selectins (e.g. E-selectins are specific to endothelial cells), vascular cell adhesion molecule type 1 (VCAM-1), and intercellular adhesion molecule type 1 (ICAM-1) [112].

One of the common mediators of endothelial cell activation is macrophage-derived cytokines, such as tumor necrosis factor alpha (TNF-α) [113]. Patients with clinical cardiovascular disease had significantly higher levels of TNF-α compared with subjects without cardiovascular disease [114]. High levels of circulating TNF-α can cause endothelial cell activation resulting in the development of numerous oxidative and inflammatory reactions and facilitating lymphocyte and monocyte adhesion to the vascular endothelium [10, 111]. Furthermore, TNF-α profoundly affects eicosanoids metabolic pathways leading to the accumulation of one or more pro-inflammatory prostaglandins.

The mechanisms by which selected fatty acids induce endothelial cell activation, oxidative stress and inflammation are not fully understood. Oxidative stress-induced transcription factors, which regulate inflammatory cytokine and adhesion molecule production, are important regulatory elements in the induction of inflammatory responses. One of these transcription factors, nuclear factor κB (NF-κB), plays a significant role in these regulatory processes [115]. Binding sites for NF-κB and related transcription factors were identified in the promoter regions of a variety of inflammatory genes [116,
such as interleukin 6 (IL-6), vascular cell adhesion molecule-1 (VCAM-1) or cyclooxygenase-2 (COX-2), all of which are up-regulated by TNF-α [118, 119].

Numerous risk factors for the development of atherosclerosis have been identified, including obesity [120] and hypertriglyceridemia [121]. Increased circulating free fatty acid levels are associated with hypertriglyceridemia and obesity [122], and high plasma free fatty acids may contribute to an environment of increased oxidative stress and inflammation in the vasculature and especially in vascular endothelial cells [123, 124]. Dietary balance of long-chain fatty acids may influence processes involving leukocyte-endothelium interactions, such as atherogenesis and inflammation [125]. Even though diets high in omega-6 fatty acids may lead to a decrease in serum cholesterol [126], replacing saturated with unsaturated omega-6 rich lipids may not be desirable because of their ability to easily oxidize. High intake of linoleic acid-rich oils or fats will lead to an increase in cellular oxidative stress and can elicit an inflammatory response [127], events which have been implicated in most chronic diseases. Omega-6 fatty acids, and especially linoleic acid can cause endothelial cell dysfunction as well as potentiate tumor necrosis factor-α (TNF-α)-mediated endothelial injury [128]. We have recently demonstrated that both the extracellular signal regulated kinase (ERK1/2) and phosphoinositide-3 kinase/ amino kinase terminal (PI3K/Akt) signaling pathways can contribute to the effect of linoleic acid on nuclear factor-kappa B (NF-κB)-dependent transcription and endothelial cell activation [105].

In contrast to omega-6 fatty acids, omega-3 fatty acids can influence cardiovascular disease pathology by beneficially modulating inflammation. Epidemiological and interventional studies have shown a dose-dependent decrease in risk of cardiovascular disease endpoints with increased dietary consumption of moderate amounts of omega-3 fatty acids, either plant or marine derived [126]. Current estimates indicate that over 90% of the omega-3 consumed by U.S. citizens is in the form of α-linolenic acid, not the longer chain omega-3 fatty acids found in fish oils [129]. Independent of their dietary source, omega-3 fatty acids contribute to cardio-protective properties, which include down-regulation of proinflammatory and proatherogenic genes, including adhesion molecules and cytokines, during early atherogenesis and possibly also during later stages of plaque development and plaque rupture [130]. For example, an α-
linolenic acid-rich oil decreased oxidative stress and CD40 ligand in patients with mild hypercholesterolemia [131], reduced levels of soluble cell adhesion molecules in plasma [132] and recurrence of coronary heart disease [133]. Also, by partially replacing omega-6 analogues in membrane phospholipids with omega-3 fatty acids, it is possible to decrease the transcriptional activation of inflammatory and pro-atherogenic genes involved in endothelial cell activation and atherosclerosis [134].

Previous studies in our laboratory have shown that linoleic acid potentiated TNF-α–mediated pro-inflammatory responses in endothelial cells [107]. Linoleic acid may further be atherogenic by causing activation of NF-κB which in turn increases the expression of E-selectin and VCAM-1 [128, 135]. On the other hand, lipids are crucial structural and functional components of cells. Even more importantly, many lipid species have distinct cellular functions. For example, ceramides, eicosanoids, diacylglycerol (DAG) and lysolipids are all second messengers which participate in various cellular events such as inflammation, growth, proliferation, differentiation and cell death. However the primary mechanism of the modification of these different metabolism pathways by the availability of different series of omega-6 and omega-3 PUFAs in the presence of TNF-α is not well known.

The present study was conducted to test the effects of linoleic acid and α-linolenic acid on TNF-α-induced prostaglandin E$_2$ (PGE$_2$) release in primary porcine pulmonary artery endothelial cells. Three markers of inflammation, oxidative stress, p38 MAPK, COX-2 that have been recognized to play a role in atherogenesis, along with transcription factor NF-κB, were assessed.

In the current study, we demonstrate that ω-3 fatty acid inhibits the ability of pro-inflammatory cytokine TNF-α to induce the expression of adhesion molecules associated with endothelial cell activation. We also show here that ω-3 fatty acid were associated with the inhibition of p38 MAPK signal transduction pathways. These results provide new insights into the modulation of TNF-α-induced inflammatory responses by dietary polyunsaturated fatty acids.
Materials and Methods

Cell culture and experimental media

Endothelial cells were isolated from porcine pulmonary arteries and cultured as previously described [30] (see detailed protocol in Appendix B). Arteries obtained during routine slaughter were donated from the College of Agriculture, University of Kentucky. The basic culture medium consisted of medium 199 (M-199) (GIBCO Laboratories, NY) containing 10% (v/v) fetal bovine serum (FBS, HyClone Laboratories, UT). The experimental media were composed of M-199 enriched with 5% (v/v) FBS and with linoleic acid (LA) or α-linolenic acid (ALA) (> 99% pure; Nu-Chek Prep, MN). Preparation of experimental media with LA and ALA were performed as described earlier [136] (see detailed protocol in Appendix B). Metabolic studies [137] indicated that the molar ratio of free fatty acid to albumin is the main factor controlling free fatty acid availability to tissues. In plasma, albumin concentration is about 600 μM, and total free fatty acid is 180 to 2000 μM thus the range of ratios of fatty acid to albumin is 0.3 to 4. In the experimental media, 20 μM fatty acids were enriched in 5% media with which the albumin concentration is about 50 to 60 μM. So the ratio of free fatty acids to albumin is within physiological and metabolic relevance and not toxic to the cells.

Human TNF-α was purchased from Sigma (Sigma-Aldrich, MO). In the p38 MAPK inhibitor study, SB203580 was dissolved in DMSO (Sterile-filtered, Sigma-Aldrich, MO) to a stock solution of 10 mM. After getting confluent, cells were first incubated with 10 μM of SB203580 (a thousand times dilution from stock solution) for 30 min followed by exposure to TNF-α. The control culture contained the same volume of DMSO.

E-selectin, VCAM-1, and COX-2 gene expression studies

RT-PCR:

Total cellular RNA was extracted from culture plates according to the RNA-STAT-60 protocol (TEL-TEST, Friendswood, TX). RNA concentration was spectrometrically determined by reading the absorbance at 260 nm. cDNA first strand was synthesized from total RNA by RT reaction. The reaction mixture contained 1 μg
RNA. Resulting cDNA was amplified by the polymerase chain reaction with enzyme nucleotides and buffers from Quiagen (Valencia, CA). The following primers for E-selectin, VCAM-1, and COX-2 were employed in the PCR. E-selectin: forward 5'-GACTCGGGCAAGTGGAATGATGAG-3'; reverse 5'-CATCACCATTCTGAGGATGGCCGAC-3'. VCAM-1: forward 5'-GGTTACCCGGTGAAAAGATGGAG-3'; reverse 5'-CACCCTGCTG CCTGTCTCT-3'. COX-2: forward 5'-GGAGAGACAGCGATAAACTGC-3', reverse 5'-GTGTGTTAAAACCGCAGCA-3'. β-actin was used as a housekeeping gene: forward 5'-GGGACCTGACCCGACTACCT-3'; reverse 5'-GGCGATGATCTTG ATCTTC-3'. The PCR products were electrophoresed on a 2% (w/v) EDTA agarose gel. Then stained with SYBR Green (Molecular Probes, Eugene, OR) and visualized by using phosphorimaging technology (FLA-5000; Fuji, Stamford, CT).

Real-time PCR:

Total RNA was extracted from endothelial cells using RNA-STAT-60 (TEL-TEST, Friendswood, TX) according to the manufacturer’s protocol. Reverse transcription was performed using the AMV reverse transcription system (Promega, Madison, WI). The levels of mRNAs and the PCR-product were then assessed by real-time PCR using 7300 Real Time PCR System (Applied Biosystems). Real-time PCR samples were mixed with SYBR Green Master Mix (Applied Biosystems) and VCAM-1 or COX-2 specific primers. The sequences for porcine VCAM-1 and COX-2 gene were designed by Primer Express Software 3.0 for real-time PCR (Applied Biosystems). VCAM-1 sequences: sense, 5'-TGGAAGACATGGCTGCCTAT-3'; antisense, 5'-ACACCACCCCAGTCACCATA TC-3'. COX-2 sequences: sense, 5'-TGCTGAAGCCCTATCGATCA-3'; antisense, 5'-TACAGCTCCATGGCATCAATG-3'. β-actin was used as a housekeeping gene in both VCAM-1 and COX-2 studies. β-actin sequences: sense, 5'-TCATCACCATCGGCAACG-3'; antisense, 5'-TTCCCTGATGTCCACGTG-3'.

Immunofluorescence study of E-selectin expression

Confluent endothelial cells were grown on glass culture slides and incubated with or without TNF-α for 4 h. After three washing in phosphate-buffered saline (PBS), cells were fixed by 70% ethyl alcohol for 10 min and air-dried. After 30 min of blocking of
non-specific binding with PBS containing 3% BSA, cells were incubated with E-selectin primary antibody for 1 h on ice at a dilution of 1:50. Then slides were washed twice in PBS then incubated simultaneously with FITC-labeled secondary antibody (1 h at room temperature, dilution 1:5; Chemicon, CA). Negative controls were prepared by incubation of the cells with anti-IgG antibody. The cells were washed twice in PBS and mounted in aqueous mounting medium before being observed using an epifluorescence Nikon Eclipse E600 microscope. The images were capture using a Spot charge coupled device camera system (Nikon, Melville, NY).

Measurement of cellular oxidative stress

Cellular oxidation was determined by 2', 7'-dichlorofluorescein (DCF) fluorescence as described earlier [138]. This method is based on the conversion of 2’, 7’-dichlorofluorescein into fluorescent 2’,7’-dichlorofluorescein by oxygen reactive species, primarily peroxyl radicals and peroxides. Please find the detailed protocol in Appendix B. After treatment cells were washed with Hanks buffer to remove serum. Then KREBS-Ringer-Glucose buffer containing DCF (100 μmol/L) (Molecular Probes, Inc., Eugene, OR) were added to microplates and incubate for 30 minutes. Blank well also received KREBS-Ringer-Glucose buffer without DCF probe. Wrap plate in aluminum foil to protect from the light. After incubation washed wells 2 to 3 times with HEPES buffer and then added HEPES to each well (1 ml for 12 well plates and 0.5 ml for 24 well plates. Wrap plate in aluminum foil before reading. A multi-well fluorescent plate reader (Molecular Devices, Sunnyvale, CA) was utilized for the imaging study. Excitation and emission wavelengths were 490 and 520 nm respectively.

Transcription factor NF-κB activation studies: electrophoretic mobility shift assay (EMSA)

Nuclear extracts containing active proteins were prepared from cells according to the method of Beg et al. [139]. Binding reactions were performed in a 20 μL volume containing 7 μg of nuclear protein extracts. Nuclear extracts were incubated for 25 min with 32P-end-labeled oligonucleotide probes containing enhancer DNA element NF-κB (5’ AGTTGAGGGGACTTTCCCAGGC 3’) (Santa Cruz, Santa Cruz, CA). Following
binding, the protein-DNA complexed and uncomplexed DNA in the mixture were resolved on native 5% polyacrylamide gels using 0.5x TBE buffer (50 mM Tris-Cl, 45 mM boric acid, 0.5 mM EDTA, pH 8.4) and visualized by autoradiography. Control reactions using 200-fold molar excess of unlabeled oligonucleotide probes or a supershift assay were performed to demonstrate the specificity of the shifted DNA-protein complexes for NF-κB.

Measurement of VCAM-1 or COX-2 protein levels, and phosphorylation of p38MAPK or cPLA₂ by western blot

Cells were harvested using cell lysis buffer as previously described [105]. Protein samples were resolved by SDS-PAGE using 10% gradient gels and transferred electrophoretically to nitrocellulose membranes using a Bio-Rad immunoblot transfer apparatus (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. The nonspecific sites on the membrane were blocked 1 h at room temperature with 5% nonfat dry milk in TBST followed by incubation with β-actin (1:5000 dilution in 5% nonfat milk), VCAM-1 (1:2000 dilution in 5% nonfat milk), COX-2 (1:2000 dilution in 5% nonfat milk), total p38 MAPK (1:1000 dilution in 5% nonfat milk), phosphor-p38 MAPK (1:1000 dilution in 5% nonfat milk), or phosphor-cPLA₂ (1:500 dilution in 5% nonfat milk) primary antibodies (Santa Cruz Biotech, CA) overnight at 4 °C. Bands were visualized using the appropriate horseradish peroxidase-conjugated secondary antibodies followed by ECL immunoblotting detection reagents (Amersham Biosci, UK). Detection and quantitative analysis were performed using a digitizing system (UN-SCAN-IT, Silk Scientific Corporation). To determine the optimal amounts of samples for linear area detection, a titration of different amounts of sample were loaded. For COX-2 and VCAM-1, 30 μg protein were loaded; for p38 MAPK, 35 μg protein were loaded; for phospho-cPLA₂, 40 μg protein were loaded.

PGE₂ determination

Cells were seeded in 60 mm petri dishes (Becton Dickinson Labware, NJ) and grown to confluence. After treatment, supernatants of cell cultures were collected into microcentrifuge tubes (Isc BioExpress, UT), centrifuged at 4 °C to remove cellular debris.
and stored at -80 °C. PGE₂ levels were assessed using a PGE₂-specific enzyme immunoassay (EIA) (Cayman Chemicals, MI) following the manufacturer’s protocol with minor modification (see detailed protocol in Appendix B). A microplate spectrophotometer SpectraMaxPro M2 (Molecular Devices Corporation, CA) was used to read the plate at 405 nm.

Statistical analysis

Values are reported as mean ± standard error of the mean (SEM) of at least three independent groups. Data were analyzed using Sigma Stat software (Jandel Corp., Wan Rafael, CA). One way ANOVA followed by post hoc least significant difference (LSD)’s pairwise multiple comparison procedure were used for statistical analysis of the original data. A statistical probability of p< 0.05 was considered significant. In figures 2.4, 2.13, and 2.15, * indicates significant difference from control group. In all the other figures, different letters represent significant differences among treatment groups. Capital or small letters represent with or without TNF-α treatment respectively as were compared separately by one way ANOVA.

Results

Determination of optimal TNF-α concentration and incubation time for the stimulation of endothelial cell activation

TNF-α is arguably the most potent inducer of a power pro-inflammatory cytokine. Previous epidemiological studies indicated that the plasma concentrations of TNF-α are increased in various pathological situations, and TNF-α levels vary widely not only among different diseases but also among different patients with the same disease. For example, in diabetic patients TNF-α can reach 19.3±7.5 pg/ml in comparison with healthy controls (11.1±5.8 pg/ml) [140], or 228 pg/ml vs. 4.2 pg/ml [141]. Another study reported a median TNF-α level at 26 pg/ml in patients with sepsis, ranging from 0 to 1000 pg/ml [142]. In patients with lymphoma, the TNF-α levels were between 5 to
380 pg/ml with the median of 20 pg/ml, and the healthy controls were between 4 to 9 pg/ml with the median of 7 pg/ml [143]. Even when the plasma levels of TNF-α are known, local concentrations of inflammatory cytokines in the endothelial microenvironment are not well defined. Some in vitro studies used relatively high concentration of TNF-α from 10 ng/ml [144] to 50 ng/ml [145]. For some apoptosis studies, TNF-α concentration also has been used at 50 ng/ml [146].

In the current study, as we were concerning about the effects of fatty acids on inflammatory responses caused by TNF-α, it was necessary to avoid challenging cells with an overwhelming amount of TNF-α. In our in vitro endothelial cell model, the binding of TNF-α to its receptor on endothelial cells is followed by rapid translocation of the transcription factor NF-κB from the cytoplasm to the nucleus. This translocation is important for stimulated transcription of the adhesion molecules E-selectin, VCAM-1, and inducible enzyme COX-2 which all possess NF-κB promoter/enhancer elements. Thus, to determine the condition of TNF-α-induced endothelial cell activation, we first did time and concentration course studies for E-selectin, VCAM-1, and COX-2.

Endothelial cells were incubated in the presence of TNF-α concentrations ranging from 0.1 to 0.5 ng/ml for 2, 4, or 6 h. Expression of E-selectin (Fig. 2.1) was assessed by RT-PCR. We observed that as low as 0.2 ng/ml of TNF-α, there was a dramatic increase of E-selectin at 4 h. When endothelial cells were treated with 0.5 ng/ml TNF-α, the expression of E-selectin rose within 2 h. But the increase of E-selectin went back to basal level in all the treatment groups after 6 h of incubation. We confirmed by immunofluorescence study that 0.5 ng/ml TNF-α increased cell surface expression of E-selectin on endothelial cells (Fig. 2.2). Furthermore, 0.5 ng/ml TNF-α at 4 h also increased VCAM-1 and COX-2 (Fig. 2.3) genes expression which were measured by RT-PCR. Thus TNF-α at the concentration of 0.5 ng/ml was determined to activate endothelial cells in the current study.

**Determination of optimal linoleic acid incubation time for endothelial cell activation**

Nutritional habits can result in modified levels of circulating metabolites, including free fatty acids. The dietary different fatty acids have been shown to be
efficient for modifying inflammatory diseases. Dietary polyunsaturated free fatty acid concentration in plasma has been reported to be 20-30 μM [147] and a concentration range of 180-2,500 μM has also been reported [148]. We next investigated the effect of physiological level of linoleic acid (20 μM) alone on endothelial cell activation. As shown in Fig. 2.4, expression of VCAM-1 mRNA rose at 18 h and continuous increasing at 24 h. Thus for inflammatory gene expression study, cells were pretreated with fatty acids for 24 h, followed by TNF-α treatment for another 4 h.

**Linoleic acid and α-linolenic acid modulate TNF-α-induced E-selectin and COX-2 expression**

To examine the effects of ω-6 and ω-3 fatty acids on TNF-α-induced endothelial activation, cells were pretreated with either linoleic acid or α-linolenic acid for 24 h, followed by exposure to TNF-α for another 4 h. As indicated in Fig. 2.5, 2.6, pretreatment with α-linoleic acid significantly decreased TNF-α-induced both E-selectin and COX-2 gene expression. For E-selectin expression, pre-incubation with linoleic acid followed by TNF-α didn’t show any different from TNF-α treatment alone (Fig. 2.5). But COX-2 mRNA expression was further amplified in the presence of linoleic acid compared with TNF-α treatment alone (Fig. 2.6). We confirmed the COX-2 data by measuring protein levels of COX-2 by western blot (Fig. 2.7).

**Linoleic acid and α-linolenic acid modulate cellular oxidative stress and NF-κB activity induced by TNF-α**

The NF-κB family of transcription factors plays a crucial role in inflammatory events induced by TNF-α. The transcription of adhesion molecules E-selectin, VCAM-1, and inducible enzyme COX-2 all possess NF-κB promoter/enhancer elements [149]. Furthermore, NF-κB is respond directly to oxidative stress [150]. We further assess effects fatty acids on TNF-α-induced oxidative stress and NF-κB activation. Exposure to TNF-α alone significantly increased cellular oxidative stress (ROS) (Fig.2.8) and NF-κB DNA binding activity (Fig. 2.9). Pretreatment with linoleic acid following by exposure to TNF-α further induced oxidative stress and activation of NF-κB compared to cultures.
treated only with TNF-α. In contrast, pretreatment with α-linolenic acid blocked the TNF-α-induced both oxidative stress and subsequent induction of NF-κB.

**Linoleic acid and α-linolenic acid modulate TNF-α-induced activation of p38 MAPK**

In mammalian cells, MAPKs are strongly activated by growth factors, environmental stresses, and inflammatory cytokines [151]. As shown in Fig. 2.10, p38 MAPK was significantly activated by TNF-α at 10 min and pre-treatment with linoleic acid followed by exposure to TNF-α further increased p38 MAPK activation, this event was blocked by pre-enrichment with α-linolenic acid.

In order to further evaluate the role of p38 MAPK in TNF-α-induced inflammatory gene expression, endothelial cells were pretreated with the specific p38 MAPK inhibitor SB203580, followed by exposure to TNF-α for an additional 8 h. As shown in Fig. 2.11 and 2.12, SB203580 significantly decreased TNF-α-induced both COX-2 and VCAM-1 protein expression. These results indicate that p38 MAPK is crucially involved in ω-6 and ω-3 fatty acids mediated TNF-α-induced COX-2 expression.

**Induction of cPLA2 phosphorylation by TNF-α**

The biosynthesis of prostaglandin E2 (PGE2), a major lipid-derived inflammatory mediator, involves three enzymatic reactions including phospholipase A2 (PLA2), cyclooxygenase (COX), and PGE2 synthase. Cytosolic phospholipase A2 (cPLA2) is the most characterized phospholipase members of PLA2 family which are responsible for remarkable selectivity toward arachidonic acid at the sn-2 position of phospholipids [152]. To assess the effect of TNF-α on PGE2 production, we first measured the activation of cPLA2. As shown in Fig. 2.13, TNF-α induced significant increase of cPLA2 phosphorylation at 8 h treatment. cPLA2 mRNA expression was not changed after TNF-α stimulation measured by Real-time PCR.
Linoleic acid and α-linolenic acid modulate TNF-α-induced PGE₂ production

We further investigated cellular PGE₂ levels after different fatty acids or TNF-α treatments. As shown in Fig. 2.14, an 18 h exposure to linoleic acid dramatically induced PGE₂ production. In contrast, α-linolenic acid did not have much induction effect.

To access the optimal TNF-α incubation time for PGE₂ production, cells were treated with TNF-α for 6, 18, and 24 h. As indicated in Fig. 2.15, a 24 h exposure to TNF-α significantly induced PGE₂ production. Compared to the TNF-α treatment group, enriching endothelial cells with linoleic acid further enhanced TNF-α-induced PGE₂ production. In contrast, cellular exposure to α-linolenic acid markedly reduced the proinflammatory effect of TNF-α (Fig. 2.16).
Cells were treated with indicated concentration of TNF-α for 2, 4, or 6 h. E-selectin gene expression was measured by RT-PCR. β-actin was used as housekeeping gene.

Cells were treated with 0.5 ng/ml TNF-α for 4 h. Immunofluorescence staining for E-selectin (FITC green fluorescence) and nuclear (DAPI blue fluorescence) were indicated. The merged images were shown to better illustrate the expression of E-selectin for each individual cell.
Figure 2.3  TNF-α-induced VCAM-1 and COX-2 expression

Cells were exposed to control culture (C), or 0.5 ng/ml TNF-α for 4 h. VCAM-1 and COX-2 gene expression were measured by RT-PCR. β-actin was used as housekeeping gene.
Cells were incubated with or without 20 μM linoleic acid (LA) for indicated time. VCAM-1 gene expression was measured by real-time PCR. β-actin was used as housekeeping gene. * indicates significant difference from control group without linoleic acid.
Figure 2.5  Effect of linoleic acid and α-linolenic acid on TNF-α-induced E-selectin gene expression

Cells were treated with 0.5 ng/ml TNF-α alone for 4 h, or pretreated with 20 μM linoleic acid (LA)/α-linolenic acid (ALA) for 24 h followed by exposure to TNF-α for additional 4 h. E-selectin gene expression was measured by real-time PCR. β-actin was used as housekeeping gene. Experiments were repeated three times. Different letters represent significant differences among treatment groups. Capital or small letters represent with or without TNF-α treatment respectively as were compared separately by one way ANOVA.
Figure 2.6 Effect of linoleic acid and α-linolenic acid on TNF-α-induced COX-2 gene expression

Cells were treated with 0.5 ng/ml TNF-α alone for 4 h, or pretreated with 20 μM linoleic acid (LA)/α-linolenic acid (ALA) for 24 h followed by exposure to TNF-α for additional 4 h. COX-2 gene expression was measured by real-time PCR. β-actin was used as housekeeping gene. Experiments were repeated three times. Values are means ± SEM. Different letters represent significant differences among treatment groups. Capital or small letters represent with or without TNF-α treatment respectively as were compared separately by one way ANOVA.
Figure 2.7  Effect of linoleic acid and α-linolenic acid on TNF-α-induced COX-2 protein expression

Cells were pretreated with 20 μM of linoleic acid or α-linolenic acid for 24 h and then exposed to 0.5 ng/ml TNF-α for an additional 8 h. COX-2 protein levels were measured by western blot. β-Actin was used as a housekeeping gene. Experiments were repeated three times, and the blots shown are representative of one of the experiments. Values are means ± SEM. Different letters represent significant difference among treatment groups.
Figure 2.8  Effect of linoleic acid and $\alpha$-linolenic acid on TNF-$\alpha$-induced oxidative stress

Cultures were pretreated in media supplemented with 20 $\mu$M linoleic acid (LA) or $\alpha$-linolenic acid (ALA) for 24 h followed by exposure to 0.5 ng/ml TNF-$\alpha$ for additional 3 h. Values are means $\pm$ SEM ($n = 3$). Different letters represent significant differences among the treatment groups.
Figure 2.9  Effect of linoleic acid and α-linolenic acid on TNF-α-induced activation of NF-κB

Cells were treated with 20 μM of linoleic acid (LA) or α-linolenic acid (ALA) for 24 h prior to exposure to 0.5 ng/ml TNF-α for an additional 6 h. Experiments were repeated three times, and the blots shown are a representative of one of the experiments. The bar graph shows the corresponding densitometric analysis of the blots. Values are means ± SEM. Different letters represent significant differences among treatment groups.
Figure 2.10 Effect of linoleic acid and α-linolenic acid on TNF-α-induced p38 MAPK activation

Cells were pretreated with 20 μM of linoleic acid (LA) or α-linolenic acid (ALA) for 24 h and then exposed to 0.5 ng/ml TNF-α for 10 min. Phosphorylated and total p38 MAPK were detected by western blot using specific dually phosphorylated p38 MAPK antibody (Thr 180/Tyr 182) or anti-total p38 MAPK antibody respectively. Experiments were repeated three times and the blots shown are a representative of one of the experiments. Values are means ± SEM. Different letters represent significant difference among treatment groups.
Figure 2.11  Effect of p38 MAPK inhibitor on TNF-α-induced COX-2 up-regulation

Cells were exposed to 0.5 ng/ml TNF-α for 8 h, or first pre-enriched with 10 μM SB203508 for 30 min followed by co-exposure to TNF-α for additional 8 h. COX-2 protein levels were measured by western blot. β-Actin was used as a housekeeping gene. Experiments were repeated three times and the blots shown are a representative of one of the experiments. Different letters represent significant differences among treatment groups.
Figure 2.12  Effect of p38 MAPK inhibitor on TNF-α- and linoleic acid-induced VCAM-1 up-regulation

Cells were exposed to 0.5 ng/ml TNF-α for 8 h or linoleic acid (LA) for 24 h, or first pre-enriched with 10 μM SB203508 for 30 min followed by co-exposure to TNF-α or LA. VCAM-1 protein levels were measured by western blot. β-Actin was used as a housekeeping gene.
Figure 2.13 Effect of TNF-α on cPLA₂ activation

Cells were treated with 0.5 ng/ml TNF-α for 4, 6, or 8 h. cPLA₂ phosphorylation was measured by western blot using specific p-cPLA₂ antibody. β-actin was used as housekeeping gene. Experiments were repeated three times. Values are means ± SEM. Different letters represent significant difference among treatment groups. * indicates significant difference from other treatment groups.
Figure 2.14  Effect of linoleic acid and α-linolenic acid on PGE$_2$ production

Cells were exposed to 20 µM linoleic acid (LA) or α-linolenic acid (ALA) 18 and 24 h. Supernatants of cell cultures were collected and PGE$_2$ levels were measured by enzyme immunoassay (EIA). Bars represent means ± SEM from three independent experiments.
Figure 2.15  Optimal TNF-α incubation time for PGE₂ production

Cells were exposed to 0.5 ng/ml TNF-α for 6, 18 and 24 h. Supernatants of cell cultures were collected and PGE₂ levels were measured by enzyme immunoassay (EIA). Bars represent means ± SEM from three independent experiments. * represents significant difference compared with the control treatment.
Figure 2.16  Effect of linoleic acid and α-linolenic acid on TNF-α-induced PGE₂ production

Cells were pretreated with 20 μM of LA or ALA for 24 h and then exposed to 0.5 ng/ml TNF-α for additional 24 h. Supernatants of cell cultures were collected and PGE₂ levels were measured by EIA. Values are means ± SEM. Different letters represent significant difference among treatment groups.
Omega-6 or omega-3 fatty acids can either enhance or reduce TNF-α-induced p38 MAPK activation, oxidative stress (ROS), and NF-κB activation. Relative activation of NF-κB will further regulate VCAM-1, as well as COX-2 enzyme activity and subsequent PGE₂ release from endothelial cells.
Discussion

Atherosclerosis is considered an inflammatory disease, which involves the interplay of prooxidative activities, induction of inflammatory cytokine, activation of vascular endothelial cells and activation of adhesion molecules, all events which promote vascular leukocyte infiltration and plaque development [6]. Inflammatory events also include the cyclooxygenase and subsequent eicosanoid pathways [153, 154]. For example, inflammatory cytokines like interleukin-1beta (IL-1-β) can induce COX-2 expression and the release of PGE2, events which may be mediated through activation of p42/44 and p38 MAPKs, and the NF-κB pathway [155]. Our data demonstrate the TNF-α can markedly induce oxidative stress, p38 MAPK, NF-κB, COX-2 and PGE2.

Sever hypertriglyceridemia has been identified as an independent risk factor for atherosclerosis, and increased circulating free fatty acid levels are associated with hypertriglyceridemia and obesity [122]. High plasma free fatty acids may contribute to an environment of increased oxidative stress and inflammation in the vasculature [123, 124], and we have previously shown that linoleic acid can markedly amplify a TNF-α-mediated endothelial inflammatory response [156]. We also have recently demonstrated that both the extracellular signal regulated kinase (ERK1/2) and phosphoinositide-3 kinase/ amino kinase terminal (PI3K/Akt) signaling pathways can contribute to the effect of linoleic acid on NF-κB-dependent transcription and endothelial cell activation [105]. In the current study, exposure to TNF-α induced oxidative stress, p38 MAPK, NF-κB, COX-2 and PGE2, which was amplified by pre-enrichment with linoleic acid. This clearly supports our hypothesis that diets high in omega-6 fatty acids, and especially in linoleic acid, are proinflammatory and thus may contribute to the early pathology of atherosclerosis.

In contrast to omega-6 fatty acids, epidemiological and interventional studies have shown a dose-dependent decrease in risk of cardiovascular disease endpoints with increased dietary consumption of moderate amounts of omega-3 fatty acids, either plant or marine derived [126]. Our data clearly demonstrate the anti-inflammatory properties of omega-3 fatty acids. In fact, our observed TNF-α induced oxidative stress, p38
MAPK, NF-κB, COX-2 and PGE2 in endothelial cells was blocked or reduced when cells were enriched relatively more with α-linolenic acid than with linoleic acid. Mechanisms of vascular inflammation are largely regulated through p38 MAPK signaling [157-160], and our data suggest that p38 MAPK is an important player in different ω-6 and ω-3 fatty acid modified TNF-α-induced inflammatory signaling cascade. The p38 MAPK inhibitor SB203580 significantly decreased TNF-α-mediated induction of COX-2 protein expression, suggesting a regulatory aspect through p38 MAPK signaling. This was supported by the evidence that p38 MAPK increases NF-κB recruitment in cells exposed to inflammatory stimuli [161].

Reactive oxygen intermediates have been reported to function as second messengers in the activation of intracellular signaling cascades, particularly the activation of the transcription factors NF-κB and AP-1 [162]. The MAPK signaling cascades are involved in the regulation and activation of these transcription factors [105]. Superoxide dismutase (SOD) has been shown to inhibit TNF-α-induced activation of p38 MAPK, NF-kB, and AP-1, thus reduces expression of VCAM-1 and ICAM-1 [144]. Implied that antioxidants have beneficial effect in pathological events involving leukocyte adhesion.

Components of the MAPK pathway have been implicated as mediators of phosphorylation of intracellular substrates such as protein kinases and transcription factors. In mammalian cells, at least three different subfamilies of MAPK have been identified: extracellular signal-regulated kinases (ERKs) with isoforms p44 MAPK (ERK1) and p42 MAPK (ERK2); stress-activated protein kinases also called c-Jun N-terminal kinases (JNKs), and the p38 MAPKs. It has been demonstrated that both JNK and p38 MAPK cascades are activated preferentially by the inflammatory cytokines such as TNF-α, as well as by a wide variety of cellular stresses such as ultraviolet light, heat shock, oxidative stress, etc. [163, 164]. Whereas activation of ERK group of MAPK is not currently considered a general event associated with TNF-α stimulation and is actually primarily involved in control of growth and differentiation [165, 166]. In general, the ERKs are mostly activated by mitogenic and proliferative stimli, whereas the JNKs and p38 MAPKs respond to environmental stress, including inflammatory cytokines [160]. Inhibition of the p38 MAPK is considered to be a promising candidate
for treatment of chronic inflammation [167]. p38 MAPK plays an important role in the regulation of NF-κB recruitment to a subset of genes activated in cells exposed to inflammatory stimuli, and a proposed mechanism is that p38 MAPK can modulate the accessibility of selected NF-κB sites in the genome [161]. P38 MAPK pathway has also been shown to involve in the induction of several other inflammatory molecules, such as COX-2, implicated in the regulation of inflammatory mediators, including PGE2 [167]. P38 MAPK has been shown to involve in TNF-α-stimulated NF-κB activation and interleukin-6 expression [168]. Our data showed that pharmacologic inhibition of p38 MAPK by SB203508 inhibited TNF-α-induced stimulation of VCAM-1, COX-2 and subsequently PGE2 synthesis. Furthermore, MAPK signaling appears to be involved in regulating omega-6 fatty acid and TNF-α-induced endothelial cell activation and modification by omega-3 fatty acids (Fig. 2.8).

Thus, data from the current study provide evidence that an increase in dietary omega-3 fatty acids contributes to an anti-inflammatory environment, which may involve cellular signaling through local repletion of selected eicosanoid species.

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Chapter Three: Involvement of Caveolae in Regulation of Endothelial Cell Activation Mediated by Fatty Acids and TNF-α

Synopsis

TNF-α transduces inflammatory responses through its receptor TNF receptor-1 (TNFR-1) which has been indicated to be enriched in plasma membrane microdomains caveolae. Hypertriglyceridemia and associated high circulating free fatty acids are important risk factors of atherosclerosis. In contrast to omega-3 fatty acids, linoleic acid, the major omega-6 unsaturated fatty acid in the American diet, may be atherogenic by amplifying an endothelial inflammatory response. We hypothesize that omega-6 and omega-3 fatty acids can differentially modulate TNF-α-induced endothelial cell activation and that functional called caveolae are required for endothelial cell activation. Caveolae are particularly abundant in endothelial cells and play a major role in endothelial trafficking and the regulation of signaling pathways associated with the pathology of vascular diseases. To test our hypothesis, endothelial cells were pre-enriched with either linoleic acid or α-linolenic acid prior to TNF-α-induced endothelial activation. Measurements included oxidative stress and NF-κB-dependent induction of COX-2 and PGE₂ under experimental conditions with intact caveolae and with cells in which caveolin-1 was silenced by siRNA. Image overlay demonstrated TNF-α-induced co-localization of TNF receptor type 1 (TNFR-1) with caveolin-1. Caveolin-1 was significantly induced by TNF-α, which was further amplified by linoleic acid and blocked by α-linolenic acid. Furthermore, silencing of the caveolin-1 gene completely blocked TNF-α-induced production of COX-2 and PGE₂ and significantly reduced the amplified response of linoleic acid plus TNF-α. These data suggest that omega-6 and omega-3 fatty acids can differentially modulate TNF-α-induced inflammatory stimuli and that caveolae and its fatty acid composition play a regulatory role during TNF-α-induced endothelial cell activation and inflammation.
Introduction

TNF-α exerts its cellular and pathological effects by binding to TNF receptor (TNFR). There are two distinct subtypes of TNFR: TNFR1 and TNFR2 [169]. TNF receptors themselves have no metabolic capability and must bind intracellular proteins to control the signaling of its target cell’s biochemistry.

Dietary ω-3 fatty acids have been shown to markedly alter the lipid composition of colonic caveolae/lipid rafts in mice fed with fish oil, relative to mice fed with ω-6 fatty acids [73]. Caveolae are 50- to 100-nm cell-surface plasma membrane invaginations that are particularly abundant in endothelial cells, where they are believed to play a major role in the regulation of endothelial vesicular trafficking and signal transduction. Caveolin-1-deficient mice suggested an important role for caveolin-1 in the pathogenesis of atherosclerosis, and vascular disease [170].

Furthermore, TNF signaling is initiated at the plasma membrane which is not homogenous. Recent evidence indicates that TNFR1 is enriched in caveolae [79] and it can form complex with caveolin-1 [171]. Caveolae are flask-shaped invaginations of plasma membrane which are composed of caveolin-1, -2 and cholesterol and glycosphingolipids. These organelles were originally discovered in the early 1950s in bladder epithelium [172], and have since been observed in adipocytes, fibroblasts, smooth and striated muscle cells, and endothelial cells show the greatest abundance [54]. Caveolae have been well accepted to function as signaling platforms. Caveolin-1 is the structural protein for caveolae which are the functional lipid rafts organelles abundant in endothelial cells. Recently, it has been more accepted that caveolae can also play a key role in cell signaling, because caveolae especially caveolin-1 has been shown to interact with many types of plasma membrane receptor proteins and concentrate these molecules with the caveolae [51].

There is evidence that fatty acids can alter localization and function of caveolae-associated signaling proteins in mouse colonic mucosa [73]. Besides their role in cellular uptake of lipophilic substances, including fatty acids [173], caveolae house an array of cell signaling molecules, and numerous genes involved in endothelial cell dysfunction and inflammation are associated with caveolae [170]. Examples of caveolae facilitated
targeting of proteins involved in pro-inflammatory signal transduction include activators of p44/p42 (ERK) MAPK pathway, H-Ras [76, 174], non-receptor tyrosine kinase c-Src [174], and the upstream regulator of NF-κB, IKK [175]. Furthermore, caveolins have been reported to co-localize with cyclooxygenase, suggesting that caveolins play a role in regulating the function of this enzyme [176, 177].

A major objective of the current study was to explore specific mechanisms involved in fatty acid-mediated activation of endothelial cells. Our current data support our hypothesis that omega-6 and omega-3 fatty acids can differentially modulate TNF-α-induced endothelial cell activation and that regulatory mechanisms are associated within caveolae and linked to caveolae function and associated gene inductions.

**Materials and Methods**

*Measurement of caveolin-1 protein level by western blot*

Cells were harvested using cell lysis buffer as previously described [105]. 15 μg protein samples were resolved by SDS-PAGE using 15% gradient gels and transferred electrophoretically to nitrocellulose membranes using a Bio-Rad immunoblot transfer apparatus (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. The nonspecific sites on the membrane were blocked 1 h at room temperature with 5% nonfat dry milk in TBST followed by incubation with caveolin-1 primary antibody at a dilution of 1:20,000 overnight at 4 °C. Caveolin-1 primary antibody was diluted 10 times from the original before using filtered PBS. β-actin was used as a house keeping gene. Bands were visualized using the appropriate horseradish peroxidase-conjugated secondary antibodies followed by ECL immunoblotting detection reagents (Amersham Biosci, UK). Hyperfilm (Amersham) was used for the visualization. Detection and quantitative analysis of the density of the bands were performed using a digitizing system (UN-SCAN-IT, Silk Scientific Corporation). To determine the optimal amounts of samples for linear area detection, a titration of different amounts of sample were loaded. For caveolin-1, 15 μg protein were loaded and for COX-2, 30 μg protein were loaded.
**Immunofluorescence microscopy**

This technique was adapted from previous protocols [178]. Cells were plated on LAB-TEKII chamber slides (Nalge Nunc International, Naperville, IL) and grown to confluence. After experimental treatments, cells were fixed in 4% (vol/vol) paraformaldehyde (in PBS) for 1 h at room temperature. After permeabilization with 0.1% (vol/vol) Triton X-100 in PBS for 5 min, cells were washed three times with PBS. Nonspecific binding sites were blocked with 5% (vol/vol) donkey serum in PBS. Cells were then incubated with anti-caveolin-1 antibody (Affinity BioReagents, Golden, CO) and anti-TNFR-1 antibody (R&D Systems, Minneapolis, MN), followed by incubation with Alexa Fluor 488-labeled donkey anti-mouse IgG antibody and Alexa Fluor 546-labeled donkey anti-goat IgG antibody respectively (Invitrogen-Molecular Probes, Carlsbad, CA). Cell nuclei were stained using Hoechst (AnaSpec Inc., San Jose, CA). Slides were mounted in Aqueous Mounting Medium with Anti-fading Agents (Biomeda Corp., Foster City, CA) and covered with coverslips. Images were captured digitally by Fluoview 300 Confocal Microscopy (Olympus America Inc., PA). In order to avoid artificial effects, the detection of the control group was set at a lower level than in Fig. 3.2. For all the other three treatment groups the strength of individual lasers, the level of contrast, and brightness were kept at the same condition as used for the control group.

**Sucrose density gradient fraction of caveolin-1 protein**

Three 100 mm culture dishes were used for each treatment group. Sucrose gradient ultracentrifugation was utilized as described in Appendix B. In brief, the sucrose gradient was created by gently overlaying lower concentrations of sucrose on higher concentrations in a centrifuge tube. Cells were washed twice with PBS and lysed with ice-cold MES-buffered saline. Sucrose was added to the lysate (final concentration of sucrose 45%), and the lysate was placed at the bottom of the ultracentrifuge tube and overlaid with lysis buffer containing 35 and 5% sucrose, respectively. After centrifugation at 39,000 rpm for 16 h, different particles travel through the gradient until they reach the point in the gradient at which their density matches that of the surrounding sucrose. Twelve 1 ml fractions from the top to the bottom were collected. Each fraction
was analyzed by Western blot for its caveolin-1 contents using specific caveolin-1 antibody.

*Detergent-free caveolae membrane purification*

Ten 100 mm culture dishes were combined for each treatment group. Detergent-free caveolae membrane purification was followed Dr. Smart’s method [179] with minor adjustment. This procedure generates a highly purified plasma membrane microdomain that is free from intracellular markers and bulk plasma membrane markers and has been used extensively to characterize caveolae membrane signaling events [180, 181]. After multiple ultracentrifugations with 5 to 15% OptiPrep gradient, the band at the 5% interface was collected and designated caveolae membranes. The cytosol fraction is obtained by collecting the overlaid material on the Percoll gradient, centrifuging again, and collecting the resulting supernatant. The plasma membranes are obtained by pooling the bottoms of both OptiPrep gradients. Each fraction was analyzed by Western blot for its caveolin-1 contents using specific caveolin-1 antibody. 1 ng protein was loaded for each sample.

*Caveolin-1 siRNA design and transfection*

Caveolin-1 gene silencing was adapted from Salani et al [182, 183]. The silence of caveolin-1 gene (GenBank Accession No. NM001753) was performed using a mix of two siRNAs directed against the following two target sequences: 5’-AACAGGGCAACATCTACAAGC-3’ and 5’-AACCAGAAGGGACACACAGTT-3’. The sequence of control siRNA was as followed: 5’-AAAGAGCGACTTTACACACTT3’. Cells were grown in 60 mm plates until 100% confluent and transfected with control siRNA or caveolin-1 siRNA at a final concentration of 40 nM using GeneSilencer (Genlantis, San Diego, CA) in Optimem I medium (Invitrogen, Carlsbad, California). Cells were incubated with 1 ml/plate serum free transfection mixtures for 4 hr, and then add 1 ml/plate of 20% FBS OptiMEM to achieve the final concentration of 10% FBS in the medium. 48 h after transfection, replace the medium by 5% FBS M199 overnight and then treated with TNF-α in the presence or absence of linoleic acid.
PGE\(_2\) determination

Cells were seeded in 60 mm culture dishes (Becton Dickinson Labware, NJ) and grown to confluence. After transfected with caveolin-1 siRNA or control siRNA, cells were pretreated with linoleic acid followed by TNF-\(\alpha\) exposure. After treatment, supernatants of cell cultures were collected into microcentrifuge tubes (Isc BioExpress, UT), centrifuged at 4 °C to remove cellular debris and stored at -80 °C. PGE\(_2\) levels were assessed using a PGE\(_2\)-specific enzyme immunoassay (EIA) (Cayman Chemicals, MI) following the manufacturer’s protocol. Absorbance at 405 nm was detected using a microplate spectrophotometer SpectraMaxPro M2 (Molecular Devices Corporation, CA).

Statistical analysis

Values are reported as mean ± standard error of the mean (SEM) of at least three independent experiments. Data were analyzed using Sigma Stat software (Jandel Corp., Wan Rafael, CA). One way ANOVA was followed by post hoc comparisons using the least significant difference (LSD) method. A statistical probability of \(p< 0.05\) was considered significant. Different letters represent significant differences among treatment groups. Capital or small letters represent caveolin-1 silencing or control silencing respectively as were compared separately by one way ANOVA.

Results

Fatty acids and TNF-\(\alpha\) modulated caveolin-1 expression

There is increasing evidence that lipid raft proteins and lipids play an important role in health and disease [184]. A major subclass of lipid rafts are caveolae, i.e., membrane domains which have been implicated in the pathology of atherosclerosis [170]. The lack of the caveolin-1 gene may provide protection against the development of atherosclerosis [185]. This may be important in understanding mechanisms of atherosclerosis, because caveolae are particularly abundant in endothelial cells, where they are believed to play a major role in the regulation of endothelial vesicular trafficking as well as the uptake of lipids and related lipophilic compounds [186]. To assess the
effects of linoleic acid and α-linolenic acid on caveolin-1 expression, endothelial cells were incubated with either linoleic acid or α-linolenic acid for 24 h. As indicated in Fig. 3.1, caveolin-1 protein level was significantly down-regulated by α-linolenic acid compared with the control culture, while there was no different in the linoleic acid treatment from control group. We next investigated the effect of TNF-α on caveolin-1 expression. As shown in Fig. 3.2, TNF-α increased caveolin-1 expression at 4 h measured by immunofluorescence study. We can also observe the basal level of caveolin-1 expression in the control culture.

Linoleic acid and α-linolenic acid modulate TNF-α-induced caveolin-1 up-regulation and the co-localization of TNFR-1 and caveolin-1

The first step in the TNF-α-induced inflammatory response is the binding of the cytokine to its cognate receptor. TNF receptor type 1 (TNFR-1) has been reported to be enriched in caveolae [79]. Caveolin-1 has also been shown to form a complex with the TNF receptor [171]. We further pre-incubated endothelial cells with either linoleic acid or α-linolenic acid, followed by activation with TNF-α. The immunofluorescence images in Fig. 3.3 demonstrated that TNF-α can increase caveolin-1 and TNFR-1 expression compared with the control group, and that pretreated cells with linoleic acid followed by TNF-α can further increase these effects compared with TNF-α treatment alone. Cells pretreated with α-linolenic acid followed by exposure to TNF-α diminished TNF-α-induced both caveolin-1 and TNFR-1 up-regulation. Similar effects of linoleic acid and α-linolenic acid on TNF-α-induced caveolin-1 expression were observed by measuring caveolin-1 protein levels by western blot (Fig. 3.4).

Linoleic acid and α-linolenic acid modulate TNF-α-induced caveolin-1 re-distribution

To further establish the caveolar localization of TNFR-1, after treated cells with different fatty acids for 24 h followed by 4 h TNF-α treatment, we used sucrose density gradient centrifugation. 12 fractions were collected and transferred to nitrocellulose membrane by Western blot. Caveolin-1 protein levels were analyzed by caveolin-1 specific antibody. As indicated in Fig. 3.5, in the control culture, caveolin-1 protein were
most enriched in fraction 4 and 5 (containing 5% sucrose, represent detergent-resistant membranes). TNF-α induced a shift of caveolin-1 protein into fraction 3 and linoleic acid further amplified this effect. In contrast, α-linolenic acid diminished TNF-α-induced the shift of caveolin-1 into fraction 3, and even decreased caveolin-1 in fraction 5 compared with control treatment. To confirm that the observation of this shift was not due to uneven transfer or other artificial effects, we analyzed fractions 3, 4, and 5 from all the four treatment groups on one western blot gel and similar results were observed (Fig. 3.5, lower panel). In fractions 7 to 9, we also observed a shift of caveolin-1 protein from fraction 8 and 9 into fraction 7 after TNF-α treatment. In the presence of linoleic acid, the TNF-α-induced shift into fraction 7 was amplified. But in the presence of α-linolenic acid, there was no change of the TNF-α-induced shift compared with TNF-α treatment alone. In fractions 10 to 12 (containing 45% sucrose) we didn’t observe the association of caveolin-1 protein.

Furthermore, the results of detergent-free caveolae membrane purification indicated that only in the presence of linoleic acid, caveolin-1 was enriched in cytosol membrane proportion (Fig. 3.6). These date suggested that linoleic acid or α-linolenic acid had profound modulation effects on caveolin-1 distribution.

Caveolin-1 silencing decreases TNF-α-induced caveolin-1 and COX-2 expression, subsequently diminishes PGE₂ production

In order to determine the role of caveolin-1 in fatty acids-modulated TNF-α-induced endothelial cell activation, we utilized small interfering RNA (siRNA) to specifically down-regulate caveolin-1. As indicated in Fig. 3.7, after 48 h transfection of caveolin-1 siRNA or control siRNA, cells were exposed to TNF-α for 4 h, in both control and TNF-α treatment groups, caveolin-1 siRNA specifically reduced caveolin-1 protein expression by approximately 80%. Similar decreasing effect by siRNA was also observed after 24 h treatment with TNF-α in the presence or absence of linoleic acid (Fig. 3.8). TNF-α-induced COX-2 expression was totally blocked by caveolin-1 silencing (Fig. 3.9) and caveolin-1 silencing also diminished the combined COX-2 induction induced by both linoleic acid and TNF-α. Similar to the COX-2 data, caveolin-1 silencing blocked
TNF-α-induced PGE₂ production and also diminished TNF-α-induced PGE₂ production in the presence of linoleic acid (Fig. 3.10). The effect of linoleic acid-induced COX-2 expression and PGE₂ production were not changed after caveolin-1 silencing. Clearly, the functional caveolae are important for TNF-α-induced up-regulation of COX-2 and subsequently production of PGE₂. Linoleic acid-induced COX-2 expression and PGE₂ production were not caveolae dependent, indicated that an alternative signaling pathway might be involved.
Figure 3.1 Effect of linoleic acid and α-linolenic acid on caveolin-1 expression

Cells were treated with 20 μM of linoleic acid (LA) or α-linolenic acid (ALA) for 24 h. Experiments were repeated three times, and the blots shown are a representative of one of the experiments. The bar graph shows the corresponding densitometric analysis of the blots. Values are means ± SEM. Different letters represent significant differences among treatment groups.
Figure 3.2  Immunofluorescence microscopy study of TNF-α-induced caveolin-1 up-regulation.

Cells were treated with 0.5 ng/ml TNF-α for 4 h. FITC green fluorescence was used for immunofluorescence staining of caveolin-1. Experiment were repeated three times and the epifluorescence micrographs shown are representative fields of one of the experiments (original magnification, X 400).
Figure 3.3 Effect of linoleic acid and α-linolenic acid on TNF-α-induced TNFR-1 and caveolin-1 localization

Cells were treated with 0.5ng/ml TNF-α alone for 4 h, or pretreated with 20 μM linoleic acid (LA) or α-linolenic acid (ALA) for 24 h followed by exposure to TNF-α for 4 h. Immunofluorescence staining for caveolin-1 (FITC green fluorescence), TNF Receptor-1 (TNFR-1) (Texas red fluorescence), and nuclear (DAPI blue fluorescence). The merged images were shown to better illustrate the partial colocalization between caveolin-1 and TNFR-1 in the same cells. Experiments were repeated three times and the epifluorescence micrographs shown are representative fields of one of the experiments (original magnification, X 400).
Figure 3.4  Effect of linoleic acid and α-linolenic acid on TNF-α-induced caveolin-1 expression

Cells were treated with 0.5ng/ml TNF-α alone for 4 h, or pretreated with 20µM linoleic acid (LA) or α-linolenic acid (ALA) for 24 h followed by exposure to TNF-α for 4 h. Caveolin-1 protein levels were detected by western blot. β-Actin was used as a housekeeping gene in the measurement. Experiments were repeated three times, and the blots shown are a representative of one of the experiments. The bar graph shows the corresponding densitometric analysis of the blots. Values are means ± SEM. Different letters represent significant differences among treatment groups.
Figure 3.5 Effects of fatty acids on TNF-α-induced caveolin-1 redistribution

Cells were treated with 0.5ng/ml TNF-α alone for 4 h, or pretreated with 20 μM linoleic acid (LA) or α-linolenic acid (ALA) for 24 h followed by exposure to TNF-α for 4 h. Caveolin-1 distribution was measured by glucose sucrose gradient. Fractions 1 to 3 containing 5% sucrose, fractions 4 to 8 containing 35% sucrose, fractions 9 to 12 containing 45% sucrose.
Figure 3.6  Effects of fatty acids on TNF-α-induced caveolin-1 redistribution

Cells were treated with 0.5ng/ml TNF-α alone for 4 h, or pretreated with 20 μM linoleic acid (LA) or α-linolenic acid (ALA) for 24 h followed by exposure to TNF-α for 4 h. Different cellular membranes were isolated using detergent-free caveolae membrane purification. This method has been well established, and different isolated membranes have been confirmed by specific markers.
Figure 3.7 Effect of caveolin-1 silencing on TNF-α-induced caveolin-1 up-regulation

Cells were first transfected with siRNA for caveolin-1 (Cav-1 siRNA) or with scrambled control siRNA for 48 h. Then cells were treated with 0.5ng/ml TNF-α for 4 h. Whole cell lysate was collected and caveolin-1 protein levels were measured by western blot.

Figure 3.8 Effect of caveolin-1 silencing on linoleic acid and TNF-α-induced caveolin-1 expression

Cells were first transfected with siRNA for caveolin-1 (Cav-1 siRNA) or with scrambled control siRNA, then treated with 0.5ng/ml TNF-α or linoleic acid (LA) alone for 24 h, or pretreated with 20μM LA for 24 h followed by exposure to TNF-α for additional 24 h. Whole cell lysate was collected and caveolin-1 protein levels were measured by western blot. β-Actin was used as a housekeeping gene. Experiments were repeated three times and the blots shown are a representative of one of the experiments.
Figure 3.9 Effect of caveolin-1 silencing on linoleic acid and TNF-α-induced COX-2 expression

Cells were first transfected with siRNA for caveolin-1 (Cav-1 siRNA) or with scrambled control siRNA. Then cells were treated with 0.5ng/ml TNF-α alone for 24 h, or pretreated with 20μM LA for 24 h followed by exposure to TNF-α for additional 24 h. COX-2 protein levels were measured by western blot under the same experimental conditions as in Fig. 3.5B. β-Actin was used as a housekeeping gene. Experiments were repeated three times, and the blots shown are representative of one of the experiments. Values are means ± SEM. Different letters represent significant difference among treatment groups. Capital or small letters represent caveolin-1 silencing or control silencing respectively as were compared separately by one way ANOVA.
Figure 3.10 Effect of caveolin-1 silencing on linoleic acid and TNF-α-induced PGE₂ production

Cells were first transfected with siRNA for caveolin-1 (Cav-1 siRNA) or with scrambled control siRNA. Then cells were treated with 0.5 ng/ml TNF-α or linoleic acid (LA) alone for 24 h, or pretreated with 20 μM LA for 24 h followed by exposure to TNF-α for additional 24 h. Supernatants of cell cultures were collected and PGE₂ levels were measured by EIA under the same experimental conditions as in Fig. 3.8 and 3.9. Experiments were repeated three times and the blots shown are a representative of one of the experiments. Values are means ± SEM. Different letters represent significant differences among treatment groups. Capital or small letters represent caveolin-1 silencing or control silencing respectively as were compared separately by one way ANOVA.
Discussion

Caveolae, specific microdomains of the plasma membrane, can concentrate different membrane receptors, signaling molecules and membrane transporters to vary extents. Caveolins are thought to function as scaffolding proteins that organize and concentrate cholesterol, glycosphingolipids and lipid-modified signaling molecules, such as endothelial nitric oxide synthase, H-ras, Src-like kinases, G protein and several hormone receptors within caveolae [187, 188]. The binding of both free cholesterol and protein by caveolae involves the same, short sequence in caveolin (residues 61 to 101) [187]. This implies that there might be a competition between lipids and proteins for the same site, within a highly ordered assembly of multiple caveolin molecules within a single caveola, in which individual polypeptides have assigned roles in the binding of either free cholesterol or protein. Several proteins of cellular lipid homeostasis are found in caveolae, including annexin II, lipoprotein scavenger receptors and CD36 [189]. These proteins do not have a caveolin-binding scaffold consensus sequence, suggesting that their primary interaction is with the lipid, not protein, moiety of caveolae [190].

Our data presented in Chapter 2 suggest that exposure to TNF-α can cause endothelial cell activation and that fatty acids (and in particular linoleic acid) can amplify TNF-α-induced endothelial activation [156], mechanisms of this interaction may be multi-layered and are not known. We further hypothesized that caveolae play a critical role in facilitating the cellular uptake and trafficking of fatty acids and trigger the initial cell signaling induced by TNF-α and lipids. We utilized siRNA-induced down-regulation of caveolin-1 which is an effective experimental approach to determine the role of caveolae in cellular signal transduction. Our data suggest that functional caveolae are necessary for TNF-α-mediated activation of endothelial cells. TNF-α markedly induced both caveolin-1 and TNFR-1, and both expressions were further induced when cells were first enriched with linoleic acid. Furthermore, silencing caveolin-1 totally blocked both TNF-α-induced COX-2 expression and cellular release of PCE₂. In human foreskin fibroblasts, a significant proportion of induced COX-2 was present in DRM fractions concomitantly with caveolin-1 [80].
It is very likely that selected fatty acids either stabilize or perturb caveolae functions, thus leading to modifications of caveolae-dependent signaling. There is evidence that fatty acids can alter localization and function of caveolae-associated signaling proteins in mouse colonic mucosa [73]. Our data suggest that omega-3 fatty acids may in part be anti-inflammatory by decreasing caveolin-1 expression or by causing dysfunctional caveolae, leading to down-regulation of caveolin-dependent down-stream signaling. Using human breast cancer cells, others have shown recently that omega-3 fatty acids can alter lipid raft composition and a decrease in epidermal growth factors [74], suggesting that selected fatty acids can modify caveolae-associated cell functions. There is evidence that caveolae play a role in lipid trafficking and uptake and transport of fatty acids [184]. Furthermore, preferential uptake of fatty acids via the FAT/CD36, a receptor exclusively located in lipid rafts such as caveolae in the plasma membrane, may explain the fatty acid effects we observed. In fact, working with mouse embryonic fibroblasts, it was recently demonstrated that mistargeting of FAT/CD36 in these cells lacking the caveolin-1 gene resulted in reduced fatty acid uptake compared with the wild-type cells [191]. Long-chain omega-3 fatty acids, such as DHA, have been shown to induce caveolar cholesterol reduction, this might due to the poor incorporation of cholesterol into phospholipids bilayers be explained by in a highly unsaturated lipid environment, [192]. In addition, α-linolenic acid has also been shown to modulate cholesterol partitioning into model membranes [193].

Our observations that linoleic acid can amplify the TNF-α-mediated induction of both caveolin-1 and COX-2 is significant because of the link between caveolins (caveolae) and the pathology of atherosclerosis [170]. Caveolin-1 has been reported to co-localize with interleukin-1beta-induced COX-2 [194], suggesting the dependence of COX-2 induction on functional caveolae. Recent evidence suggests that high-fat diets can up-regulate caveolin-1 expression in aorta of diet-induced obese rats [195], suggesting that our fatty acid data may mimic an in vivo response by activating COX-2. High-fat diets contribute to hypertriglycerideremia, and the vascular endothelium can be exposed to significant levels of free fatty acids derived from lipoprotein lipase-mediated hydrolysis of triglyceride-rich lipoproteins [128].
In summary, we provide novel data demonstrating that omega-6 and omega-3 fatty acids can differentially modulate TNF-α-induced inflammatory stimuli and that these events require functional caveolae. Furthermore, functional changes of caveolae associated with modifications by dietary fatty acids appear to affect critical phases of induction of oxidative stress-sensitive transcription factors and inducible inflammatory parameters during endothelial cell activation. Because caveolae and caveolins have been implicated in several human diseases and in particular vascular diseases, our data may have implications in understanding novel mechanisms of inflammatory diseases modulated by dietary lipids.

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Chapter Four: Changing Ratios of ω-6 to ω-3 Fatty Acids Can Differentially Modulate Polychlorinated Biphenyl Toxicity in Endothelial Cells

Synopsis

Exposure to persistent organic pollutants, such as polychlorinated biphenyls (PCBs) can cause endothelial cell activation by inducing pro-inflammatory signaling pathways. Our previous studies indicated that linoleic acid (LA, 18:2), a major omega-6 unsaturated fatty acid in the American diet, can potentiate PCB77-mediated inflammatory responses in ECs. In addition, omega-3 fatty acids (such as α-linolenic acid, ALA, 18:3) are known for their anti-inflammatory properties. We tested the hypothesis that mechanisms of PCB-induced endothelial cell activation and inflammation can be modified by different ratios of omega-6 to omega-3 fatty acids. Porcine pulmonary arterial endothelial cells were pretreated with linoleic acid, α-linolenic acid, or different ratios of these fatty acids, followed by exposure to PCB77. PCB77-induced oxidative stress and activation of the oxidative stress sensitive transcription factor nuclear factor κB were markedly increased in the presence of linoleic acid and diminished by increasing the relative amount of α-linolenic acid to linoleic acid. Similar protective effects by increasing α-linolenic acid were observed by measuring NF-κB-responsive genes, such as vascular cell adhesion molecule-1 (VCAM-1) and cyclooxygenase-2 (COX-2). COX-2 catalyzes the rate limiting step of the biosynthesis of prostaglandin E2 (PGE2). PCB77 exposure also increased PGE2 levels, which were down-regulated with relative increasing amounts of α-linolenic acid to linoleic acid. Inhibitor studies suggest that NF-κB is a critical player in the regulation of PCB-induced inflammatory markers as modulated by omega-6 and omega-3 fatty acids.

Introduction

Substantial evidence from epidemiological studies suggests that cardiovascular diseases are linked to environmental pollution. For example, there was a significant
increase in mortality from cardiovascular diseases among Swedish capacitor manufacturing workers exposed to polychlorinated biphenyls (PCBs) for at least five years [8], and most excess deaths were due to cardiovascular disease in power workers exposed to phenoxy herbicides and PCBs in waste transformer oil [9]. Furthermore, an increase in hospitalization rates was reported for coronary heart disease in populations residing near areas contaminated with persistent organic pollutants [196]. A recent study still found excessive concentrations of PCBs in the serum of Yusho victims, 35 years after accidental poisoning with PCBs in Nagasaki, Japan [197]. Interestingly, lipid peroxidation was markedly enhanced in these victims as well. These studies suggest that populations near contaminated sites are at increased risk to develop cardiovascular diseases, and in particular in the presence of additional risk factors, such as hypertriglyceridemia and an associated persistent state of oxidative stress. There is evidence linking the aryl hydrocarbon receptor (AhR) with mechanisms associated with cardiovascular diseases [198] and that AhR ligands may be atherogenic by disrupting the functions of endothelial cells in blood vessels.

A growing body of literature demonstrated that dietary components can modulate the inflammatory response in humans, thereby affecting cardiovascular risk. Evidence suggests that nutrition can influence the lipid milieu, oxidative stress and antioxidant status within cells, and thus modulate mechanisms of cytotoxicity mediated by environmental pollutants [199]. For example, certain dietary fats may increase the risk to environmental insult induced by PCBs, while fruits and vegetables, rich in antioxidant and anti-inflammatory nutrients or bioactive compounds, may provide protection [200].

Specific fatty acids rich in plant oils, such as linoleic acid (the parent omega-6 fatty acid), can amplify PCB toxicity in vascular endothelial cells [106]. There is also evidence that elevated levels of linoleic acid may enhance the cellular availability of PCBs [201]. The average American diet comprises of an excess of ω-6 fatty acids compared with ω-3 fatty acids, the ratio can reach 10 to 20:1, and it has been suggested that this imbalance could potentially promote a prothrombotic state [202]. Furthermore, coplanar PCBs can suppress delta 5 and 6 desaturase activities, thus disrupting the synthesis of fatty acid precursors for eicosanoid metabolism [203].

Our own data from plasma and livers of LDL receptor-deficient mice support the hypothesis that treatment
with PCBs can facilitate clearance of linoleic acid from plasma into vascular tissues [108]. Such a change in lipid milieu could exacerbate fatty acid- and/or PCB-induced oxidative stress and a vascular inflammatory response.

In contrast to omega-6 fatty acids, omega-3 fatty acids can influence cardiovascular disease pathology by beneficially modulating inflammation. Epidemiological and interventional studies have shown a dose-dependent decrease in risk of cardiovascular disease endpoints with increased dietary consumption of moderate amounts of omega-3 fatty acids, either plant or marine derived [126]. Cardio-protective properties of omega-3 fatty acids include down-regulation of proinflammatory and proatherogenic genes, including adhesion molecules and cytokines, during early atherogenesis and possibly also during later stages of plaque development and plaque rupture [130]. For example, an α-linolenic acid-rich oil decreased oxidative stress and CD40 ligand in patients with mild hypercholesterolemia [131], reduced levels of soluble cell adhesion molecules in plasma [132] and recurrence of coronary heart disease [133]. Randomized trials have reported that after α-linolenic acid supplementation reached the ω-6 to ω-3 fatty acids ratio 1.3:1, a significant reduction of c-reactive protein and interleutin-6 levels were observed [204].

The mechanisms by which cytokines and environmental chemicals induce endothelial cell activation, oxidative stress and inflammation are not fully understood. Oxidative stress-induced transcription factors, which regulate inflammatory cytokine and adhesion molecule production, play critical roles in the induction of inflammatory responses. One of these transcription factors, nuclear factor κB (NF-κB), plays a significant role in these regulatory processes [115]. Binding sites for NF-κB and related transcription factors were identified in the promoter regions of a variety of inflammatory genes [116, 117] such as interleukin 6 (IL-6), vascular cell adhesion molecule-1 (VCAM-1) or cyclooxygenase-2 (COX-2), all of which are up-regulated during PCB toxicity [104, 197, 205, 206].

Of increasing recognition is the paradigm that nutrition can modulate the toxicity of environmental pollutants and thus affect health and disease outcome associated with chemical insult [200]. The current study was designed to test the hypothesis that PCB-induced endothelial cell inflammation can be enhanced by omega-6 fatty acids and
antagonized by omega-3 fatty acids. We focused on omega-6 and omega-3 fatty acids, which are most commonly consumed in the average U.S. diet [129].

Materials and Methods

Cell culture and experimental media

Endothelial cells were isolated from porcine pulmonary arteries and cultured as previously described [30]. Arteries obtained during routine slaughter were donated from the College of Agriculture, University of Kentucky. The basic culture medium consisted of medium 199 (M-199) (GIBCO Laboratories, NY) containing 10% (v/v) fetal bovine serum (FBS, HyClone Laboratories, UT). The experimental media were composed of M-199 enriched with 5% (v/v) FBS and with different ratios of linoleic acid (LA) to α-linolenic acid (ALA) (> 99% pure; Nu-Chek Prep, MN). Preparation of experimental media with LA and ALA were performed as described earlier [136]. Different ratios of LA to ALA were 2:1, 1:1, and 1:2 (v/v), and the total concentration of fatty acids in all the cultures did not exceed 20 µM. Following the pretreatment with fatty acids for 18 h, cells were exposed to coplanar PCB77 for indicated times (6 to 24 h). In NF-κB inhibition studies, cells were first pre-enriched with 50 µM PDTC (EMD Biosciences, Inc., CA) for 4 h, then treated with PCB77 or LA.

PCB77 solution

PCB77 was solubilized in DMSO (sterile-filtered, Sigma-Aldrich, MO) and the final concentration in the cell culture media was 3.4 µM. This level was chosen because it has been reported in serum after acute exposure to PCBs [207, 208]. The final concentration of DMSO in the culture media did not exceed 0.03%. All vehicle controls and PCB treated cultures contained the same amount of DMSO. On the other hand, controls for fatty acid experiments did not contain DMSO.

Analysis of PGE2 synthase gene expression by real-time PCR

Total RNA was extracted from endothelial cells using RNA-STAT-60 (TEL-TEST, Friendswood, TX) according to the manufacturer’s protocol. Reverse
transcription was performed using the AMV reverse transcription system (Promega, Madison, WI). The levels of mRNAs and the PCR-product were then assessed by real-time PCR using 7300 Real Time PCR System (Applied Biosystems). Real-time PCR samples were mixed with SYBR Green Master Mix (Applied Biosystems) and PGE\textsubscript{2} specific primers. The sequences for porcine PGE\textsubscript{2} gene were designed by Primer Express Software 3.0 for real-time PCR (Applied Biosystems). PGE\textsubscript{2} sequences: sense, 5’-GCC-TGAGCGTCTGTTTTTTTA-3’; antisense, 5’-GCACCTGAATCTCTCAAGGACAT-3’.

\(\beta\)-actin was used as a housekeeping gene. \(\beta\)-actin sequences: sense, 5’-TCATCACCATCGGCAACG-3’; antisense, 5’-TTCCTGATGTCCACGTCG-3’.

**Statistical analysis**

Values are reported as mean ± standard error of the mean (SEM) of at least three independent groups. Data were analyzed using Sigma Stat software (Jandel Corp., Wan Rafael, CA). One way ANOVA followed by post hoc least significant difference (LSD)’s pairwise multiple comparison procedure were used for statistical analysis of the original data. A statistical probability of \(p< 0.05\) was considered significant. In figure 4.8, * represent significant differences among treatment groups. In all the other figures, different letters represent significant differences among treatment groups. Capital or small letters represent with or without PCB77 respectively as were compared separately by one way ANOVA.

**Results**

*Different ratios of linoleic acid to \(\alpha\)-linolenic acid modulate cellular oxidative stress induced by exposure to PCB77*

To assess the effects of changing relative amounts of linoleic acid (LA) to \(\alpha\)-linolenic acid (ALA) on oxidative stress generation in the absence and presence of PCB77, endothelial cells were incubated with different ratios of LA to ALA for 24 h without PCB77 exposure, or pre-incubated with different ratios of LA to ALA for 18 h and then exposed to PCB77 for an additional 6 h. This 6 h incubation time was
determined from our previous study [106]. As indicated in Fig. 4.1, in the absence of PCB77, LA significantly induced oxidative stress as observed by DCF fluorescence compared with control cells. When increasing the relative amount of ALA, a significant decrease in oxidative stress was observed in all groups containing more ALA than LA. In the PCB77 treatment group, oxidative stress was increased significantly compared with the control group, and pretreated cells with LA followed by PCB77 further induced oxidative stress compared with cells that were treated only with PCB77 or LA. Replacing LA with relative increasing amounts of ALA significantly reduced the additive effect of both LA and PCB on oxidative stress. Furthermore, cells pretreated with ALA followed by exposure to PCB77 blocked the oxidative stress induced with PCB77 treatment alone.

Different ratios of linoleic acid to \( \alpha \)-linolenic acid modulate PCB77-induced NF-\( \kappa \)B DNA binding activity

Oxidative stress can alter gene expression via the activation of redox sensitive transcription factors such as NF-\( \kappa \)B [209]. Previous studies in our lab have demonstrated that treatment of PCB77 for 3 h can activate NF-\( \kappa \)B, but the highest activation at approximately 6 h [106]. In the current study, we further investigated the effects of changing the relative amounts of LA to ALA on PCB77-induced NF-\( \kappa \)B activation. First, endothelial cells were incubated with different ratios of LA to ALA for 6 h. As shown in Fig. 4.2, LA significantly induced NF-\( \kappa \)B activation. When introducing ALA into the media, the activity of NF-\( \kappa \)B significantly decreased compared with cells treated with only LA. As indicated in Fig. 4.3, cells were also pretreated first with different ratios of LA to ALA for 18 h, followed by exposure to PCB77 for an additional 6 h. Compared to the control group, exposure to PCB77 significantly increased NF-\( \kappa \)B DNA binding activity and this effect of PCB77 was further increased in the presence of supplemental LA. When introducing ALA into the media, the activity of NF-\( \kappa \)B significantly decreased compared with cells treated with only LA plus PCB77. When cultures were pretreated only with ALA, a PCB77-induced increase in NF-\( \kappa \)B DNA binding was completely blocked when compared with the control group.
Different ratios of linoleic acid to α-linolenic acid modulate PCB77-induced VCAM-1 expression

NF-κB is critical in the regulation and expression of inflammatory genes, such as adhesion molecule VCAM-1. Expression of VCAM-1 on endothelial cells represents one of the early pathological changes in immune and inflammatory diseases, such as atherosclerosis [210]. We investigated whether PCB77-induced VCAM-1 expression was mediated by changing the ratios of LA to ALA. As indicated in Fig. 4.4, VCAM-1 gene expression, measured by real-time PCR, was up-regulated after exposure to PCB77. LA pre-treatment alone followed by PCB77 increased VCAM-1 gene expression, which was reduced with a relative increase in ALA. This real-time PCR data was confirmed by measuring VCAM-1 protein levels by western blot (Fig. 4.5).

PCB77 modulate three enzymes participating in PGE2 synthesis

The biosynthesis of prostaglandin E2 (PGE2), a major lipid-derived inflammatory mediator, involves three enzymatic reactions including phospholipase A2 (PLA2), COX, and PGE2 synthase. To assess the effect of PCB77 on PGE2 production, we first measured the activation of cPLA2. As shown in Fig. 4.6, PCB77 induced an increase of cPLA2 phosphorylation at 8 h treatment. Similarly, COX-2 which catalyzes the rate limiting step for PGE2 production was also increased by PCB77 at 8 h (Fig. 4.7). Furthermore, the gene expression of PGE2 synthase was significantly increased after 24 h of PCB77 treatment (Fig. 4.8).

Different ratios of linoleic acid to α-linolenic acid modulate PCB77-induced COX-2 expression and subsequent PGE2 production

COX-2 is an inducible pro-inflammatory enzyme controlled by NF-κB [211]. To assess whether different ratios of LA to ALA can modulate PCB77-induced COX-2 up-regulation, real time RT-PCR was performed on total cell RNA. Similar to the NF-κB data, PCB77-induced COX-2 mRNA levels were attenuated by increasing relative amounts of ALA (Fig. 4.9). These data were confirmed by measuring protein levels of COX-2 by western blot (Fig. 4.10).
The inducible COX-2 isoform plays a key role in inflammation and is the rate-limiting enzyme for prostaglandin synthesis. In order to assess COX-2 activity, we measured PGE\textsubscript{2} production. As shown in Fig. 4.11, PCB77 significantly induced PGE\textsubscript{2} production in a time-dependent manner. We also pretreated cells with different ratios of LA to ALA for 18 h followed by exposure to PCB77 for an additional 24 h. As indicated in Fig. 4.12, compared to the control group, LA significantly increased PGE\textsubscript{2} production, which was further enhanced by PCB77 exposure. Substituting relative amounts of LA with ALA markedly decreased the PCB-induced PGE\textsubscript{2} production.

*Inhibition of NF-κB decreases PCB77- and linoleic acid-induced VCAM-1 and COX-2 expression*

In order to confirm that NF-κB is a critical transcription factor in PCB77-induced endothelial inflammatory response as modulated by fatty acids, we studied the effect of pyrrolidine dithiocarbamate (PDTC), an antioxidant, markedly inhibited the activation of NF-κB, on both PCB77- and LA-mediated VCAM-1 and COX-2 expression by real time RT-PCR. As shown in Fig. 4.13 and 4.14, PDTC significantly decreased PCB77- and LA-induced VCAM-1 and COX-2 expression, respectively. These results clearly indicate that NF-κB is crucially involved in linoleic acid and PCB77 modulated endothelial cell activation.
Figure 4.1 Effect of different ratios of linoleic acid to α-linolenic acid on PCB77-induced oxidative stress

Cultures were incubated in media supplemented with 20 μM fatty acids, either all linoleic acid (LA), all α-linolenic acid (ALA), or different ratios of LA to ALA (R2:1, R1:1, R1:2) for 24 h. Some cultures were first pre-treated with these different ratios of fatty acids for 18 h, followed by co-exposure to 3.4 μM PCB77 for additional 6 h. Values are means ± SEM (n = 3). Statistical comparisons of the two experimental settings (without and with added PCB77) are indicated by small and capital letters. Different letters represent significant differences among the treatment groups.
Cells were treated with 20 μM of linoleic acid (LA), α-linolenic acid (ALA), or different ratios of LA to ALA (R2:1, R1:1, R1:2) for 6 h. Experiments were repeated three times, and the blots shown are a representative of one of the experiments. The bar graph shows the corresponding densitometric analysis of the blots. Values are means ± SEM. Different letters represent significant differences among treatment groups.
Figure 4.3  Effect of different ratios of linoleic acid to α-linolenic acid on PCB77-induced activation of NF-κB

Cells were treated with 20 μM of linoleic acid (LA), α-linolenic acid (ALA), or different ratios of LA to ALA (R2:1, R1:1, R1:2) for 18 h prior to exposure to 3.4 μM PCB77 for an additional 6 h. Experiments were repeated three times, and the blots shown are a representative of one of the experiments. The bar graph shows the corresponding densitometric analysis of the blots. Values are means ± SEM. Different letters represent significant differences among treatment groups.
Figure 4.4 Effect of different ratios of linoleic acid to α-linolenic acid on PCB77-induced VCAM-1 gene expression

Endothelial cells were pretreated with 20 μM of linoleic acid (LA), α-linolenic acid (ALA), or different ratios of LA to ALA (R2:1, R1:1, R1:2) for 18 h and then exposed to 3.4 μM PCB77 for additional 6 h for VCAM-1 mRNA measurement. β-actin was used as a housekeeping gene. Experiments were repeated three times. Values are means ± SEM. Different letters represent significant differences among treatment groups.
Figure 4.5 Effect of different ratios of linoleic acid to α-linolenic acid on PCB77-induced VCAM-1 protein expression

Endothelial cells were pretreated with 20 μM of linoleic acid (LA), α-linolenic acid (ALA), or different ratios of LA to ALA (R2:1, R1:1, R1:2) for 18 h and then exposed to 3.4 μM PCB77 for additional 8 h. β-actin was used as a housekeeping gene. Experiments were repeated three times, and the blots shown are a representative of one of the experiments. Values are means ± SEM. Different letters represent significant differences among treatment groups.
Figure 4.6 Effect of PCB77 on cPLA$_2$ phosphorylation

Cells were exposed to 3.4 μM PCB77 for indicated time. Phosphorylated cPLA2 was measured by phosph-cPLA$_2$ specific antibody. β-Actin was used as a housekeeping gene. Experiment was repeated twice as shown.

Figure 4.7 Effect of PCB77 on COX-2 expression

Cells were exposed to 3.4 μM PCB77 for 6 h. COX-2 protein levels were measured by western blot. β-Actin was used as a housekeeping gene. Experiment was repeated twice as shown.
Figure 4.8  Effect of PCB77 on PGE$_2$ synthase expression

Cells were exposed to 3.4 μM PCB77 for indicated time. PGE2 synthase gene expression was measured by real-time PCR. β-Actin was used as a housekeeping gene. Experiments were repeated three times. Values are means ± SEM. * represents significant difference among treatment groups.
Figure 4.9  Effect of different ratios of linoleic acid to $\alpha$-linolenic acid on PCB77-induced COX-2 gene expression

Cells were pretreated with 20 μM of linoleic acid (LA), $\alpha$-linolenic acid (ALA), or different ratios of LA to ALA (R2:1, R1:1, R1:2) for 18 h and then exposed to 3.4 μM PCB77 for additional 6 h. $\beta$-Actin was used as a housekeeping gene. Experiments were repeated three times. Values are means ± SEM. Different letters represent significant difference among treatment groups.
Figure 4.10 Effect of different ratios of linoleic acid to α-linolenic acid on PCB77-induced COX-2 protein expression

Cells were pretreated with 20 μM of linoleic acid (LA), α-linolenic acid (ALA), or different ratios of LA to ALA (R2:1, R1:1, R1:2) for 18 h and then exposed to 3.4 μM PCB77 for additional 8 h. β-Actin was used as a housekeeping gene. Experiments were repeated three times, and the blots shown are representative of one of the experiments. Values are means ± SEM. Different letters represent significant difference among treatment groups.
Figure 4.11  Optimal PCB77 incubation time for PGE$_2$ production

Cells were exposed to 3.4 µM PCB77 for 6, 18 and 24 h. Supernatants of cell cultures were collected and PGE$_2$ levels were measured by enzyme immunoassay (EIA). Bars represent means ± SEM from three independent experiments. Different letters represent significant difference among treatment groups.
Figure 4.12  Effect of different ratios of linoleic acid to α-linolenic acid on PCB77-stimulated release of PGE$_2$

Cells were incubated in media supplemented with 20 μM fatty acids, either all linoleic acid (LA), all α-linolenic acid (ALA), or different ratios of LA to ALA (R2:1, R1:1, R1:2) for 18 h. Some cultures were first pre-treated with these different ratios of fatty acids for 18 h, followed by co-exposure to 3.4 μM PCB77 for additional 24 h. PGE$_2$ levels were measured by enzyme immunoassay. Bars represent means ± SEM from three independent experiments. Statistical comparisons of the two experimental settings (without or with added PCB77) are indicated by small or capital letters.
Figure 4.13  Effect of NF-κB inhibitor on PCB77- or linoleic acid-induced VCAM-1 expression

Cells were exposed to 3.4 μM PCB77 or 20 μM linoleic acid (LA) alone for 6 h, or first pre-enriched with 50 μM PDTC for 4 h followed by coexposure to PCB77 or LA for additional 6 h. PCB77- or LA-induced VCAM-1 mRNA levels, as measured by real-time RT-PCR. β-Actin was used as a housekeeping gene. Experiments were repeated three times. Values are means ± SEM. Different letters represent significant difference among treatment groups.
Figure 4.14 Effect of NF-κB inhibitor on PCB77- or linoleic acid-induced COX-2 expression

Cells were exposed to 3.4 μM PCB77 or 20 μM linoleic acid (LA) alone for 6 h, or first pre-enriched with 50 μM PDTC for 4 h followed by coexposure to PCB77 or LA for additional 6 h. PCB77- or LA-induced COX-2 mRNA levels, as measured by real-time RT-PCR. β-Actin was used as a housekeeping gene. Experiments were repeated three times. Values are means ± SEM. Different letters represent significant difference among treatment groups.
Figure 4.15 Proposed mechanism for fatty acid-mediated modulation of endothelial cell activation induced by PCB77

Omega-6 or omega-3 fatty acids can either enhance or reduce PCB77-induced up-regulation of oxidative stress and activation of NF-κB. Relative activation of NF-κB will further regulate VCAM-1, as well as COX-2 enzyme activity and subsequent PGE₂ release from endothelial cells.
Discussion

Exposure to environmental toxicants such as persistent organic pollutants can significantly compromise health, and there is evidence that PCBs are proatherogenic. In fact, epidemiological studies with humans demonstrate a link between cardiovascular diseases and exposure to environmental pollutants. For example, an increase in hospitalization rates was reported for coronary heart disease in populations residing near areas contaminated with persistent organic pollutants [196]. Endothelial cells which line the inner layer of blood vessels are an important cell type involved in the regulation of metabolic events associated with the pathology of atherosclerosis. Dysfunction of the vascular endothelium is considered to be a critical underlying cause of the initiation of cardiovascular diseases such as atherosclerosis [6]. Environmental toxicants, once absorbed, distribute themselves to tissues, especially adipose, where they are in dynamic equilibrium with the blood. Thus, risk factors of pollutants such as PCBs are chronic and can continuously amplify pathologies of diseases that are associated with endothelial dysfunction. Data from our present study confirm that endothelial exposure to PCB77 provides a prooxidative cellular environment, sufficient to induce oxidative stress-sensitive transcription factors such as NF-κB and associated inflammatory events characteristic of early events in the pathology of atherosclerosis.

The diet is a major route of exposure to environmental toxic pollutants, such as persistent organic pollutants, including PCBs. Since many of these pollutants are fat soluble, fatty foods are not only unhealthy risk factors by themselves, but they usually contain higher levels of persistent organics than vegetable matter [109]. Thus, high-fat foods may pose as multiple risk factors in contributing to diet-derived proatherogenic lipids as well as environmental toxicants. Since omega-6 rich oils are prooxidative and can amplify PCB-mediated dysfunction of endothelial cells [106], and since omega-3 rich oils exhibit cardio-protective properties [126, 130], we were interested in comparing the effect of linoleic acid (the parent omega-6 fatty acid) and α-linolenic acid (the parent omega-3 fatty acid) on modulating PCB-mediated endothelial inflammatory events. Linoleic acid is the major fatty acid in common vegetable oils, and current estimates indicate that over 90% of the omega-3 consumed by U.S. citizens is in the form of α-
linolenic acid, not the longer chain omega-3 fatty acids found in fish oils [129]. Nevertheless, many types of fish which are an excellent source of long-chain omega-3 fatty acids are often contaminated with persistent organic pollutants such as PCBs. Whether the cardio-protective omega-3 fatty acids in fish neutralize or markedly decrease the potential health risks associated with exposure to persistent organic pollutants is not known. There appears to be some consensus that regular fish consumption should be encouraged for its overwhelming cardio-protective properties [212, 213].

Using an established cell culture model, our data clearly demonstrate that increasing the relative amount of α-linolenic acid (ALA) over linoleic acid (LA) protects against PCB-mediated endothelial activation. We have previously observed that linoleic acid can amplify PCB-mediated dysfunction of vascular endothelial cells [106]. We also have demonstrated that antioxidants such as vitamin E can protect against PCB-mediated endothelial cell activation [99]. There is increasing evidence that nutrition can dictate the lipid milieu, oxidative stress and antioxidant status within cells, and thus modulate mechanisms of cytotoxicity mediated by environmental pollutants [199]. Many environmental pollutants induce signaling pathways that respond to oxidative stress and lead to inflammatory events; and many of these same pathways are associated with the etiology and early pathology of many chronic diseases [214].

Our data support the hypothesis that the lipid milieu within the vascular endothelium can either upregulate or downregulate inflammatory events induced by PCB77. We have shown previously that 18-carbon fatty acids differing in degree of unsaturation selectively induce an inflammatory environment in human endothelial cells [215]. Of the fatty acids studied, linoleic acid stimulated NF-κB transcriptional activation the most. In addition, treatment with this fatty acid markedly enhanced mRNA levels of TNF-α, monocyte chemoattractant protein 1 (MCP-1) and adhesion molecules such as VCAM-1 [215]. In contrast, treatment with α-linolenic acid had minimal effects on these inflammatory markers. Interestingly, in the current study, a relative increase in α-linolenic acid over linoleic acid markedly reduced inflammatory markers induced by exposure to PCB77.

Mechanisms of the lipid and PCB interactions on endothelial inflammatory events are not clear, but our data suggest that NF-κB is a critical player in the overall regulation
of these events. We have previously demonstrated that PCB77-mediated activation of NF-κB can be blocked by pretreatment with pyrrolidine dithiocarbamate (PDTC), suggesting regulatory functions of PCB-induced endothelial cell activation through NF-κB signaling [99]. In the present study, we demonstrated that inhibition of NF-κB markedly reduced both PCB and linoleic acid -mediated endothelial inflammation. NF-κB binds to and affects the function of several genes encoding proteins mediating inflammation, and inhibition of NF-κB by PDTC resulted in reduced neutrophil infiltration in lungs, liver and hearts of rabbits infused with lipopolysaccharide (LPS) [216]. A recent study demonstrated that inhibition of NF-κB in aged vessels significantly attenuated inflammatory gene expression and inhibited monocyte adhesiveness [217].

Protective mechanisms of omega-3 fatty acids on down-regulation of PCB-induced vascular inflammation are not well understood. PCBs have been reported to induce arachidonic acid release in human platelets [218], and in rat amnion fibroblast cells [219] with a subsequent increase in eicosanoid formation. Our data suggest that linoleic acid can enhance and α-linolenic acid inhibit these events. One inhibitory mechanism may be a relative decrease in substrate availability for PGE2 formation during cellular enrichment with α-linolenic acid [220]. Others have reported that eicosapentaenoic acid can inhibit TNF-α induced endothelial cell inflammation via PI3K/Akt signaling pathways [221], pathways which also can regulate NF-κB DNA binding [105]. Omega-3 fatty acids also have been shown to inhibit NF-κB activation via peroxisome proliferators-activated receptor alpha signaling [222].

A recent human study demonstrated that if intake of ω-3 fatty acid (such as ALA) is sufficient, the ω-6 fatty acid intake can be increased without causing pro-inflammatory response [223]. ALA is the most commonly consumed ω-3 fatty acid in Western diets. Thus, strategies to increase ALA in the diet are important for the perspective of vascular inflammation to reduce atherosclerosis.

In summary, the current study demonstrates that diet-derived lipids can modulate PCB77 stimulated activation of endothelial cells by affecting oxidative stress sensitive signaling pathways (Fig. 4.15), including activation of NF-κB and up-regulation of inflammatory genes. Our data contribute to the paradigm that nutrition can modulate the
toxicity of environmental pollutants and thus affect health and disease outcome associated with chemical insult [200]. Whether an increase in the consumption of omega-3 polyunsaturated fatty acids can be used therapeutically against inflammation that is mediated in part by environmental pollutants warrants further study.

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Chapter Five: Summary

Conclusion

Overall, the initiation of atherosclerosis by endothelial cell activation is mediated by complex interactions between circulating nutrients, cytokines and environmental pollutants. In the current work we studied the effects of different dietary omega-6 and omega-3 fatty acids on cytokine and pollutant-induced endothelial cell activation. We have previously shown that linoleic acid, the major omega-6 fatty acid in the American diet potentiated both TNF-α and PCB-induced endothelial cell activation [106, 107, 156]. In contrast, omega-3 fatty acids from fish oil have been shown to have anti-atherogenic effects. But the beneficial effect of α-linolenic acid which is actually the major omega-3 fatty acid in the diet has not been well studied yet. Considering these findings the hypothesis was tested that the induction of endothelial cell activation may be modulated differentially by linoleic or α-linolenic acids during both TNF-α and PCB77 stimulation.

Activation of endothelial cells and associated inflammatory events are critical underlying causes of the initiation of cardiovascular diseases such as atherosclerosis [6]. The mechanisms by which selected fatty acids induce endothelial cell activation, oxidative stress and inflammation are not fully understood. Oxidative stress-induced transcription factors, which regulate inflammatory cytokine and adhesion molecule production, are important regulatory elements in the induction of inflammatory responses. One of these transcription factors, NF-κB, plays a significant role in these regulatory processes [115]. Binding sites for NF-κB and related transcription factors were identified in the promoter regions of a variety of inflammatory genes [116, 117].

Our data in chapters 2 and 4 indicate that both TNF-α and PCB77 can induce up-regulation of oxidative stress, NF-κB activation and NF-κB-dependent induction of VCAM-1, COX-2, and a subsequent increase in PGE₂ production. All of these events were amplified by pre-enrichment with linoleic acid but blocked or reduced by α-linolenic acid. Prostanoids represent a group of lipid mediators. PGE₂ is the most common prostanoid which is generated at sites of inflammation [224]. The biosynthesis of PGE₂ involves three enzymatic reactions including phospholipase A₂ (PLA₂), COX,
and terminal PGE$_2$ synthase. We demonstrated that both TNF-$\alpha$ and PCB77 can activate cPLA$_2$ at 8 hour, a similar time point when COX-2 up-regulation was observed. Both cPLA$_2$ and COX-2 have been shown to locate predominantly in the nuclear envelope [225, 226], these permit efficient functional coupling between cPLA$_2$ and downstream enzyme COX-2. Furthermore, the time course studies indicated that after 24 hours treatment, TNF-$\alpha$ and PCB77 induced significant PGE$_2$ production. This later response might partially due to the late activation of terminal PGE$_2$ synthase, as shown by our real-time PCR data that PGE$_2$ synthase expression was significantly increased by PCB77 after 24 hours treatment.

In chapter 3, we further explored the role of caveolae in fatty acids mediated TNF-$\alpha$-induced inflammatory responses in endothelial cells. Because a growing body of evidence indicates that dietary fatty acids can alter caveolae microenvironment, thereby modifying location and function of proteins in caveolae. Previous study in endothelial cell fatty acid composition of caveolae has shown that palmitic acid (16:0), palmitoleic (16:1), stearic acid (18:0), together with oleic acid (18:1) compose the 80% of total fatty acids, whereas linoleic acid (18:2) composes of 10% of total fatty acids in caveolae [71]. EPA has been shown to displaces caveolin-1 from caveolae in endothelial cells [72]. Moreover, caveolin-1, the functional protein of caveolae can bind to many types of plasma membrane receptor proteins or membrane associated enzymes and concentrate these molecules within caveolae [227]. TNFR-1 has also been shown to concentrate within the caveolae of a human endothelial cell line [79].

Interestingly, our data indicated that functional caveolae are necessary for TNF-$\alpha$-mediated activation of endothelial cells. TNF-$\alpha$ markedly induced caveolin-1 and TNFR-1, and both expressions were further induced when cells were first enriched with linoleic acid. In contrast, $\alpha$-linolenic acid diminished these effects. These data indicated that different fatty acids may modulate both TNFR-1 and caveolin-1 expression, thus altering the interaction of the two. These will in turn profoundly influence TNFR-1 dependent downstream signaling. Moreover, silencing caveolin-1 totally blocked TNF-$\alpha$-induced COX-2 up-regulation and PGE$_2$ biosynthesis. Previous studies have shown that COX-2 may be localized to caveolae-like structures [228]. COX-2 in human foreskin fibroblasts stimulated by IL-1$\beta$ was localized to plasma membrane in addition to
nuclear envelope, and image overlay showed colocalization of COX-2 with caveolin-1 [80]. These results indicated that caveolae are important cellular platform for TNF-α receptor-mediated down stream COX-2 activity. In contrast, linoleic acid-induced COX-2 up-regulation and PGE₂ production were not altered by caveolin-1 silencing. Taken together, our data indicated that lipid microdomains, and in particular caveolae, may be a novel modality mediating the effects of linoleic and α-linolenic acids in response to TNF-α-mediated inflammatory stimulations, but the inflammatory responses induced by linoleic acid appear to be independent of caveolae protein. It is very likely that selected fatty acids either stabilize or perturb caveolae functions, thus leading to modifications of caveolae-dependent signaling.

There is evidence that fatty acids can alter localization and function of caveolae-associated signaling proteins in mouse colonic mucosa [73]. These data suggests that omega-3 fatty acids may in part be anti-inflammatory by decreasing caveolin-1 expression or by causing dysfunctional caveolae, leading to down-regulation of caveolin-dependent down-stream signaling. Moreover, perturbation of membrane raft structural integrity with cholesterol-sequestering compounds had been shown to cause delocalization of eNOS from caveolae and to inhibit TNF-α-induced ROS production and protein tyrosine nitration [229].

Furthermore, we recently investigated caveolin-1-regulated mechanisms associated with PCB-induced markers of peroxynitrite formation and DNA binding of NF-κB [230] and demonstrated that caveolin-1 silencing abolished the PCB-stimulated NF-κB DNA binding, suggesting a regulatory role of caveolae in PCB-induced endothelial cell activation. Our unpublished data have also shown that PCB treatment increased formation of caveolae and PCB77 accumulated mainly in the caveolae-rich fraction and AhR, the main molecular target of PCB77 was associated with caveolin-1, and the association was increased after PCB77 treatment. Thus, the broad mediation effects of fatty acids appear to be largely due to their role as structural components of cellular membranes and are involved in many important cell-signaling pathways. This suggests that a common fundamental mechanism might be involved in the inflammatory cytokine TNF-α and environmental pollutant PCB77-triggered pro-inflammatory cascades.
In summary (Fig. 5.1), we provide novel data demonstrating that omega-6 and omega-3 fatty acids can differentially modulate TNF-α and PCB77-induced inflammatory stimuli and that these events require functional caveolae. Furthermore, functional changes of caveolae associated with modifications by dietary fatty acids appear to affect critical phases of induction of oxidative stress-sensitive transcription factors and inducible inflammatory parameters during endothelial cell activation. Because caveolae and caveolins have been implicated in several human diseases and in particular vascular diseases, our data may have implications in understanding novel mechanisms of inflammatory diseases modulated by dietary lipids. Furthermore, research findings present in this dissertation contribute to the paradigm that nutrition can modulate the toxicity of environmental pollutants and thus affect health and disease outcome associated with chemical insult [200]. Whether an increase in the consumption of omega-3 polyunsaturated fatty acids can be used therapeutically against inflammation that is mediated in part by environmental pollutants warrants further study.

**Future Directions**

The current study demonstrated the critical role of functional caveolae in fatty acid-mediated endothelial activation. Since caveolae contain sphingomyelin and initiate the conversion of sphingomyelin to ceramide which is a novel sphingomyelin-derived second messenger mediating cellular signals, further studies are needed to clarify the effects of fatty acids on caveolae composition and to explore further their role in ceramide metabolism (Fig. 5.2).

A lipid raft membrane is composed of sphingolipids and cholesterol in the exoplasmic leaflet, linked to glycerophospholipids and cholesterol in the cytoplasmic leaflet of the lipid bilayer [231]. It is possible that long chain fatty acid substituents of sphingolipids extend from the outer to the inner leaflet where they interdigitate with the saturated side chains of glycerophospholipids and establish a close association [232]. In caveolin-1 knockout mice, the transport of glycosylphosphatidylinositol-anchored proteins to the cell surface was inefficient, consistent with a role for caveolin in the transport of these lipid raft-associated proteins to the plasma membrane [233]. Caveolin-
1 null mice showed a dramatic increase in serum triacylglycerols and non-ester fatty acid levels [227] indicated that caveolae might have a primary role in regulating the lipid balance by stimulating fatty acid uptake.

Lipids are crucial structural and functional components of cells. Even more importantly, many lipid species have distinct cellular functions. For example, ceramides, eicosanoids, diacylglycerol (DAG) and lysolipids are all second messengers which participate in various cellular events such as inflammation, growth, proliferation, differentiation and cell death. Sphingomyelin is one of the major lipids in cell membranes and plasma lipoproteins. In clinical studies, sphingomyelin plasma levels have been shown to correlate with the occurrence of coronary heart disease independent of plasma cholesterol levels [234]. Previous studies indicated that the endothelium contributes to ceramide-induced relaxation possibly through an interaction between sphingomyelin hydrolysis and endothelial NO synthase within caveolae [235]. Furthermore, TNF-α has also been shown to profoundly affect sphingolipid metabolic pathways leading to the accumulation of one or more sphingolipids. For example, TNF-α transiently increases cellular ceramide by increasing the hydrolysis of sphingomyelin. Increased ceramide levels activate ERK [236, 237], JNK [237], MAPKs and NF-κB translocation [238]. Sphingomyelin and ceramide both contain one fatty acyl moiety that is linked to the sphingosine backbone by an amide bond. Whether the type of this fatty acyl moiety can be altered by dietary fatty acids in endothelial cells has not yet been studied. In our study the different polyunsaturated fatty acid-induced modification in response to TNF-α and/or PCB may be due to the changes of the microdomain polarization within sphingolipids modified by dietary fatty acids. However, the effects of PCB on ceramide signaling pathways have not been explored yet.

Thus, future studies of a more detailed involvement of caveolae in the cellular regulation of fatty acid-mediated modulation of a vascular inflammatory response may be considered.
Figure 5.1 Summery

Figure 5.2 Future direction
Appendix: Protocols

Primary Culture of Porcine Pulmonary Artery Endothelial Cells

Obtain tissue:
A. Supplies:
   1. Sterile: tweezers, scissors, razor blades, sponge gauze, specimen containers, gloves, Other supplies: 3 beakers (250 ml);
   2. Media and solutions: M199+3xP/S (warm up to 37°C), 70% ethanol
B. Obtain tissue:
   1. Take the heart of porcine during routine slaughter at the College of Agriculture. Find pulmonary artery which is next to aorta. Gently separate aorta and pulmonary artery using the other side of scissors. Try not to squeeze vessels too hard.
   2. After separation, use intestinal forceps to clamp artery at the end nearest the heart. Cut the vessel off at the edge of clamp, then with the clamps still on, dip artery in the 70% ethanol solution and then dip into M199+3xP/S.
   3. Take off the clamps, put artery into a sterile specimen cup with enough M199+3xP/S to cover the whole artery. Label the container, and place on ice carry back to the lab and put in 4°C.

Culture cells (should be done completely inside the hood):
A. Supplies
   1. Sterile: culture dishes, tweezers, allis intestinal forceps, sponge gauze, centrifuge tubes, scalpel, scissors, plugged Pasteur pipettes, plastic transfer pipets, measuring pipets, cotton swabs with wooden stick, T25 tissue culture flasks.
   2. Media and solutions: M199+3xP/S and M199+10% FBS (both warmed up to 37°C). Fill two 250 ml beakers with 70% ethanol and one 250 ml beaker with M199+3xP/S.
   3. Collagenase solution: will need 2 ml for each artery. Add 1 mg of collagenase per ml of Hanks buffer (warm up to 37°C). Vortex the solution and centrifuge at 2500 rpm for 10 min. Filter supernatant using a syringe filter unit (0.22 μM).
B. Isolation of endothelial cells
   1. Take artery from specimen container and place on Hanks moisturized sterile sponge gauze (handle artery with steriled tweezers, don’t touch it with hands). Use a Pasteur pipet to rinse the inside of the artery with M199 to get off blood clots and blood cells and avoid touching the inside of the artery. Then use a forcep and tweezers to clamp one end of the artery. Use a pasteur pipet fill from the open end of the artery with collagenase solution and clamp it up with another forcep. Wait for 5 min.
   2. During the waiting time, add 2 ml of M199+10% FBS into each of two 30 mm tissue culture dishes (swab twice for each artery), and label with cell line, date, and number of swab (1 or 2). When the 5 min are over, release forceps and dry collagenase solution into sterile sponges. Rinse inside of artery with a pasteur pipet full of M199+10%FBS and place artery on a dry, sterile gauze pad. Cut
artery open length wise with a pair of sharp sterile scissors, avoid touching other area of the artery besides the cutting line.

3. Wet a sterile cotton swab in the media from the 30 mm tissue culture dish labeled swab1. Very gently rub the swab over the inside surface of the artery. Do not put any pressure on the swab and avoid swabbing where the forceps clamped or over the same place twice or at the edge of the artery. Make about two rounds swabbing and then stir the swab around in the media and press it against the sides to release the endothelial cells into the media. Repeat this with a new swab and put into the dish labeled with swab 2.

4. Place culture dishes into the incubator. 4 h later, use the aspirator to remove the media and replace it with 2 ml of new 37°C M199+10%FBS media. Change media again at 24 or 48 h after this depends on how many cells are attached.

5. 4 or 5 days later, when the cells get confluent, pass one 30 mm dish into one T25 tissue culture flask (passage 1). When it gets confluent, pass one T25 into one T75 flask (passage 2). Later pass one T75 into one T225 or three T75 (passage 3). Usually freeze cells at passage 4 or 5. Isolated primary endothelial cells can be used up to 15 passages.

Freeze Endothelial Cells

One confluent T-75 flask can be frozen into 2 cryotubes. One T-225 flask can be frozen into 6 cryotubes.

1. For one T-75 flask: rinse cells with 10ml hanks then add 1 ml trypsin. Can do two T-75 at one time. When cells are detached, add 10ml 10% M199 and transfer into a 15ml conical tubes.

2. For one T-225 : Trypsinized cells with 1.5 ml trypsin. add 30 ml 10% M199 and transfer into three 15 ml conical tubes.

3. Centrifuge at 1200rpm for 10 min (at 37°C). Decant supernatant (gently) and add 2 ml freezing cocktail* into each conical tube. Gently mix the cells with freezing cocktail. Transfer to 2 cryotubes with 1 ml each (tubes should be properly labeled with passage, date and operator).

4. Keep tubes between two foam layers in -20°C overnight or in “Slow Freeze Apparatus (isopropano jacketed container)” for 24 h, then put in -80°C. After at least 18 h in -80°C, put cryotubes in liquid nitrogen tank.

*Freezing cocktail must be made fresh each time:
Every T-75 flask needs 2 ml of freezing cocktail, prepare a little extra (plus 2ml). Warm up everything to 37 C before using.

<table>
<thead>
<tr>
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<th>1ml</th>
<th>For 8 T75 prepare 18 ml</th>
<th>For 9 T75 prepare 20 ml</th>
<th>For 18 T75 prepare 38 ml</th>
</tr>
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<tr>
<td>28% DMSO (ml)</td>
<td>0.25</td>
<td>4.5</td>
<td>5</td>
<td>9.5</td>
</tr>
<tr>
<td>FBS (ml)</td>
<td>0.2</td>
<td>3.6</td>
<td>4</td>
<td>7.6</td>
</tr>
<tr>
<td>M199 (ml)</td>
<td>0.55</td>
<td>9.9</td>
<td>11</td>
<td>20.9</td>
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</table>

Preparation of 28% DMSO in M199:

<table>
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<tr>
<th></th>
<th>1 ml</th>
<th>4.5 ml</th>
<th>5 ml</th>
<th>10 ml</th>
</tr>
</thead>
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<tr>
<td>DMSO (ml)</td>
<td>0.28</td>
<td>1.26</td>
<td>1.4</td>
<td>2.8</td>
</tr>
<tr>
<td>M199 (ml)</td>
<td>0.72</td>
<td>2.24</td>
<td>3.6</td>
<td>7.2</td>
</tr>
</tbody>
</table>
Start a Frozen Endothelial Cell Line

1. Warm up M199+10%FBS media to 37°C and set centrifuge to 37 °C in ahead. (To achieve the temperature quicker, can set the temperature to 40°C, speed to 3000 rpm, and let run for 10 min)
2. Take one cryotube of frozen cells from liquid N2 tank or -80°C freezer at one time.
3. Add 9 ml media into a 15 ml conical tube for one cryotube. Use a 5 ml pipet get 1 ml media from the conical tube put it quickly up and down into cryotube to thaw the 1 ml freezing cocktail with endothelial cells. Put the resolved part into the conical tube and get some fresh and warm media from the same tube, up and down again until get all the liquid out from the cryotube.
4. Centrifuge at 2000 rpm for 5 min immediately. Don’t leave cells with freezing cocktail at room temperature for too long. Then use the aspirator to remove the media and freezing cocktail, put 1 ml of fresh media and gently scatter the pellet of cells. Add 9 ml of media to conical tube and centrifuge for 5 min again. Remove the media after centrifuge, repeat the scatter and mix cells with 10 ml of media. Place the cell suspension into one T75 cell culture flask. Change media 4 h after revival or change media in the following morning depends on how many cells attached.

Cell Culture and Experimental Media

A. Cell culture
   Pass the cells:
   1. It will usually take two days for cells to grow confluent. When cells are ready, use the aspirator to remove old media. Add 6 ml of Hanks for a T75 flask and 10 ml for T225. Gently tilt the flask to rinse all the cells. Remove Hanks buffer 1 after 1 min incubation at room temperature.
   2. Add trypsin (1 ml for the T75 and 2 ml for the T225 flask). Place in the incubator for 2 to 3 minutes. Then strike the side of flask to detach the cells and check under the microscope to see whether all cells are detached as little round circle.
   3. While cells are in the trypsin incubating, add medium to new flasks (7 ml for the T75 and 20 for the T225), and label with “P#” for the passage, and date.
   4. After the cells are detached gently add 12 ml of medium (for T75) or 30ml of medium (for T225), up and down avoid air bubble. Usually one T75 can be passed into 3 T75 or one T225. One T225 can be passed into 3 T225. Add 4 ml (for T75) or 10 ml (for T225) of the re-suspended cells to each new flask.
   5. Place flasks in the incubator.

Plating the cells:
   1. Ratio of cell suspension and media for plates and flasks
      100 mm culture dishes can hold up a total volume of 10 ml (3 ml of the cell suspension plus 7 ml of medium)
      60 mm dishes can hold a total volume of 3 ml (1 ml of the cell suspension plus 2 ml of medium).
      After plating, it usually takes about 48 h for cells to get confluent.
T-75 flask can be passed into three T-75, one T-225, four 100 mm plates, or twelve 60 mm plates.
T-225 flask can be passed into three T-225, twelve 100 mm plates, or thirty six 60 mm plates.

2. Plating cells on multiple well plates
No matter the multiple-well plate has how many wells, the whole plate usually can hold up to 12 ml of total media volume. This will need 4 ml of the cell suspension plus 8 ml of media. Usually use T-75 flask to plate. Always make the 12 ml of the cell and medium mixture first before divided into each well. For a 6 well plate: 12 divided by 6, so put 2 ml of the mixture into each well. For a 12 well plate: put 1 ml into each well. For a 24 well plate: put 0.5 ml et al.

B. Preparation of Media and Hanks
1. M199+AA:
   Add 2 pouches of the media (M199, Gibco Cat No 31100-035)
   Add 4.4g of NaHCO
   Add 2 L of autoclaved (dl H2O)
   Add 2ml of NaOH (6N same as 6M)
   Add 40 ml of amino acids (premade solution from Gibco)
   Mix well and adjust pH to 7.4

2. Hanks
   KCl: 0.8g
   KH2PO4: 0.12g
   NaCl: 16g
   NaHCO3: 0.7g
   Na2HPO4 * 7H2O: 0.18g
   D glucose: 2.0g
   Phenol Red: 0.02g
   Add 2 L of autoclaved H2O adjust pH to 7.4 and then filter.

3. Trypsin
   Remove 40ml of Hanks (new un-used bottle that has 400 ml total)
   Add 40 ml of trypsin to the remaining 360 ml of Hanks. Swirl and make 12 ml aliquots

4. 10 % or 5% FBS M199
   For 10% media: Take out 52 ml of the M199 media from 400ml into another bottle and add 4ml of P/S (penicillin streptomycin, Cat No 15140-122), 4 ml of L-glutamine (Cat No 25030-156), 4 ml of MEM amino acids (GIBCO Cat. No. 11130-051), and 40 ml of FBS (fetal bovine serum).
   For 5% media: Take out 32 ml of the M199 media and add 4ml of P/S (penicillin streptomycin, Cat No 15140-122), 4 ml of l-glutamine (Cat No 25030-156), 4ml of MEM amino acids (GIBCO Cat. No. 11130-051) and 20 ml of FBS (fetal bovine serum)

Preparation of Fatty Acid Enriched Media

A. Preparation of fatty acids stock solution
The pure fatty acids are purchased from NuCheck (http://www.nu-chekprep.com/home.htm). The stock solution is made in Hexane.

1. Get fatty acid from the commercial container (100mg). Record the weight of fatty acid and the molecular weight. Make a 5 ml solution in graduated flask and then calculate the final concentration: \[ N_1 \]
   \[
   \frac{X \text{ mg} \times 1000}{5 \text{ ml} \times MW}
   \]
   Result is in unit as mM.

2. Fill the graduated flask with gas N\(_2\) and carefully sealed with parafilm.

B. Fatty acids Media preparation

Fatty acid will be prepared in M199 enriched with 5% (v/v) FBS. Assuming the 100% serum has an average concentration of albumin at 600 \(\mu\)M, then the 5% FBS will give us up to 30 \(\mu\)M albumin. The ratio of free fatty acids to albumin should not exceed 3 to 1, otherwise fatty acid is toxic to cells. So, for a 5% FBS media, the highest fatty acid concentration can achieve is around 90 \(\mu\)M.

1. For 10ml plate, if the final conc. of fatty acid is 20 \(\mu\)M (0.02 mM), we need to calculate how much fatty acid solution in hexane we will need. \(V_1\)
   
   Set 1 as stock solution, 2 as experimental solution:
   \[
   V_1N_1=V_2N_2
   \]
   \[
   X\times N_1 = 10 \times 0.02
   \]
   
   \[
   X= \frac{N_1}{10 \times 0.02}
   \]
   
   \(X\) is in the unit of \(\mu\)l

2. For 12 100 ml plates, we will need 120ml media. Always prepare extra 5 ml, because we will need to filter the media and later which can cause the lost of some volume.

After finishing the calculation, take a glass tube put NaOH 30 times exceed of FA concentration. Volume of 6 N NaOH: \(V_3\)

\[
\text{FA conc.} \times 30 \times V_2
\]

6

Put NaOH (\(V_3\)) in a culture glass tube, then add FA (\(V_1\)). Carefully blow in the gas N\(_2\) for 15 min to 30 min until it totally dried (white powder).

3. Wash out FA from glass tubes into the media (\(V_2\)). Adjust pH of the media to 7.4 with HCl.

4. Filtration through the syringe filter or bottle filter (0.02 \(\mu\)m) then add to the cells.

Oxidative Stress Measurement

1. Grow cells in microplates (12, 24 or 48) well plates. Include a control groups to be used as negative and positive controls.

2. Wash cells at least two or three times with Hanks buffer to remove serum.

3. Add KREBS-Ringer-Glucose buffer containing DCF (100 \(\mu\)mol/L) to microplates. Use 1ml for 12 wells and 0.5 ml for 24 wells and incubate for 30 minutes. Blank well
receives KREBS-Ringer-Glucose buffer without DCF probe. Wrap plate in aluminum foil to protect from the light.
4. Wash 2 to 3 times with HEPES buffer.
5. Add HEPES to each well (1 ml for 12 well plates and 0.5 ml for 24 well plates. Wrap plate in aluminum foil.
Optional: Positive control receives 0.1 mmol/L H2O2 in HEPES buffer after DCF staining.
6. Set plate reader excitation to λ 490 nm and emission to λ 520 and/or 530 nm.
Light sensitive reactions: protect stocks from light by wrapping in aluminum foil when adding DCF media on cells, switch off the light in the hood.

Buffers and solutions:
HEPES buffer (500ml pH 7.4):
- NaCl: 4.237g
- KCl: 186.4mg
- MgCl2: 50.8mg
- D-glucose: 901.0mg
- HEPES: 1.1915g
- CaCl2: 110.25mg

KREBS-Ringer-Glucose Buffer:

<table>
<thead>
<tr>
<th>Components</th>
<th>To make 1 L</th>
<th>To make 500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>118 mM NaCl</td>
<td>6.9 g</td>
<td>3.448 g</td>
</tr>
<tr>
<td>4.7 mM KCl</td>
<td>0.35 g</td>
<td>0.175 g</td>
</tr>
<tr>
<td>1.3 mM CaCl2</td>
<td>0.191 g</td>
<td>0.096 g</td>
</tr>
<tr>
<td>12.0 mM MgCl2</td>
<td>2.375 g</td>
<td>1.187 g</td>
</tr>
<tr>
<td>1 mM NaH2PO4H2O</td>
<td>0.138 g</td>
<td>0.069 g</td>
</tr>
<tr>
<td>25 mM NaHCO3</td>
<td>2.1 g</td>
<td>1.050 g</td>
</tr>
<tr>
<td>11mM Glucose</td>
<td>1.982 g</td>
<td>0.991 g</td>
</tr>
</tbody>
</table>

DCF stock solution:
To make the DCF stock solution mix 9.7458 mg of DCF with 1 ml of DMSO. The DCF concentration in the stock is 20 mM.

Note on DCF stock: 20mM in EtOH or DMSO, MW of DCF 487.29g.
To make the DCF working solution, add 125 μl of the stock and complete volume to 25 ml with KREBS buffer. The DCF concentration in the working solution should be 100 μM.

Fluorescent plate reader settings:
Softmax Pro 3.1.2
Set up wavelength:
- Sensitivity: 15
- Auto Calibrate: on
- Strip
Template: set blank at 0, or set all samples.

PGE2 EIA kit

Plate cells:
Small plates will be used, so that the protein level of COX-2 and mPGE synthase can be measured.

Supernatants of cell cultures will be mixed well then collected 1 ml directly into Eppendorf’s tubes. Store at -80 C. In the day of measurement, thaw the samples, mix well then centri at 4C 12000rpm for 10 min.

Using the Kit:
Condition preparation: for the first time to use the kit. Make standard curve dilution in 5% medium (the four highest ones). And one control and one TNF-α treatment sample to check whether it’s within the range of standard curve.

Key terms:
Blank: background absorbance caused by Ellman’s reagent.
Total activity: total enzymatic activity of the AchE-linked tracer.
Non-specific binding (NSB): non-immunological binding of the tracer to the well.
Maximum binding (B0): maximum amount of the tracer that the antibody can bind in the absence of free analyte.
% bound/ maximum bound (%B/B0): ratio of the absorbance of a particular sample or standard well to that of the maximum binding.
Standard curve: a plot of the %B/B0 values versus concentration of a series of wells containing various known amounts of analyte.

A. Pre-assay preparation:
Buffer preparation (all stored at 4C, will be stable for ~2 months)
1. EIA buffer: dilute one vial of EIA buffer (vial #4) with 90 ml of Ultrapure water. Rinse the vial well.
2. Wash buffer: dilute 5 ml vial of Wash buffer (vial #5) to a total volume of 2 liters with Ultrapure water and add 1 ml of Tween 20 (vial #5a) use a syringe.

<table>
<thead>
<tr>
<th>Wash buffer (vial #5)</th>
<th>Ultrapure water (1:400 of wash buffer)</th>
<th>Tween 20 (vial #5a) (0.5ml/liter wash buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5ml</td>
<td>2 L</td>
<td>1ml</td>
</tr>
<tr>
<td>12.5ml</td>
<td>5 L</td>
<td>2.5ml</td>
</tr>
</tbody>
</table>

Collecting samples
Cell culture supernatants may be assayed directly. If the PGE2 concentration in the medium is high enough to dilute the sample 10-fold with EIA buffer, the assay can be performed without any modifications. When assaying less concentrated samples (where samples cannot be diluted with EIA buffer), dilute the standard curve in the same culture medium as that used in the experiment. This will ensure that the matrix for the standards is comparable to the samples. A standard curve is recommended to be run first to ensure that the assay will perform in a particular medium.

B. Preparation of assay-specific reagents
1. PGE2 standard
Reconstitute the contents of the PGE2 standard with 1 ml of EIA buffer. The concentration of this solution (the bulk standard) will be 10 ng/ml. Stored at 4C; this standard will be stable for up to 4 weeks.

NOTE: if assaying culture medium samples that have not been diluted with EIA buffer, culture medium should be used in place of EIA buffer for dilution of the standard curve. The diluted standards should not be stored for more than 24 hr.

2. PGE2 AChE tracer: Will be used twice in the two days measurement. Always keep the rest.

Reconstitute the 100 dtm PGE2 tracer (vial #2) with 6 ml EIA buffer. Store the reconstituted PGE2 tracer at 4C (DO NOT FREEZE!) and use within 2 weeks.

Tracer Dye Instructions: this dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the reconstituted tracer at a final dilution of 1:100 (add 60 μl of dye to 6 ml tracer or add 300 μl of dye to 30 ml of tracer).

3. PGE2 monoclonal antibody:

Reconstitute the 100 dtm PGE2 antibody (vial #1) with 6 ml EIA buffer. Store the reconstituted PGE2 antibody at 4 C. it will be stable for at least 4 weeks.

Antiserum dye instructions: this dye may be added to the antiserum, if desired, to aid in visualization of antiserum-containing wells. Add the dye to the reconstituted antiserum at a final dilution of 1:100 (add 60 μl of dye to 6 ml antiserum).

C. Performing the assay

1. Plate set up

It is not necessary to rinse the plate prior to adding the reagents. If strip plate, place the unused strips back in the plate packet and store at 2-4C. Be sure the packet is sealed with the desiccant inside.

Each set of strips must contain a minimum of two blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B0), and an eight point standard curve run in duplicate. Each sample should be assayed at two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

2. Pipet the reagents:

Before pipetting each reagent, equilibrate the pipet tip in that reagent (slowly fill the tip and gently expel the contents, repeat several times).

EIA buffer:
Add 100 μl EIA buffer to Non-specific binding (NSB) wells.
Add 50 μl EIA buffer to Maximum binding (B0) wells.

Or: If culture medium was used to dilute the standard curve, substitute 50 μl of culture medium for EIA buffer in the NSB and B0 wells:
Add 50 μl culture medium and 50 μl of EIA buffer to NSB wells.
Add 50 μl culture medium to B0 wells.

PGE2 standard:
Add 50 μl from tube #8 to both of the lowest standard wells (S8).
Add 50 μl from tube #7 to each of the next two standard wells (S7).
Continue with this procedure until all the standards are aliquoted.
The same pipet tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipet tip in that standard.

**Samples:**

Add 50 μl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

**PGE2 AChE tracer:**

Add 50 μl to each well except the total activity (TA) and the blank (Blk) wells.

**PGE2 Monoclonal antibody:**

Add 50 μl to each well except the Total Activity (TA), the Non-Specific Binding (NSB), and the Blank (Blk) wells.

**Incubate the plate:**

Cover each plate with plastic film and incubate 18 hr at 4C.

**Develop the plate:**

When ready to develop the plate, reconstitute one 100 dtn vial of Ellman’s Reagent (vial #8) with 20 ml of UltraPure water. Reconstitute Ellman’s Reagent is unstable and should be used the same day it is prepared; protect the Ellman’s Reagent from light when not in use.

Empty the wells and rinse five times with Wash Buffer. Add 200 ml of Ellman’s Reagent to each well and 5 ml of tracer to the Total Activity wells. Cover the plate with plastic film.

Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plates to develop in the dark. This assay typically develops (i.e., B0 well ≥ 0.3 A.U.) in 60-90 minutes.

**Read the plate:**

Read the plate at a wavelength between 405-420 nm (405 was used). Before reading, wipe the bottom of the plate with a clean tissue to remove finger prints. The plate may be checked periodically until the B0 wells have reached a minimum of 0.3 A.U. the plate should be read when the absorbance of the B0 wells is in the range of 0.3-1.0 A.U. If the absorbance of the wells exceeds 1.5, wash the plate, add fresh Ellman’s Reagent and let it develop again.

**Immunofluorescence**

Plate cells (100μl cells plus 300μl Medium) onto glass chamber slides leaving room for 3 extra controls (IgG, no 1°, no 1° or 2°), grow to confluence, serum starve overnight. Then start the treatment.

1) Remove media from wells.
2) Depends on antigen of interest, could fix with:
   a). 500 μL of 50:50 acetone/methanol solution for 30 minutes at RT;
   b). 500 μL of methanol for 15 min at RT;
   c). 500 μL of 4% paraformaldehyde in PBS for 1hr. Would need to quench with 0.3M/ (made a 0.2M) glycine for 10 min.

   During the fixation, turn on the heater to 37C on the shaker.
3) Wash with 1x PBS 3x for 5 minutes at 37°C shaking.
4) Depending on antigen, permeabilize with 0.2% Triton X 100 in 1x PBS for 15 min at 37°C shaking (could use less if unsure 0.01%)
5) Wash with 1x PBS 3x for 5 minutes at 37°C shaking
6) At this step you can put the slide in the fridge, wrapped in parafilm, before going on.

The second day:
7) Heat slide back up if stopped.
8) Block with 500 μL of a solution of 15 μL/mL of the serum of the secondary in 1% BSA (0.1g BSA in 9.9ml 1x PBS) in 1x PBS for 45 minutes at 37°C shaking.

<table>
<thead>
<tr>
<th>Protein of interest</th>
<th>Secondary Ab</th>
<th>Serum of the 2nd Ab</th>
<th>Dye (color)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cav-1 (mouse)</td>
<td>Donkey anti-mouse</td>
<td>Donkey (7.5μL in 500μL/well)</td>
<td>Green</td>
</tr>
<tr>
<td>TNF-receptor 1 (goat)</td>
<td>Donkey anti-goat</td>
<td>Donkey (7.5μL in 500μL/well)</td>
<td>Orange</td>
</tr>
</tbody>
</table>

9) Without washing, put the respective primaries on the indicated wells diluted in 1x PBS. Add corresponding IgG to the control well allotted at the same concentration as your primary (ex. 5 μg/mL) for 30 minutes at 37°C shaking.

<table>
<thead>
<tr>
<th>Protein of interest</th>
<th>Primary Ab conc. (dilution in 1x PBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cav-1 (mouse)</td>
<td>Mouse-200μg/ml. (need a 40x dilution: 1.25μl into 500μl PBS)</td>
</tr>
<tr>
<td>TNF-receptor 1 (goat)</td>
<td>Goat-100μg/ml. (need a 20x dilution: 25μl into 500μl PBS)</td>
</tr>
</tbody>
</table>

10) Wash the wells 2x in 1x PBS, then an additional 1x for 5 minutes at 37°C shaking
11) **Avoiding light**, add the secondary antibody (ex. 1/200) diluted in 1x PBS for 15 minutes at 37°C shaking covered in aluminum foil.

<table>
<thead>
<tr>
<th>Protein of interest</th>
<th>2nd Ab conc. (dilution in 1x PBS)</th>
<th>Dye (color)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cav-1 (mouse)</td>
<td>Alexa Fluor 488 Donkey anti-mouse: 2mg/ml (need a 1/200 dilution: 2.5μl into 500μl PBS)</td>
<td>Green</td>
</tr>
<tr>
<td>TNFRI (goat)</td>
<td>AlexaFluor 546 donkey anti-goat: 2mg/ml (need a 1/200 dilution: 2.5μl into 500μl PBS)</td>
<td>Orange</td>
</tr>
</tbody>
</table>

12) Wash the well 2x in 1x PBS, and then an additional 1x for 5 minutes at 37°C shaking
13) Add Hoechst stain at a concentration of 1 μg/mL (add 0.5μl into 500μl from the 1mg/ml Hoechst solution) for 5-10 minutes at 37°C shaking.
14) Wash the wells 3x in 1x PBS, then pull off the sides of the wells. Mount with one drop of VectaMount and covered with large cover slides avoid bubbles. Use nail polish to seal the sides. Store at 4°C in foil.
Confocal Microscopy

A. Turn on the machine:
   From the right side to the left side and then right again, last is the Middle.
   1. Right side: Burner
   2. Left: Laser black switch and turn the key, then turn on the Green and Red lasers.
   3. Right: LD laser
   4. Middle: Up LG-PS2
      Middle: BX-UCB
      Lower: FV5
   Let the machine warm up for 10 min before start experiment.
   Only when the FV5-PSU on, the laser can be turn on. FV5-LDPSU has a safety knob switch, need to be on all the time.

B. Adjust the focus of samples and check the dye by switch light path 1 through 5 by microscope.
   Our system is having 2 channels and 4 filters. Switch the filter on the left side of the machine.
   If use 60X, must use a drop of oil between the projector and the slide.
   Use the up baton on the right lower side of the microscope to make the length at the proper position.
   When check different dye, use the respective filter. Shut off the shutter after each check in order to avoid the quencher of the slide.

C. After ready for taking pictures, switch the light path to 1 (clear light). And pull out the camera light path on the right side of microscope.
   First clear the dye selection then add preferred dyes on the middle of the screen.
   APPLY
   The lower mirror set to an empty one.
   Active the laser filters on the left lower part of the screen.
   1. DAPI: blue color. Dark pink laser. DAPI is Hoechst, Blue color. Channel 1, A in B out. (LD405)
   2. FITC: green. Blue laser. FITC is green. (AR488)
   3. TEX red.

   On the left side of the channel and filter switch, a black bar is for the setting of how much light goes to each channel.
   For DAPI set the black bar to the middle position and for FITC, pull the black bar all the way out.
   There is a round bigger turning, can adjust the signal.
   Try the ONCE scanning first to see how the picture is.
   To clean up the image, always keep the Gain low at 1 and increase the PMT (voltage) to get stronger signals.
   Don’t increase the laser adjusting on the left lower part of the screen, because increase the density of laser is bad for the sample.
Can also try the XYREPEAT and adjust the focus at the same time to choose better image.

The type of scanning:
I. Normal, can give only one picture each time.
II. Sequence: can add channel, and do a continuous scanning with different dye settings. Optical Syst. Group 4. Add channel, click on optical system, add DPSI or FITC.

Click on the icon: --Δ

Δ--- which is “scan unit configuration”. Set FITC (green) and Mito (red) to group I, and DAPI to group II. Sequence: once.

The three-color bar can be used to change the image from different channel. One group can include 2 channels.

Detergent-free Purification of Caveolin Enriched Membrane Fractios (Sucrose Gradient)

1. Cells were plated in 4 100 mm plate/treatment. Cells were washed with 1x PBS and lysed with 2 ml of ice-cold MES-buffered saline (MBS; 25mM MES (morpholineethanesulfonic acid, pH 6.5) 150 mM NaCl) containing 500 mM Na2CO3.

For the preparation:
25 mM MES: 2.4405 g in 500 ml water. Adjust PH to 6.5.
Then add 4.383g NaCl into MES, achieve MBS.
Take 50 ml MBS, add 2.65g Na2CO3, get 500mM. this is lysis buffer.
90% Sucrose: 45g sucrose and make final volume 50 ml. Heat in water bath to dissolve.
35% Sucrose: 17.5 g sucrose and final volume 50 ml.
5% sucrose: 2.5 g sucrose and final volume 50 ml.

2. Homogenation was carried out with 10 strokes of a loose-fitting Dounce homogenizer (7ml). After homogenized, get samples into 15ml cornival tubes for sonication: 30 sec. for 10 times on ice. The setting is 4 and 60. The frequency should be under 20.
3. After sonication, centrifuge at 3000 rpm for 10 min at 4C.
4. Collect superant into centrifuge tubes (14x89mm), if its not exactly 2 ml, add more MBS to reach 2 ml.
5. The homogenate was adjusted to 45% sucrose by addition of 2 ml of 50ml 90% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube, and overlaid with a discontinuous sucrose gradient

Add 2 ml 90% sucrose to make the final 45% sucrose (cut off the top of the tip), add into tube slowly against walls, then vortex to mix well.
6. Add 5 ml of 35% sucrose. Use 1 ml peppit five times. The first 1 and 2 ml must be very slow, add drop by drop to avoid interrupt layers. After the 3rd ml, can be a little faster.
7. Add 3 ml of 5% sucrose. Then the tube is pretty full.
8. Measure weight of the 2 or 4 tubes (depends on sample number), put the one with more volume first on the scale, set to 0. then put the less volume ones. Achieve 0 with 5% sucrose. At least the 3 digital.

9. Get the rotor from 4C. Centrifuge 39000 rpm for 16 h.

10. The second day morning, collect fractions into 1.5ml clear tubes. Label 1 to 12. can see a white fraction which is Cav-1 enriched faction. Sometime the 12\textsuperscript{th} fraction has a little bit less or more volume.

    Lei: 15 μl + 3 μl 6x loading buffer.

Run the gel for 12 fractions on each western blot gel.
References:


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2003 MS of Food Science and Nutrition, China Agricultural University, Beijing China
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Space Nutritional Laboratory, China Research Institute of Space Medical Science
1999-2002 Research Assistant
Food Safety and Inspection Standard Laboratory, Chinese Ministry of Agriculture

Awards and Honors
2006-2007 Presidential Fellowship, University of Kentucky.
2006 Featured on NIEHS/NIH website as “Student Success Story”:
   http://www.niehs.nih.gov/research/supported/sbrp/training/training2_s9.cfm
2004-2007 Annual Travel Grant Award, University of Kentucky
   The 17th International Conference of Nutrition, Austria Vienna (Only 25 students
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2001 Second-Prize of Excellent Dissertation, The 4th National Ocean Active Compounds
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Publications

Manuscripts already published:


Manuscripts under review:

1. Lei Wang, Eun Jin Lim, Michal Toborek, Bernhard Hennig. The role of fatty acids and caveolin-1 in TNF-α-induced endothelial cell activation. (submitted to Metabolism in November 2007).

Abstracts and Presentations

1. Lei Wang, Michal Toborek Bernhard Hennig. Omega-3 but not omega-6 fatty acids protect against TNF-alpha induced endothelial cell activation. The FASEB Journal, April, 2006: B240.


3. Lei Wang, Reiterer Gudrun, Michal Toborek Bernhard Hennig. Different ratios of omega-6 to omega-3 fatty acids can modulate proinflammatory events in endothelial cells induced by TNF-alpha and environmental contaminants. The FASEB Journal, April, 2005 19(4): A442.


8. PCB Workshop, University of Illinois at Urbana-Champaign. 2004. “Nutrient-mediated protection of PCB toxicity in endothelial cells.”