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EFFECT OF N-3 AND N-6 POLYUNSATURATED FATTY ACIDS ON INFLAMMATION

by

Tao Xu

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

Approved:

Robert E. Ward Major Professor	Korry Hintze Committee Member
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UTAH STATE UNIVERSITY
Logan, Utah

2017

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ABSTRACT

Effect of n-3 and n-6 Polyunsaturated Fatty Acids on Inflammation

by

Tao Xu, Doctor of Philosophy

Utah State University, 2017

Major Professor: Dr. Robert E. Ward
Department: Nutrition, Dietetics, and Food Sciences

Intake of polyunsaturated fatty acids (PUFA) has changed drastically in the past century in the American diet and has received attention due to potential effects on chronic inflammation-related metabolic diseases. In this project, the effects of dietary PUFA content and the n-6 to n-3 ratio on inflammatory responses in the acute and chronic inflammation models were evaluated. The PUFA content was modified on a Western diet background to deliver both n-6 and n-3 intakes at the 10^{th} and 90^{th} percentile of the population in the United States, and models of acute and chronic inflammation were tested in mice model. The experimental PUFA diets had a modest effect on the response to lipopolysaccharide (LPS) in the acute inflammation model. A high n-6 to n-3 ratio promoted lipogenesis. In addition, high n-6 PUFA enhanced the inflammatory responses via the NF-κB pathway. In chronic inflammation model, low-grade inflammatory stress was induced by osmotic mini-pump delivery of LPS. A high n-6 intake increased glucose intolerance. On the other hand, high n-6 intake promotes cholesterol ester accumulation in both acute and chronic inflammation models.

(146 pages)

PUBLIC ABSTRACT

Effect of n-3 and n-6 Polyunsaturated Fatty Acids on Inflammation

Tao Xu

The purpose of this study is to evaluate the effects of dietary PUFA on inflammatory responses. Dietary PUFAs have shown to have the effects on various chronic diseases caused by inflammation. In our study, acute inflammation and chronic inflammation were developed on the mice model to test the hypotheses. The PUFA diets modified from a Western diet background to deliver both n-6 and n-3 intakes at the 10^{th} and 90^{th} percentile of the population in the United States were used in the study. The experimental PUFA diets had a modest effect on the response to LPS in the acute inflammation model. A high n-6 to n-3 ratio promoted lipogenesis. In addition, high n-6 PUFA enhanced the inflammatory responses via the NF-κB pathway. In chronic inflammation model, low-grade inflammatory stress was induced by osmotic mini-pump delivery of LPS. The PUFA diets also had a modest effect on inflammation responses in the chronic model. A high n-6 intake increased glucose intolerance. The study indicated that dietary PUFA content and ratio under physiological range of intake would only cause modest effect on inflammation *in vivo* which gives useful information for dietary guidelines for Americans. The overall cost of this research was approximately 20,000 dollars.

ACKNOWLEDGMENTS

I am very grateful to my advisor, Dr. Robert E. Ward, to give me this precious chance to learn from him. His inspiration and guidance were keys in helping me finish my project and degree program. I would also like to thank my committee members, Dr. Korry Hintze, Dr. Michael Lefevre, Dr. Lance C. Seefeldt, and Dr. Brian Gowen for their valuable time and helpful suggestions on my research.

I appreciate the help from my lab mates, Albert Zhou, Zhou Fang, and many others. At the end, I want to thank to my families and friends. Without the encouragement and support, I would not have been successful to get my Ph.D. degree.

Tao Xu

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LIST OF ABBREVIATIONS

14,15-DiHETE	(±)14,15-dihydroxy-eicosa-5,8,11,17-Tetraenoic Acid
4-HHE	4-hydroxy-2-hexenal
4-HNE	4-hydroxy-2-nonenal
8-epiPGF2a	8-Epi Prostaglandin F2 Alpha
AA	Arachidonic acid
ACACA	Acetyl-CoA carboxylase-1
Acox	Acyl-Coenzyme A oxidase
ALA	α-linolenic acid
ANOVA	Analysis of variance
AUC	Area under the curve
BHT	Butylated hydroxytoluene
CD14	Cluster of differentiation-14
CE	Cholesteryl ester
CHD	Coronary heart disease
CI	Chemical ionization
COX	Cyclooxygenase
Cpt-1	Carnitine-Palmitoyl-Coenzyme A transferase I
CRP	C-reactive protein
Ct	Cycle threshold
CVD	Cardiovascular diseases
DG	Diglycerides
DGAT2	Diacylglycerol O-acyltransferase-2

DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
ELOVL5	Elevated very long chain fatty acyl elongase-5
EPA	Eicosapentaenoic acid
FABP4	Fatty acid binding protein-4
FAME	Fatty acid methyl ester
FAS (FASN)	Fatty acid synthases
FDR	False discovery rate
FID	Flame ionization detector
GC	Gas chromatography
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HETEs	Hydroxyeicosatetraenoic acids
HH	High n-6 and high n-3 PUFA diet
HL	High n-6 and low n-3 PUFA diet
HMGB1	High-mobility group protein B1
HPTLC	High performance thin layer chromatography
IAP	Intestinal alkaline phosphatase
IκB	Inhibitor of nuclear factor kappa B
IKK	Inhibitor of nuclear factor kappa B kinase
IL	Interleukin
IFN-γ	Interferon-γ
IRAK	Interleukin-1 receptor-associated kinase
IUPAC	International Union of Pure and Applied Chemistry

KC	Keratinocyte-derived chemokine
LA	Linoleic acid
LARC	Laboratory Animal Research Center, Utah State University
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LH	Low n-6 and high n-3 PUFA diet
LL	Low n-6 and low n-3 PUFA diet
LOX	Lipoxygenase
L-Phe	L-phenylalanine
LPS	Lipopolysaccharide
LT	Leukotriene
MAPKs	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein 1 (CCL2, chemokine ligand 2)
MD2	Lymphocyte antigen 96
ME1	Malic enzyme-1
MIP-1α	Macrophage inflammatory protein 1α (CCL3, chemokine ligand 3)
MRI	Magnetic resonance imaging
MUFA	Monounsaturated fatty acids
MYD88	Myeloid differentiation primary response protein 88
n-6 & n-3	Omega-6 & Omega-3
NAFLD	Non-alcoholic fatty liver disease
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NF-κbib	Inhibitor of NF-κB-β
NHANES	National Health and Nutrition Examination Survey
NLRs	Nod-like receptors
OGTT	Oral glucose tolerance test
O-PFB-TMS	Pentafluorobenzyl-oxime, trimethylsiyl
PG	Prostaglandin
PL	Phospholipid
PPARs	Peroxisome proliferator-activated receptors
PUFA	Polyunsaturated fatty acids
RANTES	Regulated upon activation, normal T cell expressed and presumably secreted (CCL5, chemokine ligand 5)
SCD1	Stearoyl-CoA desaturase-1
SFA	Saturated fatty acids
SREBP1	Sterol regulatory element binding protein-1
TAG	Triglycerides
TIR	Toll/interleukin-1 receptor
TLC	Thin layer chromatography
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
TRAF6	TNF receptor-associated factor-6
TWD	Total Western diet
TXB_2	Thromboxane B2
USDA	U.S. Department of Agriculture
UV	Ultraviolet

USDHHS	U.S. Department of Health and Human Service
VSMCs	Vascular smooth muscle cell

CHAPTER 1

INTRODUCTION

Dietary fats

The typical American diet is composed of approximately 50% carbohydrate, 15% protein, and 35% fat (1). Compared with carbohydrates and proteins, each gram of fat provides more than double the calories (carbohydrates 4.183 kcal/g; protein 4.442 kcal/g; fat 9.461 kcal/g) (2). Another difference between fat and the other macronutrients, from a nutrition perspective, is that the lipid composition of an organism's membranes reflect the dietary lipid intake (3). In terms of dietary fat, you literally are what you eat. Therefore, compared to protein and carbohydrate, dietary fats may have a greater potential to impact physiological processes.

Many studies have been done to investigate the relationship between dietary fat composition and disease. There is substantial evidence that dietary fat is associated with cardiovascular diseases (CVD) risk (4-6). There is general consensus that reduction of dietary saturated fatty acids (SFA) could reduce the risk of coronary heart disease (CHD) by decreasing serum total cholesterol and low-density lipoprotein (LDL) cholesterol (7-9). Beyond CVD, dietary fat can impact other metabolic diseases such as non-alcoholic fatty liver disease (NAFLD), type 2 diabetes, and Alzheimer's disease (10-13).

Fatty acids

Fatty acids consist of a carboxylic acid (head) and an aliphatic tail. In humans, saturated fatty acids are synthesized up to 16-carbon (palmitic acid) by fatty acid

synthases (FAS) in the cytoplasm. The pathway for fatty acids synthesis begins with malonyl CoA and acetyl CoA which are activated by thioester bonds to FAS. Then, two-carbon units (donated by activated malonate) are added onto the chain through every cycle. CO_2 is eliminated from the added malonyl CoA in each cycle which results in fatty acids with even carbon chains (14).

Although humans can synthesize fatty acids, other fatty acids that cannot be synthesized *de novo* are required. This is due to lack of Δ12 and Δ15 desaturases, which introduce double bonds at carbons 12 and 15 (when counting from the carbonyl carbon) (15, 16). Consequently, it is necessary to acquire certain fatty acids from the diet. Those fatty acids are called essential fatty acids (17). There are two fatty acids that are essential for humans, linoleic acid (LA) and α-linolenic acid (ALA) (18). LA and ALA are both polyunsaturated fatty acids (PUFA) with 18-carbon aliphatic chains. The differences between LA and ALA are: 1) LA has two *cis* double bonds while ALA has three *cis* double bonds; 2) the first double bond of LA located at the 6^{th} carbon from the methyl end which we call omega-6 (nomenclature systems will be discussed shortly) while ALA has the first double bond located at the 3^{rd} carbon from the methyl end and is referred to as an omega-3 fatty acid (19). Using LA and ALA as substrates, longer and more highly unsaturated fatty acids can be produced by elongases and desaturases in the mitochondria and microsomes (20).

Based on the number of double bonds in the aliphatic chain, fatty acids are classified to three different groups which are saturated fatty acids (SFA) (no double bond), monounsaturated fatty acids (MUFA) (one double bond), and PUFA (more than

one double bonds) (21). For PUFA, there are two common nomenclature systems, the delta system and the omega system (or "n-minus" system). The delta system counts the position of double bonds from the carboxyl end (alpha carbon) to the first unsaturated carbon. For example, ALA is named as 18:3 $\Delta^{9,12,15}$. The omega system, conversely, counts from the terminal methyl carbon (omega carbon) to the first unsaturated carbon. Thus, ALA is named as 18:3 (ω-3) (22). Often, "n" is used in place of the "ω". The delta system is the standard chemistry nomenclature (IUPAC system) which is the most comprehensive for naming fatty acids, and is useful for those in the molecular chemistry since the name of all the fatty acids are unambiguous (23). However, from a nutrition stand point, the omega system is more commonly used because of the important physiological differences between omega-3 (n-3) and omega-6 (n-6) fatty acids (24). The reason that the omega system is more useful from a nutrition perspective is that all longer and more highly unsaturated n-3 PUFAs are synthesized from ALA and all n-6 PUFAs are from LA.

Dietary SFAs, MUFAs, and PUFAs have different effects on human health. For example, *in vivo* inflammatory responses, SFAs are generally considered to promote inflammatory process (25-28) while MUFAs are thought to have neutral or opposite effects on inflammation. On the other hand, PUFAs have more complicated interactions with the inflammatory responses which are discussed below.

PUFA

Dietary fatty acids, especially PUFAs, are widely studied not only for their relationships to different diseases but also for their essentiality in human diet. In the last

century, Americans' diets have changed drastically in terms of PUFA intake. Consumption of LA has increased from 2.79% of energy to 7.21% of energy while intake of ALA has increased from 0.39% to 0.72% (29). Data from National Health and Nutrition Examination Survey (NHANES) indicates that the range of n-6 PUFA intake of Americans is from 5.1% of total energy (10^{th} percentile population) to 10.4% of total energy (90^{th} percentile population) while n-3 PUFA intake is from 0.4% (10^{th} percentile population) to 1.1% (90^{th} percentile population) (1). Thus, dietary n-6 PUFA intakes from 5.1% to 10.4% and dietary n-3 PUFA intakes from 0.4% to 1.1% would include 80% of the population in the United States. In other words, if different PUFA intakes are affecting health in the US diet, the intake of n-3 and n-6 PUFA should be within these ranges.

Although PUFA intake is affected by many factors, one important reason that PUFA consumption has increased is due to the use of vegetable oils in cooking and in processed foods. It has been recommended to Americans to consume more PUFAs by the U.S. Department of Agriculture (USDA) and the U.S. Department of Health and Human Services (HHS) due to their cardiovascular benefits (30). In general, research with dietary PUFA has shown positive effects on CVD by lowering both total serum cholesterol and LDL cholesterol (7, 31, 32) and reducing blood pressure (33, 34).

Beyond the lowering of serum cholesterol, might the dramatic change in PUFA intake in the last century have other health effects? The overall effects on health of dietary PUFA arise from both their metabolic properties and role in cellular signaling

processes. The process of essential fatty acid elongation and desaturation is shown in Figure 1.1.

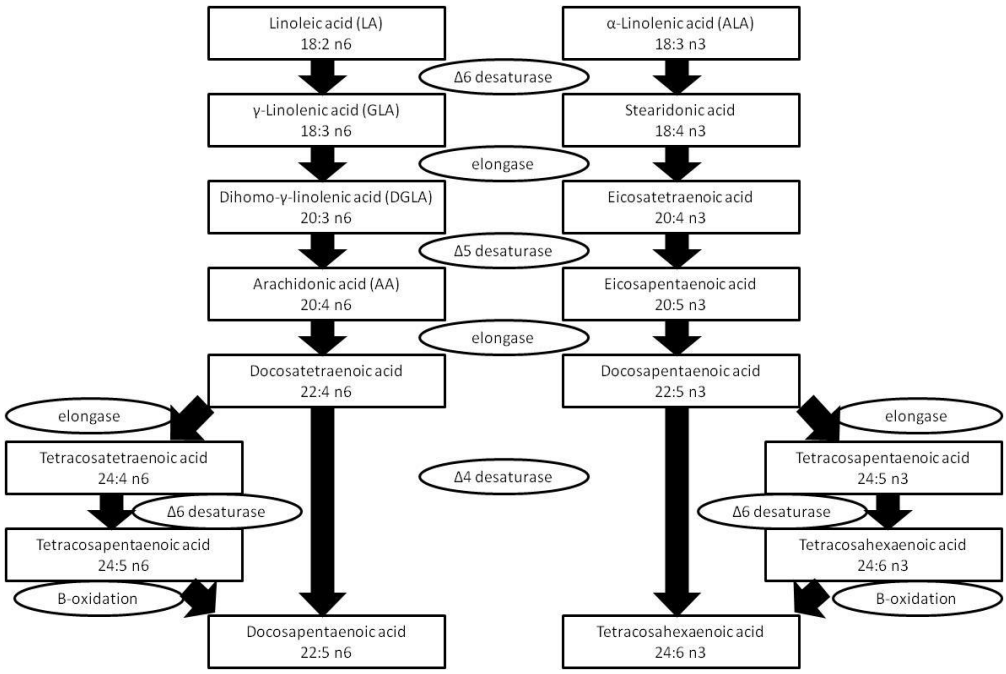

FIGURE 1.1 Biosynthesis of PUFAs from EFA

From the Figure 1.1, it can be noted that all n-6 PUFAs such as AA are derived from LA while the n-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are derived from ALA. These pathways also explain the value of the omega nomenclature system, as the position of the n-6 and n-3 double bonds does not change as the molecules are elongated and further desaturated. Since the typical American diet contains an excess of LA (n-6) over ALA (n-3), the *in vivo* concentration of Arachidonic

acid (AA) (n-6) is significantly higher than EPA (n-3) in tissue long chain PUFA which may contribute to inflammatory processes and chronic diseases (35).

Intakes of PUFA have changed in the last century as have rates of obesity and related metabolic diseases like diabetes (36, 37). Interestingly, these metabolic diseases are associated with a low-grade, chronic inflammation (38, 39). On the other hand, research has suggested another aspect of dietary PUFA may impact cardiovascular health: the n-6 to n-3 PUFA ratio should also be considered in the US dietary guidance (40, 41). Does n-6 to n-3 PUFA ratio in the diet affect the inflammatory responses in humans? To better understand these two questions, it is critical to understand the mechanism of inflammatory pathway of n-3 and n-6 PUFAs.

Inflammation and related cytokines, gene expression

Inflammation is a complex host response to eliminate the foreign invaders, such as infectious pathogens, as well as damaged tissues (42). It is a biological mechanism used to kill pathogens, initiate tissue repair, and restore homeostasis at an infection or injury site. The inflammatory process can be regulated through activation of negative feedback mechanisms such as releasing anti-inflammatory signals (cytokines), or inhibiting the generation of pro-inflammatory signaling cascades (43). On the other hand, if not controlled, excessive inflammation can cause damage to host tissues and also lead to a series of human diseases (44).

Depending on the specific process, inflammation could be categorized as acute or chronic. Acute inflammation normally occurs over short time (hours to days) whereas chronic inflammation is continuous over longer time frames. Microbial (bacterial, viral,

fungal, and parasitic) toxins are common stimuli for the initiation of acute inflammation (42). The immediate responses of acute inflammation are usually triggered by various inflammatory mediators in the tissues which recruit immune cells such as neutrophils and polymorphonuclear leukocytes (45). On the other hand, chronic inflammation is commonly caused by low grade endotoxemia such as lipopolysaccharide (LPS) (46), cellular stress and malfunction. In chronic inflammation, monocytes and long-lived macrophages are mainly involved in the immune responses (47). High-fat diets have also been shown to cause metabolic endotoxemia in animals and humans. High-fat diets can enhance translocation of LPS from the gut to the systemic circulation which eventually induces systemic chronic inflammation (48, 49).

Stimuli for the initiation of inflammation include microbial cell wall components such as LPS. LPS consists a lipid and a polysaccharide bound by a covalent bond. It is a component of the outer membrane of Gram-negative bacteria. The immune system recognizes LPS via Toll-like receptors (TLR). While gut-derived endotoxin is thought to be one factor that promotes metabolic endotoxemia, there are endogenous protection mechanisms in place to handle this potential stress. For example, intestinal alkaline phosphatase (IAP) is a protective enzyme that detoxifies LPS by removal of a phosphate group, which prevents gut-derived systemic inflammation (50). Interestingly, it was also reported that L-phenylalanine (L-Phe) inhibits endogenous IAP activity which increases serum endotoxin levels and promotes metabolic inflammation (51). L-Phe supplement in the diet could be a suitable model to cause chronic inflammation.

One of the key links between inflammation and PUFAs are eicosanoids, which are oxidized compounds generated from 20-carbon PUFAs (44). AA (20:4 n-6) is the major substrate for biosynthesis of eicosanoids (52). Figure 1.2 shows several pathways of eicosanoids syntheses which are generated from AA. *In vivo*, AA concentration can be affected by dietary intake of AA itself (53) or of its precursor, LA. However, the range of LA intakes which affects this relationship has not been defined. More importantly, AA in the cell membranes can be partially replaced by EPA which would be predicted to decreases production of AA-derived eicosanoids (54).

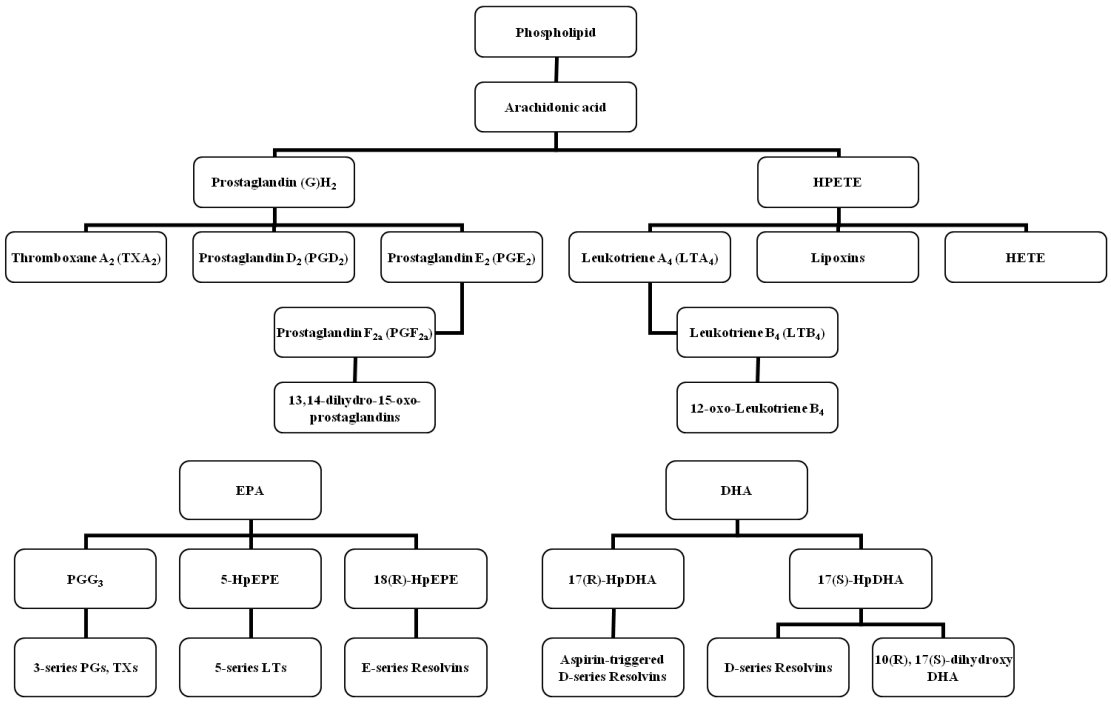

FIGURE 1.2 Effect of PUFA substrate on eicosanoid biosynthesis (55, 56)

As shown in the Figure 1.2, AA can be converted to eicosanoids resulting in different groups of compounds such as thromboxanes, leukotrienes, prostaglandins, and other oxidized PUFA metabolites. Prostaglandin-D2 (PGD_2) promotes the neutrophil recruitment process. However, EPA is an alternate substrate for cyclooxygenase (COX) which leads to the production of PGD_3 which competes for the PGD_2 receptor to inhibit the migration of neutrophils across endothelial cells (57-59). Another AA-derived eicosanoid, PGE_2 has been demonstrated to induce production of Interleukin-6 (IL-6) which is a pro-inflammatory cytokine produced by macrophages (60). However, other studies have indicated PGE_2 promotes anti-inflammatory effects through 15-lipoxygenase (15-LOX) induction to promote lipoxin generation (61, 62). Another eicosanoid, leukotriene B4 (LTB_4), is a powerful inducer of leukocyte chemotaxis and adherence which leads to pro-inflammation (63).

In the inflammatory process, 20-carbon n-3 PUFAs may serve as substrates for the biosynthesis of eicosanoids to prevent conversion of AA to pro-inflammatory eicosanoids. Numerous studies have shown that PUFA-derived mediators, such as resolvins, docosatrienes, and neuroprotectins, generated from EPA and DHA have anti-inflammatory function (64-66). Similar to PGD_3, long chain n-3 PUFAs are able to modulate prostaglandin metabolism by increasing PGE_3 (an active vasodilator and inhibitor of platelet aggregation) and LTB_5 (weak inducer of inflammation) (67).

Various cytokines and associated genes are involved in the PUFA-related inflammatory processes and are useful as biomarkers to measure inflammation. Cytokines are low-molecular-weight proteins produced by immune cells such as

macrophages, neutrophils, T- and B-lymphocytes which regulate the immune system (68). Metabolic disorders may trigger a persistent inflammatory cascade including production of eicosanoids (lipid autacoids) which recruit immune cells to the site of inflammation to secrete cytokines (69). Thus, measurement of cytokines could also be a useful endpoint of evaluating downstream inflammatory responses.

Studies have demonstrated that lipid components of the diet can modulate transmembrane TLRs which may consequently affect the vigor of immune responses (70). EPA and DHA have been shown to act as inhibitors of various TLRs and nod-like receptors (NLRs) and suppress nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) related signal transduction (27, 28, 71-73). NF-κB has dual intracellular effects on inflammation. It induces proteins promoting survival in the acute cellular stress responses (74). It is also a pro-inflammatory transcription factor which upregulates the expression of pro-inflammatory cytokines and adhesion molecules (75). In LPS-induced inflammation, LPS interacts with a heterologous receptor which contains TLR4, cluster of differentiation-14 (CD14), and lymphocyte antigen 96 (MD2) to activate the complex (76). Then, the adapter protein MYD88 is recruited to the cytoplasmic domain of TLR4 complex through homotypic binding of Toll/interleukin-1 receptor (TIR) domains (77, 78). Subsequently, recruitment of Interleukin-1 receptor-associated kinase (IRAK) and TNF (Tumor necrosis factor alpha) receptor-associated factor-6 (TRAF6) on the complex can result in the activation of a downstream kinase cascade involving inhibitor of inhibitor of nuclear factor kappa B kinase (IKK). The IKK-mediated phosphorylation of inhibitor of nuclear factor kappa B (IκB) leads to degradation of IκB by proteasome and

allows NF-κB translocate to the nucleus from cytosol which promotes the pro-inflammatory gene expression (see Fig. 1.3) (79). Eventually, the immune response involves the expression of pro-inflammatory cytokines like tumor necrosis factor α (TNF-α), IL-1α, IL-1β, IL-6, monocyte chemotactic protein 1 (MCP-1), and macrophage inflammatory protein 1α (MIP-1α) will be initiated (80-83). Liuzzo et al. also found that C-reactive protein (CRP) had pro-inflammatory effect via NF-κB pathway which can be another interesting endpoint to measure (84).

Eicosanoids have been extensively linked to the NF-κB pathway which might have a close relationship to LPS-induced inflammation. Erdinest et al. indicated that resolvin D1 (generated from DHA) had inhibitory effects on Poly I:C induction which caused decreased IκB-α expression in the human corneal epithelial cells (85). Resolvin E1 also showed anti-inflammatory effect through inducing IAP expression and enhancing epithelial IAP enzyme activity (86). IAP detoxifies LPS which attenuates LPS-induced NF-κB pathway and eventually represses NF-κB involved inflammation (87). On the other hand, AA-derived eicosanoids are more likely to promote the inflammation via activation of NF-κB pathway. Hydroxyeicosatetraenoic acids (HETEs), one of the major eicosanoids generated from AA, have been reported to promote the NF-κB pathway. For example, Ishizuka et al. demonstrated that 20-HETE activates the NF-κB pathway and increases nuclear translocation of NF-κB p65 subunit in human endothelial cells (88). 15-HETE promotes the IκB-α phosphorylation and degradation to activate the NF-κB transcription pathway (89). Eicosanoids generated from PUFAs mainly affect LPS-induced NF-κB pathway (Fig. 1.3) via IκB-α activation or deactivation (90).

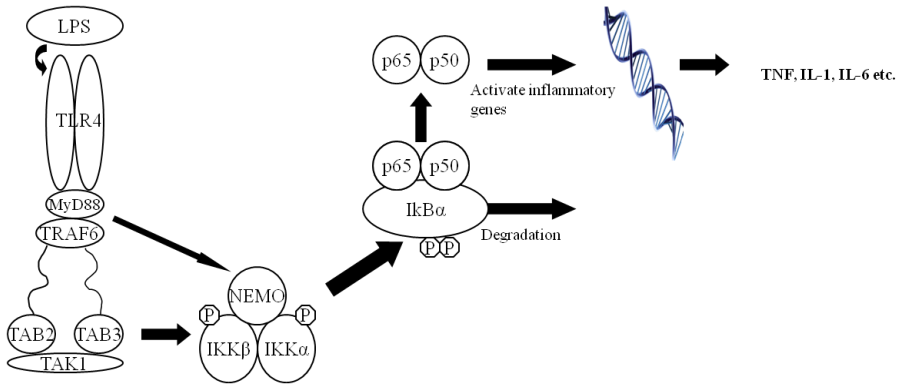

FIGURE 1.3 LPS induced NF-κB pathway (91)

Peroxisome proliferator-activated receptors (PPARs) are transcriptional factors which belong to nuclear receptors superfamily. Subtypes of PPARs include PPAR-α, PPAR-β, and PPAR-γ. PUFAs and eicosanoids are natural ligands for PPARs (92, 93). In rodents and humans, PPAR-α is expressed in tissues such as liver, skeletal muscle, and brown fat which is associated with fatty acid oxidation. In general, n-3 PUFAs act as activators of PPAR-α (94). PPAR-α agonists exert anti-inflammatory effects in vascular cells which involves inhibition of cytokine-induced expression of adhesion molecules (95). PPAR-α agonists reduce IL-6 concentration in the plasma and decrease the expression of COX-2 by repressing NF-κB signaling. PPAR-α physically interacts with NF-κB p65 subunit to form inactive complexes which interfere its transcription activity (96). Also, PPAR-α activators inhibit NF-κB-driven gene transcriptions by inducing IκB-α gene expression and its protein translocation which accelerates NF-κB nuclear deactivation (97). Other studies showed PPAR-α activation by n-3 PUFA was evidenced by enhancement in the PPAR-α-regulated Acox and Cpt-1 gene expression which are critical enzymes for β-oxidation of lipids (75). PPAR-β is expressed in myeloid cells.

Activation of PPAR-β will cause the repression of inflammatory genes such as Ccl_2 gene (C-C motif chemokine ligand 2, MCP-1) (92). PPAR-γ is expressed in white and brown adipose tissue, colon, endothelial cells, vascular smooth muscle cells (VSMCs), and macrophages. Activation of PPAR-γ results in strong suppression of various pro-inflammatory cytokines including TNF-α and IL-6 in cultured macrophages (98). One study showed that the n-3 PUFA-derived eicosanoid 15d-PGJ_3 can enhance the expression of PPAR-γ targeted genes such as fatty acid binding protein-4 gene (Fabp4) (99). Interestingly, n-6 PUFA-derived 15d-PGJ_2 (the dehydration end product of PGD_2) and 8(S)-HETE are potential agonists of PPAR-α and PPAR-γ and inhibits activation of NF-κB (100-102). In the LPS-induced inflammation, PPARs are also shown to inhibit LPS-induced high-mobility group protein B1 gene (Hmgb1) (103). In fact, the interaction of Hmgb1 and TLR4 leads to upregulation of NF-κB (104).

On the other hand, PUFAs may also affect inflammatory mediators via suppressing lipogenesis genes. For example, n-3 PUFAs repress sterol regulatory element binding protein-1 gene (Srebp-1) (105). SREBP1 promotes diacylglycerol O-acyltransferase-2 (DGAT2) which catalyzes diacylglycerol and acyl-CoA to form triglycerides, Acetyl-CoA carboxylase-1 (ACACA) which catalyzes carboxylation of acetyl-CoA to malonyl-CoA, and fatty acid synthases (FAS) activities (106). Also, studies have shown decreased activity of malic enzyme-1 (ME1) (generating NADPH for fatty acid biosynthesis) in rats fed with high n-3 PUFA diets (107). Elongation of very long chain fatty acids acyl elongase-5 (ELOVL5), an enzyme which is critical in C20-22 PUFA synthesis, may be enhanced in animals fed a high PUFA diet (C18 PUFAs) (108).

More than that, LA and ALA inhibit stearoyl-CoA desaturase-1 (SCD-1), the enzyme which adds a double bond to stearoyl-CoA and produces oleic acid (109). With high dietary n-3 PUFA level, reduced level of FAS in the rat liver was measured (107, 110).

In short, activation of NF-κB pathway from LPS triggers the inflammatory responses. N-3 PUFAs and their eicosanoids are able to attenuate the NF-κB pathway which results anti-inflammatory activity in cells while n-6 PUFAs and their eicosanoids promote this pathway. PPAR-α pathway promotes β-oxidation which is the opposite of lipogenesis. PPARs also show inhibitory effects on NF-κB pathway. Evidence indicates that eicosanoids generated from n-3 PUFAs are associated to enhancement of PPARs pathway while n-6 PUFAs inhibit the process. In lipogensis, high level of PUFAs, especially n-3 PUFAs, are thought to inhibit the fatty acid synthesis. Thus, measuring NF-κB signaling, PPARs pathway and lipogenesis related genes will be critical for us to understand the effects of PUFA contents and ratios on LPS-induced inflammation. In summary, these three pathways associated genes are listed in the Table 1.1.

TABLE 1.1 Inflammatory pathways related genes.

NF- κB pathway	PPARs pathway	Lipogensis
IκB-α	Hmgb1	Srebp-1
IL-1α	Acox1	Scd-1
IL-1β	Cpt-1	Acaca
IL-6	Ppar	Fasn
TNF-α		ME-1
Ccl2 (MCP-1)		Dgat2
Ccl3 (MIP-1α)		Elovl5
CRP		
NF-κB1		
Hmgb1		

Effects of modifying dietary PUFAs in rodent diets and preliminary studies

Before investigating the relationship between dietary PUFA intake and inflammation, it is necessary to define the model. First, what would be a suitable diet to study the relationship between inflammation and dietary PUFA intake? Secondly, does dietary PUFA %kcal, PUFA ratio, or both affect the inflammatory responses?

Many groups have used mouse models to investigate the effects of dietary n-6 to n-3 PUFA on inflammation. Lots of studies indicate high n-6 to n-3 PUFA ratio enhance the inflammatory responses in both animal and human models (111-117). On the contrary, other studies have shown that a high dietary n-6 to n-3 PUFA ratio does not show a significant increase on inflammatory biomarkers (118, 119).

In previous work in our lab, the effects of both the PUFA content and n-6 to n-3 ratio were investigated (120). AIN93-based PUFA diets with total PUFA percentage at 2.5%, 5%, 10% of kcal and n-6 to n-3 PUFA ratios at 1:1, 10:1, 20:1 were used. After 8 weeks feeding, a sub lethal dose of LPS was injected into mice to cause acute inflammation. The preliminary results indicated that the PUFA content had an effect on feed efficiency (weight gain to food intake ratio) with low dietary n-6 to n-3 PUFA ratio leading to more fat gain in mice. Furthermore, in the cytokine analysis, mice fed with lower PUFA percentages had lower concentration of interferon-γ (INF-γ), IL-5, IL-6, IL-10, IL12, IL-1β, IL-17, IL-3, and chemokine ligand 5 (RANTES). Four cytokines including IL-3, TNF-α, RANTES, and MCP-1 indicated differences between diets with different PUFA ratios. Six cytokines including IL-17, IL-3, MIP-1α, TNF-α, RANTES,

and MCP-1 showed differences because of the interaction of total PUFA percentages and PUFA ratios. The summary of cytokines is shown in the Figure 1.4.

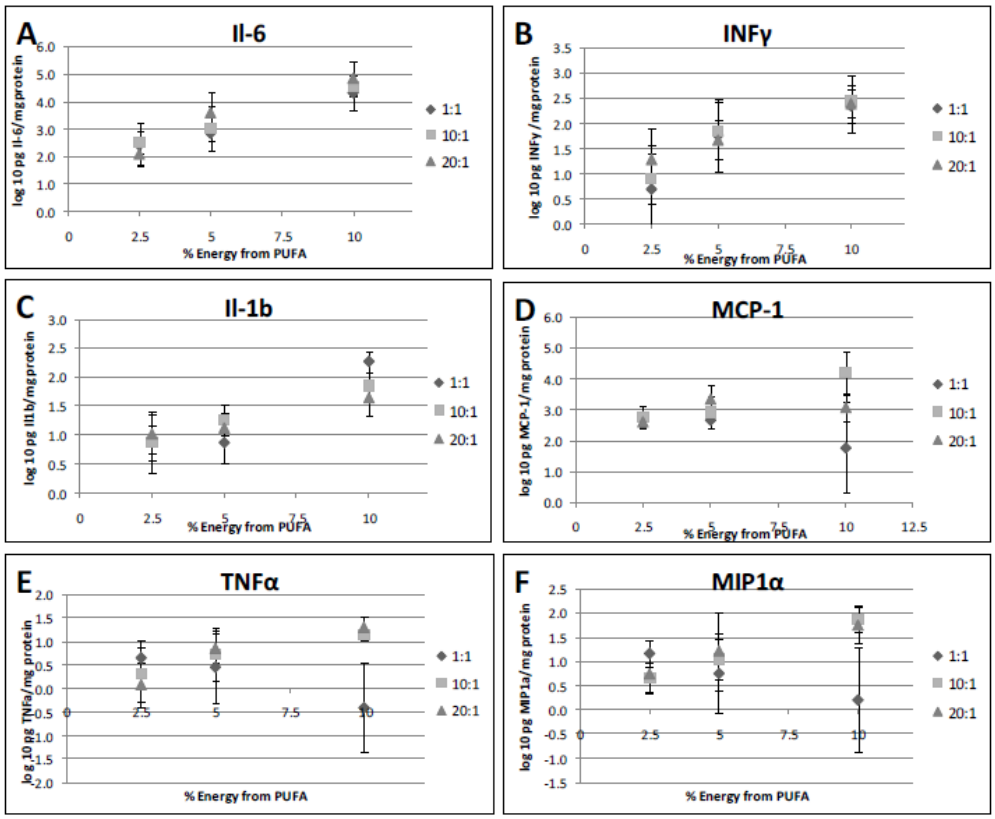

FIGURE 1.4 Log(10) transformed cytokine levels in the mice plasma. The ratios in the legend represent n-6 to n-3 PUFA ratios in the diets. The data represents mean ± SD.

As can be seen in Figure 1.4, TNF-α, MCP-1, and MIP-1α showed significantly lower levels in the 1:1 n-6 to n-3 PUFA ratio under diet with 10% energy from PUFAs, while IL-6, INF-γ, and IL-1β were only affected by total PUFA content (120). Interestingly, MCP-1 and MIP-1α both belong to C-C chemokines which induce the

recruitment and migration of leukocytes into inflammatory area (121). This suggests that dietary PUFA contents and ratios may affect different inflammatory pathways. However, we need to note that n-6 to n-3 PUFA ratio at 1:1 used in our previous study was very extreme. In our current study, we used physiologically relevant PUFA intakes, based on NHANES, which should indicate whether differential intakes are likely to affect health.

In another study conducted in the Ward Lab (unpublished), the effects of dietary PUFA percent and n-6 to n-3 ratios were tested on chronic inflammation. Many studies have shown diet induced obesity (DIO) diets, or high fat diets with a kcal from fat percentage >45%, cause obesity and chronic inflammation in mice (122-126). Thus, DIO diets with different PUFA levels (5% & 10% for n-6 PUFA and 1% & 5% for n-3 PUFA) and n-6 to n-3 PUFA ratios (1:1, 2:1, 5:1, and 10:1) were formulated to be tested in a 2 × 2 design. Mice were fed for 18 weeks, which has been shown to be sufficient to cause an obese and inflamed phenotype in most studies. Surprisingly, the results indicated no significant differences in weight gain, inflammation or any metabolic parameters measured among different PUFA diets. Although the mice fed the DIO diets had significantly more body fat than mice fed the AIN93 diet, there were no differences in insulin sensitivity, plasma cytokines or inflammatory gene expression in adipose or liver. This is not the only time high PUFA DIO diets have not shown a difference on inflammation biomarkers. Murphy et al. used a similar design by using high fat diet with n-6 to n-3 PUFA ratios at 1:1, 5:1, 10:1, and 20:1. They also concluded that reduced dietary n-6 to n-3 PUFA ratio by using ALA did not affect inflammation (119). The previous studies from other labs and our lab do not have a consistent conclusion for the

relationship between dietary PUFA intake and inflammation. However, we need to pay attention to relatively high risk of false negative results (Type II error) due to the small sample size in our previous experiment (127).

There are some methodological issues with previous studies that may explain differing results. First, the basic diet composition used in studies likely has an effect with respect to the PUFA contents. Many studies used standardized mouse diets such as the AIN76 and AIN93 diets which do not represent the typical Western diets eaten by Americans. AIN diets were optimized for rodent growth and fertility (128, 129). However, the daily meals American people eat have less than recommended amounts of micronutrients and much higher fat content. Using standardized mice diet to study effects of dietary PUFA intakes of Americans on human health may thus hide important effects. To address this issue, Drs. Ward, Hintze, and Benninghoff developed a new rodent diet formula to emulate the typical Western diet based on median American intakes of macronutrients and micronutrients (1, 130). The median intakes of American diets were investigated by NHANES for the years 2005-2006 (130). In the survey, total energy intake, sources and intakes of macronutrients, and most of the essential micronutrients were investigated. To match the median dietary macronutrients and micronutrients levels of Americans, TWD was designed and formulated using the concept of nutrient density (mass of nutrient per kcal). Compared to standard diets for mice like AIN76 and AIN93 diets which have much lower fats and optimized concentration of micronutrients, TWD is a more physiologically relevant diet as it represents what Americans are actually eating. The second concern is the PUFA compositions in the diets. In previous studies, the PUFA

sources, contents and ratios used have been extreme compared to that which is typically consumed on average. Our PUFA diets were designed based on TWD with different n-6 PUFA and n-3 PUFA contents. The levels of n-6 and n-3 PUFAs were formulated based on the intakes of 10^{th} percentile population (low limit) and intakes of 90^{th} percentile population (high limit) from NHANES. Thus, our PUFA diets can represent the realistic dietary PUFA intakes extremes that Americans are actually consuming.

Based on the design of our PUFA diets, if dietary PUFA do have effects on human inflammatory responses, differences of inflammatory biomarkers we selected would be observed among our PUFA diets with different n-6 and n-3 PUFA contents and different n-6 to n-3 PUFA ratios. If there is no significant effect, then it would lead us to another conclusion that the PUFA intakes of Americans are not likely to affect the inflammation.

Summary

In summary, to achieve our goals of this thesis, the models of *in vivo* acute and chronic inflammation were established, and the effects of dietary PUFA contents and ratios on both acute inflammation and chronic inflammation models were investigated. TWD-based PUFA diets with different n-6 and n-3 PUFA contents and different n-6 to n-3 PUFA ratios were designed and used in our inflammation models. The effects of LPS on NF-κB and PPARs signaling as well as on and were studies as a function of dietary PUFA content. Endpoints assessed included weight gain, fat gain, fatty acid profile, oral glucose tolerance test (OGTT), cytokines, and gene expression levels.

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CHAPTER 2

EFFECT OF DIETARY N-6 AND N-3 PUFA IN AN ACUTE INFLAMMATION MODEL

Introduction

Over the past 100 years, dietary PUFA intake in the US has changed significantly due to increased use of vegetable oils in cooking and processed foods. The consumption of LA has increased from 2.79% of energy to 7.21% of energy while intake of ALA has increased from 0.39% to 0.72% (1). Might these changes have an effect on physiology and health?

In general, n-6 PUFAs are considered to have pro-inflammatory properties while n-3 PUFAs are considered anti-inflammatory (2). The lipid composition of an organism's membranes roughly reflects dietary fat intake due to the functional requirements of lipids in the membranes (3). In addition, tissue PUFA levels may mediate the inflammatory responses through their metabolic products, eicosanoids. Thus, studying the effects of dietary PUFA intake on inflammation provides important information for understanding the role of fat in a healthy diet.

As there is both acute and chronic inflammation, it is of interest to determine how dietary PUFA may affect both responses. In previous work, Drs. Ward and Hintze investigated how both the percent PUFA intake and n-6 to n-3 ratio affected the cytokine response in an acute inflammatory stress in LPS-challenged mice (4). Mice were fed diets that contained either 2.5%, 5% or 10% kcal from PUFA, at n-6 to n-3 ratios of 1:1, 10:1

and 20:1 and then injected intraperitoneally with a sub lethal dose of LPS (5 mg/kg). Interestingly, the %PUFA in the diet significantly affected 11 of 12 cytokines measured, whereas the n-6 to n-3 ratio affected only 5 of 12. For 7 of 12 cytokines there was a PUFA × ratio interaction. While the results of this study indicated that dietary PUFA can affect the acute inflammatory response, there were methodological details that may limit its translatability to human PUFA intakes. First, the PUFAs intakes were not within average US ranges of human intake. Next, the background diet used in the study (AIN93G) is a low fat diet (17% kcal) and which was formulated to deliver optimum levels of vitamins and minerals.

Other groups have investigated the role of dietary PUFA in inflammatory processes in mice. For example, Broughton et al. found a low n-6 to n-3 diet ratio increased anti-inflammatory eicosanoid biosynthesis compared a high ratio (5). However, the diets were extreme in the ratios and paired short chain n-6 with long chain n-3 fatty acids. The diets used had, at one extreme, an n-6:n-3 ratio of close to infinity (i.e. almost no omega-3) and at the other extreme a 1:1 ratio, which contained far more long-chain n-3 than most Americans eat. According to NHANES for the years 2005-2006, the average n-6 PUFA intake was 7.1% of total energy and n-3 PUFA intake was 0.7% of total energy. Thus, the PUFA intake is ~8% kcal, with an average n-6:n-3 ratio of 10:1.

With diet limitations in mind, Drs. Ward, Hintze, and Benninghoff developed the Total Western Diet (TWD) which was formulated to deliver macronutrient and micronutrient intake based on the intakes of 50th percentile population in the United

States from NHANES report (6, 7). A strength of TWD, from a translation perspective, is it represents the median composition of diets that Americans are eating (7).

To investigate the possible effects of PUFA on inflammation in physiologically relevant diets, we formulated four diets on the TWD background. We reasoned that these diets should not be too extreme, and thus decided to use the 90^{th} and 10^{th} percentiles of intake for both n-6 and n-3 PUFAs. These diets allow us to investigate the individual roles of both PUFA classes and cover the majority of diets in the US.

In this study, our hypothesis was high n-3 PUFA diet would reduce acute inflammation caused by LPS, whereas high dietary n-6 PUFA would promote this process. To induce acute inflammation, a sub lethal dose of LPS, delivered intraperitoneally, was selected because a strong cytokine inflammatory response was previously measured in this model (4). To evaluate the effect of these diets on metabolism and the acute inflammatory response, we selected endpoints to measure including weight gain, fat gain, cytokine levels, the lipid profile, and oxylipin concentration. Because the main focus was inflammation, cytokine levels were the primary endpoint to evaluate the overall responses. Also, we evaluated three possible pathways (NF-κB, PPARs, and lipogenesis) via hepatic gene expression that may be involved in the inflammatory responses.

Materials and Methods

Animals and diets

The TWD and PUFA modified diets were formulated using the principle of nutrient density. First, we selected median daily intake levels from NHANES report.

Next, we normalized median nutrient consumption data to calories consumed data to calculate nutrient density measure (mass of nutrient per kcal per day). Finally, we translated human nutrient intake information to a suitable rodent diet. To achieve this step, relative contribution of total carbohydrates, proteins, and fats sources to total energy consumed was calculated to establish the basic TWD. A mixture of oils was used to design diets with different total overall PUFA and specific ratios. Casein and L-cysteine were used for the protein portion of the diet. The nutrient density for noncaloric dietary components was calculated based on median caloric intake per day from NHANES data (7). The PUFA diets in this study were formulated to achieve n-6 (LA) and n-3 (ALA) intakes at the 10^{th} and 90^{th} percentiles population according to NHANES by adjusting the different oil composition in TWD. The components of four PUFA diets are summarized in Table 2.1. The key parameters important for this study are underlined and italicized. The targets for the lipid composition are shown at the bottom of the table. In using different oil sources, the goal was to produce diets with defined n-6 and n-3 levels, while keeping the saturated fatty acid composition similar across diet. Thus, the major difference in the fatty acid composition, besides PUFA, should be in the monounsaturated fatty acid content.

Thirty-two male weanling C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were randomly separated into four groups, with balanced initial weight across the groups, and provided with one of the four diets with different PUFA levels (high n-6 & high n-3 (HH), high n-6 & low n-3 (HL), low n-6 & high n-3 (LH), and low n-6 & low n-3 (LL)) for 30 days. Mice were housed individually. Mice were weighed every four days and

food intake was measured every other day. Body composition including body fat, lean, and water was measured every two weeks for four weeks by MRI (EchoMRI-900^{TM}, Houston, TX, USA).

Acute inflammatory challenge, sacrifice, and sample collection

At day 29, all mice were injected with a sub lethal dose (5 mg/kg) of LPS solution (0.5 mg/mL) via intraperitoneal injection. Twenty hours after injection, diets were removed, and all mice were sacrificed four hours later via asphyxiation with CO_2. Blood was collected via cardiac puncture and pipetted into EDTA-lined tubes. Plasma was isolated via centrifugation of the blood for 10 minutes at 1,000 × g. The supernatant (plasma) was transferred to clean micro-centrifuge tubes, and red blood cells were kept for fatty acid analysis. Liver and adipose tissue were collected and immediately frozen at -80℃.

Plasma cytokine analysis

The plasma cytokine analysis was conducted following the manufacturer's instructions. Analysis of the cytokines in plasma was performed by 96-well Plate Assay with Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore, Billerica, MA, USA) (8). Standards were reconstituted in nanopure water and the antibody-immobilized beads were dispersed by vortexing. Subsequently, 60 µL of the bead solution from each antibody vial was mixed and brought final volume to 3.0 mL with assay buffer. Plasma was reconstituted by adding 2.0 mL assay buffer to lyophilized plasma matrix. Working standards were made by reconstituting the mouse cytokine standards with 250 µL nanopure water. A dilution series was prepared six dilution levels of standard, and the

blank standard was the assay buffer. A 96-well plate was cleaned with 200 µL of 1X wash buffer. Next, 25 µL each standard including blank standards and quality control standards were added into the wells with another 25 µL assay. After that, 25 µL matrix solution was added. Then, 25 µL of sample was added. Finally, 25 µL of mixed beads were mixed thoroughly and loaded to each well. The plate was sealed with aluminum foil and placed on the shaker overnight at 2-8℃. After incubation, solution was removed from the wells and each well was washed by 200 µL wash buffer twice. A handheld magnet was used to set the magnetic beads in the plate. After washing the plate, 25 µL detection antibodies were added into each well. The plate was incubated again on the plate shaker for 1 hour at room temperature with aluminum foil covered. After incubation, 25 µL Streptavidin-Phycoerythrin was added to each well. Plate was covered with aluminum foil again and incubated on the plate shaker for another 30 minutes at room temperature. After incubation, solution was gently removed from the well and then was washed twice. Finally, 150 µL sheath fluid was loaded to all wells and suspended the beads on the plate shaker for 5 minutes. Plate was sent to BioPlex system (Bio-Rad, Hercules, CA, USA) to measure the concentration of cytokines.

Fatty acid profiling

Fatty acid profiling of red blood cells and diets

Frozen red blood cell pellets, or diet samples, were transferred to a 16 x 125 mm screw-cap tube. Next, 0.7 mL of 10 M KOH solution and 5.3 mL methanol was added to the tube. Samples were incubated at 55℃ with shaking for 1.5 hours to saponify lipids. After hydrolysis, tubes were cooled on ice, and 0.58 mL of 12 M H_2SO_4 solution was

added to facilitate methylesterification. Samples were mixed thoroughly by inversion and incubated at 55℃ water bath shaker for 1.5 hours. After incubation, samples were cooled on ice and 3 mL hexane was added with thorough mixing via vortex for 5 minutes. Samples were centrifuged at 2,000 × g for 5 minutes and the hexane layer transferred to a GC vial (9).

The fatty acid composition was determined by gas chromatography using a Shimadzu GC-2010 (Shimadzu, Kyoto, Japan) with Agilent HP-88 column (100m x 0.25mm x 0.2μm; Agilent Technologies Inc., Santa Clara, CA, USA). Both injector and detector temperature were set at 250℃. The initial column temperature was 153℃, held for 2 minutes and subsequently temperature was increased to 174℃ at a rate of 2.3℃ per minute. Then, it was held for 0.2 minute. Temperature was continuously increased to 210℃ at a rate of 2.5℃ per minute, and was held for 5 minutes. The split ratio was 25:1. Column flow rate was 1.17 mL per minute. Fatty acids in the red blood cells or diets were identified by comparing retention time with fatty acid methyl ester (FAME) standards.

Fatty acid profiling of hepatic tissue

Approximately 200 mg frozen hepatic tissue was ground under liquid nitrogen with 50 μL of each internal standards including cholesterol esters (CE 15:0, 10 mg/mL), diglycerides (DG 17:0, 10 mg/mL), triglycerides (TAG 19:1, 10 mg/mL), free fatty acids (C21:0, 10 mg/mL), and phospholipids (PC 19:0, 10 mg/mL). Next, 4 mL of Folch reagent (chloroform/methanol at 2:1 with Butylated hydroxytoluene (BHT)) was added to sample powder. The mixture was agitated for 20 minutes to extract the lipids from liver to organic phase. Then, vacuum filtration was applied to remove the protein residue.

Next, 0.8 mL of 0.9% NaCl was added into the liquid collection and was then vortexed for 2 minutes. Mixture was centrifuged at 2000 rpm at 4℃ for 10 minutes. The organic layer (lower phase) was transferred to a pre-weighed 4 mL vial. Then, the organic layer was dried under nitrogen gas and weighed to calculate the weight of extracted lipids. Lipids were re-dissolved with 200 μL Folch reagent.

Thin layer chromatography (TLC) plate was activated at 120℃ for 10 minutes. The plate was subsequently cleaned by running it in the TLC chamber with 100 mL of solvent (chloroform/methanol at 1:1) and then plate was removed once solvent reached two third of the plate. Twenty microliters of lipid extract and lipid standards mix (including cholesteryl esters, triacylglycerols, free fatty acids, diacylglycerols, phospholipid, choline) were loaded on TLC plate. TLC plate was placed in solvent (hexane/diethyl ether/formic acid at 80:20:2) to separate lipid classes in each unknown samples. TLC plate was removed from chamber once the solvent front reached the top edge of plate. Premulin solution (0.05% premulin in acetone:water (8:2, v/v)) was sprayed on the plate to visualize the lipid bands under UV light. Locations of lipid classes were marked on the plate. Silica gel with each lipid class was scraped off from the plate and collected.

Seven hundred microliters of 10 N KOH and 6.3 mL methanol was added into the collection tubes. The mixture was placed in 55℃ water bath shaker for 1.5 hours. Tubes were cooled under tap water after incubation. Then, 580 μL of 24 N H_2SO_4 was added into the tubes and mixed thoroughly. The tubes were placed back to the same water bath shaker for another 1.5 hours. Samples were cooled down again after shaking. Two

milliliters of hexane were added into the tubes and was vortexed for 5 minutes. Mixture was centrifuged at 2,000 rpm for 10 minutes to separate the organic phase and aqueous phase. Upper phase was transferred to 2 mL GC vials without disturbing the aqueous layer. Organic phase was dried completely under nitrogen stream. Then, 100 μL of hexane was added to re-dissolve the lipids samples for GC injection.

TABLE 2.1 Components of PUFA diets.

Diet / components (g/kg)	HH	HL	LH	LL
Casein	190.00	190.00	190.00	190.00
L-Csytine	2.85	2.85	2.85	2.85
Corn starch	230.00	230.00	230.00	230.00
Maltodextrin	70.00	70.00	70.00	70.00
Sucrose	261.07	261.07	261.07	261.07
Cocoa butter	*56.43*	*54.12*	*52.80*	*52.80*
Olive oil	*23.10*	*29.70*	*79.37*	*85.50*
Soybean oil	*61.88*	*13.20*	*8.33*	*16.50*
Corn oil	*23.60*	*67.98*	*24.50*	*10.20*
Cholesterol	0.55	0.55	0.55	0.55
Cellulose	30.00	30.00	30.00	30.00
Mineral mix	35.00	35.00	35.00	35.00
Sodium Chloride	4.00	4.00	4.00	4.00
Vitamin mix	10.00	10.00	10.00	10.00
Choline bitartrate	1.40	1.40	1.40	1.40
TBHQ, antioxidant	0.03	0.03	0.03	0.03
Food color	0.10	0.10	0.10	0.10
Macronutrient (% kcal)				
Carbohydrate	50.00	50.00	50.00	50.00
Protein	15.40	15.40	15.40	15.40
Fat	34.50	34.50	34.50	34.50
PUFA (% kcal)				
n-6 PUFA	*10.40*	*10.40*	*5.10*	*5.10*
n-3 PUFA	*1.10*	*0.40*	*1.10*	*0.40*
n-6 to n-3 PUFA ratio	*9.45*	*26.00*	*4.64*	*12.75*

HH, HL, LH, and LL represent high n-6 & high n-3 PUFA diet, high n-6 & low n-3 PUFA diet, low n-6 & high n-3 PUFA diet, and low n-6 & low n-3 PUFA diet, respectively. TBHQ is tert-Butylhydroquinone.

Fatty acids oxygenated compounds analysis

Mice hepatic sample was ground with 2 mL ethanol and oxylipin standards (deuterated standard, 10 ng each). Standards included 4-hydroxy-2-hexenal (4-HHE) CD_3, 4-hydroxy-2-nonenal (4-HNE) CD_3, TXB_2 D_4, 8-epiPGF2a D4, PGE_2 D_4, LTB_4 D_4, (±)14,15-dihydroxy-eicosa-5,8,11,17-Tetraenoic Acid (14,15-DiHETE) D_4. Then, 3 mL methanol, 5 mL chloroform, and 4 mL nanopure water was added into the same tube. After vortexing and centrifuge the samples, organic phase was collected. Then, another 5 mL chloroform was added to do the second extraction. After vortexing and centrifuging the mixture, organic phase was collected again. Next, organic phase was evaporated and sample was re-dissolved in 2 mL methanol to wash the sample. After methanol was evaporated, 1 mL hexane with O-PFB-TMS was added into the sample tube. Sample tube filled with nitrogen gas was placed in the shaker at room for 30 minutes with agitation. After incubation, 5 mL hexane and 60 μL H_2SO_4 was added into the tube. Supernatant was collected after thorough mixing. Sep-Pak C18 column was used to purify the samples. Column was washed by 3 mL methanol then followed by 6 mL nanopure water. The samples were then loaded on the column. Column was washed again by 9 mL pH 3.5 HCl solution then followed by 4 mL nanopure water. Finally, column was washed by 5 mL hexane. The sample was eluted by 9 mL of methyl formate. After evaporating the solvent, samples were re-dissolved in the injection solvent for LC-MS/MS.

Gene expression analysis

The genes which we measured for mice inflammatory responses include 18s rRNA (for normalization), Acaca, Acox1, Ccl2, Ccl3, Cpt-2, CRP, Dgat-2, Elovl-5, Fasn,

Hmgb-1, Ikb-α, IL-1α, IL-1β, IL-6, Me-1, NF-κB-1, Inhibitor of NF-κB-β (NF-κbib), Ppar-α, Ppar-β, Scd-1, Srebp-1, and Tnf-α.

The RNeasy tissue mini kit (QIAGEN, Hilden, Germany) was used for RNA extraction from mice tissue. Before RNA extraction, working wash buffer solution was made from wash buffer RPE by mixing with 4 volumes of ethanol. Also, 350 µL 2-mercaptoethanol was added to the QIAzol lysis reagent (QIAGEN, Hilden, Germany). Working area was cleaned with 70% ethanol and RNaseZap (Invitrogen, Waltham, MA, USA) to remove RNase contamination.

Liver RNA extraction

Approximately 20 mg frozen hepatic tissue was ground under liquid nitrogen. After the liquid nitrogen evaporated, 600 µL lysis reagent was added, and samples were homogenized with a pestle until tissue was totally disrupted and lysed. Lysate was incubated at 4℃ for one hour and then was loaded onto a QIAshredder spin column placed in a 2 mL collection tube. Samples were centrifuged at 12,000 rpm for 2 minutes at 4°C. Next, 600 µL 70% ethanol was added to the lysate and mixed thoroughly. The lysate/ethanol mixture was incubated at 4℃ overnight. Subsequently, 600 µL of sample was transferred to spin cartridge, and centrifuged at 12,000 rpm for 15 seconds at room temperature. Steps were repeated till all sample solution was loaded. Next, the spin cartridge was washed with 350 µL wash buffer I. Subsequently, 100 µL DNase I working solution was added to column. After quick centrifugation, the column was held at room temperature for 20 minutes. The spin cartridge was washed again and was placed into a new collection tube. Then, the spin cartridge was washed by 500 µL working wash buffer

solution twice and placed into a recovery tube. Finally, 50 μL RNase-free water was added to the spin cartridge to cover the whole surface of membrane. Spin cartridge was centrifuged at 12,000 rpm for 2 minutes after short incubation to collect the RNA in the recovery tube.

Adipose RNA extraction

Approximately 100 mg frozen adipose tissue was ground under liquid nitrogen. Steps for lysis, homogenization and loading samples to the spin column were same as described in the liver RNA extraction. Next, 200 μL chloroform was added to lysate and spin column was shaken vigorously for 15 seconds. Spin column was centrifuged for 15 minutes at 12,000 rpm at 4℃ after short sitting under room temperature. Aqueous phase was then transferred to a fresh tube. Approximate 600 μL of 70% ethanol was added to the sample, and then sample was mixed thoroughly by vortexing. The remaining procedures were same as described in the liver RNA extraction.

Measurement of RNA concentration

Nanodrop 1000 spectrometer (Thermo Scientific, Walthem, MA, USA) was used for measuring RNA concentration from extraction. RNA-40 was chosen on the software. About 2 μL nanopure water was loaded on the clean lower measurement pedestal to zero the absorbance. Then, 2 μL RNA sample was loaded to get the reading of RNA concentration.

RNA to cDNA conversion

Based on the concentration of RNA measured by nanodrop spectrometer, RNA concentration was adjusted to 2000 ng/μL in 9 μL solution by RNase-free water. Next, 11

μL RT master was mixed with 9 μL RNA sample in the 200 μL micro-centrifuge tube. The micro-centrifuge tube was then placed in the PCR cycler. RT reaction was held at 37℃ for 60 minutes, and then terminated at 95℃ for 5 minutes followed by cooling at 4℃.

Real Time quantitative PCR

cDNA samples were pre-amplified before real time quantitative PCR for 14 cycles to increase the original concentration. Fluidigm 48.48 chip (Fluidigm, San Francisco, CA, USA) was applied for real time quantitative PCR. Control line fluid was injected into each accumulater on the chip. Next, the chip was placed into the Integrated Fluidic Circuit (IFC) Controller MX to prime the control line fluid into the chip. In the meantime, 3 μL 20x TaqMan Gene Expression Assay (Applied Biosystems, Waltham, MA, USA) and 3 μL 2x Assay Loading Reagent (Fluidigm, San Francisco, CA, USA) was mixed in a 96 well microplate. Also, 180 μL TaqMan Universal PCR Master Mix (2x) (Applied Biosystems, Waltham, MA, USA) and 18 μL 20x GE Sample Loading Reagent (Fluidigm, San Francisco, CA, USA) was mixed to prepare pre-mix solution. After that, 3.3 μL pre-mix solution and 2.7 μL cDNA sample was mixed in a 96 well microplate by multi-channel pipette. After chip was primed, 5 μL of each gene primer mix was loaded in the assay inlets. Also, 5 μL of each sample mix was loaded in the sample inlets. Then, the chip was placed back to the IFC Controller MX to load the samples and assays into the chip. After process was finished, chip was placed on Biomark data collection system (Fluidigm, San Francisco, CA, USA) to run real time quantitative PCR.

Gene expression data analysis

The data from real time quantitative PCR is shown as Ct value (threshold cycle for gene amplification) for each gene from each sample. The analytical method used in this study to present relative gene expression levels is comparative Ct method and is known as the known as $2^{-\Delta\Delta Ct}$ method (10, 11). In PCR, the gene amplification is described as follows.

$X_n = X_0 * (1+E_x)^n$

Where X_n is the amount of target genes at cycle n of PCR reaction; X_0 is the initial amount of target genes; Ex is the efficiency of gene amplification; n is the number of PCR cycles.

Once threshold cycle (Ct) is reached, the amount of target genes can be described as follow.

$X_t = X_0 * (1+E_x)^{Ct,x} = K_x$

Where Xt is the amount of genes at the number of cycle reaches threshold; Ct,x is the threshold cycle for target gene (x) amplification; K_x is a constant.

Similar as target genes, a reference gene (18s rRNA) is used in the same calculation model.

$R_t = R_0 * (1+E_R)^{Ct,r} = K_R$

Where Rt is the amount of reference gene at the number of cycle reaches threshold; R_0 is initial amount of reference gene; E_R is the efficiency of reference gene amplification; Ct,r is the threshold cycle for reference gene (R) amplification; K_R is a constant.

Although the value of X_t and R_t can be affected by many factors, it is assumed that the efficiencies of target and reference genes are the same under same PCR processing ($E_x=E_R=E$). The relative expression level of target gene compared with reference gene can be written as follow.

$X_t/R_t = X_0/R_0 * (1+E)^{Ct,x-Ct,r} = K$

Here, ΔCt is calculated by $C_{t,x}-C_{t,r}$. Thus, the equation can be written as follow.

$X_N * (1+E)^{\Delta Ct} = K$

Where X_N is normalized initial amount of target gene (X_0/R_0); ΔCt is the difference of threshold between target gene and reference gene. Transform the equation to get new expression.

$X_N = K * (1+E)^{-\Delta Ct}$

If there is a control group in the experiment, X_N could be normalized to control group to calculate the relative increment of target gene in the treatment group compared with control group. The equation can be rewritten as follow (t and c means treatment and control group in the equation).

$X_{N,t}/X_{N,c} = K * (1+E)^{-\Delta Ct,t-(-\Delta Ct,c)} = K * (1+E)^{-\Delta\Delta Ct}$

Here $-\Delta\Delta Ct = -(\Delta C_{t,t} - \Delta C_{t,c})$. $\Delta\Delta Ct$ is obtained by normalizing to control group. In this study, we used average ΔCt in the dietary group LH as control since it is expected to have the lowest inflammatory gene expression level based on published researches.

As we mentioned that PCR process is exponential amplification from initial amount of gene, the amount of gene after amplification could be calculated as $2^{-\Delta\Delta Ct}$.

$2^{-\Delta\Delta Ct}$ represents the fold changes of target genes compared to reference gene and control group.

By using this method, we could see that fold changes of genes in the control group are supposed to be very close to one ($2^0 = 1$). However, because of the high variation in the biological samples, $-\Delta\Delta Ct$ from each sample could be somehow away from zero since each sample will not be exact same as average under same treatment. This variation will lead to average of $2^{-\Delta\Delta Ct}$ being away from one.

Statistical analysis

A 2×2 factorial design was used to evaluate the main effects of n-6 PUFAs and n-3 PUFAs with the interaction effect based on n-6 to n-3 PUFA ratio on inflammatory responses. Shapiro-Wilk test was used to test the normality of data (logarithm transformation was done if the original data was not normal distributed). A False discovery rate (FDR) was applied and was set at 0.05. The FDR reduces the risk of false positives by adjusting the p-values due to multiple comparisons. Analyses were performed with SPSS software version 19 (IBM Corporation, Armonk, New York, USA) and SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Data was reported as mean ± standard deviation.

Results

Diet composition

The main fatty acid compositional parameters of the diets are shown in Table 2.2. In general, the measured values were very close to the targets. Thus, there was ~10% n-6 PUFA in the HH and HL diets, and ~5% in the LH and LL diets. For n-3 PUFA, the

values were close to 1% in the HH and LH diets, and 0.4% in the HL and LL diets. Due to the high n-6 content, both the HH and HL diets contained over 10% total PUFA, whereas LH and LL diets were lower than 6.2%. Importantly, all diets contained a similar amount of saturated fatty acids (~10.5%), and the balance was achieved by changing the monounsaturated fatty acid content. This is important as dietary saturated fatty acids may affect inflammatory processes independently.

TABLE 2.2 Measured lipid values for experimental diets.

	HH	**HL**	**LH**	**LL**
			% kcal	
Saturates	10.6 ± 0.1	10.1 ± 0.1	10.5 ± 0.1	10.4 ± 0.1
MUFA	12.1 ± 0.1	13.6 ± 0.1	17.8 ± 0.1	18.8 ± 0.1
PUFA	11.8 ± 0.1	10.6 ± 0.1	6.2 ± 0.1	5.4 ± 0.1
n-6	10.7 ± 0.1	10.2 ± 0.1	5.1 ± 0.1	4.9 ± 0.1
n-3	1.1 ± 0.1	0.4 ± 0.1	1.1 ± 0.1	0.4 ± 0.1
n-6 to n3 ratio	9.6	24.0	4.6	11.6

HH, HL, LH, and LL represent high n-6 & high n-3 PUFA diet, high n-6 & low n-3 PUFA diet, low n-6 & high n-3 diet, and low n-6 & low n-3 PUFA diet, respectively. MUFA represents monounsaturated fatty acids.

Food intake, body weight, and body fat composition

Mice fed with high n-6 PUFA diets (HH and HL) had a significantly higher food intake (Fig. 2.1). In addition, mice fed the high n-6 diets gained significantly more weight (Fig. 2.2). Furthermore, mice fed the high n-6 diets had significantly higher fat gain (Fig. 2.3). Interestingly, mice fed the low n-3 diets gained less lean weight than mice fed the high n-3 diets (Fig. 2.4).

Fatty acid profile of red blood cells

The red blood cell (RBC) PUFA content is shown in the Table 2.3. The three dominant PUFAs are 18:2n6, 20:4n6, and 22:6n3. According to the 2x2 ANOVA with adjustment for the FDR, n-6 intake affected concentration of 18:2n6, 20:3n6, 22:5n6, 22:5n3 and 22:6n3. With the exception of 20:3n6, the results indicate that more n-6 increased both 18:2n6 (present in the diet) as well as its elongation and desaturation products, and reduced n-3 elongation and desaturation products. The surprise effect is that the high n-6 diets decreased the amount of 20:3n6 relative to the low n-6 diets. On the other hand, high n-3 intake affected levels of 20:2n6, 22:5n6, 20:5n3, 22:5n3, and 22:6n3. There were no effects from an n-6 to n-3 PUFA interaction. The n-3 index, which is the sum of the percentage of EPA and DHA in RBCs, and which has been correlated with several health parameters in humans, was higher in the LH group compared to the others. Since mice in the LH diet group had higher DPA and DHA than mice in the HH group, it appears reducing the n-6 intake promotes elongation of linolenic acid.

Plasma cytokine analysis (post-LPS challenge)

Cytokines analyzed in plasma included IL-1α, IL-1β, IL-3, IL-5, IL-6, IL-10, IL-12, IL-17, IFN-γ, MCP-1, MIP-1α, Granulocyte-macrophage colony-stimulating factor (GM-CSF), Keratinocyte-derived chemokine (KC), TNF-α, RANTES. As the data was not normally distributed, a logarithmic transformation was applied. Using 2x2 factorial analysis, the differences (p-value less than 0.05) between groups are shown in the Table 2.4. There was an increase in the concentration of IL-1α, IL-1β, IL-10, IL-17, MIP-1α, and RANTES that was driven by the n-3 PUFA content of the diet. On the other hand,

higher MCP-1, TNF-α, and KC levels were affected by n-6 PUFA and n-3 PUFA interaction (high n-3 and low n-6 PUFA in the diet). The higher IL-6 and IFN-γ concentration was led by n-3 PUFA and n-6 x n-3 PUFA interaction (high n-3 and low n-6 PUFA in the diet). However, when a False Discovery Rate (FDR) adjustment for multiple testing was applied at a p value of 0.05 (to reduce type I error), there were no significant differences for any cytokines.

Fatty acid profile of liver

The lipid composition of the liver tissue was measured to investigate the effects of dietary fat, and to determine if there is a relationship with both gene expression and oxylipin production. The total lipid content of each major lipid classes, phospholipids (PL), triacylglycerol (TAG), diglycerides (DG), free fatty acids (FFA) and cholesterol esters (CE) are shown in the Table 2.5. There was no difference between dietary groups in overall PC, TAG, and DG. On the other hand, n-6 PUFA intake affected both the FFA and CE levels in the liver.

In liver, PL are the primary lipid class from which PUFA are cleaved in order to generate eicosanoids (12), and TAG may also provide substrates. The PUFA profiles of PL and TAG are shown in Tables 2.6 and 2.7. In the PL profile, the levels of 18:2n6 and 20:2n6 are significantly higher in the high n-6 PUFA groups. Conversely, for the n-3 diets there were higher 18:3n3 levels. EPA was affected by both low dietary n-6 and high dietary n-3. From TAG PUFA profile, higher 20:4n6 level was driven by both high n-6 PUFA content and low n-3 PUFA content.

Analysis of oxygenated compounds of fatty acids

Oxygenated fatty acids (oxylipins) are intermediates in the inflammatory process and are metabolites of the long chain n-6 and n-3 fatty acids. Hepatic oxylipins are shown in Table 2.8. PGE_2, a major oxylipin derived from AA, was higher in the HL group (affected by high n-6 PUFA content and low n-3 PUFA content) and there was more 14-OH DHA according to the n-3 content of the diet. However, these differences were not significant after adjusted p-value for multiple comparisons.

Tissue gene expression

The results of hepatic gene expression are shown in Table 2.9. Expression levels were normalized to the 18s rRNA gene in the LH dietary group, which we hypothesized to have lowest gene expression level among all groups. Next, the fold change of each gene in the other dietary groups was compared. Generally, the fold change of genes in the control is supposed to be close to one when data variance is small. However, the variances in our data were large which results in the fold change in the control group (LH group) not being exactly one. A 2x2 factorial analysis was run to access the effects of dietary PUFA on gene expression across all dietary groups. There were increases in expression of genes associated with lipogenesis such including Acaca, Dgat2, Elovl5, Me1, and Srebp-1, which were driven by the high n-6 diets. Furthermore, genes related to NF-κB pathway, Ccl2, Ccl3, IL-1b, IL-6, nfkb1, and Tnf-α also had increased expression in the high n-6 diets. For PPAR-α pathway, Acox1, Cpt-2, Hmgb1, and Ppar-α were enhanced in the mice fed with high n-6 PUFA diet. On the other hand, dietary n-3 PUFA did not show any effects on lipogenesis, NF-κB, and PPAR-α related gene expression.

The mice fed with HL diet also showed significantly higher lipogenesis related Fasn expression level than those fed with LL diet.

Hepatic cholesterol esters

While there were no differences in the content of PL, TAG or DG in the liver, there were large differences in the CE and FFA content. The fatty acid profile of CE is listed in the Table 2.10. The dominant fatty acid in the cholesterol ester fraction is C14:0 (myristic acid), and it makes up over 50% of the total fatty acids. The n-6 content of the diet appears to lower several fatty acids in the cholesterol esters including 14:0, 14:1n5, 16:0, 16:1n7, 18:1n9 and 20:3n6.

The hepatic free fatty acid profile is shown in Table 2.11. Like the CE fraction, there are several differences driven by the n-6 content of the diet, but no independent effects of n-3 fatty acids. There are significant differences in 12:0, 16:0, 17:0, 18:0 and 18:1n9.

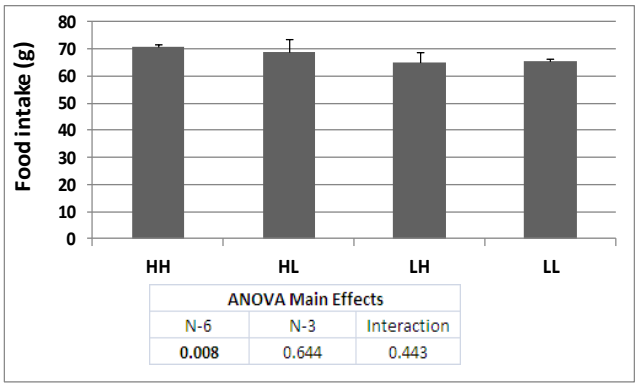

FIGURE 2.1 Food intake across the different dietary groups. The data represents mean ± SD. Means with different superscripts are significantly different ($p<0.05$).

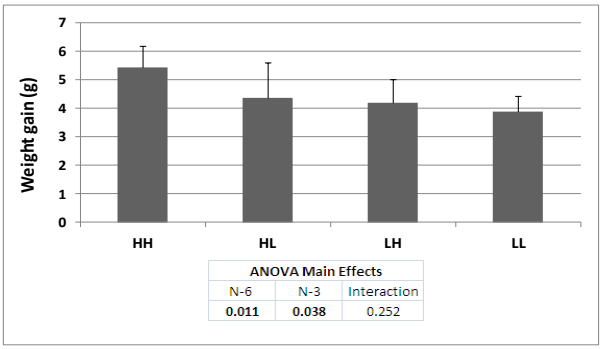

FIGURE 2.2 Weight gain across the different dietary groups. The data represents mean ± SD. Means with different superscripts are significantly different ($p<0.05$).

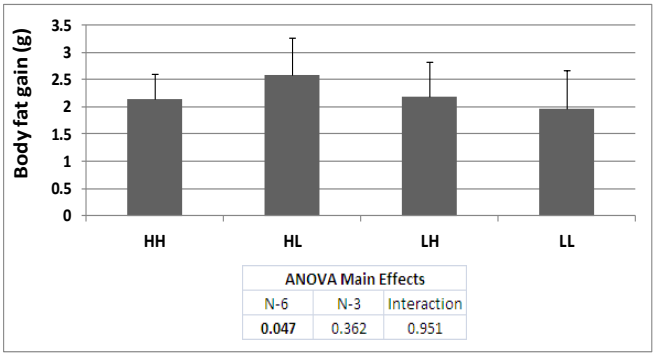

FIGURE 2.3 Fat gain across the different dietary groups. The data represents mean ± SD. Means with different superscripts are significantly different ($p<0.05$).

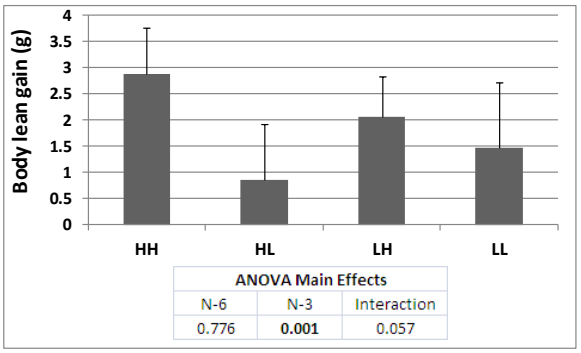

FIGURE 2.4 Lean gain across the different dietary groups. The data represents mean ± SD. Means with different superscripts are significantly different ($p<0.05$).

TABLE 2.3 Fatty acid composition of mice red blood cells from four PUFA diets.

	Diets				Adjusted *p* value (FDR)		
PUFA	**HH**	**HL**	**LH**	**LL**	**n-6**	**n-3**	**Interaction**
			Omega-6				
C18:2 n6 (LA)	12.26±3.44	13.38±2.71	9.14±0.71	9.33±2.19	0.01	NS	NS
C20:2 n6 (Eicosadienoic acid)	0.35±0.06	0.38±0.06	0.27±0.04	0.38±0.11	NS	0.05	NS
C20:3 n6 (DGLA)	1.37±0.29	1.48±0.23	1.83±0.16	1.63±0.27	0.02	NS	NS
C20:4 n6 (AA)	13.89±2.56	16.20±1.31	13.70±1.75	14.03±2.20	NS	NS	NS
C22:4 n6 (Adrenic acid)	1.45±0.41	1.59±0.38	1.22±0.23	1.27±0.31	NS	NS	NS
C22:5 n6 (Osbond acid)	0.80±0.26^{b}	1.26±0.24^{a}	0.57±0.07^{b}	0.80±0.16^{b}	<0.01	<0.01	NS
			Omega-3				
C18:3 n3 (ALA)	0.35±0.16	0.21±0.05	0.35±0.12	0.22±0.07	NS	0.03	NS
C20:5 n3 (EPA)	0.68±0.39	0.35±0.13	1.00±0.28	0.51±0.14	NS	<0.01	NS
C22:5 n3 (DPA)	0.85±0.18^{b}	0.68±0.19^{b}	1.22±0.25^{a}	0.81±0.16^{b}	0.02	<0.01	NS
C22:6 n3 (DHA)	5.77±0.69^{b}	5.68±0.59^{b}	7.35±0.71^{a}	5.67±1.08^{b}	0.05	0.03	NS
n-3 index	6.45±1.08^{b}	6.03±0.72^{b}	8.34±0.99^{a}	6.17±1.22^{b}	<0.01	<0.01	0.01

The n-3 index is the sum of EPA and DHA. The data represents mean (percentage) ± SD. Means with different superscripts are significantly different ($p<0.05$). NS means not significantly different.

TABLE 2.4 Log(10) transformed plasma cytokine concentration across four dietary groups.

Cytokines (pg/mL)	HH	HL	LH	LL	*p*-value		
					n-6	n-3	Interaction
IL-10	2.96±0.33	2.83±0.26	2.86±0.20	2.52±0.22	NS	0.03	NS
IL-17	2.61±0.87	2.12±0.73	2.91±0.17	1.70±1.15	NS	0.01	NS
IL-1α	2.23±0.38	1.93±0.24	2.19±0.34	1.88±0.31	NS	0.02	NS
IL-1β	1.69±0.34	1.34±0.28	1.68±0.24	1.26±0.50	NS	0.01	NS
MIP-1α	2.80±0.33	2.61±0.33	2.84±0.30	2.38±0.44	NS	0.04	NS
RANTES	3.89±0.84	3.47±0.36	4.19±0.53	3.09±0.72	NS	<0.01	NS
IFN-γ	2.86±0.67	2.67±0.55	3.54±0.32	2.04±1.29	NS	0.01	0.05
IL-6	4.44±0.44	4.54±0.21	4.70±0.04	3.71±0.84	NS	0.04	0.01
KC	4.44±0.20	4.45±0.13	4.54±0.09	4.14±0.39	NS	NS	0.04
MCP-1	4.05±0.45	4.13±0.19	4.32±0.16	3.69±0.47	NS	NS	0.02
TNF-α	2.01±0.38	2.04±0.13	2.20±0.17	1.63±0.50	NS	NS	0.04
IL-3	0.46±0.48	0.41±0.13	0.41±0.31	0.26±0.49	NS	NS	NS
IL-5	1.49±0.46	1.49±0.26	1.76±0.27	1.36±0.21	NS	NS	NS
IL-12	1.50±0.38	0.97±0.46	1.52±0.29	1.53±0.38	NS	NS	NS
GM-GSF	2.52±0.12	2.47±0.19	2.57±0.18	2.41±0.18	NS	NS	NS

The data represents mean ± SD. NS means not significantly different.

TABLE 2.5 Total liver lipid compositions from all lipid classes.

	Diets				*p* values for 2*2 ANOVA		
Fatty acid (mg/g)	HH	HL	LH	LL	n6	n3	Interaction
PL	61.03±5.48	62.13±3.16	60.67±8.74	63.37±4.88	NS	NS	NS
TAG	24.42±15.49	28.59±11.00	19.70±9.46	31.27±10.99	NS	NS	NS
DG	0.91±0.36	0.84±0.20	1.06±0.37	1.13±0.29	NS	NS	NS
FFA	0.44±0.10	0.48±0.08	0.81±0.43	0.82±0.29	<0.01	NS	NS
CE	16.26±4.62	14.30±3.77	20.35±6.37	25.92±3.28	<0.01	NS	NS
Total	102.66±14.79	107.33±17.30	102.59±17.53	122.51±18.33	NS	NS	NS

The data represents mean ± SD. NS means not significantly different.

TABLE 2.6 PL PUFA composition in liver.

	Diets				Adjusted *p* value (FDR)		
PUFA (mg/g)	**HH**	**HL**	**LH**	**LL**	**n-6**	**n-3**	**Interaction**
C18:2 n6 (LA)	9.44±0.99	9.95±0.37	7.99±1.55	7.67±0.76	<0.01	NS	NS
C18:3 n6 (GLA)	0.12±0.03	0.13±0.03	0.09±0.04	0.09±0.04	NS	NS	NS
C20:2 n6 (Eicosadienoic acid)	0.14±0.03	0.14±0.02	0.09±0.02	0.10±0.02	<0.01	NS	NS
C20:3 n6 (DGLA)	0.61±0.16	0.59±0.11	0.76±0.35	0.79±0.16	NS	NS	NS
C20:4 n6 (AA)	10.18±1.70	11.19±0.80	9.52±1.60	10.61±0.61	NS	NS	NS
C18:3 n3 (ALA)	0.15±0.09	0.07±0.02	0.19±0.05	0.08±0.02	NS	<0.01	NS
C20:5 n3 (EPA)	0.15 ± 0.03^{b}	0.06 ± 0.01^{b}	0.38 ± 0.10^{a}	0.16 ± 0.04^{b}	<0.01	<0.01	NS
C22:5 n3 (DPA)	0.25±0.11	0.23±0.04	0.23±0.12	0.23±0.10	NS	NS	NS
C22:6 n3 (DHA)	7.32±0.57	6.47±0.95	6.76±1.66	7.17±1.15	NS	NS	NS

The data represents mean ± SD. Data with different superscripts are significantly different ($p<0.05$). NS means not significantly different.

TABLE 2.7 TAG PUFA composition in liver.

	Diets				Adjusted *p* value (FDR)		
PUFA (mg/g)	**HH**	**HL**	**LH**	**LL**	**n-6**	**n-3**	**Interaction**
C18:2 n6 (LA)	5.78±4.26	6.89±2.67	2.69±1.56	4.26±1.82	NS	NS	NS
C18:3 n6 (GLA)	0.11±0.08	0.14±0.04	0.05±0.03	0.09±0.04	NS	NS	NS
C20:2 n6 (Eicosadienoic acid)	0.06±0.02	0.08±0.05	0.04±0.01	0.08±0.04	NS	NS	NS
C20:3 n6 (DGLA)	0.15±0.04	0.22±0.07	0.12±0.03	0.17±0.05	NS	NS	NS
C20:4 n6 (AA)	$0.46\pm0.16^{a,b}$	0.63 ± 0.17^{a}	0.21 ± 0.08^{c}	$0.39\pm0.09^{b,c}$	<0.01	0.03	NS
C18:3 n3 (ALA)	0.31±0.24	0.18±0.07	0.25±0.16	0.19±0.10	NS	NS	NS
C20:5 n3 (EPA)	0.08±0.04	0.05±0.02	0.08±0.04	0.05±0.03	NS	NS	NS
C22:5 n3 (DPA)	0.14±0.08	0.10±0.03	0.12±0.06	0.09±0.03	NS	NS	NS
C22:6 n3 (DHA)	0.76±0.32	0.64±0.19	0.61±0.28	0.63±0.16	NS	NS	NS

The data represents mean ± SD. Data with different superscripts are significantly different ($p<0.05$). NS means not significantly different.

TABLE 2.8 Oxylipins in the liver.

	Diets				Adjusted *p* value (FDR)		
Oxylipin (ρmol/g)	**HH**	**HL**	**LH**	**LL**	**n-6**	**n-3**	**Interaction**
15-HETE	3.05±1.71	2.00±1.51	1.13±1.31	1.29±1.76	NS	NS	NS
12-HETE	21.04±20.37	8.23±9.72	9.81±6.00	4.28±2.92	NS	NS	NS
5-HETE	0.95±0.73	1.07±0.98	0.49±0.48	0.82±1.28	NS	NS	NS
4-OH DHA	0.77±0.58	0.77±0.63	0.59±0.70	0.64±0.77	NS	NS	NS
7-OH DHA	0.13±0.04	0.08±0.04	0.08±0.05	0.09±0.06	NS	NS	NS
14-OH DHA	3.60±3.33	1.18±1.05	2.87±1.58	1.12±0.94	NS	NS	NS
17-OH DHA	1.44±0.85	0.67±0.37	0.79±0.67	0.86±0.78	NS	NS	NS
11-OH DHA	0.30±0.14	0.19±0.14	0.15±0.08	0.15±0.08	NS	NS	NS
PGE_2	$0.12±0.04^b$	$0.37±0.23^a$	$0.10±0.06^b$	$0.15±0.10^b$	NS	NS	NS
$PGF_{2\alpha}$	0.21±0.07	0.25±0.17	0.24±0.14	0.26±0.26	NS	NS	NS
TXB_2	0.24±0.21	0.19±0.20	0.35±0.30	0.15±0.11	NS	NS	NS

Abbreviation: 15-hydroxyeicosatetraenoic acid (15-HETE), 12-hydroxyeicosatetraenoic acid (12-HETE), 5-hydroxyeicosatetraenoic acid (5-HETE), 4-hydroxy-docosahexaenoic acid (4-OH DHA), 7-hydroxy-docosahexaenoic acid (7-OH DHA), 14-hydroxy-docosahexaenoic acid (14-OH DHA), 17-hydroxy-docosahexaenoic acid (17-OH DHA), 11-hydroxy-docosahexaenoic acid (11-OH DHA), prostaglandin E2 (PGE_2), prostaglandin F2α ($PGF_{2\alpha}$), thromboxane B2 (TXB_2). The data represents mean ± SD. Data with different superscripts are significantly different ($p<0.05$). NS means not significantly different.

TABLE 2.9 Hepatic gene expression.

	Diets				Adjusted *p* value (FDR)		
Genes (log2 fold change)	**HH**	**HL**	**LH**	**LL**	**n-6**	**n-3**	**Interaction**
NF-κB pathway							
Ccl2	2.26±1.28	1.86±1.26	1.56±1.07	0.49±0.31	0.03	NS	NS
Ccl3	2.15±1.04	1.49±0.80	1.39±0.92	0.74±0.35	0.03	NS	NS
CRP	5.50±7.52	6.80±7.30	3.20±3.46	2.46±2.11	NS	NS	NS
IL-1α	2.61±2.05	1.82±0.95	1.10±0.46	0.40±0.31	NS	NS	NS
IL-1β	2.67±1.48	1.69±0.82	1.50±0.78	0.76±0.35	0.02	NS	NS
IL-6	3.77±2.93	1.75±1.14	1.47±1.01	0.46±0.44	0.03	NS	NS
Nfκb1	1.52±0.82	1.68±0.77	1.16±0.56	0.67±0.26	0.02	NS	NS
Nfκbib	1.30±1.31	1.10±0.57	1.29±1.03	0.61±0.32	NS	NS	NS
Tnf- α	2.03±1.10	1.55±0.93	1.27±0.83	0.45±0.33	0.02	NS	NS
Lipogenesis							
Acaca	1.77±1.41	3.44±2.29	1.46±1.04	0.76±0.59	0.03	NS	NS
Dgat2	2.23±1.69	3.44±1.81	1.81±1.01	1.19±1.28	0.04	NS	NS
Elovl5	2.52±1.12	2.20±1.12	1.78±1.00	0.67±0.25	0.03	NS	NS
Fasn	$1.09\pm0.48^{a,b}$	2.05 ± 0.78^{a}	$1.43\pm0.87^{a,b}$	0.79 ± 0.46^{b}	NS	NS	0.03
Me1	1.46±0.71	2.59±0.95	1.36±1.01	1.09±0.70	0.04	NS	NS
Scd-1	1.61±0.93	2.19±0.85	1.92±1.21	1.06±0.75	NS	NS	NS
Srebp-1	1.68±0.92	1.57±0.48	1.21±0.59	0.82±0.32	0.04	NS	NS
PPAR-α pathway							
Acox1	1.64±0.63	2.83±1.10	1.69±1.01	1.26±0.52	0.03	NS	NS
Cpt-2	1.82±0.93	3.02±1.12	1.51±0.94	1.53±0.82	0.03	NS	NS
Hmgb1	2.37±1.99	2.40±1.39	1.26±0.70	0.53±0.70	0.03	NS	NS
Ppar-α	2.23±0.94	3.22±1.75	2.06±1.14	1.24±0.59	0.03	NS	NS
Ppar-d	1.50±1.40	0.95±0.39	1.07±0.37	0.99±1.10	NS	NS	NS

The data represents mean ± SD. Data with different superscripts are significantly different ($p<0.05$). NS means not significantly different.

TABLE 2.10 Fatty acid content of CE in the liver.

	Diets				*p* values for 2*2 ANOVA		
PUFA	**HH**	**HL**	**LH**	**LL**	**n-6**	**n-3**	**Interaction**
C14:0 (Myristic acid)	9.92±3.39	9.27±2.56	12.17±3.81	15.09±2.10	<0.01	NS	NS
C14:1 n5 (Myristoleic acid)	1.06±0.36	0.89±0.29	1.10±0.28	1.43±0.32	0.04	NS	NS
C16:0 (Palmitic acid)	1.40±0.34	1.11±0.18	1.48±0.44	1.85±0.36	<0.01	NS	NS
C16:1 n7 (Palmitoleic acid)	0.21±0.29	0.09±0.07	0.27±0.16	0.38±0.24	0.05	NS	NS
C18:0 (Stearic acid)	0.65±0.36	0.40±0.11	0.65±0.41	1.02±0.52	NS	NS	NS
C18:1 n9 (Oleic acid)	0.82±0.47	0.64±0.53	2.09±1.19	3.02±1.90	<0.01	NS	NS
C18:2 n6 (LA)	0.33±0.21	0.27±0.22	0.29±0.12	0.40±0.19	NS	NS	NS
C18:3 n3 (ALA)	0.04±0.03	0.03±0.02	0.07±0.05	0.05±0.03	NS	NS	NS
C20:3 n6 (DGLA)	0.06±0.03	0.06±0.02	0.11±0.07	0.13±0.07	<0.01	NS	NS
C20:4 n6 (AA)	0.17±0.06	0.18±0.07	0.11±0.04	0.19±0.07	NS	NS	NS

The data represents mean ± SD. NS means not significantly different.

TABLE 2.11 Fatty acid content of FFA in the liver.

	Diets				*p* values for 2*2 ANOVA		
PUFA	**HH**	**HL**	**LH**	**LL**	**n-6**	**n-3**	**Interaction**
C12:0 (Lauric acid)	0.02±0.01	0.03±0.01	0.04±0.02	0.05±0.02	<0.01	NS	NS
C14:0 (Myristic acid)	0.04±0.02	0.05±0.03	0.08±0.07	0.08±0.07	NS	NS	NS
C15:0 (Pentadecanoic acid)	0.01±0.00	0.01±0.00	0.02±0.02	0.01±0.01	NS	NS	NS
C16:0 (Palmitic acid)	0.11±0.04	0.11±0.03	0.22±0.13	0.22±0.08	<0.01	NS	NS
C17:0 (Margaric acid)	0.02±0.01	0.02±0.01	0.03±0.01	0.04±0.01	<0.01	NS	NS
C18:0 (Stearic acid)	0.09±0.04	0.11±0.06	0.15±0.06	0.17±0.06	0.02	NS	NS
C18:1 n9 (Oleic acid)	0.03±0.01	0.02±0.01	0.05±0.05	0.04±0.02	0.04	NS	NS
C18:2 n6 (LA)	0.01±0.01	0.01±0.00	0.01±0.01	0.01±0.01	NS	NS	NS

The data represents mean ± SD. NS means not significantly different.

Discussion

In this project, our goal was to investigate whether modifying the PUFA content of the TWD, within physiological ranges, would affect acute inflammation induced by a sublethal dose of LPS. Mice fed the high n-6 PUFA diets had significantly higher food intake and gained more weight. The increased weight was due to both fat and lean tissue. However, the increased mass in mice fed the HL diet was only due to increased fat. This suggests that the HH diet promotes greater weight through growth while the HL diet appears to promote increased weight via adipogenesis. A possible mechanism is that

dietary PUFA enhance muscle mammalian target of rapamycin (mTOR) pathway to increase lean gain (13). On the other hand, it has been shown that high n-6 to n-3 PUFA ratio decreases long-chain n-3 PUFA in humans. This, in turn, has been shown to enhance the SREBP-1c to PPAR-α ratio, favor lipogenesis over fatty acid oxidation (14, 15).

The fatty acid composition of red blood cells is a good indicator of dietary fatty acid intake (16), and in this study was used to monitor how the different diets affected their composition. The main focus was on AA, EPA, and DHA because they are the precursors of eicosanoids which are thought to drive inflammatory responses directly. According to the data, higher levels of EPA and DHA were driven by high intake of n-3 PUFA (ALA). Moreover, DHA content increased under low n-6 PUFA diet. AA, major precursor for n-6 PUFA related eicosanoids, surprisingly, was not affected by dietary PUFA contents and ratios. This result implies that there is a competitive process for the elongation and desaturation of ALA to EPA and DHA, as a lower level of n-6 PUFA in the diet resulted in more DHA, when similar amounts of ALA were fed. This result is consistent with the inhibition of LA on the biosynthesis of n-3 long chain PUFAs via competing the rate-limiting enzymes of long chain PUFA synthesis such as Δ5 and Δ6 desaturase (17-20).

An interesting biomarker changed by the completion for elongation and desaturation of ALA is the n-3 index. It was higher in the LH group than the other groups. In humans, the n-3 index has shown a correlation with susceptibility to heart disease, with a value over 8% being cardio protective, while a value less than 4% is consider a risk

factor (21). Mice in LH diet had a n-3 index significantly higher than mice fed the other diets, and at 8.34%, this level would be associated with protection against the development of CHD in humans. Collectively, these data suggest that the overall body long chain PUFA is affected by the levels fed in the diets, and may affect downstream inflammatory processes.

Cytokines were the primary endpoints measured to assess the inflammatory response to the LPS injection. From cytokine analysis, after adjusting the p-value for multiple tests, we found there were no differences in the cytokines. Although there were some significant differences in the 2x2 factorial design, the effect was not sufficiently robust to withstand correction for multiple tests. Thus, the PUFA contents and n-6 to n-3 PUFA ratios did not affect the mice plasma cytokine levels. Although Schmocker et al. (22) reported that n-3 PUFAs could alleviate acute inflammation caused by LPS, the n-6 to n-3 PUFA ratio (AA:EPA) in the mice was very extreme (at 1:1). In our study, by feeding our PUFA diets, the AA to EPA ratio in the RBC was from 14:1 to 46:1 which is lower than their study, and more importantly, closer to levels in humans consuming a Western Diet. A study done by Pluess et al. (23) found that dietary n-3 PUFA blunted LPS-induced acute inflammation. However, they used fish oil which is very high in EPA and DHA. Diets such as these, with very high long chain n-3 PUFA, do not represent the n-3 PUFA intake of Americans, result in super physiological levels of membrane long chain PUFA, and thus are limited in their translatability. While the feeding period of this study was short, there were significant and meaningful differences in the lipid composition of tissues, like RBCs, that are used to assess whole body PUFA metabolism

in human studies. The lack of a PUFA effect on the cytokine response does not support a role for PUFA in significantly affecting the acute inflammatory response.

Beyond cytokines, several other inflammation-related measures were investigated. In a manner related to the RBC profile, the hepatic PL PUFA profile and eicosanoids response to LPS was determined. According to the PL profile, LA was significantly higher by the n-6 diet content and ALA by the n-3 PUFA content. EPA concentration in PL in the liver was affected by both n-6 and n-3 PUFA levels in the diets. Higher dietary n-3 PUFA and lower dietary n-6 PUFA could lead to higher EPA level in the PL in liver. From posthoc analysis, LH group mice showed significantly higher EPA level than the rest groups. The result implies that long-chain n-3 PUFA biosynthesis is affected by competitive inhibition from n-6 PUFA (LA) which is same as we observed in the red blood cell lipid profiling. In the TAG PUFA profile, similar result was also found that AA biosynthesis may be affected by competitive inhibition from ALA in the diet.

In the oxylipin analysis, PGE_2 was higher in the mice fed the HL diets, but the effect was not significant after adjusting for multiple testing. PGE_2 is an AA-derived eicosanoid which has pro-inflammatory effects such as inducing production of IL-6 and increasing vascular permeability (24, 25). PGE_2 may be involved into the inflammatory responses by stimulating NF-κB pathway (26). Although we did not see difference of AA between the dietary groups in PL, there was difference in TAG AA level across the groups. High AA in the TAG in liver is constant with the increased level of PGE_2 level in liver, even though the effect was not significant after multiple testing. Interestingly, some studies have suggested that TAG may also be a source of lipids for oxylipin biosynthesis

in inflammatory cells (27). In our study, AA in the TAG rather than in the PL may lead to higher level of PGE_2 in the HL group. The result may also suggest that a higher n-6 to n-3 PUFA ratio in the diet may lead the fatty acids metabolic flux to form more n-6 PUFA derived eicosanoids in liver.

In the hepatic gene expression data, we can see that most of NF-κB pathway related genes especially Nfkb1 were enhanced by high dietary n-6 PUFAs. Ccl2 and Ccl3 genes play important roles in promotion of recruitment and migration of immune cells such as neutrophil and macrophages to inflammation site (28, 29). Increased Ccl2 and Ccl3 expression in high n-6 PUFA groups suggests dietary n-6 PUFA may promote immune cell recruitment or migration during acute inflammation. Furthermore, IL-1β, IL-6, and Tnf-α genes are downstream genes which are stimulated after NF-κB translocation which implies high dietary n-6 PUFA may promote NF-κB translocation which initializes NF-κB downstream (30).

PPAR-α pathway is associated to lipid oxidation which is inversely correlated with inflammatory responses (31). From PPAR-α pathway, the results indicated that high dietary n-6 PUFA led to higher expression of Acox-1, Cpt-2, Hmgb1, and Ppar-α which indicates high dietary n-6 PUFA levels promote gene expression in the PPAR-α pathway. The result is not consistent with our hypothesis, but one caveat is that these animals were under LPS stress. Research indicated that dietary long-chain n-3 PUFAs could enhance PPAR-α pathway with increased Acox1, Ppar-α expression in the mice (32, 33). Scientists also found increased Cpt-2 gene expression in rabbits after treatment with high n-3 PUFA diet (34). On the other hand, oral long-chain n-3 PUFA supplements have

shown the effect of reducing the expression of Hmgb1. However, some papers indicated that PUFAs (LA and ALA) could function as ligands for PPAR-α pathway (35, 36). Thus, based on what we observed from our data, we believe that PPAR-α pathway is positively related with overall PUFA level in the diets.

From the pathway of lipogenesis, we can see that high n-6 PUFA diets could lead to increased expression of lipogenesis related genes including Acaca, Dgat2, Elovl5, Me1, and Srebp-1. Expression of Fasn, which encodes the fatty acid synthetase to catalyze the biosynthesis of palmitic acid from acetyl-CoA and malonyl-CoA, was significantly increased in the HL group compared with LL which means dietary high n-6 to n-3 PUFA ratio could enhance Fasn expression. Other studies showed that dietary n-3 PUFA could reduce expression levels of lipogenesis related genes such as Acaca, Fasn, Scd-1, and Srebp-1 in rodent liver (37-39). Furthermore, scientists also pointed out that DHA treated C57BL mice had lower FAS and ME activity levels (40). However, some other studies indicated that dietary PUFAs (both n-6 and n-3 PUFAs) could inhibit lipogenesis by inhibiting lipogenic gene expression (41, 42). In this study, we found high n-6 PUFA diets were positively related to lipogenesis in the mice. Moreover, high n-6 to n-3 PUFA ratio may also lead to lipogenesis by increasing the gene expression level of Fasn.

From both PPAR-α and lipogenesis pathways, we know that higher overall dietary PUFA would increase lipid oxidation. However, if n-6 and n-3 PUFAs are not balanced, over-intake of n-6 PUFA would lead to more lipogenesis than lipid oxidation.

From lipid profiles in liver, there was no difference in total PL, TAG, and DG. However, both CE and FFA were significantly affected by the content of n-6 in the diet.

There was significantly more CE in the low n-6 diets, and the majority was due an increase in myristic acid (14:0). The other prominent difference was with oleic acid (18:1n9) which was also lower. Is the difference in hepatic CE due to specific fatty acids, or due to an overall reduction across all fatty acids? When the fatty acid content is expressed in percentage, the only notable difference is in oleic acid. Abbey et al. (43) found that replacement of dietary SFAs with PUFAs could lower the total plasma cholesterol and LDL cholesterol. Berry et al. compared efficiency of reducing LDL cholesterol between dietary MUFAs and PUFAs. He found that PUFAs were more effective on reducing LDL cholesterol than MUFAs (44). Our PUFA diets have same amount of SFAs and total fats. HH and HL have higher PUFA contents but lower MUFA contents compared to LH and LL diets. Thus, the reason that lower levels of CE in the mice fed with HH and HL diets could be PUFA have better ability to reduce LDL cholesterol. For the FFA, there was approximately double the amount in the low n-6 diets. In terms of percentage, there is no clear effect across any specific fatty acid, indicating that increased n-6 PUFA reduces the overall FFA content.

In summary, in our acute inflammation model, high n-6 PUFA diets appear to promote acute inflammation caused by sublethal dose of LPS injection. However, the effect is modest. As most of the suggested effects were lost with control of the FDR, it may be that this study was underpowered to detect significant differences. To our knowledge, this is the first study to investigate the effect of dietary PUFA on acute inflammation using diets with PUFA formulas within physiological ranges consumed by

Americans. The diets did cause the predicted changes in tissues of eicosanoid precursors, and also recapitulated the well-known cholesterol lowering effect of n-6 PUFA.

High dietary n-6 PUFA led to higher food intake which consequentially caused higher weight gain in the mice. We found that mice fed with HH diet had increased weight gain because of increased fat and lean while the mice fed with HL diet only had increased fat. The results suggest that high n-6 PUFA with low n-3 PUFA in the diet may cause fat accumulation through enhanced lipogenesis. Cytokine analysis showed that there were no differences of inflammatory cytokine concentration. The reason may be moderate PUFA contents and ratios in our diets which could not affect the acute inflammation in short feeding period. In the lipid profile of red blood cells, higher levels of EPA and DHA were driven by high intake of n-3 PUFA. DHA level was also enhanced under low n-6 PUFA diet which implies there is a competitive inhibition from n-6 PUFA on DHA biosynthesis. In the liver lipid profiles, there was no difference in overall fatty acid levels in major lipid classes, PL and TAG. In PL, LA and ALA were significantly higher in the high n-6 PUFA dietary groups and high n-3 PUFA dietary groups, respectively, due to their original higher concentration in the diets. Higher dietary n-3 PUFA and lower dietary n-6 PUFA could lead to higher EPA level in the PL in liver which also suggests EPA biosynthesis is affected by competitive inhibition like what we observed in the red blood cells. In the TAG, increased AA was seen in the HL group which suggests that AA biosynthesis is also affected by competitive inhibition from ALA in the diet. Higher level of PGE_2, AA-derived eicosanoid, was driven by high n-6 PUFA and low n-3 PUFA in the diets. In our study, the source of PGE_2 may be from TAG

instead of PC due to higher AA level in the HL group in the TAG. Our oxylipin result suggests that higher n-6 to n-3 PUFA ratio in the diet may lead fat metabolic flux to form more AA-derived eicosanoids in the liver. From gene expression data, high dietary n-6 PUFA could promote expression level of lipogenesis and NF-κB related genes which are positively related to inflammation. Higher overall PUFA content could stimulate PPAR-α pathway which is associated to fat oxidation. Except the effect on inflammation, dietary PUFA showed a better ability to reduce LDL cholesterol in the liver than dietary MUFAs.

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CHAPTER 3

CHRONIC INFLAMMATORY STRESS VIA LOW-DOSE LIPOPOLYSACCHARIDE INFUSION

Introduction

Previously, we evaluated the effects of modifying dietary PUFA, within physiologically relevant ranges, in an acute inflammation model by injection of sublethal dose LPS into mice. Yet, metabolic inflammation, common in those consuming modern, Western Diets, is thought to result from a low-grade inflammatory stress from LPS. In this chapter, the implementation of a low-grade, chronic stress was explored via methods to increase plasma LPS.

Interestingly, scientists have found that IAP, detoxifies luminal endotoxin including LPS, and prevents gut-derived systemic inflammation (1). Some studies have reported that the detoxification of LPS by IAP is also due to the dephosphorylation of the lipid A moiety (2, 3). In addition, IAP is induced by LPS and then detoxifies LPS to suppress excessive TLRs and TNF signaling (4). However, it has been reported that dietary L-Phe inhibits endogenous IAP activity which is associated with an increase in serum endotoxin levels by, presumably be inhibiting its dephosphorylation (3). There is some indication that gut levels of IAP may be induced by dietary-derived compounds. For example, a group reported indicated that Resolvin E1, a metabolite of EPA, induces IAP expression in intestinal epithelial cells (5). Thus, a higher level of n-3 PUFA may increase IAP expression which facilitates LPS detoxification.

The goal for this experiment was to evaluate methods to cause chronic inflammation in mice. This included testing whether L-Phe promotes inflammation, presumably via inhibiting IAP activity. In addition, a low-grade inflammatory stress was induced via osmotic mimipump installed in the mice and delivering LPS based on the method of Cani et al (6). Hereby, we have two hypotheses in this study. First hypothesis is stronger inflammatory responses would be observed in the mice installed with pump with LPS compared to pump-control mice (saline in the pump). The second hypothesis is that mice fed with L-Phe contained drinking water would show increased inflammatory responses compare to control mice. The primary endpoint for inflammatory responses is levels of cytokines. Other endpoints measurements included weight gain, glucose tolerance, inflammation related gene expression levels.

Materials and Methods

Animals and diets

Forty male weanling mice (C57BL/6) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and fed chow until all reached at least 20 g in weight. Mice were allocated into one of four groups (10 mice each group) with balanced initial weight across the groups. These were control, pump-control (mini osmotic pumps filled with saline), pump-LPS group (mini osmotic pumps filled with 200 μL 1.7 μg/μL LPS solution), and L-Phe group (10 mM L-Phe in drinking water) (1). Mice were fed the TWD for 6 weeks (starting three days before pump implantation). Mice were housed individually. Mice were weighed every four days and diet intake measured every other day. Body composition of mice not fit with pumps was measured every two weeks.

All mice were fed the TWD. The TWD contains 50.1% energy (kcal) from carbohydrate, 34.5% energy from fat and 15.4% energy from protein. The n-6 to n-3 PUFA ratio is roughly 10:1 which represent the Americans' average PUFA intake. The components of TWD are summarized in the Table 3.1.

TABLE 3.1 Components of TWD.

Components of TWD	g/kg
Casein	190.00
L-Csytine	2.85
Corn starch	230.00
Maltodextrin	70.00
Sucrose	261.32
Olive oil	28.00
Soybean oil	31.40
Corn oil	16.50
Lard	28.00
Beef tallow	24.80
Andydrous milkfat	36.30
Cholesterol	0.40
Cellulose	30.00
Mineral mix	35.00
Sodium Chloride	4.00
Vitamin mix	10.00
Choline bitartrate	1.40
TBHQ, antioxidant	0.03

Chronic inflammatory challenge, mice sacrifice, and sample collection

Mice in the pump control and pump LPS groups were implanted subcutaneously with an osmotic mini-pump (Alzet Model 2006; Alza, Palo Alto, CA). The pumps were filled with either NaCl (0.9%) or LPS solution to infuse 300 $\mu g \bullet kg^{-1} \bullet day^{-1}$ for 6 weeks (200 μL of 1.7 μg/μL LPS was loaded in the pump reservoir) in the mice. Before the

surgery, all the surgical equipment and the working area were sanitized by Nolvasan solution. Mice were anaesthetized by ketamine (supplied by Dr. Olsen in LARC). A section of skin approximately 1 cm away from the tail was sanitized with betadine and a small cut was made to open the skin with scissors. The minipump was inserted under the skin, and a stapler was used to close the incision. Next, the area was cleaned again with betadine. The mice were left on a heating blanket to maintain body temperature. Once mice were able to move by themselves, they were returned to their cages.

Oral glucose tolerance test (OGTT)

The OGTT was conducted one week before sacrifice. Six mice from each treatment group were randomly selected for OGTT. Mice were moved to clean cages with a paper towel on the bottom instead of corn cob bedding, but the water bottle was kept. The weight of each mouse (weight of minipump were included) was taken and used to calculate the dose of glucose (2 g/kg body weight) needed. The mice were fasted for six hours prior to the test.

Eight minutes before a mouse was tested, anesthetic cream was applied to the tip of each mouse tail and allowed to take effect. Next, a mouse was placed on the restraining device. The tail was cleaned with an ethanol soaked wipe. Using razor blade, a cut was made at 0.5 mm from the end of the tail. Two microliters of blood was collected from the tail with a pipette. After blood collection, the bleeding was stopped by pressure applied with a clean tissue. Three glucose measurements were made with a glucose meter and results at each time point was averaged from the three measurements. Once fasting data had been collected, the mice were gavaged with the appropriate dose of

glucose, based on body weight. After fifteen minutes, blood glucose was measured again as described previously. Overall, glucose measurements were made at 15, 30, 60, 90, and 120 minutes.

After 6 weeks on the treatment, mice were sacrificed and tissues collected as described in the Chapter 2.

Plasma cytokine analysis

The following cytokines, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-17, MCP-1, IFN-γ, TNF-α, MIP-1α, GM-CSF, and RANTES were measured in plasma by using Q-PlexTM array (multiplex ELISA arrays) kit (Quansys Biosciences, Logan, UT) according to manufacturer's directions. The images were captured by Quansys Q-viewer^{TM} Imager.

Calibrator (standards) was dissolved into 500 µL sample diluents and then a series of dilutions were made to prepare an eight-point standard curve with seven different concentrations and one blank in a low-binding 96-well plate. Thirty microliters of pre-thawed plasma sample were mixed with 30 µL sample diluents in the same plate. Next, 50 µL of standards and sample mixtures were transferred to the Q-Plex array 96-well plate. Sealed Q-Plex plate was placed on shaker for 1 hour at 500 rpm at room temperature. After incubation, plate was washed with 200 µL of 1X working wash buffer three times. Then, 50 µL detection mix was added into wells and the sealed plate was placed back to shaker for another hour. The plate was washed three times again and then 50 µL Streptavidin-HRP 1X was added into each well. After 30 minutes incubation, plate was washed six times. Fifty microliters of pre-mixed substrate A and substrate B was

added into each well right after washing. Finally, Q-viewer imager was applied to capture the image of the plate to calculate cytokine concentration.

Gene expression analysis

In this study, the genes we measured in the mice liver include 18s rRNA, Acaca, Acox1, Ccl2, Ccl3, Cpt-2, CRP, Dgat-2, Elovl-5, Fasn, Hmgb-1, Ikb-α, IL-1α, IL-1β, IL-6, ME-1, NF-κB-1, NF-κbib, Ppar-α, Ppar-β, Scd-1, Srebp-1, Tnf-α. The procedures of measurements and data analysis were same as described in the Chapter 2.

Statistical analysis

Two hypotheses we have were analyzed by two samples t-test which was performed with SPSS software version 19 (IBM Corporation, Armonk, New York, USA). Data are reported as mean ± standard deviation.

Results

Food intake, body weight, and body fat composition

The food intake data is shown in Figure 3.1. There is no difference of food intake between mice in the pump control group and pump LPS group. On the other hand, there is also no difference between mice in the control group and L-Phe group. For the weight gain, mice in the pump-LPS group gained significantly more weight than mice in the pump control group (Fig. 3.2). The body fat percentage and fat gain between control and L-Phe groups were not different (Fig. 3.3). Since it was not possible to assess body composition on mice in the pump groups, adipose tissues were collected and weighed after dissection. The weights of adipose tissue were not significantly different between pump control and pump LPS group (Fig. 3.4).

Oral glucose tolerance test

There were no differences in blood glucose level between pump control group and pump LPS group at any time point. In addition, there were no differences in overall glucose level (AUC) between these two groups. When we compared control group and L-Phe group, there was also no difference for each time point and AUC (Table 3.2). After normalizing the glucose level based on baseline (glucose level at 0 minute), there were still no difference in AUC, but pump control group had significantly higher blood glucose level at 120 minutes than pump LPS group (Table 3.3).

Plasma cytokine analysis

Cytokines analyzed in this experiment were IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-17, MCP-1, IFN-γ, TNF-α, MIP-1α, GM-CSF, RANTES. Most of the cytokines measured were under the detection limit. Only IL-1β, IL-3, IL-6, and RANTES were consistently detected but there was no significant difference between the pump control and pump LPS groups for these four cytokines. Furthermore, there was also no difference between control and L-Phe groups (Table 3.4).

Tissue gene expression

Acaca, Acox1, Ccl2, Ccl3, Cpt-2, CRP, Dgat2, Elovl5, Fasn, Hmgb1, IL-1α, IL-1β, IL-6, ME1, NF-κB1, Nf-κbib, Ppar-α, Ppar-d, Scd-1, Srebp-1, Tnf-α genes were measured in this study. 18s rRNA was used to normalizing the genes across the samples. Normalized genes in the control group were considered as control to calculate the fold changes of each gene compared to other treatment groups. Fold changes are shown in the Table 3.5. The results indicate that there were no significant differences in all

inflammation related genes between pump control group and pump LPS group. Moreover, there were no difference in genes between control group and L-Phe group.

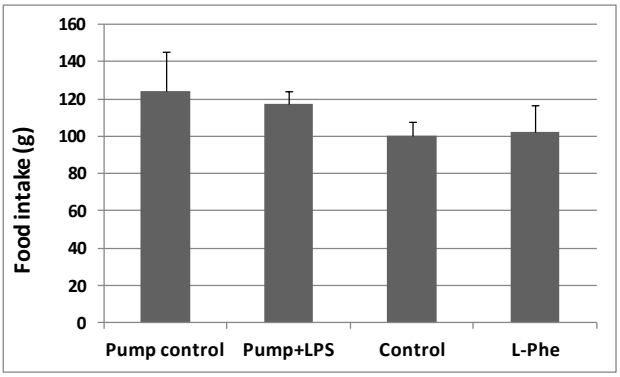

FIGURE 3.1 Total food intake of mice across different treatment groups. The data represents mean ± SD. Means with different superscripts are significantly different ($p<0.05$).

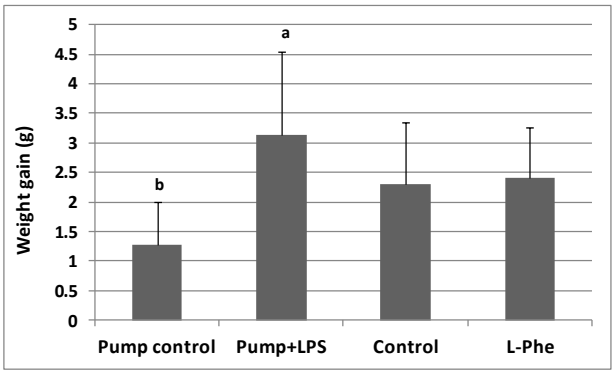

FIGURE 3.2 Mice weight gain across the different treatment groups. The data represents mean ± SD. Means with different superscripts are significantly different ($p<0.05$).

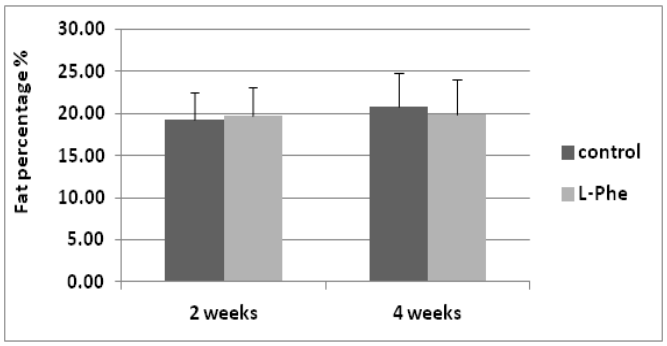

FIGURE 3.3 Mice body fat percentage between control group and L-Phe group. The data represents mean ± SD.

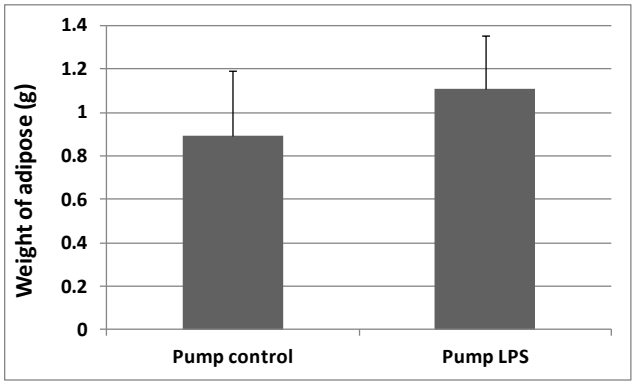

FIGURE 3.4 Weight of adipose tissues of mice in the pump control and pump LPS groups. The data represents mean ± SD.

TABLE 3.2 Blood glucose levels at each time point and overall blood glucose level in OGTT across different dietary PUFA groups.

Time (min)	Pump control	Pump LPS	Control	L-Phe
0	124.5±33.2	137.7±16.5	134.5±15.7	120.7±33.4
15	235.3±55.0	280.6±54.0	247.5±39.7	240.8±31.6
30	243.4±62.7	247.5±38.2	244.8±39.8	251.4±48.5
60	201.9±27.5	213.9±28.0	185.6±40.5	220.4±84.8
90	177.8±31.0	145.8±22.0	152.1±29.6	167.4±33.6
120	160.4±47.1	125.1±16.3	146.1±45.3	170.5±49.0
AUC	23737.3±3815.5	23479.2±2425.1	22544.2±3182.9	24355.0±5298.9

The data represents mean ± SD.

TABLE 3.3 Normalized blood glucose levels at each time point and overall blood glucose level in OGTT across different dietary PUFA groups.

Time (min)	Pump control	Pump LPS	Control	L-Phe
15	110.8±48.0	142.9±59.6	113.0±39.9	120.1±31.7
30	118.9±62.3	109.8±50.6	110.3±51.7	130.7±42.7
60	77.4±47.9	76.2±36.7	51.1±48.1	99.7±85.0
90	53.3±39.4	8.1±32.2	17.6±36.3	46.7±25.4
120	35.9±44.0[a]	-12.6±18.3[b]	11.6±51.1	49.8±43.4
AUC	8797.1±4389.3	6952.6±3728.6	6487.6±4145.5	9882.6±4812.0

The total glucose level in 120 minutes was normalized by glucose level at 0 minute. The data represents mean ± SD. Means with different superscripts are significantly different ($p<0.05$).

TABLE 3.4 Plasma cytokine concentration across four dietary groups.

Cytokine concentration (pg/mL)	Pump control	Pump LPS	Control	L-Phe
IL-1β	56.40±15.88	77.68±21.36	61.21±17.63	46.67±18.18
IL-3	11.80±1.29	14.62±5.78	12.28±1.76	13.17±2.01
IL-6	23.29±5.68	34.01±13.57	29.99±16.40	20.60±4.43
RANTES	16.86±6.50	23.62±11.18	23.20±12.97	30.03±20.36

The data represents mean ± SD.

TABLE 3.5 Mice liver gene expression.

Genes (log2 fold change)	Pump control	Pump LPS	Control	L-Phe
		NF-κB pathway		
Ccl_2	2.60±2.01	2.41±1.99	1.29±0.88	2.40±2.08
Ccl_3	2.83±1.74	4.14±4.64	1.31±0.89	3.36±3.38
CRP	2.25±1.37	1.71±1.33	1.34±0.97	1.51±2.01
IL-1α	2.13±1.40	1.78±1.53	1.35±1.03	1.50±1.51
IL-1β	2.75±2.20	3.77±4.04	1.29±0.90	0.99±0.79
IL-6	1.63±1.10	1.02±0.92	1.57±1.26	0.66±0.54
Nfκb1	2.45±1.36	2.07±1.59	1.27±0.80	2.08±2.36
Nfκbib	2.32±1.21	2.15±1.68	1.22±0.75	1.72±1.87
Tnf-α	2.43±1.69	3.54±4.22	1.30±0.88	2.44±2.13
		Lipogenesis		
Acaca	2.21±0.94	2.22±2.35	1.41±1.07	2.62±3.63
Dgat2	1.93±0.84	1.54±1.02	1.26±0.78	1.97±2.69
Elovl5	1.92±1.29	2.00±1.41	1.30±0.87	1.58±1.36
Fasn	1.50±0.58	2.88±4.46	1.73±1.96	2.67±2.67
Me1	1.15±0.42	1.40±1.41	1.43±1.12	1.89±2.42
Scd-1	2.97±1.98	2.98±2.31	1.35±0.95	2.06±2.11
Srebp-1	1.74±0.90	1.62±0.99	1.27±0.82	1.98±2.20
		PPAR-α pathway		
Acox1	1.93±1.13	1.70±1.27	1.28±0.81	1.69±2.05
Cpt-2	1.67±0.88	1.42±1.03	1.29±0.89	1.86±2.53
Hmgb1	2.23±1.29	1.77±1.37	1.32±0.94	1.85±2.45
Ppar-α	1.85±1.06	1.41±0.95	1.25±0.75	1.95±2.72
Ppar-d	2.53±1.45	1.91±1.34	1.25±0.74	2.33±2.92

The data represents mean ± SD. Data with different superscripts are significantly different ($p<0.05$). NS means not significantly different.

Discussion

The focus of this experiment was to evaluate a chronic inflammatory stress model and to determine if L-Phe had an effect on inflammation. In this study, there were two control groups. One group did not receive a minipump and was fed the same diet as other treatment. The other control group was pump-control group with saline in the pump which evaluated the possible effect from minipump installed in the mice body. The TWD was used in this experiment as it was used in the first experiment and third studies. Thus, using TWD as a standard diet here will give us more accurate and reasonable evaluation on later experiment which is studying the effect of typical American diets with different n-3 and n-6 PUFA levels and ratios on inflammatory responses.

Our first hypothesis in this study is slowly released LPS via minipump would increase the inflammatory responses in the mice.

The diet intake and weight gain data indicate that mice in the pump LPS group had more weight gain than pump control group when their food intakes are roughly equal which is expected in our hypothesis. However, the adipose tissue weights collected from dissection were not significantly different. In a similar study done by Cani, mice implanted with minipump filled with saline or LPS were studied. The mice in the pump LPS group had higher weight gain compared with pump control group. Their result is consistent with what we observed. In Cani's study, slowly released LPS from osmotic minipump was designed to mimic the metabolic endotoxemia obtained from high fat diet feeding (72% fat). They suggested that bacterially related factor like LPS is responsible for high-fat diet induced obesity via binding to CD14 (6).

In OGTT, since the blood glucose levels at 0 minute (baseline) were different across the dietary groups, normalized blood glucose levels should be used to calculate AUC. The results indicate that mice in the pump control group had higher blood glucose concentration at 120 minutes, but not the other time points and AUC. The results show that slowly released LPS did not lead to insulin resistance and glucose intolerance. The result is also comparable to Cani's study (6). Scientists reported that TLR4 was involved into insulin resistance caused by LPS (7). To continuously study the mechanism, understanding the regulation of TLR4 could be important.

Plasma cytokine level is primary endpoint to measure the inflammatory responses. In this experiment, there were only four cytokines present at high enough levels to be reliably detected. Compared with the cytokine levels in the acute inflammation study, overall low concentration of cytokines could imply weak inflammatory responses in the mice. The possible reason for the weak inflammation could be slowly released LPS would only cause weak inflammation. Another possible reason for low cytokine levels is not enough LPS from minipump releases to tissue and blood. In Cani's study, they indwelled an intrafemoral catheter into the femoral vein in the mice which may improve infiltration of LPS into blood (6, 8).

In terms of hepatic gene expression, although mice in the pump LPS group have relatively higher inflammation related gene expression levels, there is no difference between pump control and pump LPS groups which suggests that over expressions of these genes are caused by minipump implantation instead of slowly released LPS.

Our second hypothesis is that supplement of L-Phe in the drinking water could enhance inflammatory responses. Although previous studies have suggested that L-Phe may deactivate IAP which would inhibits absorption of endotoxin (1, 3), our results showed that there were no significant difference between control group and L-Phe group on weight gain and dietary intake, blood glucose concentration, cytokine levels, and inflammation related gene expression levels. It is possible that the diets were not sufficiently high in fat to promote endotoxin absorption, or perhaps there is another explanation. Further study on *in vivo* IAP activity and IAP coded gene expression could be helpful to investigate the mechanism of inhibition from L-Phe on IAP. Nevertheless, inclusion of L-Phe in the drinking water does not appear to promote metabolic dysregulation.

In summary, the chronic inflammation model we tested could only cause modest inflammatory responses *in vivo*. More weight gain in pump-LPS group suggests possible chronic inflammation via CD14-dependent mechanism. Overall, this model could be a useful model for inducing chronic inflammation for further studying the effects of our PUFA diets on chronic inflammatory responses. For the other goal we want to test in this experiment, L-Phe could not cause inflammation in mice through inhibition of IAP. To induce chronic inflammation by L-Phe, a longer period of feeding or higher concentration of L-Phe supplements in the drinking water may be necessary. Measurements of IAP activity and expression levels of IAP related genes would help us understand more mechanism of IAP inhibition from L-Phe.

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CHAPTER 4

EFFECT OF MODIFYING DIETARY N-6 AND N-3 PUFA ON METABOLISM AND INFLAMMATION IN CHRONIC INFLAMMATION MOUSE MODEL

Introduction

In Chapter 2, the rationale was given for using TWD modified PUFA diets in our experiments to study the effect of dietary PUFA on inflammation. As the high fat diet fed to mice in a previous study in the Ward Lab did not induce chronic inflammation, an alternate model was tested. Although the results indicated that implantation of an osmotic mini-pump with LPS induced a weak inflammatory response in the mice (increased weight gain), yet, by that time this study had already been conducted. Thus, this chapter describes a study to evaluate the role of the n-6 to n-3 ratio and total PUFA content on the inflammatory response in mice subjected to a chronic stress. The same TWD-based n-6 and n-3 diets were used as in Chapter 2.

Low-grade, chronic LPS administration may not lead to an increase in systemic cytokine levels due to endotoxin tolerance which is a defense mechanism of the immune system (1). Fischer applied osmotic minipumps in the mice for chronic LPS infusion and their results indicated that there was no weight gain (2) or fat mass changes (3). However, chronic exposure to LPS caused hyperglycemia and insulin resistance (3). Effects of low-grade LPS on inflammation related signaling pathways were reported. Ma et al. found that low dose LPS infusion in the rat brain activated transcription factors such as NF-κB and cyclic AMP responses element which are regulated by mitogen-activated protein

kinases (MAPKs) and IκB-α (4). Trifilieff et al found that PPAR-α showed anti-inflammatory activity by interfering Th2 pathways in chronic inflammation induced by LPS in the mice lung (5). It has also been shown that chronic inflammation induced by a low-grade dose of LPS results in similar metabolic effects of a high fat diet, which promotes weight gain by increasing the concentration of phosphorylated NF-κB and IKK (3). Based on results from these studies, LPS infusion enhances NF-κB and lipogenesis pathways and suppressed the PPAR-α pathway. Thus, we hypothesized, high dietary n-6 PUFA will promote an inflammatory response and will be accompanied with increased NF-κB pathway activation and lipogenesis. Conversely, high dietary n-3 PUFA will suppress the NF-κB pathway and lipogenesis while increasing PPAR-α pathway.

The goal of this experiment was to evaluate the effect of n-6 to n-3 PUFA ratio and their concentration on chronic inflammation. The diets used in this study were the same TWD-based PUFA diets used in Chapter 2. The NF-κB pathway, PPARs pathway, and lipogenesis are known to be affected by the inflammatory stress of low-grade LPS infusion, and are hypothesized to be differentially affected as a function of dietary PUFA.

Materials and Methods

Animals and diets

Forty weanling male C57BL/6 mice (Jackson Labs, Bar Harbor, ME, USA) were randomly separated into four groups with balanced initial weight across the groups. Mice were housed individually and fed with the same HH, HL, LH, and LL PUFA diets for 6 weeks. Mice were weighed every four days and diet intake was measured every other day.

Chronic inflammatory challenge, mice sacrifice, and sample collection

Mice with body weight over 20 g were implanted subcutaneously with an osmotic mini-pump filled with LPS solution. The implantation procedures and pump parameters were the same as described in the Chapter 3, as was the sacrifice and tissue collection.

Oral glucose tolerance test

The OGTT was conducted one week before sacrifice. Six mice from each treatment group were randomly selected for OGTT. The procedures of OGTT were described in the Chapter 3.

Plasma and adipose cytokines analysis

The concentration of cytokines, which includes IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-17, MCP-1, IFN-γ, TNF-α, MIP-1α, GM-CSF, RANTES, in the plasma were measured by using Q-PlexTM array as described in the Chapter 3.

Fatty acid profile

The fatty acid profile of red blood cells and livers was determined using procedures described in Chapter 2.

Fatty acids oxygenated compounds analysis

Fatty acids oxygenated compounds in the mice liver were analyzed by LC-MS/MS. Extraction method and analytical procedures are described in the Chapter 2.

Gene expression analysis

Genes measured in liver and adipose tissue include 18s rRNA, Acaca, Acox1, Ccl2, Ccl3, Cpt-2, CRP, Dgat-2, Elovl-5, Fasn, Hmgb-1, Ikb-α, IL-1α, IL-1β, IL-6, ME-

1, NF-κB-1, NF-κbib, Ppar-α, Ppar-β, Scd-1, Srebp-1, and Tnf-α. The procedures are described in the Chapter 2.

Statistical analysis

A 2x2 factorial design was applied to evaluate the main effects of n-6 PUFAs and n-3 PUFAs with the interaction effect based on n-6 to n-3 PUFA ratio on inflammatory responses. Shapiro-Wilk test was used to test the normality of data and a log transformation was applied if the original data was not normal distributed. A FDR was used and controlled at 0.05, which reduces the risk of false positives, by adjusting p-values of analysis due to multiple comparisons. Analyses were performed with SPSS software version 19 (IBM Corporation, Armonk, New York, USA) and SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Data was reported as mean ± standard deviation.

Results

Food intake, body weight and feed efficiency

In this experiment, there was significantly higher food intake in mice fed the low n-6 PUFA diets (LH and LL) (Fig. 4.1). There was no difference across the dietary groups for weight gain (Fig. 4.2). It was not possible to assess body composition by MRI due to the minipumps. Feed efficiency was calculated as the ratio of weight gain to food intake (Fig. 4.3). This parameter allows for the normalization of growth to food intake. According to the data, there was an n-6 PUFA diets effect for higher feed efficiency.

Oral glucose tolerance test

For the OGTT, blood glucose levels are shown as a function of time and treatments in Table 1. At baseline, mice fed with high n-6 PUFA diets had significantly

lower blood glucose than those fed with low n-6 PUFA diets. However, at 60 minutes, mice in the high n-6 group had higher blood glucose. Integrating all the data to produce an area under the curve (AUC), mice in the HH group had a tendency to have a higher overall blood glucose than the mice fed with LH diet (α=0.091), according to a post-hoc analysis. There was an interaction effect at both 30 and 60 minutes for mice fed the high n-6 diets, in that the levels were higher in the HH group, compared to the HL group at both time points.

As noted from data, mice fed the low n-6 diets had a significantly higher baseline glucose level. To assess how glucose was metabolized and account for the high baseline level, a second analysis was preformed on the data after normalization to baseline values. Table 4.2 showed blood glucose levels at each time point and overall blood glucose level. As we can see from the data, from 30 minute to 120 minute, mice fed with high n-6 PUFA diets had significantly higher blood glucose levels than those fed with low n-6 PUFA diets. Furthermore, mice in the high n-6 PUFA groups also showed higher overall blood glucose level compared with the other groups.

Fatty acid profile of red blood cells

The percentage of red blood cell PUFAs are listed in the Table 4.3. The three dominant PUFAs were LA, AA, and DHA. According to the statistical analysis, after adjustment for multiple comparisons, there were several differences. For example, there was more LA and eicosadienoic acid (C20:2 n6) in the mice fed with high n-6 PUFA diets. Surprisingly, there was more DGLA (C20:3 n6) in the mice with low n-6 PUFA diets, which we also found in Chapter 2. There were no differences in the AA level

between groups. On the other hand, there was more ALA and EPA in the mice fed with high n-3 PUFA diets. Higher levels of DPA (C22:5 n3) and DHA have been shown in the LH group. N-3 index in the mice red blood cells indicated that n-3 long-chain PUFA levels were affected by both PUFA content (high n-3 PUFA level) and ratio (lower n-6 to n-3 PUFA ratio).

Plasma cytokine analysis

The following cytokines were analyzed in plasma; IL-1α, IL-1β, IL-3, IL-5, IL-6, IL-10, IL-12, IL-17, IFN-γ, MCP-1, MIP-1α, GM-GSF, KC, TNF-α, and RANTES. However, most of the analytes were below the detection limit, and only two cytokines (MCP-1 and RANTES) at a high enough concentration to be quantified. A log transformation was applied to the data as the cytokine levels were not normally distributed. The statistical analysis indicated that there was no significant difference (Table 4.4).

Hepatic phospholipids profile

The lipid composition of the liver tissue was measured to investigate the effects of the PUFA intakes, and to determine if there is a relationship with gene expression and oxylipin production. The major focus was on PL profile (Table 4.5), as these molecules provide the substrates for oxylipin synthesis and possibly gene expression.

In the PL PUFA profile of the liver, there were higher levels of LA and eicosadienoic acid (C20:2 n6) in mice fed with high n-6 PUFA diets while a higher DGLA level was observed in the mice with low n-6 PUFA diets. On the other hand, there were lower levels of AA in the liver of mice with high n-3 PUFA diets. Moreover, there

was higher ALA and DPA in the mice with high n-3 PUFA diets. In addition, higher EPA and DHA levels in the liver were observed in the dietary high n-3 PUFA and low n-6 PUFA groups. EPA level was also affected by n-6 to n-3 PUFA ratio which higher ratio leads to lower EPA level in the PL. The levels of long chain n-6 and n-3 PUFAs which affected by dietary PUFAs could consequently affect the eicosanoids concentration in the liver.

Analysis of oxygenated compounds of fatty acids

Data for the hepatic oxylipins is shown in Table 4.6. The concentration was normalized by external standard. Although there were some differences in the hepatic long chain PUFA precursors, there were no differences in oxylipins according to the 2-way ANOVA, after correction for multiple comparisons.

Tissue gene expression

Genes normalized to 18s rRNA in the LH group were set to control and log2 fold changes of each gene in the other dietary groups were compared. The results of hepatic gene expression are shown in the Table 4.7. From Table 4.7, although there are some differences in gene expression from 2-way ANOVA, there was no difference shown after correction for multiple comparisons based on different inflammation related pathways.

Hepatic cholesterol esters

Although not a primary endpoint in the study, the contents of five hepatic lipid classes in the liver were measured, and the data is shown in the Table 4.8. There is a clearly lower CE level in the liver in mice fed the high n-6 PUFA diets. In fact, mice fed the low n-6 diets had approximately 40% more hepatic CE than mice fed the high n-6

diets. This result is consistent with the results from Chapter 2 where the diets had the same effect on the CE fraction in the liver.

To better understand this effect, the fatty acid profile of CE was determined and it is shown in the Table 4.9. Similar to the results in Chapter 2, there are several fatty acids that appear to be affected by the n-6 content of the diet. However, it appears that oleic acid (18:1n9) is the principal fatty acid that is significantly higher in the low n-6 diets that can explain up to 13% of the difference.

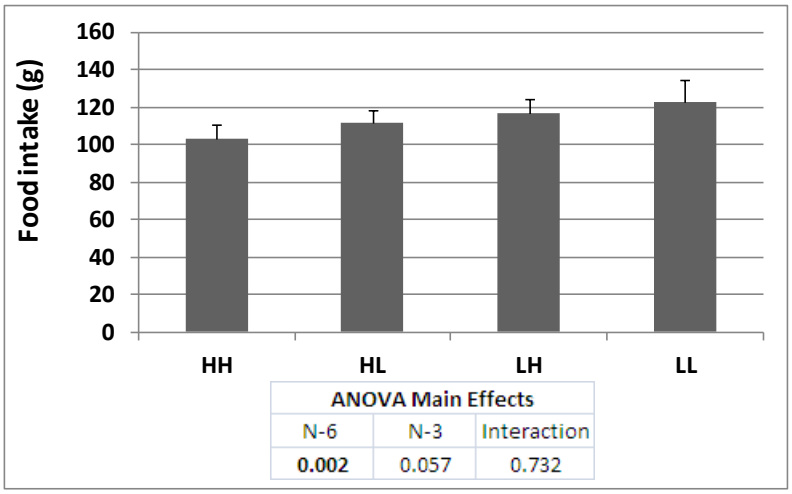

FIGURE 4.1 Food intake across the different dietary groups. The data represents mean ± SD.

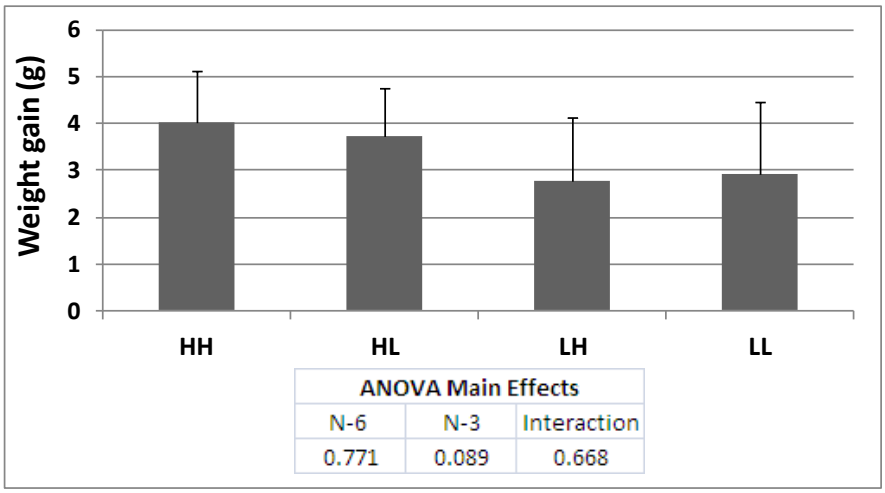

FIGURE 4.2 Mice weight gain across the different dietary groups. The data represents mean ± SD.

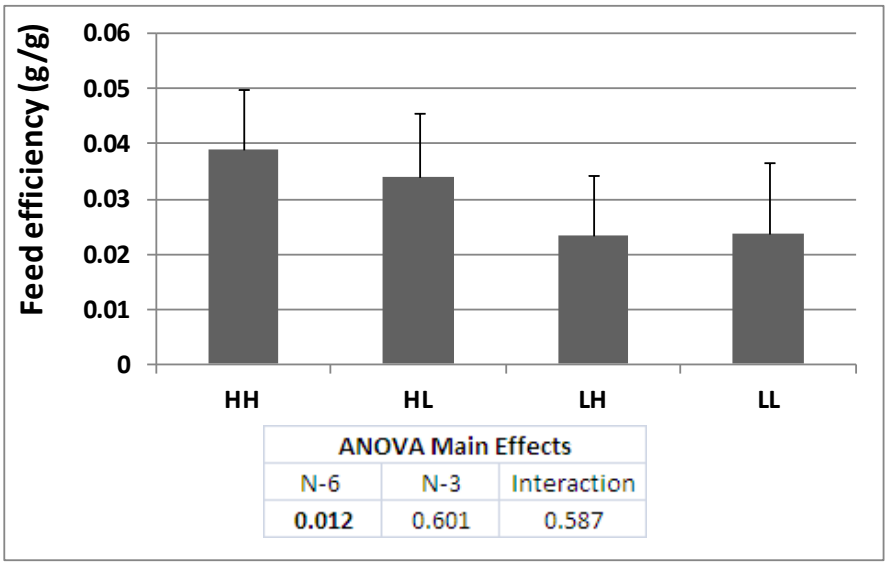

FIGURE 4.3 Feed efficiency across the different dietary groups. The data represents mean ± SD.

TABLE 4.1 Blood glucose levels at each time point and overall blood glucose level in OGTT across different dietary PUFA groups.

Time (min)	HH	HL	LH	LL	*p*-value for 2*2 ANOVA		
					n-6	n-3	Interaction
0	96.3±11.5	106.3±18.2	137.9±14.7	152.8±23.3	<0.01	NS	NS
15	245.1±44.1	250.3±35.1	254.0±33.1	276.8±42.9	NS	NS	NS
30	262.9±39.0[a]	228.0±38.2	200.9±39.1[b]	234.6±27.4	NS	NS	0.03
60	213.3±52.5[a]	175.9±32.0	147.4±24.8[b]	175.7±29.1	0.04	NS	0.05
90	152.7±39.6	144.7±25.1	135.2±10.7	140.9±25.2	NS	NS	NS
120	141.1±24.3	126.6±14.9	112.1±14.2	126.0±16.3	NS	NS	NS
AUC	23411.5 ±3111.9	21199.0 ±2195.9	19523.5 ±2225.1	21961.8 ±2549.9	NS	NS	0.04

The data represents mean ± SD. Means with different superscripts are significantly different ($p<0.05$). NS means not significantly different.

TABLE 4.2 Normalized blood glucose levels at each time point and overall blood glucose level in OGTT across different dietary PUFA groups.

Time (min)	HH	HL	LH	LL	*p*-value for 2*2 ANOVA		
					n-6	n-3	Interaction
15	148.9±39.5	144.0±27.8	116.1±32.2	124.0±25.8	NS	NS	NS
30	166.6±31.8	121.6±48.3	62.9±38.7	81.8±30.8	<0.01	NS	NS
60	117.1±52.0	69.6±37.9	9.5±17.3	22.9±15.2	<0.01	NS	NS
90	56.4±43.0	38.3±35.4	-2.7±7.6	-11.9±30.1	<0.01	NS	NS
120	44.9±23.5	20.3±25.4	-25.9±7.4	-26.8±19.5	<0.01	NS	NS
AUC	11858.3±2868.3	8439.0±3250.0	2971.4±1720.5	3628.4±1840.6	<0.01	NS	NS

The total glucose level in 120 minutes was normalized by glucose level at 0 minute. NS means not significantly different.

TABLE 4.3 Fatty acid composition of red blood cells from four PUFA diets.

	Diets				Adjusted *p* value (FDR)		
PUFA	**HH**	**HL**	**LH**	**LL**	**n-6**	**n-3**	**Interaction**
			Omega-6				
C18:2 n6 (LA)	13.67±0.74	11.67±0.72	9.89±1.49	9.58±1.25	0.01	NS	NS
C20:2 n6 (Eicosadienoic acid)	0.25±0.01	0.28±0.03	0.22±0.03	0.20±0.05	0.05	NS	NS
C20:3 n6 (DGLA)	1.21±0.09	1.25±0.17	1.56±0.18	1.45±0.20	0.02	NS	NS
C20:4 n6 (AA)	15.47±1.39	17.69±2.16	15.90±0.58	18.16±2.17	NS	NS	NS
			Omega-3				
C18:3 n3 (ALA)	0.34±0.08	0.11±0.04	0.22±0.01	0.10±0.03	NS	0.03	NS
C20:5 n3 (EPA)	0.24±0.02	0.10±0.02	0.69±0.08	0.18±0.04	NS	<0.01	NS
C22:5 n3 (DPA)	0.63±0.05	0.36±0.06	0.96±0.13	0.43±0.08	0.02	<0.01	NS
C22:6 n3 (DHA)	5.49±0.51	4.35±0.53	7.78±0.17	5.24±0.67	0.05	0.03	NS
n-3 index	5.73 ± 0.51^{b}	4.45 ± 0.55^{b}	8.48 ± 0.24^{a}	5.42 ± 0.71^{b}	<0.01	<0.01	<0.01

N-3 index is the sum of EPA and DHA. The data represents mean (percentage) ± SD. Means of n-3 index with different superscripts are significantly different ($p<0.05$). NS means not significantly different.

TABLE 4.4 Log(10) transformed plasma cytokine concentration across four dietary groups.

Log10 concentration (pg/mL)	**HH**	**HL**	**LH**	**LL**	Adjusted *p* value (FDR)		
					n-6	**n-3**	**Interaction**
MCP-1	3.32±3.08	2.79±2.44	3.56±2.44	2.72±1.80	NS	NS	NS
RANTES	10.48±7.04	9.50±7.74	11.43±6.69	10.03±6.38	NS	NS	NS

The data represents mean ± SD. NS means not significantly different.

TABLE 4.5 PL PUFA composition in liver.

	Diets				Adjusted *p* value (FDR)		
PUFA (mg/g)	**HH**	**HL**	**LH**	**LL**	**n-6**	**n-3**	**Interaction**
C18:2 n6 (LA)	9.40±0.84	7.50±2.00	7.05±0.33	7.18±1.05	0.03	NS	NS
C18:3 n6 (GLA)	0.12±0.01	0.18±0.12	0.08±0.03	0.15±0.03	NS	NS	NS
C20:2 n6 (Eicosadienoic acid)	0.20±0.03	0.19±0.03	0.15±0.04	0.14±0.03	<0.01	NS	NS
C20:3 n6 (DGLA)	1.00±0.05	0.83±0.26	1.21±0.15	1.15±0.22	<0.01	NS	NS
C20:4 n6 (AA)	11.49±1.46	12.09±2.66	9.11±1.02	13.52±1.01	NS	<0.01	NS
C18:3 n3 (ALA)	0.10±0.03	0.03±0.01	0.08±0.03	0.03±0.01	NS	<0.01	NS
C20:5 n3 (EPA)	0.18 ± 0.02^{b}	0.04 ± 0.02^{b}	0.44 ± 0.06^{a}	0.13 ± 0.04^{b}	<0.01	<0.01	<0.01
C22:5 n3 (DPA)	0.25±0.02	0.14±0.03	0.24±0.03	0.17±0.03	NS	<0.01	NS
C22:6 n3 (DHA)	5.77±0.96	3.93±0.56	5.97±0.92	5.81±0.53	0.01	0.01	NS

The data represents mean ± SD. Data with different superscripts are significantly different ($p<0.05$). NS means not significantly different.

TABLE 4.6 Oxylipins in the liver.

	Diets				Adjusted *p* value (FDR)		
Oxylipin (ρmol/g)	**HH**	**HL**	**LH**	**LL**	**n-6**	**n-3**	**Interaction**
15-HETE	0.22±0.28	0.21±0.14	0.13±0.09	0.20±0.13	NS	NS	NS
12-HETE	0.94±0.74	1.77±1.58	3.91±4.24	0.67±0.34	NS	NS	NS
5-HETE	0.07±0.08	0.09±0.04	0.06±0.08	0.11±0.06	NS	NS	NS
4-OH DHA	0.06±0.05	0.05±0.04	0.05±0.08	0.04±0.03	NS	NS	NS
14-OH DHA	0.16±0.15	0.19±0.17	0.44±0.26	0.08±0.05	NS	NS	NS
17-OH DHA	0.06±0.05	0.06±0.03	0.04±0.02	0.05±0.03	NS	NS	NS
PGE_2	0.03±0.01	0.03±0.02	0.05±0.04	0.06±0.00	NS	NS	NS
$PGF_{2\alpha}$	0.09±0.06	0.09±0.06	0.14±0.17	0.05±0.03	NS	NS	NS
TXB_2	0.08±0.09	0.11±0.09	0.29±0.43	0.04±0.03	NS	NS	NS

Abbreviation: 15-hydroxyeicosatetraenoic acid (15-HETE), 12-hydroxyeicosatetraenoic acid (12-HETE), 5-hydroxyeicosatetraenoic acid (5-HETE), 4-hydroxy-docosahexaenoic acid (4-OH DHA), 7-hydroxy-docosahexaenoic acid (7-OH DHA), 14-hydroxy-docosahexaenoic acid (14-OH DHA), 17-hydroxy-docosahexaenoic acid (17-OH DHA), 11-hydroxy-docosahexaenoic acid (11-OH DHA), prostaglandin E2 (PGE_2), prostaglandin F2α ($PGF_{2\alpha}$), thromboxane B2 (TXB_2). The data represents mean ± SD. Data with different superscripts are significantly different ($p<0.05$). NS means not significantly different.

TABLE 4.7 Hepatic gene expression.

	Diets				Adjusted *p* value (FDR)		
Genes (log2 fold change)	**HH**	**HL**	**LH**	**LL**	**n-6**	**n-3**	**Interaction**
NF-κB pathway							
Ccl2	2.02±1.44	1.71±1.23	1.91±2.64	0.56±0.71	NS	NS	NS
Ccl3	1.46±0.81	1.36±0.89	1.60±1.76	1.01±1.23	NS	NS	NS
CRP	2.24±1.24	1.84±1.46	1.24±1.03	1.66±0.68	NS	NS	NS
IL-1α	1.47±0.76	1.61±1.30	1.15±0.78	1.11±1.29	NS	NS	NS
IL-1β	2.31±1.25	2.06±1.52	1.22±0.88	1.42±1.79	NS	NS	NS
IL-6	1.96±2.12	2.58±2.09	1.43±1.41	1.22±0.98	NS	NS	NS
Nfκb1	1.94±0.80	1.98±1.27	1.26±1.07	1.34±1.08	NS	NS	NS
Nfκbib	2.07±0.95	1.97±1.31	1.30±1.19	2.19±1.14	NS	NS	NS
Tnf-α	1.77±1.15	1.68±1.46	1.51±1.56	1.56±2.35	NS	NS	NS
Lipogenesis							
Acaca	2.84±1.49	2.15±1.47	1.24±1.04	2.11±2.67	NS	NS	NS
Dgat2	2.46±0.96	2.53±1.40	1.17±0.80	2.37±1.67	NS	NS	NS
Elovl5	3.09±1.64	2.53±1.83	1.41±1.51	1.98±1.55	NS	NS	NS
Fasn	5.07±3.11	2.41±1.39	1.32±1.21	2.31±4.27	NS	NS	NS
Me1	4.65±2.26	2.57±1.78	1.13±0.66	1.97±2.02	NS	NS	NS
Scd-1	2.86±1.13	2.27±1.53	1.19±0.85	2.70±2.00	NS	NS	NS
Srebp-1	2.65±1.17	2.18±1.33	1.22±0.93	1.95±1.31	NS	NS	NS
PPAR-α pathway							
Acox1	3.23±1.28	2.03±1.48	1.11±0.58	3.19±1.74	NS	NS	NS
Cpt-2	2.47±1.17	2.30±1.62	1.16±0.78	2.11±1.06	NS	NS	NS
Hmgb1	1.06±0.46	2.17±1.49	1.33±1.26	1.03±0.87	NS	NS	NS
Ppar-α	2.58±1.10	2.14±1.79	1.10±0.51	2.61±1.83	NS	NS	NS
Ppar-d	2.20±1.22	2.41±1.13	1.23±0.99	1.92±1.07	NS	NS	NS

The data represents mean ± SD. NS means not significantly different.

TABLE 4.8 Total liver lipid compositions from all lipid classes.

	Diets				*p* values for 2*2 ANOVA		
Fatty acid (mg/g)	**HH**	**HL**	**LH**	**LL**	**n6**	**n3**	**Interaction**
PC	59.38±5.71	56.09±4.80	56.38±3.24	63.98±3.32	NS	NS	0.01
TAG	15.04±5.77	20.16±7.26	15.79±9.00	14.01±10.35	NS	NS	NS
DG	1.62±0.54	1.49±0.23	1.67±0.70	1.68±0.54	NS	NS	NS
FFA	1.04±0.44	0.76±0.21	1.04±0.62	1.26±0.51	NS	NS	NS
CE	18.86±7.34	19.52±1.35	26.87±8.29	28.22±6.44	<0.01	NS	NS
Total	95.94±9.54	98.02±8.21	101.75±17.06	109.14±10.06	NS	NS	NS

The data represents mean ± SD. NS means not significantly different.

TABLE 4.9 Fatty acid content of CE in the liver.

	Diets				*p* values for 2*2 ANOVA		
PUFA	**HH**	**HL**	**LH**	**LL**	**n-6**	**n-3**	**Interaction**
C14:0 (Myristic acid)	8.31±6.98	7.31±3.62	11.48±7.00	10.86±7.02	NS	NS	NS
C14:1 n5 (Myristoleic acid)	0.64±0.60	0.58±0.24	0.86±0.44	0.80±0.43	NS	NS	NS
C16:0 (Palmitic acid)	1.86±0.57	2.52±1.63	2.62±1.22	2.75±1.01	NS	NS	NS
C16:1 n7 (Palmitoleic acid)	0.34±0.07	0.46±0.14	0.64±0.39	0.71±0.51	NS	NS	NS
C18:0 (Stearic acid)	1.19±1.11	1.76±1.94	1.18±0.58	1.89±1.18	NS	NS	NS
C18:1 n9 (Oleic acid)	2.16±0.66	2.84±1.03	4.91±2.44	6.21±4.68	0.02	NS	NS
C18:2 n6 (LA)	0.70±0.18	0.73±0.21	0.59±0.18	0.64±0.41	NS	NS	NS
C18:3 n3 (ALA)	0.11±0.03	0.09±0.02	0.16±0.03	0.13±0.04	0.01	NS	NS
C20:3 n6 (DGLA)	0.12±0.07	0.13±0.07	0.19±0.08	0.25±0.08	<0.01	NS	NS
C20:4 n6 (AA)	0.19±0.07	0.27±0.07	0.15±0.04	0.27±0.12	NS	0.01	NS

The data represents mean ± SD. NS means not significantly different.

Discussion

In this experiment, our goal was to investigate if the TWD-based PUFA diets with different n-6 to n-3 PUFA ratios would have effects on chronic inflammation caused by low-grade LPS stress. From the previous study, we examined feasibility and mechanism of causing chronic inflammation through osmotic minipump release of LPS. The test study of the minipump delivery system indicated higher weight gain in the pump-LPS group mice than in the pump-control group, which shows the LPS effects on metabolism. Thus, the mini-pumps LPS delivery system was used in mice with different PUFA diets to investigate the effects on chronic inflammation.

Interestingly, mice fed with low n-6 PUFA diets consumed more food, yet there was no difference in weight gain. This was due to the fact that these mice had a lower feed efficiency. Previous work is in agreement, and has shown that n-6 PUFA enriched diet increase feed efficiency in rodents. However, they used diet with 46.99% LA and 0.95% ALA from fat (28.8% fat in the diet) which is not within the physiological range of human diets (6). Our result supported that n-6 PUFA enriched diet under physiological range of intake could still increase feed efficiency. Although there are some other studies showed that only dietary n-3 PUFA increased feed efficiency (7), our result suggested that higher n-6 PUFA level is the factor that increased feed efficiency. Interestingly, this finding is consistent with the results in the acute inflammation study (Chapter 2). Furthermore, minimum level of n-3 PUFAs in the diet may also be critical for animal growth. Scientists found that approximate 1% n-3 PUFA in the dry diet is necessary for animal growth (8, 9). On the other hand, we found the average daily food intake in the

chronic inflammation experiment (2.84 g per day) is significantly higher than the food intake in acute inflammation model (2.41 g per day) (p value is 0.05). The result suggests that the mice implanted with mini-pump may consume extra energy compared with healthy mice.

In the OGTT, since the baseline glucose levels at 0 minute (baseline) were different, a normalized value should be used to calculate the AUC. By normalizing baseline glucose, we could also see how effectively the mice can handle the incoming glucose load. According to the normalized data, mice fed high n-6 diets had higher glucose levels after 30 minute and a higher AUC than the mice fed low n-6 diets. Thus, at ranges of intake that are physiologically relevant to the US population, n-6 hinders glucose tolerance under chronic LPS stress. Many studies have shown that high dietary n-6 PUFA or a high n-6:n-3 ratio promotes insulin resistance and glucose intolerance while a high n-3 intake reduces these responses (10-15). Our results indicated that the ratio may not affect glucose tolerance since HH and LL diets have similar ratios. This result is also supported by some research articles which they found dietary PUFA ratio did not influence insulin sensitivity (16, 17).

The fatty acid composition in the red blood cells is a good indicator of dietary fatty acid intake (18). According to the RBC profile, LA and ALA are directly linked to the contents in the diets. Intermediates from PUFA elongation were also affected by PUFA contents in the diets, yet they are not thought to participate in cell signaling processes, like longer chain PUFA. EPA, one of the major precursors of n-3 PUFA derived eicosanoid, was positively associated to dietary n-3 PUFA level. Moreover, we

found LH group had highest EPA level which suggests possible competitive inhibition from LA would decrease EPA elongation. DHA, which is the end product of n-3 PUFA elongation, was higher in the LH vs. HH group, which suggests that there is competitive inhibition for its biosynthesis from ALA. Research has shown that LA inhibits the biosynthesis of n-3 long chain PUFAs via competing the rate-limiting enzymes of long chain PUFA synthesis such as Δ5 and Δ6 desaturase (19-22). DHA is the major driver of the n-3 index, and a value for mice in the LH diet (> 8% of RBC fatty acids) reached a level associated with cardio protection in humans (23).

As the primary endpoints for inflammatory responses, the concentration of cytokines in plasma samples appear to be low compared to the cytokine levels in the acute inflammation study and most were below the detection limit. From cytokine analysis, there is no difference of detected cytokines (MCP-1 and RANTES) across the dietary groups. Cytokines are important biomarkers of inflammatory responses. Low level of cytokine represents low inflammatory response. In this chronic inflammation model, we found much lower cytokine levels than our acute inflammation model which implies that slowly released LPS from mini-pumps did not cause strong inflammatory responses. The possible reason for weak inflammatory responses could be not enough amounts of LPS released by mini-pumps get into the mice circulation system to cause inflammatory responses. Also, this can be caused by endotoxin tolerance mechanism in the immune cells. This result is similar as we observed in the Chapter 3 which means the pro- or anti-inflammation effects from our dietary PUFAs may not be so effective to cause strong inflammatory responses in short feeding period.

Since we did not see differences from cytokines between the dietary groups, other measurements related to inflammation were investigated. The PUFA profile of PL indicated levels of LA and ALA were driven by the PUFA contents in the diet. Lower AA in the high n-3 PUFA groups shows competitive inhibition from ALA. Long-chain n-3 PUFAs (EPA and DHA) were directly related to dietary n-3 PUFA content and were competitively inhibited by dietary high n-6 PUFA.

Although we saw higher AA in hepatic PL in the low n-3 PUFA diet groups, there was no difference of AA derived eicosanoids. In addition, there was no difference in long chain n-3 derived oxylipins between groups even though EPA and DHA were significantly higher in the LH and HH groups. Furthermore, the concentrations of eicosanoids in the liver were very low compared with our acute inflammation study. As we all know, eicosanoids are important inflammation mediators (24). Low levels of eicosanoids suggest the inflammatory responses are weak in this chronic inflammation model. Result from oxylipin analysis is consistent with cytokine data.

In liver inflammatory gene expression data, after controlling for FDR, we found no difference for any genes related to the NF-κB, PPAR-α, or lipogenesis pathways between the groups. These results are consistent with the cytokine and oxylipin data and indicate the diets did not differentially affect the inflammatory response.

The only lipid class in the liver to be affected by the diets was CE, and this result is consistent with the acute inflammation study. CEs were lower in the mice fed with high n-6 PUFA diets. Previous studies in humans have consistently shown that higher overall dietary PUFA levels reduce plasma cholesterol (25). While this measure was not a

primary objective of this study, it does show that changing the fat composition of the typical US diet, within physiological ranges, does have an effect consistent with human diets.

In summary, changing the PUFA content of the typical Western Diet, within physiologic ranges actually consumed by Americans, did result in changes in tissue composition of substrates for inflammatory signaling (AA, EPA and DHA). Yet, the infused LPS only caused a modest inflammatory response in the mice as measured by cytokines and eicosanoids, and there were no significant differences between treatments. The reason could be (1) slowly released LPS from minipump may not fully get into mice circulation system; (2) PUFA contents and ratios in our diets were not extreme; (3) relatively short feeding period.

Despite the lack of an effect on inflammation, changing the PUFA content of the diet did have significant and interesting effects on other aspects of metabolism. For example, PUFA intakes affected feed efficiency. In addition, PUFA intakes affected glucose metabolism, as measured by fasting glucose and also reduced insulin sensitivity. Lastly, higher n-6 levels in the diet were associated with a significant decrease in hepatic cholesterol esters.

Literature Cited

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CHAPTER 5

CONCLUSION

In the last century, Americans' diets have changed drastically in terms of PUFA intake. Consumption of LA has increased from 2.79% of energy to 7.21% of energy while intake of ALA has increased from 0.39% to 0.72%. PUFA consumption has increased due to the use of vegetable oils in cooking and in processed foods. Changes of PUFA intake have been related to obesity and metabolic diseases which are associated with low-grade, chronic inflammation. The overall goal of this project was to study the effects of dietary PUFA intake on inflammation. To achieve this goal, TWD based PUFA diets were formulated to represent the 90^{th} and 10^{th} percentiles of intake for both n-6 and n-3 PUFAs from NHANES report which cover the majority of diets in the United States.

The first hypothesis we had for this project was high n-3 PUFA diet would reduce acute inflammation caused by LPS, whereas high dietary n-6 PUFA would promote this process. The results from the first experiment indicate that our PUFA diets had modest effects on the response to LPS due to the fact that PUFA diets did not have extreme PUFA contents and ratios. However, some responses under acute inflammation were observed. HH diet had increased weight gain because of increased fat and lean while the mice fed with HL diet only had increased fat. That means high n-6 PUFA with low n-3 PUFA in the diet may cause fat accumulation through enhanced lipogenesis. On the other hand, high dietary n-6 PUFA could promote expression level of lipogenesis and NF-κB related genes which are positively related to inflammation. Higher overall PUFA content

could stimulate PPAR-α pathway which is associated to fat oxidation. Except for the effect on inflammation, dietary PUFA showed a better ability to reduce LDL cholesterol in the liver than dietary MUFAs.

For the second hypothesis for the thesis, we assumed a chronic inflammation would be induced by slowly release of LPS in the mice through osmotic mini-pump. Also, L-Phe supplement in the drinking water would lead to inhibition of IAP activity which could cause chronic inflammation. The results showed only modest inflammation was caused by slowly release of LPS. On the other hand, L-Phe supplement did not induce chronic inflammation.

The third hypothesis we had was long term, low concentration of LPS would cause chronic inflammation, and a high n-6 PUFA intake would promote the inflammatory process while high dietary n-3 PUFA would relief the inflammation. From the results in the experiment, our chronic inflammation model only caused modest inflammatory responses. However, some differences were observed. For example, high dietary n-6 level promoted glucose intolerance and reduced insulin sensitivity. Furthermore, higher n-6 levels in the diet were associated with a significant decrease in hepatic cholesterol esters.

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Education Background

Utah State University, Logan, Utah

Ph.D. in Nutrition and Food Sciences, Aug. 2012-Dec. 2016

Advisor: Robert E. Ward

Thesis: "Effect of n-3 and n-6 polyunsaturated fatty acids on inflammation"

University of Louisville, Louisville, Kentucky

M.S. in Chemistry, Aug. 2009-Aug. 2012

Advisor: Teresa Whei-Mei Fan

Thesis: "FT-ICR MS based analysis on carbonyl metabolites in the cell extracts"

Beijing University of Chemical Technology, Beijing, China

M.E. in Cereals, Oils and Vegetable Protein Engineering, Sep. 2006-Jul. 2009

Advisor: Peng Zhang

Thesis: “Study on producing L-ornithine by Arginase in microorganism”

Beijing University of Chemical Technology, Beijing, China

B.E. in Bioengineering, Sep.2002-Jul. 2006

Advisor: Peng Zhang

Thesis: “The screening and culture of strains producing citrulline”

Work Experience

Projects

- *Effect of n-3 and n-6 polyunsaturated fatty acids on inflammation*

  Scientist, Utah State University

  Project: Evaluated effect of dietary polyunsaturated fatty acids on inflammation in mice model. Investigated relationship between dietary PUFA, PUFA-associated inflammation intermediates (eicosanoids/oxylipins), and inflammatory responses under both acute and chronic inflammation models. The results of project provide useful reference for American dietary guideline.

- *Peach fruit quality analysis in relation to organic and conventional cultivation techniques*

  Research Assistant, Utah State University

  Project: Analyzed multiple quality parameters on peach. Detected and identified volatile metabolites related to peach flavor by using headspace solid phase micro extraction and GC-MS. Sensory evaluation was also applied to judge the likeness of peach under different cultivation techniques.

- *A carbonyl capture approach for profiling oxidized metabolites in cell extracts*

  Scientist, University of Louisville

  Project: Synthesized quaternary ammonium aminooxy reagent for aldehydes and ketones detection. Aminooxy reagent has strong affinity to aldehydes and ketones and will form stable compounds which could be analyzed by FT-ICR-MS. The study provides a noval and effective method for aldehydes and ketones detection which has potential to be used in disease diagnose with specific aldehydes and ketones biomarkers.

- *Stable isotope-resolved metabolomics for lung cancer chemotherapy*

  Research Assistant, University of Louisville

  Project: Investigated metabolic pathways affected by the medicine in the lung cancer cells. The fluxes of metabolic pathways are studied based on C^{13} isotope-labeled technology which monitors the changes of metabolites through the changes of positions and locations of C^{13}.

- *Metabolomics in microenvironmental interaction between cancer cells and macrophages*

  Research Assistant, University of Louisville

  Project: Study on metabolic pathways of immunotherapy in the lung cancer cells and the interactions between immune cells and cancer cells. Developed method to measure the nitrate and nitrite released from immune cells. Evaluated changes of metabolites in the microenviroment of cancer cells and macrophages which could provide useful information for lung cancer immunotherapy.

- *Produce amino acids by microorganism*

Scientist, Beijing University of Chemical Technology

Project: (1) Screened the bacterium mutant with high activity of arginase for L-ornithine production (Chinese patent). (2) Screened the bacterium mutant with high activity of arginine deiminase for L-citrulline production (Chinese patent). The new strains have high efficiencies to produce amino acids with strong resistance to other microbe contamination.

- *Synthesis of site–specific, high activity DNA cleavage reagents*

  Research Assistant, Beijing University of Chemical Technology

  Project: Synthesized intermediate product of polyamide-Cyclen-Zn2+ conjugates for enhancing DNA-cleaving activity. The artificial DNA cleavage reagents could help scientists to understand the mechanism of DNase.

- *Enhancing oil recovery ratio by selected microorganism*

  Research Assistant, Beijing University of Chemical Technology

  Project: Screened microorganism mutant which could emulsify and reduce the viscosity of crude oil quickly and effectively. The study provides a novel way to enhance the oil recovery ratio in crude oil industry.

Teaching experience

- Food chemistry lab (Utah State University)

  Prepared and taught food chemistry lab 20 students on safe lab procedures to ensure successful analysis.

- General and analytical chemistry lecture and lab (University of Louisville)

Facilited lectures for over 100 students to teach and educate on various chemistry topics. Taught 20 students on various chemical analysis (GC-MS, fluorescence analysis, etc.) with safe lab procedures.

- Biochemistry lab (Beijing University of Chemical Technology)

  Taught over 30 students various biochemical analysis (gel electrophoresis, size-exclusion chromatography, enzyme extraction and activity measurement, molecular cloning etc.)

Publications

[1] Stephanie Mattingly, **Tao Xu** (co-first author), Michael Nantz et al. A carbonyl capture approach for profiling oxidized metabolites in cell extracts [J]. *Metabolomics*, 2012, 8(6): 989-996.

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